Prostaglandin F$_{2\alpha}$-Mediated Luteolytic and Luteotrophic Effects on the Human Granulosa-Luteal Cell

by

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B.Sc., Simon Fraser University, 1991
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Reproductive and Developmental Sciences Program)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
March 1997

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Department of obstetrics + gynaecology

The University of British Columbia
Vancouver, Canada

Date 29 April 1997
These studies examined the effects of prostaglandin-F₂α (PGF₂α) on progesterone and 17β-estradiol (estradiol) production, as well as DNA and PGF₂α-receptor (PGF₂α-R) mRNA levels, in the human granulosa-luteal cell (GLC). Additionally, the interactions of PGF₂α with human chorionic gonadotrophin (hCG), gonadotrophin-releasing hormone (GnRH) and prostaglandin E₂ (PGE₂) were examined, with respect to progesterone and estradiol production. In one study, cells were collected from small (<12 mm) and large (>12 mm) follicles separately, permitting the examination of follicle size-dependent alterations in steroidogenesis. Pharmacological techniques were utilized to elucidate the signal transduction pathways involved in the anti-gonadotrophic effects of PGF₂α. Moreover, these experiments were performed on GLCs cultured for one (D₁), eight (D₈) and/or twelve to fourteen days (D₁₂-₁₄), in order to reveal culture time-dependent alterations in cellular response.

Briefly, GLCs collected from patients undergoing in vitro fertilization (IVF), were cultured for the time periods described above, followed by a 24 h treatment period. After the treatment period media were collected and assayed for progesterone and estradiol, while cells were extracted for DNA or total RNA.

It was found that human GLC responses to PGF₂α are culture time- and concentration-dependent, with PGF₂α being either luteolytic or luteotrophic, depending on culture and treatment conditions. Moreover, GLC responses to hCG and PGF₂α varied with follicle size, suggesting that these hormones’ actions are targeted toward more mature follicles. Furthermore, GnRH potentiates the luteolytic effects of PGF₂α, while it acts as a permissive factor for the luteotrophic effects. A complex interaction between PGF₂α and PGE₂ was also seen. The luteolytic effects of PGF₂α are mediated through a pertussis toxin-sensitive G-protein (possibly G₁, Gₚ or both). PGF₂α inhibits cholera toxin-, isoproterenol- and forskolin-, but not db-cAMP-stimulated progesterone production suggesting that this G-protein is exerting its actions on the adenylate cyclase pathway at the level of adenylate cyclase, but not distal to it. Additionally, PGF₂α is capable of autoregulating its receptor mRNA levels, and thus its ability to regulate steroidogenesis in the human GLC. Prostaglandin F₂α-R mRNA levels were found to be inversely related to progesterone and estradiol production.

In conclusion, PGF₂α is a multi-functional hormone which acts through complex signal transduction pathways and interactions with confounding hormones, to exert both luteotrophic and luteolytic effects.
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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</tr>
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<td>AA</td>
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</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
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</tr>
<tr>
<td>C</td>
<td>Celcius</td>
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</tr>
<tr>
<td>CA</td>
<td>California</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>cDNA</td>
<td>Complimentary DNA</td>
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<td>CL</td>
<td>Corpus luteum</td>
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<td>Cox-I</td>
<td>Cyclooxygenase I (constitutive)</td>
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<td>Cyclooxygenase II (inducible)</td>
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<td>CTX</td>
<td>Cholera Toxin</td>
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<td>2D</td>
<td>Two dimensional</td>
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<td>D₁</td>
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<td>D₈</td>
<td>Precultured for 8 days</td>
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<td>D₁₂-₁₄</td>
<td>Precultured for 12-14 fourteen days</td>
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<td>DAG</td>
<td>Diaclyglycerol or diglyceride</td>
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<td>db-cAMP</td>
<td>Dibutryl-cyclic-adenosine monophosphate</td>
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<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
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<td>dCTP</td>
<td>Deoxycytosine-triphosphate</td>
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<td>dNTPs</td>
<td>Deoxynucleotide-triphosphate(s)</td>
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<td>DP</td>
<td>Prostaglandin D₂ receptor</td>
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<td>dpi</td>
<td>Dots per inch</td>
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<td>FSH</td>
<td>Follicle stimulating hormone</td>
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<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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DMEM  Dulbecco’s Modified Eagle’s Medium  
DNA  Deoxyribonucleic acid  
dNTPs  Deoxynucleotide-triphosphates  
DTT  Dithiothreitol  
E₂  Estradiol  
EDTA  Ethylenediaminetetraacetic acid  
EP₁  Prostaglandin E₂ receptor (isoform 1)  
EP₃  Prostaglandin E₂ receptor (isoform 3)  
Estradiol  17β-estradiol  
FBS  Foetal bovine serum  
For  Forskolin  
FP  Prostaglandin F₂α receptor  
g  Grams  
GLB  Gel loading buffer  
GLC  Granulosa-luteal cell  
GnRH  Gonadotrophin-releasing hormone  
GTP  Guanosine triphosphate  
G-protein  GTP dependent protein  
Gα  G-protein alpha subunit(s)  
GαS  Gα stimulatory  
Gα₁,₂  Gα inhibitory (isoform 1 or 2)  
Gα₃  Gα inhibitory (isoform 3)  
Gαp  Gα placental  
Gαq  Gα placental (q isoform)  
Gα₁₁  Gα placental (11 isoform)  
Gα₀  Gα olfactory  
GRB  Gel running buffer  
GTC  Guanosine thiocyanate Lysis buffer  
h  Hours  
hCG  Human chorionic gonadotrophin  
Indo  Indomethacin
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</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol trisphosphate</td>
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<td>IsoP, or Iso</td>
<td>Isoproterenol</td>
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<td>Liter</td>
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<td>LH</td>
<td>Luteinizing hormone</td>
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<td>LT</td>
<td>Leukotrienes</td>
</tr>
<tr>
<td>M199</td>
<td>Medium 199</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliters</td>
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<tr>
<td>min</td>
<td>Minutes</td>
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<tr>
<td>MD</td>
<td>Maryland</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MO</td>
<td>Missouri</td>
</tr>
<tr>
<td>m.w.</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>M</td>
<td>Moles/Liter</td>
</tr>
<tr>
<td>NADP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Hydrogenated NADP&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
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<td>New Hampshire</td>
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<tr>
<td>nM</td>
<td>Nanomolar</td>
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<tr>
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<td>New York</td>
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<td>Optical density</td>
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<tr>
<td>P&lt;sub&gt;4&lt;/sub&gt;</td>
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<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase activating polypeptide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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</table>
PBS  Phosphate-buffered saline
PG   Prostaglandin
PGE₂  Prostaglandin E₂
PGF₂α  Prostaglandin F₂α
PGF₂α-R  Prostaglandin F₂α receptor
PGG₂  Prostaglandin G₂
PGH₂  Prostaglandin H
PGI₂  Prostaglandin I₂
PI   Phosphoinositide
PKA  Protein kinase A
PKC  Protein kinase C
PKCi  Protein kinase C inhibitor
           Bisindolylmaleimide
PL   Prolactin
PLA₁  Phospholipase A₁
PLA₂  Phospholipase A₂
PLB  Phospholipase B
PLC  Phospholipase C
PLD  Phospholipase D
pM  Picomolar
pMole  Pico moles
PTX  Pertussis toxin
RIA  Radioimmunoassay
RNA  Ribonucleic acid
RT  Reverse transcription
s  Seconds
SCC  P450-side chain cleavage enzyme
SDS  Sodium dodecyl sulfate
Sigma  Sigma Chemical Company, St. Louis, MO.
SSC  
Sodium chloride and sodium cytrate buffer

TBE  
Tris borate EDTA

TRIS  
Tris(hydroxymethyl)aminomethane

Tx  
Thromboxanes

UV  
Ultraviolet

v/v  
Volume per volume

w/v  
Weight per volume

x  
Times (or multiplied by)

x g  
Times gravity

PQ  
Quebec

Abbreviations Starting with Greek Characters

\( \alpha \)  
Alpha

\( \beta \)  
Beta

\( \gamma \)  
Gamma

\( \mu M \)  
Micromolar (10^{-6} molar)
ACKNOWLEDGMENTS

My gratitude to Dr. Leung for letting me explore my ideas while acting as a sounding board cannot be overstated. I would further like to thank him for allowing me the opportunity to gain experience by helping review papers, acting as chairperson for the departmental seminars and supervising three undergraduate students. This has truly been an educational experience. Many thanks to Dr. Ho Yuen for encouragement and instructive suggestions. I would also like to thank Dr. Auersperg for demonstrating so much enthusiasm after a good number of years of science. I hope that I may maintain such a passion for science. Dr. Rajamahendran’s comments during my committee meetings have helped me tighten up my language. The chairman of my committee Dr. Lee helped strengthen this thesis by reinforcing my awareness of the some of the caveats of the human granulosa-luteal cell model. Additionally, Dr. Buchan, in the Department of Physiology, is largely responsible for the style and approach to science contained within this thesis, as her prolific advice during my Masters thesis still resides within my mind. I hope that she would be pleased with this manuscript.

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This thesis is dedicated to my family.
I – BACKGROUND

A. The Classical Neuro-Endocrine Pathway of Gonadal Regulation

Classically, regulation of ovarian steroidogenesis was seen as a purely hypothalamo-pituitary axis phenomena (Fig. 1). The classical neuro-endocrine pathway acts as follows. Various inputs such as corticotrophin releasing hormone, dopamine, endorphin, estradiol, norepinephrine, pheromones, serotonin and the light/dark cycle are integrated in the arcuate and preoptic nuclei [Advis et al., 1978; Balthazart et al., 1981; Donham et al., 1993; Dufour et al., 1988; Laatikainen, 1991; Rotsztejn et al., 1976; Sawyer, 1975; Yen et al., 1977]. These influences regulate the secretion of gonadotrophin-releasing hormone (GnRH) from neuron-like cells, originating in these nuclei, and terminating in the anterior pituitary. Gonadotrophin-releasing hormone is a decapeptide that is clipped from a larger propeptide [Hsueh et al., 1983; Nillius et al., 1974]. Through a receptor-dependent mechanism, GnRH acts on gonadotrophs to stimulate the release of the gonadotrophins, follicle stimulating hormone (FSH) and lutenizing hormone (LH) [Baldwin et al., 1984; Joshi et al., 1993; Rommler et al., 1979]. Follicle stimulating hormone and LH are collected by the portal system of the anterior pituitary and distributed, via the efferent veins, into the general circulation where they eventually reach their target the ovaries [Sawyer, 1975].

Lutenizing hormone is secreted in pulses, with the period between peaks being 1 to 7 hours depending on the phase of the menstrual cycle [Filicori et al., 1979]. The pulsatile nature of LH release is probably due to pulsatile GnRH secretion or GnRH-receptor number fluctuations, rather than gonadal feedback [Baldwin et al., 1984; I naudi et al, 1992; Schuiling and Gnodde, 1976]. During the follicular phase estradiol levels increase in response to FSH. When the developing follicle is fully mature, the estradiol levels reach a threshold which initiates an LH peak and triggers ovulation.

Follicle stimulating hormone is released from a single pool, in a pulsatile manner, with a lower amplitude than LH [Filicori et al., 1979]. The release of FSH is less sensitive to GnRH than LH [Hall et al., 1992], and can further be regulated by estradiol.
Figure 1. The hypothalamopituitary axis. Various stimulatory (→) and inhibitory (←) neural inputs regulate the secretion of gonadotrophin-releasing hormone (GnRH) from the hypothalamus. GnRH in turn stimulates the production and secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. Circulating FSH and LH stimulate progesterone, estradiol and inhibin production from the ovaries. Inhibin exerts negative feedback on the pituitary and possibly the hypothalamus. Additionally, follistatin (an ovarian product) inhibits the actions of inhibin (not diagrammed). Estradiol can positively or negatively feedback on the pituitary and hypothalamus depending on temporal and concentration conditions.
Both FSH and LH are glycoproteins which share a common α-subunit (m.w. 14,000; 96 AA). Additionally, FSH and LH each have a unique β-subunit, which is noncovalently linked to the α-subunit [Combarnous, 1988; Gray, 1988; Ryan et al., 1987; Wierman et al., 1988]. These peptides possess carbohydrate moieties which account for 15 percent of their weight, and are involved in receptor binding [Combarnous, 1988; Gray, 1988; Ryan et al., 1987; Wierman et al., 1988].

Once FSH and LH reach their primary target in the female—the ovaries—their actions diverge. In the granulosa cell FSH is responsible for stimulating mitosis, aromatase activity and inducing LH-receptor expression and membrane presentation. These actions serve to ripen or prepare the developing follicle for ovulation. This FSH-induced increase in LH-receptors primes the granulosa cell for the LH surge just prior to ovulation. Lutenizing hormones primary action on the granulosa cell is an increase in progesterone synthesis. Furthermore, in the theca cell LH promotes mitosis and progesterone and androgen synthesis. With the granulosa and theca cells working in concert, estradiol is released into the intracellular space where it feeds back on both cell types. Estradiol promotes FSH-receptor and estradiol-receptor expression on the granulosa cell, and LH-receptor expression on the theca cell, further enhancing the actions of these hormones. Additionally, estradiol feeds back on the anterior pituitary to increase GnRH secretion as well as the pituitary response to it [Burger, 1981]. The increase in GnRH and the pituitary's sensitivity to it, increases LH secretion and decreases FSH secretion. This feedback further promotes the ripening of the follicle in preparation for ovulation. Ovulation is induced by LH in concert with numerous peptides, steroids, prostaglandins, leukotrienes and neurotransmitters, including but not limited to: collagenase, epidermal growth factor, relaxin, GnRH, vasoactive intestinal polypeptide, progesterone, prostaglandin F2α, prostaglandin E2 and possibly prostaglandin I2. For further information on the mechanisms of ovulation, see the following reviews: Channing et al., 1980; Haour and Lang, 1978; Leung and Steele, 1992; Suzuki and Takahashi, 1974; Turgeon, 1980; Wu and Prazak, 1974; Yen, 1977.
B. Pregnancy

Following ovulation, the ovum is transported down the fallopian tubes where fertilization occurs, usually within 12 to 24 hours post-ovulation. If fertilization has been successful the zygote will pass through the fallopian tubes (2-3 days) and implant in the uterus (approx. 3 more days). The key hormones in promoting and maintaining pregnancy are estradiol and progesterone (reviewed below, p. 4). The post-ovulatory follicle differentiates into the corpus luteum following the ovulatory phase. Granulosa cells differentiate into luteal cells account for about 80 percent of the corpus luteum (large luteal cells), with the remainder of luteal cells being derived from the theca interna (small luteal cells). The corpus luteum is the primary source of sex steroids during the luteal phase. Moreover, if fertilization occurs, the luteal phase is maintained beyond its 14 day lifespan by conceptus and/or placental derived hCG, which stimulates steroidogenesis through a cAMP dependent mechanism (Fig. 2). The corpus luteum is maintained until placental derived progesterone levels are adequate to maintain pregnancy, after which time it regresses. The regressed corpus luteum either in pregnancy or in the menstrual cycle is called the corpus albicans.
Figure 2. Model of signal transduction pathway for human chorionic gonadotrophin (hCG) stimulated steroidogenesis, in human luteal cells. G - stimulatory G-protein; AC - adenylate cyclase (AC); cAMP - cyclic adenosine monophosphate; and PKA - protein kinase A.
C. The Sex Steroids

The Progestins and Estrogens

The key sex steroid hormones are the progestins and the estrogens. Progestins are known as the pro-gestational hormones for their ability to maintain, prepare for and promote pregnancy. The progestins include progesterone, 17α-OH-progesterone and 20α-OH-progesterone, of which progesterone is the most potent. The estrogens are responsible for the secondary sex characteristics of the female, follicle maturation, and are behavioural modifiers in animals and possibly humans. In animals, the estrogens are reported to promote estrous behaviour (or mating behaviour), hence the name estrogen (a derivative of ‘estrous-genic’). The estrogens include 17β-estradiol (commonly known as estradiol or E₂) and estrone, of which estradiol is the most potent.

The Synthesis of Progesterone and Estradiol

The sex steroids are synthesized in the ovarian granulosa, luteal and thecal cells where they are known to have paracrine and/or autocrine actions in addition to their peripheral endocrine effects. Progesterone and estradiol are synthesized from cholesterol, which may be obtained from dietary sources or synthesized from two acetyl-CoA molecules, by a series of enzymatic reactions [Stryder 1988; Schroepfer 1982; Fielding 1985; Nebert and Gonzales 1987; Granner 1988]. The side chain of cholesterol is cleaved by P450-side chain cleavage enzyme (P450-SCC) or 20,22-desmolase to produce pregnenolone (Fig. 3). Pregnenolone may then be converted to progesterone by a complex of 3β-ol-dehydrogenase and Δ4, 5-isomerase. Through a series of enzymatic reactions, progesterone or pregnenolone may be converted to estradiol. One of the key enzymes in this conversion is aromatase. Aromatase and P450-SCC are highly regulated enzymes, as discussed below. For a more complete description of these synthetic pathways, please refer to Figure 3.

The Two-Cell Model of Steroid Biosynthesis

In the human ovary it requires the co-operation of two different cell types, the theca interna cell and the granulosa cell, to produce estrogen [Moon et al., 1978; Moon et al., 1981; Tsang et al., 1982; Moon and Duleba 1982; Takahashi et al., 1984]. This two-cell model of steroidogenesis is depicted in Figure 4. Briefly, LH stimulates cAMP production in the theca
Figure 3. The synthetic pathway of the female sex steroids progesterone and estradiol, from cholesterol. The enzymes involved in sex steroidogenesis include: 1) P450 side chain cleavage enzyme or 20,22-desmolase, 2) 17-hydroxylase, 3) 17,20-desmolase, 4) 17β-OH-steroid dehydrogenase, 5) 3β-ol-dehydrogenase and Δ4,5-isomerase, and 6) aromatase. In the studies presented herewithin, androstenedione is added to the culture medium to provide an aromatizable substrate for the production of estradiol.
Figure 4. The two cell model of steroidogenesis in the human ovary. Luteinizing hormone (LH) stimulates cAMP production in the theca interna and granulosa cells, while follicle stimulating hormone (FSH) stimulates cAMP production in the granulosa cell. Progesterone (P₄) is produced via a cAMP-mediated increase in desmolase (1) activity, in both cell types. However only the theca interna cells are able to convert progesterone to aromatizable androgens. In order for the granulosa cell to produce estrogens an exogenous source of aromatizable androgens is necessary. These exogenous androgens are provided through diffusion from the theca interna cells to the granulosa cells. The conversion of androgens to estrogens is achieved by cAMP-mediated increase in aromatase (2) activity.
interna and granulosa cells, while FSH stimulates cAMP production in the granulosa cell. Progesterone is produced in both cell types via a cAMP-mediated increase in desmolase activity. However, only the theca interna cells are able to convert progesterone to aromatizable androgens. In order for the granulosa cell to produce estrogens, an exogenous source of aromatizable androgens is necessary. These exogenous androgens are provided through diffusion from the theca interna cells to the granulosa cells. In an in vitro culture system, it is necessary to provide granulosa cells with exogenous androgens (usually androstenedione or testosterone), if one wishes to measure estradiol production in response to stimuli. The conversion of androgens to estrogens is achieved by cAMP-mediated increase in aromatase activity.

Sex Steroid Receptors

Following synthesis, the sex steroids have local effects within the ovary, as well as endocrine effects throughout the body and hypothalmo-pituitary axis [Goebelsmann 1979; McCarty et al., 1983; McNatty et al., 1979a and b; Schroepfer 1982; Rasmussen and Yen 1983; Nebert and Gonzalez 1987]. The majority of progestins and estrogens circulate bound to binding proteins including albumin, cortisol binding protein and sex steroid binding protein. Only one to two percent of these steroids circulate in their free form.

Due to the hydrophobic nature of steroid hormones, they readily pass through cellular membranes, both from their sites of production and into their sites of action. Thus, these hormones do not have membrane receptors. This has the advantage that it eliminates the need for a secondary messenger system. Steroid receptors belong to a super-family of receptors which also include the thyroid hormone, retinoic acid and vitamin D receptors [McCarty et al., 1983]. The progestin and estrogen receptors each possess a DNA binding domain and a ligand binding domain. Following binding of the steroid to its receptor, the receptor-steroid complex attaches to its DNA acceptor site. This complex forms a site for the binding of RNA polymerase to the chromosome, and results in the production of RNA transcripts and their associated proteins. These de novo proteins are responsible for steroid-mediated cellular actions.
Sex Steroid Sites of Action

The regulation of the human menstrual cycle, conception and pregnancy by progesterone and estradiol is a body-wide process involving the brain, pituitary, ovary, uterus, fallopian tubes, vagina, breasts and other tissues. The following is a brief review of the effects of progesterone and estradiol on these tissues. As the hypothalamic-pituitary-gonadal axis has already been reviewed, this section will not discuss them further [see Mahesh 1985; Franz 1988; Tonetta 1989; and Genuth 1988 for further review]. Likewise, there are too many sex steroid-dependent functions throughout the body to discuss them all in the context of this thesis.

1. The Fallopian Tubes

Following ovulation estradiol assists in the capture and transportation of the ovum down the fallopian tube [Spilman and Harper 1975; Genuth 1988; Janzen 1995]. Estradiol is responsible for the widening and undulatory movement of the fimbria which assists in catching the ovum and directing it into the fallopian tube. The number of cilia on the surface of fallopian tube epithelial cells is increased by estradiol. Once in one of the fallopian tube, the ovum is transported toward the uterus by an estradiol-dependent beating of epithelial cilia and fallopian tube contractions. During the luteal phase, progesterone maximizes the ciliary beating and increases nutrient secretion into the lumen of the fallopian tubes. These nutrients may help to maintain the viability of both the ovum, sperm and eventually the zygote if fertilization occurs.

2. The Uterus

Elevated estradiol levels during the follicular phase are responsible for an increase in endometrial thickness (3- to 5-fold), and elevated levels of watery, strand-like mucus [Bazer et al., 1979; Janne 1981]. The increase in endometrial thickness may be in preparation for implantation, and establishes a nutritive base for the new conceptus. Elevated levels of fluid, strand-like mucous create channels to allow sperm to pass freely through the cervix into the uterus. Thus, estradiol is responsible for creating a uterine environment conducive to fertilization and implantation.
On the contrary, elevated progesterone levels reduce mitotic activity and the proliferation of the endometrium, although it is responsible for maintenance of the decidual lining [Genuth 1988]. Progesterone increases glycogen accumulation in vacuoles at the base of endometrial cells, and stimulates the movement of these vacuoles towards the lumen during the luteal phase. These glycogen stores provide an energy rich environment for the zygote within the lumen during implantation. Progesterone reduces the levels of mucus, and changes the mucus from fluid to viscous. These changes assist in implantation of the conceptus within the uterus.

3. The Vagina

Estradiol assists in successful copulation by improving vaginal conditions such as increasing mucous secretions, mucus fluidity, epithelial thickness (protective), vaginal plasticity and external genitalia size [Genuth 1988]. Following the ovulatory phase when it would be less appropriate for copulation to occur, progesterone reduces secretions, secretion fluidity and the numbers of cornified cells [Genuth 1988].

4. The Breasts

In preparation for pregnancy, estradiol promotes the development of the breasts by increasing fat deposits (i.e. energy stores) and the number of lobules [Mauvais et al., 1986; Mauvais et al., 1986; Mauvais et al., 1987]. These changes are in concert with progesterone-mediated alveoli formation. Thus, should pregnancy occur, the breasts will be partially prepared to fulfill their role as a primary nutrient dispensary for the neonate.

5. Other Progesterone-Dependent Actions

A number of other tissues are dependent on the sex steroids for their reproductive functions [Siiteri 1987]. Progesterone acts as a primary substrate for the production of cortisol and aldosterone by the foetal adrenal gland. Additionally, the crucial inhibition of the maternal immune response to foetal antigens is regulated by progesterone [Genuth 1988]. Progesterone also suppresses uterine contractions and expulsion of the foetus from the uterus. Progesterone also acts as a pyrogen, through a thyroid gland mediated increase in metabolism, which elevates body temperature. Behavioural effects have also been reported [Barfield et al., 1984].
D. The Eicosanoids

Prostaglandins, Thromboxanes and Leukotrienes

Membrane phospholipids can be metabolized into a class of hormones called the eicosanoids [Smith 1985; Mayes 1988]. The eicosanoids are further broken up into one of three sub-families, including the prostaglandins (PG), thromboxanes (Tx) and leukotrienes (LT). These hormone sub-families contain a number of hormones each designated by a letter such as A, B, C, et cetera. This character is further followed by a subscript number indicating the number of double bonds contained in the hormone. Furthermore, there are three groups within each of these three eicosanoid sub-families: those with one, two or three double bonds (Table 1). For example, the double bonded form of prostaglandin E is abbreviated PGE$_2$.

Phospholipases and Arachidonic Acid

The main precursor to eicosanoid synthesis is a twenty carbon, four double-bond fatty acid called 5, 8, 11, 14-eicosatetraenoic acid, commonly known as arachidonic acid (AA). The primary enzyme responsible for the production of AA is phospholipase A$_2$ (PLA$_2$), although a number of other lipases are capable of producing AA from glycerophospholipid precursors [Waite 1985; Dennis 1983].

Phospholipase A$_2$ is a hydrophobic, membrane-bound esterase which is active at the water-lipid interphase [Waite 1985]. The family of phospholipases consists of at least five members including phospholipase A$_1$, A$_2$ (B), C and D, each of which cleaves phospholipids at a unique site (Fig. 5) [Mayes 1988]. Normal saturation kinetics do not apply to membrane-bound phospholipases, as they do to soluble esterases. Compared to soluble esterases, phospholipases are exposed to extremely high concentrations of substrate molecules (phospholipids), which are pre-oriented toward the catalytic site due to their polarity [Waite 1985]. Moreover, phospholipase enzyme products are hydrophilic, a property which enhances their diffusion away from the enzyme and the hydrophobic membrane, thus reducing product inhibition of substrate catalysis. Phospholipases can be greater than 1000 times more active than soluble esterases, due to their aforementioned properties.
<table>
<thead>
<tr>
<th>Fatty Acid Precursor</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>8, 11, 14-Eicosatrienoate</td>
<td>13,16,19-Eicosatrienoate</td>
<td>Arachidonic acid</td>
<td>5,8,11,14,17-Eicosapentaenoate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme*</th>
<th>COX</th>
<th>Lipox</th>
<th>COX</th>
<th>Lipox</th>
<th>COX</th>
<th>Lipox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eicosanoid</td>
<td>PGE₁</td>
<td>TxA₁</td>
<td>LTA₃</td>
<td>PGD₂</td>
<td>TxA₂</td>
<td>LTA₄</td>
</tr>
<tr>
<td></td>
<td>PGF₁</td>
<td>LTC₃</td>
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<tr>
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<td></td>
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<tr>
<td></td>
<td>PGI₂</td>
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<td></td>
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</table>

PG - Prostaglandin; Tx - thromboxanes; LT - leukotrienes. Group I, II, and III possess 1, 2 and 3 double bonds, respectively. * the key enzyme responsible for metabolism from the above fatty acid precursor, including: cyclooxygenase (COX) and lipoxygenase (Lipox).
In a number of systems, the reported pathway for the activation of PLA$_2$ involves a receptor mediated rise in intracellular calcium, which activates phospholipase C (PLC). Phosphatidyl inositol (PI) cleavage by PLC produces diacylglyceride which can either be converted directly to AA by glyceride lipase(s), or may stimulate diacylglyceride (diglyceride) dependent-protein kinase C (PKC) which in turn activates PLA$_2$, via removal of tonic inhibition by a protein inhibitor (Fig. 6) [Waite 1985]. Other factors influencing the activation of PLA$_2$ include membrane charge (and associated enzyme pH), density of phospholipids and membrane fluidity. Factors which affect these three parameters will alter PLA$_2$ activity [Waite 1985] and AA production. Finally, anti-inflammatory corticoids can block the PLA$_2$ activity.

**Eicosanoid Production from Arachidonic Acid**

Two isoforms of cyclooxygenase (COX-I, constitutive and COX-II, inducible) are capable of converting arachidonic acid to prostaglandin G$_2$ (PGG$_2$; Fig. 7). Cyclooxygenase I and II are selectively inhibitable by numerous anti-inflammatory agents. Inhibitors of COX-I include acetylsalicylate and indomethacin [Vane 1971; Roth and Siok 1978], while dexamethasone and other modern nonsteroidal anti-inflammatory agents inhibit COX-II [McCarthy 1995]. Hydroperoxidase converts PGG$_2$ to prostaglandin H$_2$ (PGH$_2$), the precursor to group II or double bonded prostaglandins and thromboxanes. PGH-PGE isomerase converts PGH$_2$ to PGE$_2$, which can be further converted PGF$_{2\alpha}$ by E-2-9 ketoreductase. Theoretically, PGF$_{2\alpha}$ could be produced directly from PGH$_2$ by a reductase, although this pathway has not been demonstrated [Smith 1985].
Figure 5. Phospholipase cleavage (hydrolysis) sites on phospholipids. Phospholipases are capable of hydrolysing the number one acyl bond, number two acyl bond, glycerophosphate bond or the base group. The number one acyl bond is hydrolysed by phospholipase A₁ (PLA₁) or PLB, while the number two acyl bond is hydrolysed by phospholipase A₂ or B (PLA₂ or PLB). The phosphodiesterases, phospholipase C (PLC) and phospholipase D (PLD) hydrolyse the glycerophosphate bond and base group, respectively.
Figure 6. Arachidonic acid production in a model system. This model is based on research performed on platelets. In platelets calcium is mobilized via some external stimuli. Elevated calcium levels activate phospholipase C (PLC) which liberates diglyceride from phosphatidyl inositol. Glyceride lipase can convert diglyceride to arachidonic acid directly. Alternately, diglyceride may activate phospholipase A$_2$ (PLA$_2$), which converts phosphatidyl choline to arachidonic acid.
Figure 7. Synthesis of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from arachidonic acid. Enzymes are in red. Arachidonic acid may be converted to prostaglandin G$_2$ (PGG$_2$) by cyclooxygenase I (constitutive) or cyclooxygenase II (inducible). Hydroperoxidase converts PGG$_2$ into PGH$_2$. Prostaglandin E$_2$ is produced from PGH$_2$ by PGH-PGE isomerase. The enzyme E-2-9 ketoreductase converts PGE$_2$ to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by hydroxylation of the ketone group of PGE$_2$. Theoretically, a reductase could produce PGF$_{2\alpha}$ by reducing PGH$_2$, although this pathway has never been demonstrated.
Prostanoid Receptors

Numerous prostanoid receptors have been cloned from mammalian tissues. These receptors include the PGD$_2$ receptor (DP), the PGE$_2$ receptors (EP$_1$, EP$_2$ and EP$_3$-family), the PGF$_{2\alpha}$ receptor (FP) and the prostacyclin or PGI$_2$ receptor (IP) [Lake et al., 1994; Abramovitz et al., 1994; Adam et al., 1994; Boie et al., 1994 and 1995; Funk et al., 1993]. Based on sequence analysis, these receptors all appear to belong to the seven-transmembrane G-protein coupled receptor family.

The DP, IP and EP$_3$-family of receptors are all coupled to cAMP regulation [Adam et al., 1994; Boie et al., 1994 and 1995; An et al., 1994], while the DP, FP, EP$_1$ and EP$_3$-family of receptors are coupled to rises in intracellular calcium [Abramovitz et al., 1994b; Adam et al., 1994; Boie et al., 1995; Funk et al., 1993; An et al., 1994]. Additionally, the human EP$_3$-family of receptors is capable of inhibiting cAMP production through a pertussis toxin-sensitive G-protein [An et al., 1994].

Prostaglandins as Autocrine/Paracrine Factors

Prostaglandins are believed to be autocrine or paracrine hormones. There are numerous lines of evidence pointing to the local nature of prostaglandin actions, these include the following [Smith et al., 1985]:

1) Prostaglandins have a short half life (minutes) in vivo, which probably prevents them from having effects systemically. This short half life is mainly due to local degradation by prostaglandin dehydrogenase(s), and systemic degradation by the lung.
2) Prostaglandins are secreted in short (1-5 min) bursts, likely preventing systemic hormone levels from becoming elevated.
3) Most cells that secrete prostaglandins also possess receptors for these hormones, suggesting that they are acting locally.
4) Almost every tissue produces prostaglandins and prostanoid receptors, although these prostanoids produce radically different actions from one tissue to another.
Inhibition and Degradation of Prostaglandins

Cyclooxygenase I and II are short lived enzymes as they are capable of undergoing self-catalyzed destruction. Thus they have been dubbed a suicide enzymes [Smith and Borgeat 1985]. This self catalyzed destruction acts as a negative feedback mechanism on prostaglandin synthesis.

As mentioned above, prostaglandins have very short half lives. The rapid degradation of prostaglandins is due to molecular instability, local degradation by tissue specific hydroxyprostaglandin dehydrogenases and systemic degradation in the lung and kidney [Smith and Borgeat 1985]. It has been reported that circulating PGE$_1$, PGE$_2$ and PGF$_{2\alpha}$ are degraded on their first pass through the lung. The lung acts as a filter, by removing virtually all active prostaglandins from the circulatory system. Degradation is achieved by removal of the hydroxyl group at carbon 15 by a NADPH-dependent 15-OH prostaglandin dehydrogenase. Removal of this hydroxyl group reduces the biological activity to ten percent of its original level. Prostaglandin D$_2$ and PGI$_2$ are dehydroxylated by another 15-OH dehydrogenase which is specific to these prostaglandins. This second enzyme is found in the kidney.

Further degradation of these prostaglandins occurs via the reduction of the $\Delta^{13}$ double bond by an NADPH-dependent $\Delta^{13}$ reductase, resulting in 15-keto-13, dihydroprostaglandins which are biologically inactive. Oxidation in the liver and excretion in the urine complete the process.
E. Prostaglandin F\(_{2\alpha}\) in Reproduction

Localization of Prostaglandin F\(_{2\alpha}\)

Prostaglandin F\(_{2\alpha}\) has been detected in the human decidua, amnion, pregnant myometrium and ovary [Satoh et al., 1981; Aksel et al., 1977]. In the human ovary, PGF\(_{2\alpha}\) has been localized to the follicle and theca-, granulosa- and luteal-cells [Aksel et al., 1977; Patwardhan and Lanthier, 1981; Plunkett et al., 1975]. Further, the presence of PGF\(_{2\alpha}\) has been detected in the human follicle at all stages of the reproductive cycle [Patwardhan and Lanthier, 1981]. Additionally, PGF\(_{2\alpha}\) synthesis has been detected in human luteal and stromal tissues where arachidonic acid derived PGE\(_2\) is converted to PGF\(_{2\alpha}\), via E-2-9-ketoreductase [Watson et al., 1979; Endo et al., 1988].

Regulation of PGF\(_{2\alpha}\) Production in the Ovary

In the ovary, PGF\(_{2\alpha}\) production is regulated by a number of ovarian hormones including luteinizing hormone, human chorionic gonadotrophin (hCG), interleukin-1, and tumor necrosis factor [Patwardhan and Lanthier, 1981; Plunkett et al., 1975; Mitsuhashi, 1981; Watanabe et al., 1993; Zolti et al., 1990]. In the rabbit, oxytocin has been suggested as another secretagogue [Fuchs, 1988]. Thus far, propranolol and norepinephrine are known to be receptor-mediated inhibitors of PGF\(_{2\alpha}\) production in the human ovary [Bennegard et al., 1984]. However, hCG or cAMP pretreatment has been shown to inhibit the antigonadotrophic actions of cloprostenol (PGF\(_{2\alpha}\) analog) in the luteal cell [Michael and Webley, 1991b].

Functions of PGF\(_{2\alpha}\)

Prostaglandin F\(_{2\alpha}\) has been shown to mediate functional luteolysis and luteal regression, in the mammalian ovary [Michael and Webley, 1991b; Jalkanen et al., 1987; Korda et al., 1975; Grinwich et al., 1976; Moon et al., 1986; Hanzen, 1984; Richardson and Masson, 1980]. However, the presence of PGF\(_{2\alpha}\) in the ovary only roughly correlates with this action, as PGF\(_{2\alpha}\) levels are highest in mid- rather than late luteal-phase in the human. This discrepancy has been accounted for with the examination of PGE\(_2\), which is known to counteract PGF\(_{2\alpha}\) induced luteolysis. Prostaglandin E\(_2\) levels in mid-luteal phase are high while they are not in late-luteal
phase. Thus it is postulated that during the mid-luteal phase, the ratio of \( \text{PGF}_{2\alpha} : \text{PGE}_2 \) is low and not suitable for luteolysis, although in the late-luteal phase this ratio is high allowing for luteolysis in the human [Pathwardhan and Lanthier, 1985]. Prostaglandin \( F_{2\alpha} \) is known to inhibit LH-, hCG- and PGE2-stimulated progesterone production (functional luteolysis). Potential mechanisms for functional luteolysis include the inhibition of LH/hCG receptor levels and/or binding [Luborsky et al., 1984], a reduction in adenylate cyclase activation [Dorflinger et al., 1984], increased progesterone catabolism through 20-alpha-hydroxysteroid dehydrogenase [Moon et al., 1986] and possibly an increase in cAMP phosphodiesterase activity via PKC [Lahav et al., 1989; Michael and Webley, 1991a]. Luteal regression is believed to be effected through a PGF\(_{2\alpha}\)-mediated reduction in blood flow to the corpus luteum and apoptotic cell resorption [Hanzen, 1984; Khan et al., 1989; Richardson and Masson 1980; Quirk et al., 1995].

The luteotrophic action of PGF\(_{2\alpha}\) appears to be time-, concentration- and species-dependent. These actions are reported to be strongest in the mid-luteal phase and during pregnancy of investigated species [Khan et al., 1989; Michael and Webley, 1993; Webley et al., 1989; Suginami et al., 1976]. Moreover, \textit{in vitro} and \textit{in vivo} studies have demonstrated the luteotrophic effects of PGF\(_{2\alpha}\) in the presence of gonadotrophin [Suginami et al., 1976], suggesting that the mere presence of gonadotrophins is not sufficient to initiate a luteolytic response from PGF\(_{2\alpha}\).

\textit{PGF}_{2\alpha} \text{ in Pregnancy}

Studies have demonstrated that temporal and confounding relationships of ovarian hormones may be important in preventing CL regression, should pregnancy occur [Michael and Webley, 1991b]. For example, PGF\(_{2\alpha}\) is well accepted as being able to inhibit hCG-stimulated progesterone production in studies where these two hormones are administered together. However, when hCG treatment precedes PGF\(_{2\alpha}\), this luteolytic effect is not seen [Michael and Webley, 1991b]. Similarly, prolactin, LH and FSH, alone and in combination, were not capable of blocking PGF\(_{2\alpha}\)-induced luteolysis. However, pretreatment with prolactin, FSH plus LH prevented PGF\(_{2\alpha}\)-induced luteolysis in 11/14 hamsters [Harris and Murphy, 1981]. The blockade of luteolysis by pretreatment with hCG is suggested as being a means by which the placenta rescues the corpus luteum (CL) from PGF\(_{2\alpha}\)-mediated regression [Webley et al., 1991], thus allowing pregnancy to proceed.
Prostanoid Receptors in Reproductive Tissues

Prostaglandin F$_{2\alpha}$ lowers both gonadotrophin- and prostaglandin E$_2$-stimulated rises in cAMP, as well as increases intracellular calcium and inositol phosphates in reproductive tissues [Davis et al., 1989; Currie et al., 1992; Pepperell et al., 1989; Lahav et al., 1987]. It is unknown if the actions of PGF$_{2\alpha}$ are exerted through a single or multiple-receptors. Prostaglandin F$_{2\alpha}$ and PGE$_2$ are both present and active in the human granulosa and luteal cells [Grinwich et al., 1976; Richardson and Masson, 1980; Pathwardhan and Lanthier, 1985; Satoh et al., 1981; Watson et al., 1979]. Thus, it is probable that multiple prostanoid receptors exist in these cells. Furthermore, the currently cloned prostanoid receptors all possess varying degrees of cross-reactivity with PGE$_2$ and PGF$_{2\alpha}$ [Lake et al., 1994; Abramovitz et al., 1994; Adam et al., 1994; Boie et al., 1994 and 1995; Funk et al., 1993; An et al., 1994].

Ligand binding studies have demonstrated that the human PGF$_{2\alpha}$-receptor binds PGF$_{2\alpha}$ with an equilibrium dissociation constant (Kd) of approximately 1 to 1.63 nM [Abramovitz et al., 1994; Lake et al., 1994]. The binding characteristics of the rat PGF$_{2\alpha}$-R suggest a two site model, with a high affinity site (Kd = 3.9 nM) and a lower affinity site (Kd = 34 nM) [Lake et al., 1994].

**PGF$_{2\alpha}$ Signal Transduction**

Prostaglandin F$_{2\alpha}$-receptor cDNA sequences appear to suggest a G-protein coupled receptor [Lake et al., 1994; Abramovitz et al., 1994], as with other cloned prostanoid receptors [Adam et al., 1994; Boie et al., 1994 and 1995; Funk et al., 1993], although pharmacological studies toward this end have not been done in the human ovary. Immunocytochemical studies have localized four different G-protein alpha subunits to the human granulosa-luteal cell including $G_{\alpha_S}$, $G_{\alpha_i3}$, $G_{\alpha_{i1,2}}$ and $G_{\alpha_p}$ (namely $G_{\alpha_q}$ and $G_{\alpha_{11}}$), but not $G_{\alpha_O}$ [Lopez et al., 1995]. Furthermore, it has been demonstrated in these cells that cAMP production is regulated by the ratio of $G_{\alpha_S}$ and $G_{\alpha_i}$, while rises in inositol phosphates and intracellular calcium appear to be regulated by $G_{\alpha_p}$ (namely $G_{\alpha_q}$ and $G_{\alpha_{11}}$) and $G_{\alpha_i}$ [Lopez et al., 1995].
Exposure of mammalian granulosa or luteal cells to PGF$_{2\alpha}$ has been shown to stimulate phospholipase-C and its downstream pathways [Dorflinger et al., 1984; Abayasekara et al., 1993; Davis et al., 1989; Currie et al., 1992; Michael et al., 1993]. It has been suggested that PGF$_{2\alpha}$ is inhibiting cAMP- and progesterone-production via this rise in inositol phosphates and/or calcium [Leung, 1985; Steele and Leung, 1993]. A direct link between these two pathways has not been clearly established, as numerous reports have demonstrated PGF$_{2\alpha}$-mediated luteolysis in the presence of inositol phosphate, calcium and calmodulin inhibitors [Jalkanen, 1987; Michael and Webley, 1993; Pepperell et al., 1989; Lahav et al., 1987]. Moreover, PGF$_{2\alpha}$ and GnRH stimulate phospholipase-C (PLC) in young, and mid but not in old corpora lutea, suggesting that inositol phospholipid metabolism by itself is not sufficient to explain the luteolytic effects of these hormones [Lahav et al., 1988; Endo et al., 1992]. Further confusing the issue, there are reports of PLC products stimulating progesterone production. Luteinizing hormone can stimulate [Davis et al., 1989; Richards et al., 1995], and has been shown to even potentiate PGF$_{2\alpha}$-stimulated IP$_3$ production [Davis et al., 1989]. Thus, the possibility of these messengers being responsible for the luteotrophic effects of PGF$_{2\alpha}$ also exists.

Prostaglandin-F$_{2\alpha}$ is known to increase PKC [Abayasekara et al., 1993a,b] and intracellular calcium levels [Currie et al., 1992]. Additionally, PKC activators have been shown to reduce hCG-stimulated cAMP levels. These results suggest that PGF$_{2\alpha}$ exerts its inhibition of hCG-stimulated cAMP and progesterone production via PKC [Abayasekara et al., 1993a,b]. Furthermore, it is believed that inhibition of hCG-stimulated cAMP levels may occur at the level of G$_s$, as cholera toxin stimulated progesterone production is blocked by PGF$_{2\alpha}$.

**Clinical Applications of PGF$_{2\alpha}$**

In the female, PGF$_{2\alpha}$ has been utilized for contraception and the induction of abortion or parturition [Concannon and Hansel 1977; Lau et al., 1980; Cameron and Baird 1988; Baird et al., 1988]. Conversely, cyclooxygenase inhibitors such as indomethacin have been used effectively to arrest premature labour and delivery [Manauh and Novoy 1976; Fuchs et al., 1976].

Prostaglandin F$_{2\alpha}$ is capable of contraceptive effects in the human as well as in some other mammals [Singh and Dominic, 1986; Bilinska and Wojtusiak, 1988; Orlicky and Williams, 1992; Chinoy et al., 1980]. Investigation has revealed the presence of PGF$_{2\alpha}$-receptors on the
Leydig cell, although not on cells of the tunica albuginea, subcapsular- or peritubular-stroma, peritubular boundary tissue, vasculature, spermatogonia, spermatocytes, spermatids, spermatozoa or Sertoli cells [Orlicky and Williams, 1992].

In the mouse, suppressed spermatogenesis and a significant reduction in the weights of the testis, epididymis and accessory sex glands have been reported following PGF$_{2\alpha}$ administration [Singh and Dominic, 1986]. Moreover, seminiferous tubules were found to be devoid of spermatozoa, while Leydig cells showed atrophy. Interestingly, these regressive changes were reversible, as 56 days after drug withdrawal a normal state was achieved [Singh and Dominic, 1986]. Prostaglandin F$_{2\alpha}$ treated rats exhibited reduced testicular- and epididymal-weight, while the weight of their seminal vesicle and ventral prostate increased. Additionally, altered morphology and reduced density- and motility-spermatozoa were seen [Chinoy et al., 1980].

Aside from morphological changes, Leydig cell-androgen production has been reported to be reduced by a PGF$_{2\alpha}$-mediated inhibition of delta 5,3 beta-hydroxysteroid dehydrogenase activity [Bilinska and Wojtusiak, 1988].
F. Gonadotrophin-Releasing Hormone

GnRH Functions

GnRH- and GnRH-receptor mRNA have recently been isolated in the human granulosa cell, indicating that GnRH probably has important local actions within the ovary [Peng et al., 1994]. GnRH is a decapeptide that was first discovered in the hypothalmo-pituitary axis. As mentioned above GnRH is the primary mediator of gonadotrophin release. Gonadotrophin-releasing hormone has also been shown to have luteolytic as well as luteotrophic effects [Leung 1985] in some mammals. Buserelin (a GnRH agonist) has been reported to block hCG, PGE\textsubscript{2} epinephrine and cholera-toxin stimulated progesterone production, as well as potentiating PGF\textsubscript{2\alpha}-inhibition of cAMP production [Massicotte, 1984]. On the contrary, GnRH administration has been utilized to maintain pregnancy or enhance fertility in the cow [Farin and Estill 1993; Funston and Seidel 1995].

GnRH Localization

In humans and other mammals, at least two molecular forms of GnRH have been demonstrated in the brain, ovary and other tissues [King et al., 1990; Ireland et al., 1988; Aten et al., 1987; Behrman et al., 1989; King et al., 1989]. The amount of GnRH in luteal tissues is reported as being proportional to the weight of these tissues, although the concentration of GnRH peptides drops as the corpus luteum develops. While GnRH and/or GnRH peptides are found in numerous nonovarian tissues, in cattle they appear to be relatively concentrated in granulosa cells [Ireland et al., 1988] and pituitary.

GnRH Receptor

Gonadotrophin-releasing hormone is capable of reducing progesterone production and interrupting reproductive cycles and pregnancy in the rat [Clayton et al., 1979]. These actions have been attributed to specific high-affinity receptors present in luteal cell membranes [Clayton et al., 1979; Latouche et al., 1989]. Additionally, this action appears to be autocrine in nature as both GnRH- and GnRH receptor (GnRH-R)-mRNA have been detected within the human
granulosa-luteal cell [Peng et al., 1994]. Moreover, GnRH is reported to autoregulate its own mRNA level as well as those of GnRH-R. Conversely, hCG has been shown to down-regulate GnRH receptor mRNA levels. Messenger RNA for GnRH has also been cloned from the rat corpus luteum, where it was found to have an identical sequence to the rat anterior pituitary GnRH receptor [Whitelaw et al., 1995]. Furthermore, the expression of GnRH-R gene in granulosa cells is purported to be individually regulated for each follicle, to persist in the corpus luteum and is expressed in atretic follicles [Whitelaw et al., 1995; Minaretzis et al., 1995]. In fact atretic follicles appear to exhibit the greatest degree of GnRH-R gene expression, suggesting that GnRH is important in the induction of follicular atresia [Bauer and Jameson, 1995].

**GnRH Signal Transduction**

In the pituitary gonadotroph, GnRH is known to stimulate polyphosphoinositide breakdown [Kiesel et al., 1986]. On the other hand, both GnRH and NaF-stimulated LH release can occur in the absence of inositol phosphate production [Hawes et al., 1992], suggesting that inositol triphosphate is not an essential second messenger for the release of LH. The question remains which second messengers are necessary for the release of LH from the gonadotroph. Phosphatidic acid, a phospholipase D product, has been reported to increase dose- and time-dependently (2-3 fold; 1-2 min) following GnRH analogue administration in alpha T3-1 cells [Netiv et al., 1991].

Pituitary adenylate cyclase activating polypeptide (PACAP)-stimulated cAMP production is inhibited by GnRH in the alpha T3-1 gonadotroph cell line, although GnRH did not inhibit PACAP binding to gonadotrophs nor forskolin- or cholera toxin-stimulated cAMP production. Thus it has been suggested that the inhibitory effects are exerted at early stages in the signal transduction pathway distal to receptor occupancy but preceeding cAMP production [McArdle et al., 1994], possibly at the level of a G-protein.

Gonadotrophin-releasing hormone and PGF$_{2\alpha}$ both inhibit cAMP production in the corpus luteum. Phosphatidyl inositol (PI) and phosphatidic acid (PA) turnover occurs rapidly (2 and 5 min respectively) with a mean effective dose of 15 and 100 nM for GnRH and PGF$_{2\alpha}$, respectively [Leung, 1985; Davis et al., 1984; Davis et al., 1986]. When co-treatment with the hormones is performed, their effects appear to be additive. Incidentally, A23187 (a pore-forming calcium ionophore) also causes a dramatic increase in PA and PI turnover. Dibutryl-cAMP and
8-Br-cAMP attenuate GnRH and PGF\(_{2\alpha}\) stimulated PA and PI turnover. The biproducts of PLC activity (IP3 and DAG) mobilize intracellular calcium, activate PKC and release arachidonic acid [Davis et al., 1986; Shinohara et al., 1985]. The similarity of GnRH and PGF\(_{2\alpha}\) responses has led to the suggestion that they may share post-receptor signalling mechanisms [Leung, 1985].

**GnRH Mechanism of Action**

Studies in the rat have demonstrated GnRH-mediated inhibition of progesterone production through increased activity of 20-alpha-hydroxysteroid dehydrogenase, inhibition of pregnenolone production and reduced activity of P450SCC and 3-beta-hydroxysteroid dehydrogenase activity [Jones et al, 1983; Srivastava et al., 1994]. The mechanisms by which GnRH exerts its luteotrophic effects are not reported in the literature.

**Clinical Applications of GnRH**

Potent and long-lasting GnRH analogues (super-active agonists) originally developed with fertility promotion in mind have, in fact, proven to have anti-fertility properties in the male and female [Molcho et al., 1984; Bhasin et al, 1984; Nillius, 1985]. These compounds have been applied to numerous therapeutic applications in the female including contraception, treatment of central precocious puberty, and sex steroid-dependent benign and malignant diseases of the reproductive organs [Nillius, 1985]. One of the most common uses of GnRH agonists is the down-regulation of pituitary function in preparation for IVF treatment. Inhibition of ovulation by continuous GnRH agonist administration appears to be safe, reliable and reversible in women [Nillius, 1985]. However, attempts to inhibit luteal function, induce luteolysis or early abortion have not been very successful [Nillius, 1985].

In the human male, high dose GnRH administration interrupts testicular function leading to azoospermia. However, the incompleteness of this azoospermia and unacceptable side effects (loss of libido and potency) rule out the use of GnRH as a male contraceptive [Nillius, 1985]. There have, however, been reports of reduced side effects with co-administration of testosterone [Bhasin et al, 1984; Nillius, 1985].

Interestingly, GnRH is also capable of improving rather than impairing fertility in some species such as the bovine [Farin and Estill 1993; Funston and Seidel 1995].
II – HYPOTHESIS

Prostaglandin F$_{2\alpha}$ is a multi-functional hormone capable of luteolytic and luteotrophic effects in the human granulosa-luteal cell. Moreover, these effects are time-, concentration- and confounding factor-dependent.

III – SPECIFIC OBJECTIVES

A. To define the steroidogenic response of human GLCs to PGF$_{2\alpha}$ with respect to the effects of time in culture, hormone concentration and follicle-size.

B. To examine the potential interactions of PGF$_{2\alpha}$ and GnRH with respect to steroidogenesis.

C. To examine the potential interactions of PGF$_{2\alpha}$ and PGE$_2$ with respect to steroidogenesis.

D. To define the signal transduction pathways involved in PGF$_{2\alpha}$-mediated luteolysis. Additionally, to define the signal transduction pathway(s) or mechanism(s) by which PGF$_{2\alpha}$ exerts its luteotrophic actions.

E. To examine the regulation of PGF$_{2\alpha}$-R mRNA levels by PGF$_{2\alpha}$.

For a diagramatic depiction of the specific objectives which these studies sought to satisfy please refer to Figure 8. For the rationale (p. 3), results (p. 54), discussion (p. 117) and a synopsis of the findings (p. 143) for each of these objectives refer to the corresponding character (i.e. A, B, C, D & E) in the respective section.
Figure 8. A diagramatic depiction of the specific objectives to be satisfied in these studies. Note that the characters A, B, C, D and E refer to the specific objectives presented above (p. 1). These studies sought to examine the following: A) the effects of PGF$_{2\alpha}$ on progesterone and estradiol production; B) the potential interactions of GnRH and PGF$_{2\alpha}$ on steroidogenesis; C) the potential interactions of PGE$_2$ and PGF$_{2\alpha}$ on steroidogenesis; D) the signal transduction pathways involved in PGF$_{2\alpha}$-mediated luteolysis; and E) the effects of PGF$_{2\alpha}$ on PGF$_{2\alpha}$-receptor mRNA levels. For the rationale (p. 3), results (p. 54), discussion (p. 117) and a synopsis of the findings (p. 143) for each of these objectives please refer to the corresponding character (i.e. A, B, C, D & E) in the respective section.
IV - RATIONALE

Progesterone and estradiol are key hormones in the regulation of all aspects of the reproductive cycle and pregnancy (as reviewed above, p. 12). Thus the examination of the regulation of these two hormones by PGF$_{2\alpha}$ should reveal, in a very real sense, its effects on reproduction as a whole. If PGF$_{2\alpha}$ were to regulate either of these two hormones in any significant fashion, this would suggest that this hormone is a very important regulator of the human female reproductive system.

Reports on the effects of PGF$_{2\alpha}$ on estradiol production are scant to non-existent. Thus the underlying studies report estradiol in addition to progesterone responses wherever possible (i.e. sample volume permitting).

The rationale for each group of studies corresponding to the specific objectives follows.

A. The Effects of PGF$_{2\alpha}$ on Steroidogenesis

Prostaglandin F$_{2\alpha}$-receptors have been demonstrated in and have been recently cloned from human ovarian cells. These findings suggest that PGF$_{2\alpha}$ may play an important role in the regulation of ovarian function. However, very few functional studies have been performed in the human granulosa cell. Thus the role of PGF$_{2\alpha}$ remains unclear. The conditions under which the luteotrophic and luteolytic functions of PGF$_{2\alpha}$ exist have not been adequately defined. Furthermore, the majority of previous reports examined the effects of PGF$_{2\alpha}$ in the $\mu$M range of concentrations, while the reported equilibrium dissociation constants (Kd) of cloned prostanoid receptors fall within the nM range [Abramovitz et al., 1994; Lake et al., 1994]. Therefore, these studies utilized PGF$_{2\alpha}$ at concentrations ranging from 1 pM to 1 $\mu$M in order to provide a more complete understanding of the nature of estradiol and progesterone responses to PGF$_{2\alpha}$. There exists the potential that PGF$_{2\alpha}$ is not only important in corpus luteum regression, but also that its temporal relationship to hCG may play a role in the maintenance of early pregnancy. Not only is an understanding of PGF$_{2\alpha}$ important for basic science, but it could also be important clinically.
**B. PGF$_{2\alpha}$ and GnRH Interaction Studies**

Historically, GnRH has been considered a modulator of gonadotrophin secretion from the gonadotroph. As such, GnRH analogs have been used extensively in both experimental and clinical settings for the modulation of the hypothalamopituitary axis in various situations including: IVF [Pellicer et al., 1992; Gonen et al., 1991; Segars et al., 1990], contraception [Fraser, 1993] and control of amenorrhoea [Martin et al., 1990]. It is only recently that GnRH has been identified in the human ovary, and suggested as a potential local regulator of human ovarian function [Oikawa et al., 1990; Peng et al., 1994]. In order to understand any unwanted side-effects of GnRH use in these applications, it is important to further elucidate the local actions of GnRH in the ovary and human granulosa cell.

Gonadotrophin-releasing hormone is believed to share common functions (both luteolytic and luteotrophic actions) and signal transduction pathways (IP$_3$ and PKC) with PGF$_{2\alpha}$. As the focus of these studies has been to examine the effects of PGF$_{2\alpha}$ in the human ovary, GnRH has been examined primarily in its relationship to potential interactions with PGF$_{2\alpha}$.

**C. PGF$_{2\alpha}$ and PGE$_2$ Interaction Studies**

As described above, in the human granulosa-luteal cell PGF$_{2\alpha}$ and PGE$_2$ exert opposing actions on cAMP-levels and progesterone-production [Grinwich et al., 1976; Richardson and Masson, 1980; Pathwardhan and Lanthier, 1985; Satoh et al., 1981; Watson et al., 1979]. Prostaglandin F$_{2\alpha}$ and PGE$_2$ can decrease or increase cAMP-levels and progesterone-production, respectively. Prostaglandin F$_{2\alpha}$ is reported to be at its highest concentration during the mid-luteal phase, although it is reported to be luteolytic during the late-luteal phase. The temporal discrepancy between these two events is accounted for by the levels of PGE$_2$ during these two phases. It has been suggested that high levels of PGE$_2$ during the mid-luteal phase may prevent premature corpus luteum regression. However, this explanation fails to account for the fact that PGF$_{2\alpha}$-levels are (perhaps ‘unnecessarily’) at their highest during the mid-luteal phase when conception and implantation occur. A more comprehensive explanation for the elevated levels of PGF$_{2\alpha}$ during the mid-luteal phase may be necessary. Thus, these studies examined the interactions of PGF$_{2\alpha}$ and PGE$_2$ with respect to steroidogenesis in human GLC *in vitro*.
D. Signal Transduction Studies

In order to fully understand the actions of a hormone, it is essential to know the mode of these actions. Therefore, these studies undertook to examine the signal transduction pathways involved in PGF$_{2\alpha}$-mediated luteolysis and luteotrophism. The post-receptor events involved in the luteotrophic and luteolytic actions of PGF$_{2\alpha}$ are at present speculative.

As the PGF$_{2\alpha}$-receptor [Lake et al., 1994; Abramovitz et al., 1994] is known to belong to the seven transmembrane G-protein coupled receptor family, studies focused on the potential role of G-proteins in the mediation of luteolysis and luteotrophism. Prostaglandin F$_{2\alpha}$ has been shown to lower gonadotrophin- and PGE$_2$-stimulated progesterone production (through a lowering of cAMP levels), and G-proteins are known to regulate cAMP levels within these cells. This study examined the role of G-proteins in mediating the effects of PGF$_{2\alpha}$. Pertussis-toxin (PTX) and cholera-toxin (CTX) were utilized to elucidate the potential role of G-proteins in the anti-gonadotrophic actions of PGF$_{2\alpha}$. In order to determine the action(s) of PGF$_{2\alpha}$ distal to G-proteins in the signal transduction cascade, these studies examined the ability of PGF$_{2\alpha}$ to inhibit progesterone production induced by activators of the adenylate-cyclase, and by cyclic adenosine monophosphate (cAMP) analogues.

Previous studies have demonstrated a correlation between the effects of PGF$_{2\alpha}$ and a rise in inositol phosphate metabolism [Leung, 1985; Steele and Leung, 1993]. Moreover, a number of studies have demonstrated altered responses to PGF$_{2\alpha}$ in the presence of PKC modulators. However, there is much controversy in the literature over the importance of inositol phosphates and PKC in the luteolytic effects of PGF$_{2\alpha}$ [Jalkanen, 1987; Michael and Webley, 1993; Pepperell et al., 1989; Lahav et al., 1987]. The underlying studies sought to confirm or disaffirm the existence of of PKC-mediated alteration in the luteolytic effects of PGF$_{2\alpha}$, although an exhaustive examination of this pathway was not performed. An explanation for the apparent discrepancies in the literature is proposed based on these studies and the known pathways by which prostaglandins are known to act in other systems.
E. PGF$_{2\alpha}$-R mRNA Studies

Prostaglandin F$_{2\alpha}$ is known to act through receptor-mediated mechanisms. Thus the regulation of PGF$_{2\alpha}$-R levels is as important as the regulation of PGF$_{2\alpha}$ itself. Receptor binding studies have previously demonstrated the presence of PGF$_{2\alpha}$-R in the rat and bovine luteal cell [Brambaifa et al., 1984; Bussmann et al., 1989]. Moreover, the existence of PGF$_{2\alpha}$-R mRNA has recently been demonstrated in the human granulosa-luteal cell [Ristimaki et al., 1997]. However, there have been no reports on the regulation of PGF$_{2\alpha}$-R mRNA levels in response to PGF$_{2\alpha}$. Thus these studies examined the ability of PGF$_{2\alpha}$ to regulate PGF$_{2\alpha}$-R mRNA levels.
V – MATERIALS AND METHODS

A. Granulosa-Luteal Cell Collection and Culture

The use of human GLC was approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects of the University of British Columbia. Granulosa-luteal cells were harvested in conjunction with oocyte collection in the University of British Columbia's in vitro fertilization program. Throughout the pre-collection period, follicular development was monitored using estradiol assays and ultrasonography. After pituitary down-regulation with a GnRH analogue (Synarel, Syntex; Montreal, PQ) and when estradiol levels were less than 150 pmol/l, follicular development was stimulated with hMG (Humegon 75 IU FSH and 75 IU LH, Organon, Scarborough, ON; or Fertinorm 75 IU FSH, Serono, Oakville, ON). When three or more follicles reached a diameter greater than 16-18 mm, and estradiol levels were greater than 5000 pmole/l, final maturation was induced with hCG (10,000 IU; Serono). Thirty-two to thirty-six hours later oocytes were harvested using a transvaginal approach. Granulosa-luteal cells were harvested from the follicular fluid following oocyte identification and removal.

Following centrifugation (1,000 x g) of the follicular contents, the supernatant was decanted and cells were resuspended in medium 199 (M199; Gibco-BRL Life Technologies, Burlington, ON) supplemented with 10% fetal bovine serum (FBS, Gibco). This step was repeated to provide a second wash. Following the second wash, the resuspended cells were layered on top of a mixture of Percoll (40%; Sigma, St. Louis, MO) diluted in M199. This gradient was centrifuged (1,700 x g), for 10 min at 22 C. Following collection from the M199/Percoll interphase, granulosa cells were washed and resuspended (10⁵-10⁶ cells/0.5 ml) in M199, supplemented with 10% FBS, sodium penicillin (100 IU/ml; Gibco) and streptomycin (100 μg/ml; Gibco), and plated on 48-well plates (Corning, NY; 0.5 ml cell suspension/well).

Cells to be used in one-day (D₁) pre-cultured experiments were cultured for 24 h and then used. However, cells to be used in eight-day (D₈) and twelve to fourteen-day (D₁₂-₁₄) pre-culture experiments had media changed every 2-3 days until the cells had been cultured for a total of 8 or 12-14 days, respectively. A pre-incubation (wash; 1 h) with fresh M199 was performed prior to experimental incubations in order to rinse the cells. All incubations were at 37 C, in a humidified, water-jacketed incubator (5% CO₂; Forma Scientific Inc, Mississauga, ON). The methods utilized in these studies are depicted in Figure 9.
Methods

GLC Collection
  ↓
Wash
  ↓
40% Percoll
  ↓
Wash
  ↓
Plating
(48 Well; M199; 10% FBS)
  ↓
Static Incubation
(24 h; M199;
Androstenedione 5x10^-7 M)

↓
RIA (E₂, P₄)

↓
DNA

RNA

Figure 9. Schematic of methods utilized in studies presented herewithin. In short, human granulosa luteal cells (GLC) are collected during oocyte collection from patients undergoing in vitro fertilization. Cells were washed twice and then separated from red blood cells on a 40% Percoll density gradient (in Medium 199), after which cells were washed twice and plated at 10^3 to 10^4 cells/well (on a 48 well tissue culture plate), in medium supplemented with 10% foetal bovine serum (FBS). After culturing for one, eight or twelve to fourteen days of culture cells were preincubated (washed) for 1 h in fresh medium and then subjected to hormonal or pharmacological treatments in medium (24 h), supplemented with substrate for estradiol production (androstenedione 5 x 10^-7 M). Supernatant was then collected and stored (-20 C) until assayed for progesterone and estradiol. Cells were either extracted for DNA or total RNA which were assayed with a Hoechst dye DNA assay or reverse-transcription/semi-quantitative polymerase chain reaction, respectively.
B. Incubation Experiments

All treatment regimens were performed in serum free Medium-199 or Dulbecco's Minimum Essential Medium) supplemented with androstenedione (5 x 10^{-7} M; precursor for estradiol formation). Following a 24 hour treatment, media were removed and stored at -20°C until assayed for progesterone or estradiol concentrations. The hormones and pharmacological agents utilized in these studies are presented in Table 2. The concentrations of these agents utilized were selected based on their known pharmacology.

The duration of these release experiments posses potential problems with receptor down-regulation or desensitization, however, this treatment duration was chosen to increase the probability of attaining measurable steroid levels in the release media.

Viability was checked post-experiment by the ability of cells to exclude trypan blue. Viability as approximated by this method was greater than 95% at all culture-time periods and under all treatment regimens.

The following experiments were performed:

1. Human Chorionic Gonadotrophin Concentration Response Curve: D_8 cultured GLCs were treated with vehicle or hCG (0.001 to 10 IU/ml).

2. Culture Time- and Concentration-Dependent Responses to PGF_{2\alpha} and GnRH: Day_1, D_8 and D_{12-14} cultured human GLCs were treated with vehicle, hCG (1 IU/ml) or hCG plus PGF_{2\alpha} (10^{-11} to 10^{-6} M). A similar experiment was performed with GnRH in place of PGF_{2\alpha}.

3. Follicle Size Dependent Changes in hCG and PGF_{2\alpha} Responses: Cells were also separated based on follicle size (> and < 12 mm in diameter) and subjected treatment with vehicle, hCG (1 IU/ml), PGF_{2\alpha} (10^{-6} M), or hCG plus PGF_{2\alpha} (10^{-11} to), at D_1. Ideally, follicles should have been separated into more categories. However due to clinical limitations this was not possible.
### Table 2. Hormones and pharmacological agents utilized in these studies.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Abbrev.</th>
<th>Class</th>
<th>Target(s)</th>
<th>Concentration(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Androstenedione</td>
<td>None used</td>
<td>Steroid Hormone Precursor</td>
<td>Estradiol Biosynthetic Pathway</td>
<td>$5 \times 10^{-7}$ M</td>
</tr>
<tr>
<td>2</td>
<td>Bisindolylmaleimide</td>
<td>PKCι</td>
<td>Enzyme Antagonist</td>
<td>Protein kinase-C</td>
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</tr>
<tr>
<td>2</td>
<td>Cholera Toxin</td>
<td>CTX</td>
<td>Bacterial Toxin</td>
<td>G Protein α-subunit Gs</td>
<td>$1\mu$g/ml</td>
</tr>
<tr>
<td>3</td>
<td>Dibutylcyclic-Adenosine Monophosphate</td>
<td>db-cAMP</td>
<td>Second Messenger Analogue</td>
<td>Protein Kinase A</td>
<td>$10^{-5}$ M</td>
</tr>
<tr>
<td>4</td>
<td>Forskolin</td>
<td>For</td>
<td>Enzyme Activator</td>
<td>Adenylate Cyclase</td>
<td>$10^{-5}$ M</td>
</tr>
<tr>
<td>5</td>
<td>Gonadotrophin Releasing hormone</td>
<td>GnRH</td>
<td>Peptide Hormone</td>
<td>GnRH Receptor</td>
<td>$10^{-10}$ to $10^{-5}$</td>
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<tr>
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<td>Human Chorionic Gonadotrophin</td>
<td>hCG</td>
<td>Peptide Hormone</td>
<td>LH/hCG Receptor</td>
<td>0.001 to 1 IU/ml</td>
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<tr>
<td>7</td>
<td>Indomethacin</td>
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<td>Cyclooxygenase I; Prostaglandin Dehydrogenase</td>
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<tr>
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<td>Iso or IsoP</td>
<td>Catecholamine Hormone Antagonist</td>
<td>β-adrenergic Receptor</td>
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<td>PTX</td>
<td>Bacterial Toxin</td>
<td>G Protein α-subunit(s): Gi, Gp</td>
<td>50 ng/ml</td>
</tr>
<tr>
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<td>Prostaglandin E$_2$</td>
<td>PGE$_2$</td>
<td>Eicosanoid Hormone</td>
<td>PGE$_2$ Receptor and other Prostanoid Receptors</td>
<td>$10^{-12}$ to $10^{-6}$ M</td>
</tr>
<tr>
<td>11</td>
<td>Prostaglandin F$_{2α}$</td>
<td>PGF$_{2α}$</td>
<td>Eicosanoid Hormone</td>
<td>PGF$_{2α}$ Receptor and other Prostanoid Receptors</td>
<td>$10^{-12}$ to $10^{-6}$ M</td>
</tr>
</tbody>
</table>

* Toullec et al., 1991; McCarthy 1995.
4. Interaction of PGF$_{2\alpha}$ and GnRH: Day$_{1}$ and D$_{8}$ GLCs were treated with vehicle, PGF$_{2\alpha}$ (10$^{-9}$ M), GnRH (10$^{-6}$ M) or PGF$_{2\alpha}$ plus GnRH, in the absence or presence of human chorionic gonadotrophin (hCG). In a second experiment (D$_{1}$ cells), vehicle, PGF$_{2\alpha}$ (10$^{-11}$ to 10$^{-6}$ M) and GnRH (10$^{-10}$ to 10$^{-5}$ M) concentration-response curves were crossed into a matrix of 49 separate treatments which were assayed for progesterone. Results were plotted in three dimensions with GnRH, PGF$_{2\alpha}$ and progesterone-response each on a axis. Similarly, results were also plotted as a contour map with GnRH and PGF$_{2\alpha}$ each on a separate axis and progesterone response represented by shading and colour. Moreover, ‘slices’ of the three dimensional matrix were plotted in two dimensions and analyzed statistically.

5. Interaction of PGF$_{2\alpha}$ and PGE$_{2}$: Day$_{8}$ GLCs were treated with vehicle, PGF$_{2\alpha}$ (10$^{-11}$ to 10$^{-6}$ M) and PGE$_{2}$ (10$^{-10}$ to 10$^{-5}$ M) concentration-response curves which were crossed into a matrix of 49 separate treatments. Media were assayed for progesterone. Results were plotted in three dimensions, with PGF$_{2\alpha}$, PGE$_{2}$ and progesterone-response each on a separate axis. Similarly, results were also plotted as a contour map, with PGF$_{2\alpha}$ and PGE$_{2}$ each on a separate axis and progesterone response represented by shading and colour. Moreover, ‘slices’ of the three dimensional matrix were plotted in two dimensions and analyzed statistically, as above.

6. PTX and CTX Effects on PGF$_{2\alpha}$ Mediated Luteolysis: Day$_{1}$ and D$_{8}$ cells used for G-protein studies were pre-treated (18 h) with M199 supplemented with vehicle, PTX (50 ng/ml), CTX (1 //g/ml), or PTX plus CTX. Following the pre-treatment period, cells were exposed to M199 containing vehicle, PTX, CTX or PTX plus CTX; plus vehicle, hCG (1 IU/ml), PGF$_{2\alpha}$ (10$^{-6}$ M), or hCG plus PGF$_{2\alpha}$, for 24 h. In another set of experiments cells were treated with M199 containing vehicle, IsoP (10$^{-5}$ M), PGF$_{2\alpha}$ (10$^{-6}$ M), or IsoP plus PGF$_{2\alpha}$. Finally, cells were exposed to M199 containing vehicle or PGF$_{2\alpha}$ (10$^{-6}$ M), plus or minus forskolin (10$^{-6}$ M) or Db-cAMP (10$^{-5}$ M).
7. Forskolin and Db-cAMP: Day_8 cultured human GLCs were treated for 24 hours with vehicle and PGF_{2\alpha} (10^{-6} \text{ M}) with and without forskolin (10^{-6} \text{ M}) or dibutyl cAMP (db-cAMP; 10^{-5} \text{ M}).

8. Progesterone and Estradiol Production per Cell or DNA Level: Plots were made of the basal progesterone- and estradiol-production from human GLC versus total cell numbers plated or DNA levels per well. This experiment was performed to determine if there was any correlation between steroid production and cell numbers or DNA levels.

9. Morphology of Human GLCs with Culture Time: Photographs of human GLCs at day zero, one, eight, twelve and sixteen were taken, in order to present the general morphology of cells at these culture times.

Following studies 2 and 3, it was apparent that culture-time radically altered the responses to hormone treatment. Thus, particular attention was paid to culture-time when deciding which response was to be examined with a particular experiment. For example, a luteotrophic response to PGF_{2\alpha} was absent in D_1 cultured GLCs. Therefore, this time period was particularly appropriate for examining the ability of GnRH to elicit a luteotrophic response to PGF_{2\alpha}.

C. Microscopy

Cells were routinely checked following plating, prior to experiments and following experiments for viability (as described above) and general appearance with a Nikon TMS inverted tissue culture microscope. Moreover, photographs of cells at different culture periods were taken with either a Nikon N2000 or Contax 167 MT camera body mounted on this microscope, using Fuji Provia (100 ASA) or Fujichrome Tungsten (400 ASA) film.

Slides were scanned with a Power Macintosh 6100AV (72 MB RAM) using a Nikon Coolscan II and printed on a photoenhanced Macintosh Colour Stylewriter 2500 using photograde paper (at > 720 dpi). Colour synchronization was set to automatic photograde.
D. Radioimmunoassay of Progesterone and Estradiol

The progesterone and estradiol concentrations in culture media were determined by specific RIAs, as previously described [Li et al., 1993; Rodway et al., 1990; Leung & Armstrong, 1979], with the following modification: phosphate buffered saline was replaced by a phosphate buffer containing Na$_2$HPO$_4$ (0.04 M) and NaH$_2$PO$_4$ (0.04 M) at pH 7.4. Typical standard curves for these progesterone and estradiol assays are presented in Figure 10.

Progesterone-RIA was performed as follows. Briefly, the assay used rabbit progesterone antiserum (P$_4$-2; Kindly provided by D.T. Armstrong, University of Western Ontario) raised against 4-pregnen-6β-ol-3,20-dione hemisuccinate:bovine serum albumin conjugate (Steraloids, Wilton, NH). The final antiserum concentration was 50 µg/ml. A standard competition method was employed utilizing progesterone (Sigma) standards, and $^3$H-progesterone at 10,000 cpm/tube (Amersham, Oakville, ON). The range of the assay standards was from 1 to 128 ng/ml. A 0.04 M phosphate buffer (pH 7.4) was used for diluting samples and controls with a final assay volume of 600 µl/tube. Phosphate buffer with dextran (0.025% w/v) and charcoal (0.25% w/v) was used to separate free progesterone from bound. Free progesterone in the supernatant was diluted in 3.0 ml of scintiverse (Fisher) scintillation cocktail and counted for 60 sec on a Wallac 1217 Rackbeta-counter.

The RIA was sensitive to 1.5 ng/ml, as determined by taking the progesterone concentration two times the standard deviation below the zero-binding value. Samples were assayed in duplicate. Intra- and inter-assay coefficients of variation were less than 11%.

Estradiol-RIA used specific rabbit antiserum (D.T. Armstrong) raised against estratriene-3,17β-diol-6-carboxymethyl-oxime:BSA conjugate (Steraloids). The final antiserum dilution was 1:200,000 w/v in phosphate buffer. As with the progesterone-RIA, a standard competition method was employed, utilizing estradiol (Sigma) standards and $^3$H-estradiol (Amersham, Oakville, ON) at 10,000 cpm/tube. The estradiol-RIA was performed as described above for the progesterone-RIA. Furthermore, the range and sensitivity was similar to the progesterone-RIA. Intra- and inter-assay coefficients of variation were less than 10%.
Figure 10. Typical progesterone (A) and estradiol (B) radioimmunoassay standard curves. Counts per minute (CPM).
**E. Hoechst Dye DNA Assay**

DNA quantification was performed using a modified version of Mates method [1986]. Briefly, following the treatment period, media were removed and replaced with trypsin TRTPK (50 μg/ml; Sigma) in a final volume of 500 μl in phosphate buffered saline (PBS as defined below). The plate was stored frozen at (-70 C) until assayed for DNA. At the time of assay, the plate was thawed at room temperature and incubated for 30 min to allow the trypsin to lyse the cells. During this incubation period, pre-prepared (see below) Hoechst dye stock (Bisbenzimide; 20 μg/ml in H2O; Sigma) was thawed (from -20 C) and diluted (10x in PBS). Following the incubation period, Hoechst dye solution was added to each well (at 500 μl/well), mixed and incubated for 5 min before well contents were measured with a spectrofluorometer (Aminco Rowman Spectrophotom Fluorometer, American Instrument Co., Silver Springs, MD) for fluorescence. Excitation and emission wavelengths were 354 and 458 nM, respectively. DNA was quantified by extrapolation from known standards (calf thymus DNA; Sigma) which were prepared by serial dilution (in phosphate buffered saline) over a range of 2.5 to 1000 ng/ml. Standards (1 ml) contained Hoechst dye diluted in similar fashion to samples above. Fluorescence was measured as above, with standards being measured in triplicate.

Hoechst dye stock (20 μg/ml) was slowly dissolved in distilled water, aliquotted (5 ml), wrapped in foil, and then stored at -20 C until use. Foil wrapping was necessary as bisbenzimide is light sensitive and will quench with time.

Phosphate buffered saline (PBS) was composed of Na2HPO4 (7.1 g), NaCl (116.88 g), and EDTA (0.84 g), dissolved in 750 ml of water, and then made up to final volume (1.0 l) and pH (7.4). PBS was stored at room temperature until use.

A typical standard curve for this assay is presented in Figure 11.
Figure 11. Typical hoechst dye deoxyribonucleic acid (DNA) assay standard curve. Optical density (OD).
F. RNA Extraction Procedure

Following experiments, some plates were stored (at -70C) until extracted for total RNA with an RNaid kit (Bio 101, La Jolla, CA). The extraction procedure was performed as outlined in the kits instructions. Lysis buffer (100 μl, as defined below) was added to each well, mixed with a pipette tip and left on ice for 5 min. The buffer with lysed cells was then transferred to a microcentrifuge tube (1.5 ml; Canlab). Sodium acetate (0.2M; 10 μl; pH 4.0) and phenol (100 μl) were added and vortexed. Chloroform:isoamyl alcohol (24:1; 100 μl) was added, vortexed and the preparation was then left on ice (15 min). Following this incubation, the tubes were spun (10,000 g; 20 min; 4 C) with the top phase being collected afterwards. RNA was present in the top phase, while protein and DNA remained in the lower phases. Thus, care was taken not to remove any of the interphase as this would introduce contamination into the RNA extract. A second extraction with chloroform:isoamyl alcohol (24:1; 100 μl) was performed and spun (2 min) with the top phase again being carefully removed and placed in a new microtube.

Vortexed RNAMatrix (10 μl) was added to each tube, vortexed (30 s) and incubated (5 min; RT) with occasional mixing to allow RNA adsorption. Tubes were then centrifuged (1 min; 10,000 g) to pellet the RNA/RNAMatrix complex. Supernatant was removed and saved for possible readsoption. Tubes with the pellet were briefly re-centrifuged and the remaining supernatant was carefully removed with a small bore pipette tip. Following this, the pellet was resuspended in the provided RNA wash solution (500 μl), spun (1 min; 10,000 g), supernatant was removed, and this step was repeated 1 more time. The microfuge-tubes with the pellets were then placed in the speed-vac micro centrifuge(1 min).

Finally, the pellet was resuspended in DEPC treated water (15-100 μl) and incubated (55 C; 5 min) to elute RNA. A final spin (1 min; 10,000 g) was performed to pellet the RNA Matrix while leaving the RNA in solution which was transferred to a final microfuge tube (0.5 ml). The solution was then subjected to spectrophotometric analysis to quantify total RNA.

Lysis buffer was composed of guanidine thyocyanate (4.0 M), sodium citrate (pH 7.0; 5 mM), sarcosyl (0.5 % w/v) and β-mercapto-ethanol (0.7% v/v) in diethylpyrocarbonate (DEPC) treated water.
G. RNA Gel

In order to check the relative efficacy of the RNA extraction procedure and the integrity of the RNA, the extraction products were run on an RNA gel. The RNA gel was composed of agarose (1.0%) dissolved in dH_2O (21.6 ml). Additionally, RNA gel-running buffer (GRB-R; 3 ml; as defined below) and formaldehyde (5.34 ml) were added, and the solution was allowed to cool (5 min) before pouring into a gel tray. RNA samples were loaded (1-2 μg in 10 μl) along with GLB-R (3 μl), and the gel was run (100 V; 50 min). Staining of the gel with ethidium bromide revealed two RNA bands (18 and 28 S). The gel was then photographed with polaroid 665 positive/negative film.

GLB-R was composed of glycerol (50%), EDTA (1 mM), bromophenol blue (0.4%), xylene cyanol (0.4%) and ethidium bromide. The GRB (10x) consisted of MOPS (0.2 M), NaOAc (80 mM) and EDTA (10 mM) in dH_2O (total volume 1.0 l).

H. Reverse Transcription of RNA to cDNA

A fixed quantity of total RNA, between 1-3 μg depending on the amount available (following RNA extraction) was made up in DEPC treated water (8 μl), heated (70 C; 10 min) and then spun down (5 min; 10,000 g). DTT (1 μl), oligo-dT (1 μl) and bulk mixture (5 μl) were added, followed by an incubation (37 C; 1 h; Pharmacia First Strand cDNA Kit, Upsala, Sweden). The preparation was boiled for (10 min), spun down and frozen (-20 C) until use. Total RNA levels were determined by spectrophotometric estimation. The spectrophotometer was validated by repeatedly measuring a known quantity of DNA and calculating the error between measurements (see results, p. 110).
I. Polymerase Chain Reaction (PCR)

Complementary DNA obtained from reverse transcription reactions were amplified by PCR such that relative changes in PGF$_2$-$\alpha$-receptor expression could be examined. The procedure was performed as follows. A fixed quantity of complementary DNA (cDNA) between 1 to 5 µl depending on availability for each experiment was mixed with a sense and antisense primer (1 µl of each; Table 3), Master Mix (22 µl; as defined below) and Taq polymerase (0.2 µl) in a microcentrifuge tube (0.5 ml; Canlab). Vegetable oil was then dropped on top of the mixture and the tube was capped. PCR was performed for each gene as specified in Table 4, with the primers described in Table 5.

Master Mix was composed of 10x PCR buffer (1/10 vol) plus deoxynucleotide-triphosphates (dNTPs; 0.179 µmol/ml). Ten times PCR buffer consisted of Tris-HCl (100 mM; pH 8.3), KCl (500 mM), MgCl$_2$ (15 mM) and gelatin (0.1%) in ddH$_2$O. Radiolabelled PCR contained 4.0 nCi of $^{32}$P-dCTP.

J. DNA Gel

Polymerase chain reaction products were run on an agarose gel composed of the following. Agarose (1.0%) was dissolved in a Tris-Borate-EDTA buffer (TBE) by boiling for 2 minutes. When the agarose solution had cooled (5-10 min) it was poured into a gel tray and a comb was inserted until the gel had solidified (approx. 20 min). The gel was then submersed in TBE, and cDNA samples (10 µl, with 5-20 µg DNA) mixed with DNA gel-loading-buffer (GLB-D; 3 µl) were loaded. After loading, a DNA ladder (Gibco BRL) was loaded on the outside lanes of the gel, and the gel was run (120-140 V). The gel was removed, stained with ethidium bromide and photographed with a Polaroid camera under ultraviolet light. PCR products appear as fluorescent bands.

TBE (5x) was composed of TRIS-base (10.8 g), boric acid (5.5 g) and EDTA (0.5 M; pH 8.0) dissolved in dH$_2$O (final volume 1 l). Furthermore, GLB-D consists of glycerol (50 ml), EDTA (0.5 M; 20 ml), bromophenol blue (0.1 g), xylene cyanol (0.1 g) and H$_2$O (20 ml).
Table 3. Primer combinations and expected product size following PCR.

<table>
<thead>
<tr>
<th>Sense</th>
<th>Antisense</th>
<th>Predicted Product Size (bp)</th>
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</thead>
<tbody>
<tr>
<td>hPGF+</td>
<td>hPGF-</td>
<td>802</td>
</tr>
<tr>
<td>rPGF+</td>
<td>rPGF-</td>
<td>720</td>
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<tr>
<td>Act+</td>
<td>Act-</td>
<td>524</td>
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</table>

bp - base pairs
Table 4. PCR conditions utilized for genes examined.

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<th>Gene</th>
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<th>Annealing</th>
<th>Polymerization</th>
<th>Cycles</th>
<th>Extension</th>
<th>Cycle Expt.</th>
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<tbody>
<tr>
<td></td>
<td>Temp</td>
<td>Time</td>
<td>Temp</td>
<td>Time</td>
<td>Temp</td>
<td>Time</td>
<td></td>
</tr>
<tr>
<td>hPGF&lt;sub&gt;2α&lt;/sub&gt;-R</td>
<td>96</td>
<td>30</td>
<td>57</td>
<td>30</td>
<td>72</td>
<td>1:30</td>
<td>40</td>
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<tr>
<td>rPGF&lt;sub&gt;2α&lt;/sub&gt;-R</td>
<td>96</td>
<td>30</td>
<td>50</td>
<td>30</td>
<td>72</td>
<td>1:30</td>
<td>40</td>
</tr>
<tr>
<td>β-Actin</td>
<td>96</td>
<td>30</td>
<td>55</td>
<td>30</td>
<td>72</td>
<td>1:30</td>
<td>30</td>
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</table>

All temperatures are given in degrees C, while times are in minutes:seconds
<table>
<thead>
<tr>
<th>Gene</th>
<th>+/-</th>
<th>Primer sequence (5' to 3')</th>
<th>Name</th>
<th>MW</th>
<th>Ref</th>
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</thead>
<tbody>
<tr>
<td>human PGF&lt;sub&gt;α&lt;/sub&gt; Receptor</td>
<td>+</td>
<td>CTC ATG AAG GCA TAT CAG AG</td>
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<td></td>
<td>-</td>
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<td>hPGF-</td>
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<tr>
<td>human PGF&lt;sub&gt;α&lt;/sub&gt; Receptor</td>
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<td>Rat PGF&lt;sub&gt;α&lt;/sub&gt; Receptor</td>
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<td>GAC CTG ACT GAC TCA CTC AT</td>
<td>Act-</td>
<td>6037</td>
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</table>

+ = sense; - = antisense. * - utilized as an internal probe for blots. MW - Molecular weight. 1 - Abramovitz et al., 1994. 2 - Lake et al., 1994. 3 - Ng et al., 1985,
**K. Southern Blot Hybridization**

Southern blot hybridization allows for the verification of PCR products by hybridizing a probe designed to bind to the internal portion of the predicted PCR product. The procedure used was as follows. An agarose gel containing the expected PCR product was denatured by immersion and agitation (15 min; RT) in a solution containing NaCl (1.5 M) and NaOH (0.5 M). Sodium hydroxide was then neutralized with a solution of NaCl (3 M) and Tris (0.5 M) at pH 8.0. Three washes with a sodium chloride/sodium citrate buffer (SSC; as defined below) followed (5 min each), after which an overnight transfer to a nylon membrane was performed (Fig. 12). The SSC buffer was composed of sodium chloride (26.3 g/l), and sodium citrate (13.2 g/l), in dH₂O (pH 7.0).

Following transfer of the gel to a nylon membrane, the membrane was washed (SSC), dried wrapped in Saran Wrap™, and exposed to UV light (2 min). The membrane then was stored (4 C) until hybridization, which was performed with a radiolabelled oligonucleotide, specific to the inner sequence of the predicted PCR product (Table 5).

Radiolabelling of the oligonucleotide was performed by a kination reaction, as follows. Primer (10 pmol; 1 μl), T₄ kinase buffer (1 μl; 10x), dH₂O (2 μl), γ²P-ATP (5 μl) and T₄ kinase (10 U; 1μl) were mixed and incubated (1 h; 37 C). The probe was then boiled (2 min), and spun (1 min; 10,000 g).

Just prior to hybridization the nylon membrane was removed from the refrigerator and preincubated in a prehybridization solution. The probe was then diluted in a hybridization solution and hybridized (40 C; over night). The following day the membrane was washed repeatedly in SSC (Table 6). The washed membrane was blotted, re-wrapped in Saran Wrap™, and then autoradiographed for 20 min to several days (at -70 C) depending on signal strength.

**L. Densitometry of Photographed Gels and Autoradiographs**

RNA and DNA gels stained with ethidium bromide (200 μg/100 ml gel; Sigma) could be visualized with UV illumination (Photoprep, Bio/Can Scientific, Mississauga, ON). However, quantification of products required gels to be photographed with a negative film (polaroid, 665). Negatives and autoradiographs from Southern blotting were scanned with a transluminescence video densitometer (Model 620, Bio-Rad Laboratories Inc.). Scanning software was utilized to calculate the relative optical density of each product band. In order to reduce variability, three scans of each film were performed and the means of the three scans were plotted.
Figure 12. Setup for overnight transfer of gel products to a nylon membrane for further Southern blot hybridization.
Table 6. Southern Blot SSC Washes.

<table>
<thead>
<tr>
<th>Wash</th>
<th>Duration (Minutes)</th>
<th>Temperature (Celcius)</th>
<th>SSC Dilution</th>
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</thead>
<tbody>
<tr>
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SSC-sodium chloride/sodium citrate buffer.
M. Analysis of Results

The results were presented as percentage of control values or by representative experiment. Graph bars represent the mean ± SEM of experiments performed on cells from different patients ('n' refers to patient numbers). Statistical analysis utilized one-way ANOVA followed by a Fischer or Scheffe post-hoc test. Statistical analysis was performed on mean standard score data and plotted in percentage of control data [Lewis 1984; Fisher and van Belle 1993; Grimm and Yarnold 1996; Porkess 1991]. Different characters above graph bars signify statistical difference.
VI - RESULTS

**Preliminary Results**

*Basal and hCG-Stimulated Steroidogenesis from human GLCs*

1. Basal Steroid Secretion per Cell or Level of DNA/Well

No correlation between cell plating number and basal progesterone production was seen in D₈ cultured human GLCs (n=17). Furthermore, up to 5000-fold differences in basal progesterone production from individual patients were observed (Fig. 13A and B). Moreover, when progesterone production was plotted against extracted DNA levels, no correlation was seen between culture-well DNA content and progesterone production (n=19; Fig. 14A and B).

Similar results were seen when basal estradiol production was plotted against cell plating numbers (Fig. 15A and B; n=11) or extracted DNA levels (Fig. 16A and B; n=17). Please see the discussion section for possible explanations for these results (p. 117).

Cell viability as determined by trypan blue dye exclusion was greater than 95% in these experiments, a result further supported by the ability of these cells to respond (with steroid production) to experimental stimuli such as hCG and PGF₂α (not shown).

2. hCG-Stimulated Progesterone Production in Cells from 3 Different Patients

Progesterone responses to hCG (0.001 IU/ml) in D₈ precultured human GLCs from three different patients produced significant stimulatory responses (p<0.05) of similar magnitude (approximately 4-fold; Fig. 17). However, the basal concentrations of progesterone varied up to 100-fold between experiments performed on cells from different patients, although all three experiments were performed on cells plated at 10⁴ cells/well. For example, cells from patient 1 (Fig. 17A) produced basal progesterone levels of approximately 10 ng/ml, while hCG-stimulated progesterone levels were approximately 45 ng/ml. Basal and hCG-stimulated progesterone production were approximately 1 and 4 ng/ml, respectively in cells from patient 2 (Fig. 17B). Finally, basal and hCG-stimulated progesterone production were approximately 110 and 420 ng/ml, respectively in cells from patient 3 (Fig. 17C). Thus, although very different basal levels were seen in all three cases, the relative responses to hCG were similar.

(see p. 117 for relevant discussion)
Figure 13. A. Basal progesterone production (ng/ml; over 24 h) versus cells/well, in D₈ pre-cultured human granulosa-luteal cells (GLCs). B. Basal progesterone production of individual patients plotted in ng/ml per 1000 cells plated, in D₈ pre-cultured human GLCs. Note that no correlation was seen between plated cell numbers and progesterone production between patients.
Patient

Figure 14. A. Basal progesterone production (ng/ml; over 24 h) versus DNA content per well (ng/ml), in D_8 pre-cultured human granulosa-luteal cells (GLCs). B. Basal progesterone production of individual patients plotted in ng/ml per 100 ng of DNA, in D_8 pre-cultured human GLCs. Note that no correlation was seen between extracted DNA levels and progesterone production.
Figure 15. **A.** Basal estradiol production (ng/ml; over 24 h) versus cells/well, in D8 pre-cultured human granulosa-luteal cells (GLCs). **B.** Basal estradiol production of individual patients plotted in ng/ml per 1000 cells plated, in D8 pre-cultured human GLCs. Note that no correlation was seen between plated cell numbers and estradiol production.
Figure 16. A. Basal estradiol production (ng/ml; over 24 h) versus DNA content per well (in ng/ml), in D_8 pre-cultured human granulosa-luteal cells (GLCs). B. Basal estradiol production of individual patients plotted in ng/ml per 100 ng of DNA, in D_8 pre-cultured human GLCs. Note that no correlation was seen between extracted DNA levels and estradiol production.
Figure 17. Comparison of progesterone responses to hCG treatment (0.001 IU/ml for 24 h), in D8 pre-cultured human granulosa-luteal cells from three different patients (A, B and C). Progesterone production was significantly stimulated in all three experiments (a≠b, p<0.05). Moreover, all three experiments responded with similar amplitudes (approximately 4 fold). However, the magnitude of progesterone concentrations varied up to 100 fold between experiments performed on cells from different patients. All three experiments were performed on cells plated at 10^4 cells/well.
Human GLC Morphology with Culture Time

Morphology slides presented within this section were taken from cells of a single patient which were plated at 10^4 cells per well and cultured as described above. The morphology of cultured human GLCs plated on 48-well plates changed with culture time. Cells that were freshly plated (2-3 h of culture) appeared to be evenly distributed about the surface of the well (Fig. 18A; 66 x mag). Additionally, cells did not appear to be highly associated with one another, although some cell aggregates were present. Individual cells appeared round and smooth.

Following one day of culture (D_1), GLCs were unevenly distributed throughout the well with clusters of cells being present and empty unpopulated regions throughout the well (Fig. 18B; 66 x mag). Cells were either round and smooth or elongated. Many cells possessed cytoplasmic projections which appeared to form associations with neighbouring cells.

Long term cultures (D_8) of GLC resulted in highly associated cells which were primarily present in clusters with very few cells existing outside of these aggregates (Fig. 19A; 200 x mag). Cells that were not part of a tightly associated aggregate formed contacts with aggregated cells with cytoplasmic projections. Individual cells again appeared round, although not smooth. Cells appeared luteinized, as they were highly irregular and granulated, when compared to early cultures. Even longer term cultures (D_12) resulted in cells that were even more associated and irregular in shape than those of D_8 cultures (Fig. 19B; 200 x mag).

Cells cultured for 1 to 12 days remained viable as evidenced by trypan blue dye exclusion (> 95%) and their ability to respond to experimental stimuli with steroid production. However, cells maintained in culture for sixteen or more days were no longer viable or responsive to stimuli and appeared to be luteolysed (Fig. 20; 66 x mag).

Similar culture-time dependent morphological changes were witnessed in all other cultures of human GLCs presented herewithin.

(see p. 120 for relevant discussion)
Figure 18. A. Human granulosa-luteal cells (GLCs) that were freshly plated (2-3 h of culture) appeared to be evenly distributed throughout the surface of the culture well (40 x mag). Additionally, cells did not appear to be highly associated with one another, although some cell aggregates were present. Individual cells appeared round and smooth. B. Following one day of culture GLCs were unevenly distributed, with cell clusters and empty unpopulated regions being distributed throughout the well (66 x mag). Cells appeared to be smooth. Many cells possessed cytoplasmic projections which appeared to form associations with neighbouring cells.
Figure 19. A. Eight day cultures of human granulosa-luteal cells (GLCs) resulted in highly associated cells, which were primarily present in clusters, with very few cells existing outside of these aggregates (200 x mag). Cells that were not part of a tightly associated aggregate formed contacts with aggregated cells with cytoplasmic projections. Individual cells again appeared round, although not smooth. Cells appeared luteinized, as they appeared blebbed and granulated (or vacuolated), when compared to early cultures. B. Twelve day cultured GLCs were highly associated and irregular in shape (200 x mag).
Figure 20. Sixteen day cultured human granulosa-luteal cells were no longer viable or responsive to stimuli and appeared to be luteolyzed (66 x mag).
A. The Effects of PGF\textsubscript{2\alpha} on Steroidogenesis in the Absence and Presence of hCG

Effects of PGF\textsubscript{2\alpha} on Steroidogenesis

1. Progesterone and Estradiol Production in Response to PGF\textsubscript{2\alpha}

Briefly, progesterone production in response to PGF\textsubscript{2\alpha} changed with culture time from inhibition (Fig. 21A) to stimulation (Fig. 21B; biphasic) in D\textsubscript{1} and D\textsubscript{12-14} cultured GLCs, respectively. While cells at D\textsubscript{8} of culture were in a state of transition with inhibition, stimulation or intermediate responses being possible (Fig. 22).

Progesterone production was significantly inhibited (50\% of control; a\ne c, p<0.001) in a linear fashion by PGF\textsubscript{2\alpha} in D\textsubscript{1} cultured GLCs (Fig. 21A; n=4). Conversely, in D\textsubscript{12-14} cultured GLCs PGF\textsubscript{2\alpha} significantly stimulated progesterone production (Fig. 21B; 200\% of control; a\ne c; p<0.001; n=5), with maximal stimulation at mid-range concentrations (10^{-8} to 10^{-10} M). Day\textsubscript{8} cultured GLCs were in a state of transition between D\textsubscript{1} and D\textsubscript{14} cells, with four responses being present (Fig. 22; total n=9): inhibition (n=2), no response (not shown; n=1), linear stimulation (n=3) and bimodal stimulation (n=3).

In D\textsubscript{1} (n=6) and D\textsubscript{8} (n=5) pre-cultured human GLCs, PGF\textsubscript{2\alpha} had no effect and stimulated estradiol production, respectively (Fig. 23A). The stimulatory response was significant at low (10^{-12} to 10^{-8} M; a\ne b; p<0.05) and high concentrations of PGF\textsubscript{2\alpha} (10^{-7} to 10^{-6} M; a\ne c; p<0.0001).

2. DNA Levels in Response to PGF\textsubscript{2\alpha}

DNA levels of GLC's remained unchanged by PGF\textsubscript{2\alpha} treatment in D\textsubscript{8} GLCs (Fig. 23B; n=3), suggesting that responses were due to alterations in steroid production rather than changes in the number of cells per well.

(see p. 121 for relevant discussion)
Figure 21. Progesterone production in response to PGF$_{2\alpha}$ treatment (for 24 h), in one-day (A; n=4; a$\neq$c, p<0.001 by ANOVA) and twelve to fourteen-day (B; n=4; a$\neq$c, p<0.001 by ANOVA) pre-cultured human granulosa-luteal cells (GLCs). In one and twelve to fourteen day pre-cultured human GLCs, PGF$_{2\alpha}$ inhibited and stimulated progesterone production, respectively.
Figure 22. Progesterone production in response to PGF$_{2\alpha}$ treatment (for 24 h), in eight day precultured human granulosa-luteal cells, four different progesterone-responses to PGF$_{2\alpha}$ were seen in nine separate experiments, including: linear stimulation (■; n=3), bell curve-like stimulation (□; n=3), inhibition (△; n=2) and no response (not shown; n=1).
Figure 23. A. Estradiol production in response to PGF$_{2\alpha}$ treatment (for 24 h) in one (white; n=6) and eight-day (gray; n=5; a≠b, p<0.04 and a≠c, p<0.0001 by ANOVA) pre-cultured human granulosa-luteal cells. DNA content (B; n=3) in response to PGF$_{2\alpha}$, in eight day pre-cultured human granulosa-luteal cells.
Effects of PGF\textsubscript{2α} on hCG-Stimulated Steroidogenesis

1. Progesterone Production in Response to hCG Treatment

As shown in Figure 24, human chorionic gonadotrophin (0.001 to 10 IU/ml) significantly stimulated progesterone production up to six fold from human GLCs. The highest level of statistical significance was seen with 0.01 to 1 IU/ml (hCG) treated cells (p<0.001; n=4), However, cells treated with lower (0.001 IU/ml) and higher (10 IU/ml) concentrations of hCG still responded significantly (p<0.05).

2. Follicle Size-Dependent Regulation of Steroidogenesis by hCG and PGF\textsubscript{2α}

Cells from four patients were separated into small (< 12 mm) and large (> 12 mm)-follicle size groups and subjected to hCG (1 IU/ml) and hCG plus PGF\textsubscript{2α} (10^{-6} M) treatment at D\textsubscript{1} (Fig. 25; n=4). Human chorionic gonadotrophin failed to significantly stimulate progesterone or estradiol production in GLCs collected from small follicles (p>0.05). In contrast, hCG stimulated progesterone (p<0.001) and estradiol (p<0.02) production in GLCs collected from large follicles. In addition, PGF\textsubscript{2α} inhibited hCG-stimulated progesterone and estradiol production in GLCs from large follicles (p<0.03), while it did not in cells from small follicles.

3. The Effects of PGF\textsubscript{2α} on hCG-Stimulated Steroidogenesis

In the presence of hCG, culture-time dependent changes in progesterone responses to PGF\textsubscript{2α} were observed. Prostaglandin F\textsubscript{2α} (10^{-6} M) inhibited hCG-stimulated progesterone production in D\textsubscript{1} (Fig. 26A; p<0.05; n=5) and D\textsubscript{8} (Fig. 26B; p<0.01; n=6), although not in D\textsubscript{14} (Fig. 27; n=4) cultured GLCs. Alternately, PGF\textsubscript{2α} (10^{-9} M) potentiated hCG-stimulated progesterone production in D\textsubscript{8} (p<0.01; n=6; 3 fold) and D\textsubscript{14} (p<0.05; n=4; 1.5 fold), although not in D\textsubscript{1} (n=4) cultured GLCs.

A similar trend was seen with estradiol production. Prostaglandin F\textsubscript{2α} (10^{-6} M) inhibited hCG-stimulated estradiol production in D\textsubscript{1} (Fig. 28A; p<0.05; n=8) and D\textsubscript{8} (Fig. 28B; p<0.05; n=5) cultured GLCs. Alternately, PGF\textsubscript{2α} (10^{-9} M) potentiated hCG-stimulated estradiol production in D\textsubscript{8} (p<0.01; n=5; 1.5 fold), although not in D\textsubscript{1} (n=4) cultured GLCs.
Figure 24. Human chorionic gonadotrophin (hCG) stimulated progesterone production from human granulosa-luteal cells treated for 24 h following eight days of culture (n=4; a≠b or b≠c, p<0.05; a≠c, p<0.001 by ANOVA).
Figure 25. Follicle size-dependent responses to human chorionic gonadotrophin (hCG; 1 IU/ml) and prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) treatment (for 24 h). A. In 1 day pre-cultured human granulosa-luteal cells, PGF$_{2\alpha}$ inhibited (n=4; a≠b, p<0.001 and b≠c, p<0.03 by ANOVA) hCG-stimulated progesterone production from cells collected from large follicles (H: > 12 mm). However, cells collected from small follicles (E3; < 12 mm), were unable to respond to hCG. B. Similar results were seen for hCG-stimulated estradiol production (n=4; a≠b, p<0.03 by ANOVA).
Figure 26. The effects of prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$) on hCG-stimulated progesterone production (over 24 h) from one-day (A; n=5; a≠b≠c≠d, p<0.05 by ANOVA) and eight-day (B; n=6; a≠b≠c, p<0.01 by ANOVA) pre-cultured human granulosa-luteal cells.
Figure 27. The effects of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) on hCG-stimulated progesterone production (over 24 h) from twelve to fourteen-day (n=4; a≠b≠c, p<0.05 by ANOVA) pre-cultured human granulosa-luteal cells.
Figure 28. The effects of prostaglandin F$_2$α (PGF$_2$α) on hCG-stimulated estradiol production (over 24 h) from one-day (A; n=8; a≠b≠c, p<0.05 by ANOVA) and eight-day (B; n=5; a≠b≠c, p<0.05 by ANOVA) pre-cultured human granulosa-luteal cells.
Effects of GnRH on hCG-Stimulated Steroidogenesis

Gonadotrophin-releasing hormone (10^{-6} M) inhibited hCG-stimulated progesterone production in D_1 (Fig. 29A; n=6; p<0.05) and D_8 (Fig. 29B; n=5; p<0.05) cultured GLCs. Alternately, GnRH (10^{-8} M) potentiated hCG-stimulated progesterone production in D_8 (Fig. 29B; n=5; p<0.05; a≠b≠c), although not in D_1 (n=6) cultured GLCs.

A similar trend was seen with estradiol production. Gonadotrophin-releasing hormone (10^{-6} M) inhibited hCG-stimulated estradiol production in D_8 (Fig. 30A; n=4; p<0.05) cultured GLCs. Alternately, GnRH (10^{-9} M) potentiated hCG-stimulated estradiol production in D_8 cultured GLCs (n=5; p<0.01). DNA levels were unaltered by any of the treatments (Fig. 30B; n=3; p>0.05).
Figure 29. The effects of gonadotrophin-releasing hormone (GnRH) on hCG-stimulated progesterone production (over 24 h) from one-day (A; n=4; a=b=c, p<0.05 by ANOVA) and eight-day (B; n=5; a≠b≠c≠d, p<0.05 by ANOVA) pre-cultured human granulosa-luteal cells.
Figure 30. A. The effects of gonadotrophin-releasing hormone (GnRH) on hCG-stimulated estradiol production (over 24 h) from eight-day (n=4; a≠b≠c, p<0.05 by ANOVA) pre-cultured human granulosa-luteal cells. B. DNA levels were unaltered by any of the above treatments (n=2; p>0.05 by ANOVA).
B. The Interaction of PGF<sub>2α</sub> with GnRH

Progesterone Response to GnRH and/or PGF<sub>2α</sub> with or without hCG.

Neither PGF<sub>2α</sub> (10<sup>-9</sup> M) nor GnRH (10<sup>-6</sup> M) significantly altered progesterone production, in D<sub>1</sub> human GLCs (Fig. 31A; n=5). However, the combination of PGF<sub>2α</sub> plus GnRH significantly stimulated progesterone production (a<b, p<0.05). Human chorionic gonadotrophin (1 IU/ml) also significantly stimulated progesterone production (2.5-3 fold; a<b, p<0.05). Conversely, gonadotrophin-releasing hormone alone was unable to inhibit hCG-stimulated progesterone production, although it did potentiate PGF<sub>2α</sub>-mediated inhibition (a<b, p<0.05).

In D<sub>8</sub> pre-cultured GLCs a significant luteotrophic response to PGF<sub>2α</sub> (10<sup>-9</sup> M) was present (Fig. 31B; n=4; p<0.05). However, no luteotrophic response to GnRH (10<sup>-6</sup> M) was observed, although GnRH potentiated the PGF<sub>2α</sub>-mediated luteotrophic response (p<0.05). Both GnRH and PGF<sub>2α</sub> significantly inhibited hCG-stimulated progesterone production (b<c, p<0.05), while their combination potentiated inhibition beyond levels of either hormone alone (p<0.05).

Estradiol Response to GnRH and/or PGF<sub>2α</sub> with or without hCG.

In D<sub>1</sub> human granulosa luteal cells (Fig. 32; n=3), neither PGF<sub>2α</sub> (10<sup>-9</sup> M) nor GnRH (10<sup>-6</sup> M) significantly altered estradiol production. However, the combination of PGF<sub>2α</sub> plus GnRH significantly stimulated estradiol production (p<0.01). Human chorionic gonadotrophin (1 IU/ml) also significantly stimulated estradiol production (p<0.05). Gonadotrophin-releasing hormone alone was unable to inhibit hCG-stimulated progesterone production, although it did potentiate PGF<sub>2α</sub>-mediated inhibition (p<0.05).

Progesterone Response to GnRH with or without PGF<sub>2α</sub>

Vehicle, PGF<sub>2α</sub> (10<sup>-11</sup> to 10<sup>-6</sup> M) and GnRH (10<sup>-10</sup> to 10<sup>-5</sup> M) concentration-response curves were crossed into a matrix of 49 separate treatments. Results were plotted in three dimensions, with GnRH, PGF<sub>2α</sub> and progesterone-response each on one axis (Fig. 33A and B
[mirror image of A]). Results were also plotted and as a contour map with GnRH and PGF$_{2\alpha}$ each on a separate axis and progesterone response represented by shading (Fig. 34A) and colour (Fig. 34B). In D$_1$ human GLCs, maximal stimulation of progesterone-production (2-3 fold) was seen when middle concentrations of PGF$_{2\alpha}$ (10$^{-9}$ M; p<0.05) interacted with high concentrations of GnRH (10$^{-6}$ to 10$^{-5}$ M). In the presence of high concentrations of GnRH (10$^{-6}$ M), PGF$_{2\alpha}$ stimulated progesterone production in a bell curve-like fashion as middle concentrations significantly stimulated while low and high concentrations did not (Fig. 35; p<0.05). In the presence of middle concentrations of PGF$_{2\alpha}$ (10$^{-9}$ M), GnRH significantly stimulated progesterone-production in a linear concentration-dependent manner (Fig. 36; p<0.05).

**Estradiol Response to GnRH with or without PGF$_{2\alpha}$**

In D$_1$ human GLCs, maximal stimulation of estradiol-production (4-fold) was seen when high concentrations of PGF$_{2\alpha}$ (10$^{-6}$ M; p<0.05) interacted with high concentrations of GnRH (10$^{-6}$ to 10$^{-5}$ M). These data are presented in three dimensional graph (Fig. 37; n=6) and contour format (Fig. 38), as for progesterone data above. In the presence of high concentrations of GnRH (Fig. 39; 10$^{-5}$ M), PGF$_{2\alpha}$ significantly and linearly stimulated estradiol production (p<0.05), although PGF$_{2\alpha}$ was ineffective in the absence of GnRH. On the other hand, in the absence and presence of PGF$_{2\alpha}$ (10$^{-6}$ M), GnRH significantly stimulated estradiol production (Fig. 40A and B; p<0.05). The nature of GnRH stimulated estradiol production was, however, different in the presence and absence of PGF$_{2\alpha}$, as the response shifted from a bell curve-like stimulation to a linear one with the addition of PGF$_{2\alpha}$.

**DNA Levels in Response to GnRH and PGF$_{2\alpha}$ Treatment**

DNA Levels were unaltered by treatment with either GnRH (10$^{-10}$ to 10$^{-5}$ M), PGF$_{2\alpha}$ (10$^{-11}$ to 10$^{-6}$ M) or hCG, suggesting that responses seen were due to alterations in steroid production rather than changes in cell numbers (data not shown).
Effects of Indomethacin on PGF$_{2\alpha}$ and GnRH Stimulated Steroidogenesis

In D$_8$ cultured human GLCs, progesterone was significantly stimulated in a bell curve-like fashion by PGF$_{2\alpha}$ (Fig. 41). Maximal stimulation of progesterone production was at 1 nM of PGF$_{2\alpha}$ ($p<0.05$). However, co-incubation with indomethacin ($10^{-6}$ M) reversed this effect, and PGF$_{2\alpha}$ instead inhibited or had no effect on progesterone production (depending on concentration). Similar results were seen in cells from two other patients. Cells remained viable in the presence of indomethacin, as suggested by their ability to exclude trypan blue.

Progesterone production in D$_1$ cultured human GLCs was unaffected ($p>0.05$) by vehicle, GnRH ($10^{-10}$ to $10^{-6}$ M) and/or PGF$_{2\alpha}$ ($10^{-11}$ to $10^{-6}$) when cells were co-incubated with indomethacin ($10^{-6}$ M; $n=4$; Fig. 42). Cells remained viable in the presence of indomethacin, as suggested by their ability to exclude trypan blue. Compare these results and those presented in Figure 33. Note that hCG-stimulated progesterone production was seen in these cells (not shown).

(see p. 125 for relevant discussion)
Figure 31. Progesterone production in response to vehicle (Cont), gonadotrophin-releasing hormone (GnRH; $10^{-6}$ M), prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$; $10^{-9}$ M) and GnRH plus PGF$_{2\alpha}$ treatment (over 24 h), in the presence and absence of human chorionic gonadotrophin (hCG), in one-day (A; n=5) and eight-day (B; n=4) pre-cultured human granulosa-luteal cells. Graph bars represent mean ± SEM of experiments performed on separate patients (a≠b≠c, p<0.05 by ANOVA).
Figure 32. Estradiol production in response to vehicle (Cont), gonadotrophin-releasing hormone (GnRH; 10^{-6} M), prostaglandin F_{2\alpha} (PGF; 10^{-9} M) and GnRH plus PGF treatment (over 24 h), in the presence and absence of human chorionic gonadotrophin (hCG), in one day (n=3) pre-cultured human granulosa-luteal cells. Graph bars represent mean ± SEM of experiments performed on separate patients (a≠b≠c, p<0.05 by ANOVA).
Figure 33. Three dimensional plot of progesterone production in response to vehicle (C), gonadotrophin-releasing hormone (GnRH; $10^{-10}$ to $10^{-5}$ M) and/or prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; $10^{-11}$ to $10^{-6}$ M) treatment (over 24 h), in one day pre-cultured human granulosa-luteal cells. A mirror image (B) provides a view of the back side of the image (A). These Figures represent the mean of seven separate experiments performed on seven separate patients.
Figure 34. Black and white (A) and colour (B) contour plot of progesterone production in response to vehicle (C), gonadotrophin-releasing hormone (GnRH; $10^{-10}$ to $10^{-5}$ M) and/or prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; $10^{-11}$ to $10^{-6}$ M) treatment (over 24 h), in one day pre-cultured human granulosa-luteal cells. Progesterone production of 50, 100, 200 and 300% of the control level are symbolized by: A, B, C, D, E, F, and G, respectively. These Figures represent the mean of seven separate experiments performed on seven separate patients.
Figure 35. Effects of prostaglandin F$_2$α (PGF$_{2α}$; 10$^{-11}$ to 10$^{-6}$ M), in the absence (——) and presence (———) of gonadotrophin-releasing hormone (GnRH; 10$^{-6}$ M) treatment (over 24 h), in one day pre-cultured human granulosa-luteal cells. In the presence of GnRH, PGF$_{2α}$ stimulated progesterone production in a bell curve-like fashion, with significant stimulation at middle concentrations (10$^{-9}$ and 10$^{-8}$ M PGF$_{2α}$; a≠b, p<0.05, by ANOVA). While in the absence of GnRH, PGF$_{2α}$ did not significantly alter progesterone production. Progesterone production in response to GnRH alone (10$^{-6}$ M) was not significantly different from the control response. This Figure represents the mean ± sem of seven separate experiments performed on seven separate patients, and is a two dimensional slice of three dimensional matrix presented in Figure 33A.
Figure 36. Effects of gonadotrophin-releasing hormone (GnRH; $10^{-10}$ to $10^{-5}$ M), in the absence (---) and presence (-----) of prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$; $10^{-9}$ M) treatment (over 24 h), in one day pre-cultured human granulosa-luteal cells. In the presence of PGF$_{2\alpha}$, GnRH stimulated progesterone production in a linear concentration-dependent fashion, with significant stimulation at upper concentrations ($10^{-8}$ to $10^{-5}$ M; a=b, p<0.05 by ANOVA). While in the absence of PGF$_{2\alpha}$, GnRH had no effect on progesterone production. Progesterone production in response to PGF$_{2\alpha}$ alone ($10^{-9}$ M) was not significantly different from the control response. This Figure represents the mean ± SEM of seven separate experiments performed on seven separate patients, and is a two dimensional slice of three dimensional matrix presented in Figure 33A.
Figure 37. Three dimensional plot of estradiol production (over 24 h) in response to vehicle (C), gonadotrophin-releasing hormone (GnRH; $10^{-10}$ to $10^{-5}$ M) and/or prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; $10^{-11}$ to $10^{-6}$ M), in one day pre-cultured human granulosa-luteal cells. This Figure represents the mean of six separate experiments performed on six separate patients.
Figure 38. Black and white (A) and colour (B) contour plot of estradiol production (over 24 h) in response to vehicle (C), gonadotrophin-releasing hormone (GnRH; $10^{-10}$ to $10^{-5}$ M) and/or prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; $10^{-11}$ to $10^{-6}$ M), in one day pre-cultured human granulosa-luteal cells. Estradiol production of 100, 200, 300 and 400% of the control level are symbolized by: □, ■, □□□□□□ and □□□□□□□□□□, respectively. These Figures represent the mean of six separate experiments performed on six separate patients.
Figure 39. The interaction of gonadotrophin-releasing hormone (GnRH; $10^{-5}$ M) and prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) on estradiol response (over 24 h), in one day pre-cultured human granulosa-luteal cells. In the presence (---$\rightarrow$--) of GnRH ($10^{-5}$ M), PGF$_{2\alpha}$ significantly stimulated estradiol production (n=6; a$\neq$b, $p<0.05$ by ANOVA), however PGF$_{2\alpha}$ was ineffective in the absence (---$\rightarrow$--) of GnRH. These data represent a two dimensional slice of the three dimensional graph presented in Figure 37.
Figure 40. Estradiol response (over 24 h) to gonadotrophin-releasing hormone (GnRH; $10^{-5}$ M) in the absence (A) and presence (B) of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; $10^{-6}$ M), in one day precultured human granulosa-luteal cells. In the presence of PGF$_{2\alpha}$, GnRH significantly stimulated estradiol production ($n=6$; $a$ vs $b$ vs $c$, $p<0.05$ by ANOVA). These data represent a 2D slice those in Figure 37.
Figure 41. Progesterone response (over 24 h) to PGF$_{2\alpha}$ in the absence (■) and presence (□) of indomethacin (10$^{-6}$ M), in eight day pre-cultured human granulosa-luteal cells. In the absence of indomethacin, PGF$_{2\alpha}$ significantly and in a bell curve-like fashion stimulated progesterone production ($p<0.05$; control vs PGF$_{2\alpha}$, 10$^{-9}$ M). However, in the presence of indomethacin, PGF$_{2\alpha}$ either inhibited ($p<0.05$; control vs PGF$_{2\alpha}$, 10$^{-10}$ to 10$^{-6}$ M) or had no effect on progesterone production. This Figure represents the response of cells from one patient. Similar results were seen in cells from two other patients. Cells remained viable in the presence of indomethacin, as suggested by their ability to exclude tryphan blue.
Figure 42. Three dimensional plot of progesterone production (over 24 h) in response to vehicle (C), gonadotrophin-releasing hormone (GnRH; $10^{-10}$ to $10^{-6}$ M) and/or prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; $10^{-11}$ to $10^{-6}$ M) in the presence of indomethacin ($10^{-6}$ M), in one day pre-cultured human granulosa-luteal cells. This Figure represents the mean of four separate experiments performed on cells from four separate patients. No significant difference between treatments was seen ($p>0.05$). Cells remained viable in the presence of indomethacin, as suggested by their ability to exclude tryphan blue.
C. Progesterone Response to PGF$_{2\alpha}$ plus PGE$_2$

The following study reveals a complex regulation of progesterone production in response to vehicle PGF$_{2\alpha}$ (10$^{-11}$ to 10$^{-6}$ M) and/or PGE$_2$ (10$^{-11}$ to 10$^{-6}$ M) concentration-response curves in eight day cultured human GLCs. Prostaglandin F$_{2\alpha}$ and PGE$_2$ concentration-response curves were crossed into a matrix of 49 separate treatments. Results were plotted in three dimensions with PGF$_{2\alpha}$, PGE$_2$ and progesterone-response each on a separate axis (Fig. 43). Additionally, data were plotted in contour map form (Fig. 44). Moreover, two dimensional slices of the three dimensional matrix were plotted and analyzed statistically (Fig. 45). Briefly, progesterone was significantly stimulated in a bell curve-like manner by PGF$_{2\alpha}$ with maximal stimulation at 1 nM (p<0.05). A similar response to PGE$_2$ was seen although the bell curve was shifted right. Maximal PGE$_2$-mediated stimulation of progesterone production was seen at 10 to 100 nM (Fig. 45). However, in the presence of PGE$_2$ (10$^{-7}$ M), PGF$_{2\alpha}$ significantly inhibited progesterone production (p<0.05) in an inverse bell curve-like manner, with maximal inhibition at (10$^{-10}$ to 10$^{-8}$ M, PGF$_{2\alpha}$; Fig. 46).

(see p. 131 for relevant discussion)
Figure 43. Progesterone production (over 24 h) in response to vehicle, prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; 10$^{-11}$ to 10$^{-6}$ M) and/or prostaglandin E$_2$ (PGE$_2$; 10$^{-11}$ to 10$^{-6}$ M) concentration response curves, in eight day pre-cultured human granulosa-luteal cells. A mirror image (B) provides a view of the back side of the image (A). This Figure represents the mean of four separate experiments performed on cells from four different patients.
Figure 44. Black and white (A) and colour (B) contour plots of progesterone production (over 24 h) in response to vehicle, prostaglandin F$_2$$\alpha$ (PGF$_2$$\alpha$; $10^{-11}$ to $10^{-6}$ M) and/or prostaglandin E$_2$ (PGE$_2$; $10^{-11}$ to $10^{-6}$ M) concentration response curves (n=4), in eight day pre-cultured human granulosa-luteal cells. Progesterone production of 100, 200, and 300% of control level are symbolized by: □, ■, ▪, and □, respectively. This Figure represents the mean of four separate experiments performed on cells from four different patients, and is derived from the same data as those presented in Figure 43.
Figure 45. Prostaglandin F$_{2\alpha}$ (A; PGF$_{2\alpha}$) and prostaglandin E$_2$ (B; PGE$_2$) concentration response curves (10$^{-11}$ to 10$^{-6}$ M), in eight day pre-cultured human granulosa-luteal cells. Progesterone was significantly stimulated (over 24 h) in a bell curve-like manner by PGF$_{2\alpha}$ with maximal stimulation at 1 nM (a=b$\neq$c, p<0.05 by ANOVA). A similar response to PGE$_2$ was seen although the bell curve was shifted right. Maximal PGE$_2$-mediated stimulation of progesterone production was seen at 10 to 100 nM. These data represent a two dimensional slice of the data presented in Figure 43.
Figure 46. The effects of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; $10^{-11}$ to $10^{-6}$ M), in the presence of prostaglandin E$_2$ (PGE$_2$; $10^{-7}$ M). Progesterone production (over 24 h) was significantly inhibited (a≠b, p<0.05 by ANOVA) in an inverse bell curve-like manner by PGF$_{2\alpha}$ ($10^{-10}$ to $10^{-8}$ M), in eight day pre-cultured human granulosa-luteal cells. These data represent a two dimensional slice of the data presented in Figure 43.
D. Signal Transduction of PGF$_{2\alpha}$-Mediated Luteolysis

**Effects of PGF$_{2\alpha}$ on hCG-Stimulated Steroidogenesis**

Progesterone (Fig. 47A) and estradiol (Fig. 47B) production were stimulated by hCG (1 IU/ml; p<0.05 control vs. hCG) in eight-day cultured human granulosa-luteal cells, although hCG-stimulated progesterone and estradiol production were both attenuated in the presence of PGF$_{2\alpha}$ (10$^{-6}$ M; p>0.05 control vs hCG plus PGF$_{2\alpha}$).

**Effects of PGF$_{2\alpha}$ on Isoproterenol Stimulated Progesterone Production**

The β-adrenergic agonist isoproterenol (10$^{-5}$ M) was capable of stimulating progesterone production from eight-day cultured human granulosa-luteal cells (Fig. 48A; p<0.05; control vs isoproterenol). As with hCG, isoproterenol-stimulated progesterone production was blocked by the addition of PGF$_{2\alpha}$ (10$^{-6}$ M) to culture media (p>0.05; control vs. isoproterenol/PGF$_{2\alpha}$; p<0.05 isoproterenol vs isoproterenol/PGF$_{2\alpha}$). Isoproterenol also stimulated estradiol production from human granulosa-luteal cells (Fig. 48B; p<0.05, control vs. isoproterenol). The ability of PGF$_{2\alpha}$ to inhibit isoproterenol-stimulated estradiol production was not examined in these studies. Please note that PGF$_{2\alpha}$ transiently stimulated progesterone production (compare Fig. 47A with Fig. 48A). The cause of this transience is under study in another project.

**Effects of PTX on Anti-gonadotrophs Actions of PGF$_{2\alpha}$**

Treatment of eight-day cultured human granulosa luteal cells with hCG (1 IU/ml) significantly stimulated progesterone production (Fig. 47A and Fig. 49A; p<0.05 versus control). Furthermore, this stimulation was inhibited by co-treatment with PGF$_{2\alpha}$ (10$^{-6}$ M; p<0.05 versus hCG and p>0.05 versus control). However, in the presence of PTX (50 ng/ml), PGF$_{2\alpha}$-mediated inhibition of hCG-stimulated progesterone production was blocked (p<0.05 versus control and p>0.05 versus PTX/hCG treated cells). Similar progesterone responses were seen from one-day cultured cells under the same conditions (not shown). Estradiol production from eight-day cultured human granulosa-luteal cells paralleled progesterone responses under the above treatment conditions (Fig. 49B). DNA levels remained unchanged by this treatment regime (Fig. 50) suggesting that steroid responses were not due to altered cell numbers in these experiments.
Effects of PGF$_{2\alpha}$ on CTX Stimulated Steroidogenesis

Cholera toxin (1 $\mu$g/ml) significantly stimulated progesterone production from eight-day cultured human granulosa-luteal cells (Fig. 51A; p<0.05, CTX versus control). Subsequently, PGF$_{2\alpha}$ (10$^{-6}$ M) was able to block the stimulatory effect of CTX (p<0.05, CTX versus CTX plus PGF$_{2\alpha}$; and p>0.05 for control versus CTX/PGF$_{2\alpha}$). However, co-treatment with PTX (50 ng/ml) partially reversed this effect. Estradiol production in response to these treatments followed a similar profile (Fig. 51B).

Effects of PGF$_{2\alpha}$ on Forskolin Stimulated Progesterone Production

Forskolin (10$^{-5}$ M) significantly stimulated progesterone production from eight-day cultured human granulosa-luteal cells (Fig. 52A and 52B; p<0.05, forskolin vs. control). PGF$_{2\alpha}$ (10$^{-6}$ M) was able to block the stimulatory effect of forskolin (p<0.05, forskolin vs. forskolin plus PGF$_{2\alpha}$; p>0.05 control vs. forskolin plus PGF$_{2\alpha}$). Please note that PGF$_{2\alpha}$ alternately stimulated (p<0.05, control vs. PGF$_{2\alpha}$) or did not stimulate progesterone production on its own (compare Fig. 47A and 48B), although this transience did not alter the inhibitory properties of PGF$_{2\alpha}$.

Effects of PGF$_{2\alpha}$ on cAMP Stimulated Progesterone Production

Db-cAMP was capable of stimulating progesterone production from eight-day cultured human granulosa-luteal cells (p<0.05, control vs. Db-cAMP), although in these experiments, Db-cAMP-stimulated progesterone production was not inhibited by PGF$_{2\alpha}$ (Fig. 53).

The Effects of a PKC Inhibitor on PGF$_{2\alpha}$-Mediated Inhibition of hCG-Stimulated Progesterone Production.

Human chorionic gonadotrophin (1 IU/ml) significantly stimulated progesterone production in D$_8$ cultured human granulosa-luteal cells (Fig. 54; n=4). Moreover, the highly specific protein kinase-C inhibitor bisindolylmaleimide (50 nM) significantly inhibited (p<0.05) PGF$_{2\alpha}$-mediated inhibition of hCG-stimulated progesterone production in these cells.

(see p. 132 for relevant discussion)
Figure 47. Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; $10^{-6}$ M)-mediated inhibition of human chorionic gonadotrophin (hCG; 1 IU/ml)-stimulated progesterone (A) and estradiol (B) production (over 24 h), in eight day pre-cultured human granulosa-luteal cells. Data represent the mean ± SEM of triplicate measures (a≠b; p<0.05; by ANOVA). Similar results were seen in fourteen separate experiments performed on cells from fourteen other patients.
Figure 48. Prostaglandin F$_2\alpha$ (PGF$_{2\alpha}$; 10$^{-6}$ M)-mediated inhibition of isoproterenol (Iso; 10$^{-5}$ M)-stimulated progesterone production (over 24 h), in eight day pre-cultured human granulosa-luteal cells. Data represent the mean ± SEM of triplicate measures (a=b; p<0.05; by ANOVA). Similar results were seen in three separate experiments performed on cells from three other patients.
Figure 49. The effects of pertussis toxin (PTX 50 ng/ml) on prostaglandin F$_{2\alpha}$ (PG; 10$^{-6}$ M)-mediated inhibition of human chorionic gonadotrophin (hCG; 1 IU/ml)-stimulated progesterone (A) and estradiol (B) production (over 24 h), in eight day pre-cultured human granulosa-luteal cells. Data represent the mean ± SEM of triplicate measures (a=b; p<0.05; by ANOVA). Similar results were seen in five separate experiments performed on cells from five other patients.
Figure 50. The effects of pertussis toxin (PTX 50 ng/ml), prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; $10^{-6}$ M) and human chorionic gonadotrophin (hCG; 1 IU/ml) on DNA levels (over 24 h), in eight day precultured human granulosa-luteal cells. Data represent the mean ± SEM of triplicate measures ($p>0.05$). Similar results were seen in two separate experiments performed on cells from two other patients.
Figure 51. The effects of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; 10$^{-6}$ M) on cholera toxin (CTX; 1 µg/ml) and CTX plus pertussis toxin (PTX; 50 ng/ml) stimulated progesterone (A) and estradiol (B) production (over 24 h), in eight day pre-cultured human granulosa-luteal cells. Data represent the mean ± SEM of triplicate measures (a=b; p<0.05; by ANOVA). Similar results were seen in five separate experiments performed on cells from five other patients.
Figure 52. Prostaglandin F\(_{2\alpha}\) (PGF\(_{2\alpha}\); 10\(^{-6}\) M)-mediated inhibition of forskolin (10\(^{-6}\) M)-stimulated progesterone production (over 24 h), in eight day pre-cultured human granulosa-luteal cells (A and B). Data represent the mean ± SEM of triplicate measures (a≠b≠c; p<0.05; by ANOVA). Similar results were seen in three separate experiments performed on cells from three other patients. Note: transient stimulatory effect of PGF\(_{2\alpha}\) (10\(^{-6}\) M, A vs B)
Figure 53. The effects of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; 10$^{-6}$ M) on dibutryl cAMP (Db-cAMP; 10$^{-5}$ M)-stimulated progesterone production (over 24 h), in eight day pre-cultured human granulosa-luteal cells. Data represent the mean ± SEM of triplicate measures (a≠b; p<0.05; by ANOVA). Similar results were seen in three separate experiments performed on cells from three other patients. Additionally, similar results seen with experiments utilizing 8-bromo-cAMP, on cells from two other patients.
Figure 54. The effects of a protein kinase-C inhibitor (PKCi; bisindolylmaleimide 50 nM) on vehicle (■), human chorionic gonadotrophin (hCG; □) or hCG plus prostaglandin F2α (h/P; ▼)-mediated inhibition of hCG-stimulated progesterone production (over 24 h; n=4; a ≠ b ≠ c, p<0.05 by ANOVA), in eight day pre-cultured human granulosa-luteal cells.
E. Effects of hCG and PGF$_{2\alpha}$ on PGF$_{2\alpha}$-R-mRNA

*Spectrophotometric Estimation of Known DNA Levels in Solution*

Known quantities of DNA were estimated with spectrophotometric analysis in order to validate the spectrophotometer as a tool for approximating DNA and/or RNA levels in samples to be reverse transcribed. Concentrations of DNA between 5 and 5000 ng/ml were sampled. Overall, the spectrophotometric estimation of DNA levels were within 133.8 ± 5.0% of the actual DNA concentration. At high concentrations (1250 - 5000 ng/ml), this estimation improved to 100.8 ± 0.2% of the actual DNA concentration. See Table 7 for a complete listing of the results.

*RNA Integrity and Relative Quantity*

The integrity and relative quantity of total RNA samples extracted from human GLCs were checked by denaturing (formaldehyde) agarose (1.5%) gel electrophoresis. The presence of 28 and 18 S bands suggested that RNA was intact. Moreover, the apparent consistency of signal strength from one sample to the next suggested that similar efficiency of extraction was obtained for all samples. Data from two different experiments are presented here (Fig. 55 A and B). Similar results were found in other experiments.

*PCR Cycle Experiment*

Polymerase chain reaction cycle experiments for PGF$_{2\alpha}$-R and β-Actin cDNA were performed in order determine the optimal number of cycles for a given concentration range and species of cDNA (Fig. 56). At the concentrations of cDNA utilized in these experiments, PCR amplification of product was relatively linear over the range of cycles tested. Amplification of cDNA for and PGF$_{2\alpha}$-R and β-Actin was performed using 40 and 30 cycles, respectively, based on the results of these experiments.
Amplification of PGF$_{2\alpha}$-R and β-Actin cDNAs in Human GLCs

Prostaglandin F$_{2\alpha}$-R cDNA was amplified from human GLCs (obtained from 2 different patients) with two different sets of oligonucleotide primers (hPGF+/− and rPGF+/−). Products of the expected size (802 and 720 bp) were amplified by both primers (hPGF+/− and rPGF+/−; Fig. 57A and B). Additionally, oligonucleotide primers for β-actin cDNA (Act+/−) successfully amplified a product of the expected size (524 bp) from human GLCs obtained from 3 different patients (Fig. 57C).

Confirmation of PGF$_{2\alpha}$-R cDNA in Human Granulosa-Luteal and Placental Cells

Amplification of PGF$_{2\alpha}$-R cDNA using PCR incorporating $^{32}$P-dCTP revealed the presence of products in samples obtained from human GLCs from three separate patients and in placental cells from two separate patients (Fig. 58A). However, PCR failed to detect PGF$_{2\alpha}$-R cDNA in human leukocyte cDNA samples from two patients. The photograph of this gel was further validated when lanes from this experiment were cut and counted with a β-counter. Similar results were demonstrated using this technique.

Regulation of PGF$_{2\alpha}$-R cDNA by hCG and PGF$_{2\alpha}$

One-day cultured human GLCs were incubated with vehicle, hCG (1 IU/ml) or hCG plus PGF$_{2\alpha}$ (10$^{-11}$ to 10$^{-6}$ M). The effects of these treatments on PGF$_{2\alpha}$-R and β-Actin cDNA levels were examined by RT-PCR (Fig. 59A), densitometry (Fig. 59B) and Southern blot hybridization (Fig. 60A and B).

Briefly, PGF$_{2\alpha}$-R message was down-regulated by hCG. However, PGF$_{2\alpha}$ at low (10$^{-11}$ M) and high (10$^{-6}$ M) concentrations prevented this down-regulation. On the contrary, the middle concentration of PGF$_{2\alpha}$ (10$^{-9}$ M) potentiated hCG-mediated down-regulation of PGF$_{2\alpha}$-R message (Fig. 59A). Densitometric analysis revealed significant inhibition of PGF$_{2\alpha}$-R mRNA levels in cells treated with hCG and hCG plus PGF$_{2\alpha}$ (n=3; p<0.05 by ANOVA; 10$^{-9}$ M; Fig. 59B). The housekeeping gene β-actin was unaffected by any of the above treatments (not shown).
Southern blot hybridization of a semi-quantitative PCR experiment (presented in Figure 59A), with an oligonucleotide probe confirmed the identity of the PCR products (Fig. 60A). Moreover, densitometric analysis of the autoradiogram revealed a pattern of mRNA regulation similar to that found in Figure 59B. In short, hCG inhibited PGF$_{2\alpha}$-R mRNA message in this experiment. Additionally, in presence of hCG, PGF$_{2\alpha}$ (10$^{-11}$ to 10$^{-7}$ M) inhibited PGF$_{2\alpha}$-R mRNA message, potentiating hCG-mediated inhibition at concentrations of (10$^{-10}$ to 10$^{-8}$ M, PGF$_{2\alpha}$).

(see p. 142 for relevant discussion)
<table>
<thead>
<tr>
<th>DNA by Weight * (ng/ml)</th>
<th>Spectrophotometer Estimation (% Actual Conc ± SEM)</th>
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<tr>
<td>5 to 5000</td>
<td>133.8 ± 5.0</td>
</tr>
<tr>
<td>5 to 40</td>
<td>183.6 ± 12.0</td>
</tr>
<tr>
<td>80 to 625</td>
<td>111.1 ± 1.6</td>
</tr>
<tr>
<td>1250 to 5000</td>
<td>100.8 ± 0.2</td>
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* Salmon sperm DNA serially diluted in 2x dilutions) from 5000 to 5 ng/ml.
Figure 55. RNA integrity gel. Agarose (1.5 %) and formaldehyde denaturing gel of RNA samples (1 μg/sample) extracted from one day pre-cultured human granulosa-luteal cells. The presence of 28 and 18 S bands suggests that RNA integrity was good. RNA from two experiments is presented here (A and B).
Figure 56. Polymerase chain reaction (PCR) cycle experiments for prostaglandin $F_{2\alpha}$-receptor (A; PGF$_{2\alpha}$-R) and $\beta$-Actin (B) complementary DNA (cDNA). At the concentrations of cDNA utilized in these experiments PCR amplification of product was relatively linear over the range of cycles tested. Based on these experiments PCR amplification of cDNA for and PGF$_{2\alpha}$-R and $\beta$-Actin was performed using 40 and 30 cycles, respectively.
Figure 57. Polymerase chain reaction amplification of PGF$_{2\alpha}$-R and β-actin cDNA. Two different sets of oligonucleotide primers were utilized to amplify prostaglandin F$_{2\alpha}$-receptor cDNA, and one set was utilized to amplify β-actin cDNA, from one day pre-cultured human granulosa-luteal cells. These primers were hPGF+ and hPGF- (A), rPGF+ and rPGF+ (B) and Act+ and Act- (C). All three sets of oligonucleotides were able to amplify products of the predicted size, from human granulosa-luteal cells (GLC) from up to three different patients.
Figure 58. Polymerase chain reaction amplification of PGF$_{2\alpha}$-receptor cDNA from human granulosa-luteal cells, placenta and leukocytes. A. Amplification of the prostaglandin F$_{2\alpha}$-receptor cDNA with the oligonucleotides hPGF+ and hPGF-, in the presence of $^{32}$P-dCTP. Polymerase chain reaction products of the predicted size were amplified from three different human granulosa-luteal cell (GLC1, 2 and 3; uncultured samples) and two different human placenta (PL1 and 2; uncultured samples) cDNA samples. Conversely, gel lanes loaded with molecular weight ladder (LD) or two different Leu cDNA samples (uncultured samples), did not show visible amplification of product. B. Gel bands (from A) separated and counted with a $\beta$-counter.
Figure 59. The effects of vehicle, human chorionic gonadotrophin (hCG) and prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) on PGF$_{2\alpha}$-receptor (PGF$_{2\alpha}$-R) mRNA levels (over 24 h), in one day pre-cultured human granulosa-luteal cells from three separate patients (A, B and C). Following the treatment period cells were extracted for RNA, which was reverse transcribed (RT) to cDNA and subjected to semi-quantitative polymerase chain reaction (PCR). PGF$_{2\alpha}$-R message was down regulated by hCG, however, PGF$_{2\alpha}$ at low ($10^{-11}$ M) and high ($10^{-6}$ M) concentrations prevented this down-regulation. On the contrary, the middle concentration of PGF$_{2\alpha}$ ($10^{-9}$ M) potentiated hCG-mediated down-regulation of PGF$_{2\alpha}$-R message. The house keeping gene β-actin was unaffected by any of the above treatments (not shown). Photographs were subjected to densitometric analysis, and normalized to β-actin responses and averaged (D). Significant inhibition of PGF$_{2\alpha}$-R mRNA levels was seen in cells treated with hCG and hCG plus PGF$_{2\alpha}$ (a=b=c. p<0.05 by ANOVA; $10^{-9}$ M).
Figure 60. A. Southern blot hybridization of a semi-quantitative PCR experiment, with an oligonucleotide probe for PGF$_{2\alpha}$-R, in cells from one day pre-cultured human granulosa-luteal cells. These data confirm the identity of the PCR products presented in (Fig. 59 A). B. Densitometric analysis of the autoradiogram presented in A. Human chorionic gonadotrophin (hCG) inhibited PGF$_{2\alpha}$-R mRNA message in this experiment. Moreover, in the presence of hCG, PGF$_{2\alpha}$ ($10^{-11}$ to $10^{-7}$ M) inhibited PGF$_{2\alpha}$-R mRNA message, potentiating hCG-mediated inhibition at concentrations of ($10^{-10}$ to $10^{-8}$ M, PGF$_{2\alpha}$).
Caveats of the Human Granulosa-Luteal Cell Model

Variability in Basal Steroidogenesis in the Human GLC Model

Granulosa-luteal cells obtained during oocyte collection from superovulated in vitro fertilization patients are a very difficult model to work with as they exhibit a high degree of variability in their basal levels of steroidogenesis (Fig. 13-16).

In part, these differences can be accounted for by varying proportions of cells obtained from different-sized follicles (Fig. 25) since the number and maturity of follicles punctured varies greatly with patients. Additionally, the possibility of contaminant epithelial or immune cell involvement in the PGF$_{2\alpha}$-mediated responses of luteal cells may further complicate matters, should this exist. Epithelial cells are reported to permit or even enhance PGF$_{2\alpha}$-mediated responses in luteal cells in the placenta [Alecozay et al., 1991]. Thus, differences in follicle puncture, cell collection and purification can all contribute to variability in steroidogenic responses.

Other potential sources of variability include the following: 1) the population of patients from which cells are collected have a much higher probability of infertility than the general population; 2) patient responses to super-ovulation are highly variable, suggesting important biological differences; and 3) the differences in time between follicle puncture and cell plating.

Throughout these experiments cells were plated at a density of $10^3$ to $10^4$ cells/well. Within this range of cell densities, no significant density-dependent difference was seen in the steroidogenic responses to hCG or hCG plus PGF$_{2\alpha}$ between patients (differences within a patient were not examined). Moreover, basal steroidogenesis did not change in response to cell density. However, in a report by Bari-Ami and Gitay-Goren [1993], basal steroidogenesis showed cell density and culture-time dependent changes in progesterone and estradiol production such that increases in cell density from $2.5\times10^3$ to $1\times10^4$ cells/well could increase progesterone production 1.3-fold. A further increase in cell density from $1\times10^4$ to $8\times10^4$ cells/well could decrease progesterone production 3.7-fold. They also reported density and culture time-dependent changes in estradiol production. Culture condition, cell manipulation or other factors may account for the discrepancies in these two studies.
It should be noted, however, that in most cases, although the basal levels of steroidogenesis varied greatly, the relative responses to stimuli were quite similar when comparing responses from different patients (see Fig. 17). Thus, although the problem of variability exists, standardization of data can still render this model useful for determining relative trends and mechanisms of action. Data standardization can be achieved by either experimental or statistical means.

Experimental methods normally include taking the ratio of the data from one physiological parameter over some other form of data. For example: peptide secretion from endocrine cells is often represented over the total amount of stored peptide. Taking the ratio of a response over DNA levels or cellular protein content is also common. In this model, experimental methods of standardization are not satisfactory, as response per cell or DNA level is too variable. Therefore, a statistical method of standardization is required.

Statistical standardization methods include converting data to a percentage of the control level, log transformations or conversion to standard mean scores [Lewis 1984]. All of these methods have advantages and disadvantages. Log transformations are not completely satisfactory with respect to this model as basal levels can vary many-fold. Thus, these studies utilized percentage of control and standard mean score transformations.

Although the human GLC model is quite variable in the basal levels of steroidogenesis, the relative responses to stimuli are similar in cells from different patients. Thus, this model can be effectively utilized to determine relative responses to stimuli and the mechanisms of these responses. Moreover, this model provides the only source of human granulosa-luteal cells ethically available in high enough quantity to study effectively.

Cell Numbers and Low Level RNA Expression

Although human GLCs are readily available in high enough quantity for morphological or pharmacological study, the numbers of cells obtained are barely adequate for molecular biological techniques. This is especially true when examining genes which are expressed at low levels. Reverse transcription-PCR allowed for semi-quantitative examination of genes of low level expression, due to the amplification obtained through PCR. Even with this amplification PGF$_{2\alpha}$-R mRNA was difficult to detect. Total RNA levels extracted from cells were normally
between 1 to 2 μg per sample, a level too low to be useful for Northern blot hybridization. Thus, although Northern blot analysis would have been an easier and more direct quantification method, the levels of PGF₂α-R mRNA did not permit this procedure.

Rather than not study the regulation of PGF₂α-R mRNA, RT-PCR was utilized to provide some useful insights that would not otherwise be possible.

A Question of Physiological Concentration?

What is a physiological concentration? This is a difficult question to answer. In these studies, concentrations of PGF₂α, PGE₂ and GnRH ranged from pM to μM levels. This range of concentrations was utilized because in vivo data in the human is unobtainable. Moreover, the concentrations which a receptor 'sees' are virtually impossible to determine. Tight junctions, secretion patterns, local degradation, binding proteins, receptor affinity and other factors can greatly alter the effective concentration of a hormone at its site of action.

The female reproductive system is not a homeostatic system, it is a cycling system. Herein lies its appeal to many scientists. In a cycling system, one physiological concentration cannot be assigned to most hormones. For example, progesterone and estradiol concentrations vary greatly throughout the menstrual cycle. Which concentration is physiological? At any given point in time, the physiological concentration of a hormone may change. Additionally, functions can be attributed to a hormone’s absence as well as its presence. Finally, as the affinity of the PGF₂α-R is quite high (Kd of 1 nM), it is reasonable to expect that this hormone may have physiological effects at concentrations which are several-fold lower or higher than this Kd. This turned out to be the case.

Summary

Notwithstanding these limitations, it must be noted that this model is a valuable one. There are very few human tissues which are so obtainable for scientific examination. The author believes that it is a scientist’s responsibility to work around the limitations of such a valuable model and learn as much as one can from it.
Morphology of Human Granulosa-Luteal Cells in Culture

The morphological characteristics of human GLCs change dramatically with culture time. Moreover, these changes correspond well with those reported for cells undergoing luteinization. Granulosa cells exposed to luteotrophins change from their characteristic polygonal shape to a round one, occasionally projecting finger-like processes which may attach to adjacent cells [Soto et al., 1986]. These characteristics are very similar to those of freshly cultured and one-day cultured human GLC, as seen in these studies. As cells luteinize, they are reported to form tighter associations [Ratamales et al., 1994], increased vacuolation and blebbing [Quirk et al., 1995]. All of these reported characteristics were seen in eight-day and twelve-day cultures of human GLCs, suggesting that these cells are luteinizing in culture. This notion is certainly supported by the functional differentiation seen with culture time.

An increase in luteal cell blebbing, characteristic of cells undergoing apoptosis, has also been reported in human granulosa-luteal cells [Quirk et al., 1995]. Interestingly, in these studies it was found that an increase in cell irregularity and blebbing was seen as culture time progressed. Moreover, cells disrupted by 16 to 18 days of culture appeared to have undergone apoptosis. This disruption was associated with a loss in functional response.

Similar morphological characteristics have been reported in other mammals including porcine, bovine, feline and rat models [Gregoraszczuk and Krzysztofowicz 1989; Roth et al., 1995; Chegini et al., 1984; Fields et al., 1992; Meidan et al., 1990; Fields et al., 1985; Yuh et al., 1986; Nelson et al., 1992]. Luteal cell vacuolation, blebbing of various sizes, ruffles and lipid vacuoles are also reported in these species. In the rat, differences between small and large luteal cells are also reported, such that small luteal cells appear stellate while large luteal cells do not flatten out completely (probably due to large lipid droplets) [Nelson et al., 1992]. In one-day human GLCs, two cell populations appeared to be present which corresponded remarkably well with those reported in the rat.

In summary, human GLCs appeared to undergo morphological luteinization and possibly apoptosis with culture time. These results support the human granulosa-luteal cell as a good model for the study of luteinization.
effects of PGF$_{2\alpha}$ on Human Granulosa-Luteal Cells in the Absence and Presence of hCG

Wide ranging concentration-response studies (1 pM to 1µM PGF$_{2\alpha}$ or GnRH) were performed in both the presence and absence of hCG, in short, medium and long term cultures of human GLCs. The importance of these parameters was highlighted by the concentration- and culture time-dependent differential responses to PGF$_{2\alpha}$ under these conditions. Profoundly different progesterone and estradiol production in response to PGF$_{2\alpha}$ was seen when comparing D$_1$, D$_8$ and D$_{12-14}$ cultured human GLCs. In the case of GnRH, differences were observed from D$_1$ to D$_8$ cells. These findings emphasize the importance of maintaining awareness of culture time in experiments using highly differentiated GLCs.

Further, the basal effects of PGF$_{2\alpha}$ suggest that superovulation-derived human GLCs continue to undergo luteinization in vitro, as they paralleled previous results examining early- and mid-luteal phase cells [Khan et al., 1989]. The effects of PGF$_{2\alpha}$ on progesterone production in GLCs differed with culture time. Prostaglandin F$_{2\alpha}$ inhibited in day D$_1$, but stimulated progesterone production in D$_{12-14}$ cultured GLCs. The cells were found to be less defined in their responses to PGF$_{2\alpha}$ in D$_8$, as they appeared to be in a state of transition between their inhibitory and stimulatory responses. Early-luteal and D$_1$ GLCs both demonstrated inhibition, while mid-luteal and D$_8$ GLCs demonstrated stimulation of basal-progesterone production in response to PGF$_{2\alpha}$ [Khan et al., 1989]. This further supports the suitability of IVF derived cells as a model to study human ovarian cell function.

Thus, PGF$_{2\alpha}$ was capable of either inhibiting or stimulating progesterone production depending on concentration and culture conditions.

**Follicle Size**

Differential responses to hCG and PGF$_{2\alpha}$ based on follicle size were seen in D$_1$ GLCs. When the results from four separate experiments were pooled, it became clear that the magnitude of the hCG-induced steroidogenic response was reduced in small versus large follicles. The response was significant in large follicles, but not in small follicles. Previous studies have demonstrated that the number of hCG receptors increases with follicle size [Kammerman and
Ross, 1975; May and Schomberg, 1984; Hillier et al., 1980]. These results suggest that there could be differential steroidogenic responses in cells from different patients, due to differing proportions of small and large follicles. This hypothesis is supported by a previous report which found that follicles yielding mature cumulus-oocyte complexes (COC) represent a non-homogenous population in which GLCs from follicles yielding type A-B COC (cumulus cells aggregated into clumps) are less luteinized than GLCs from follicles yielding type C-D COCs (cumulus cells homogeneously spread out) [Gitay-Goren et al., 1990].

**Concentration and Culture Time Dependent Responses**

The concentration range of PGF$_{2\alpha}$ and GnRH used in these studies resulted in bell curve like inhibition of hCG-stimulated progesterone and estradiol production (see Fig. 26, 28, 30-31). This bimodal nature is not unusual for prostaglandin actions [Cohen and Rimon, 1992; Sano and Shichi, 1993; Puschel et al., 1993; Hargrove et al., 1975]. For example, in the rat and rabbit testicular tissues, bimodal responses to PGE$_2$ and PGE$_1$ have been reported, respectively [Cohen and Rimon, 1992; Hargrove et al., 1975]. Rat epididymal adipocytes displayed a PGE$_2$-mediated inhibition and stimulation of cAMP at concentrations of 10 mM and >10 mM, respectively. In the rabbit, testicular contractions were stimulated and inhibited by PGE$_1$ at concentrations of 1–10 nM and 100 nM, respectively. Non-reproductive tissues also have been shown to exhibit bimodal responses to prostaglandins [Sano and Shichi, 1993; Puschel et al., 1993]. In rat hepatocytes, PGE$_2$ can act in a glycogenolytic and in a antiglycogenolytic fashion at concentrations of 10 $\mu$M and 1 nM, respectively. It is reported that these glycogenolytic and antiglycogenolytic actions are likely mediated through the inositol triphosphate and cAMP pathways, respectively. The potential for multiple G-protein coupling to PGF$_{2\alpha}$ receptors is also present as seen in the gonadotroph [Hawes et al., 1993; Barnes and Conn, 1993]. This possibility is supported by the identification of four different G-protein alpha subunits in the human GLC, including $G_{\alphaS}$, $G_{\alphai3}$, $G_{\alphai1,2}$ and $G_{\alphaq,11}$ [Lopez et al., 1995]. Similarly, multiple-receptors have been suggested as an explanation for bimodal prostaglandin responses in the porcine ciliary epithelium [Sano and Shichi, 1993].
In the presence of hCG, culture time (presumably luteinization)-dependent alterations in the steroidogenic responses to PGF$_{2\alpha}$ and GnRH have also been observed. The general trend of the concentration-dependent response to PGF$_{2\alpha}$ or GnRH was retained with culture time although it shifted in an upward (stimulatory) fashion, retaining its anti-gonadotrophic effects only at the highest concentration tested (1 $\mu$M), in D$_8$ GLCs. Potentiation (1.5- to 3-fold) of hCG-stimulated progesterone production was seen D$_8$ GLCs cultured in the presence of PGF$_{2\alpha}$ or GnRH (at 1 nM; Fig. 3B and 6B). The ability of PGF$_{2\alpha}$ to potentiate hCG-stimulated progesterone production in D$_8$ (presumably mid-luteal like) cells, may have implications for early pregnancy. Further support for this idea resides in the literature, as hCG pretreatment has been shown to prevent the anti-gonadotrophic actions of PGF$_{2\alpha}$ [Michael and Webley, 1991b]. It has been suggested that hCG produced by the new conceptus may prevent corpus luteum regression by this mechanism. Furthermore, PGF$_{2\alpha}$ concentrations in the human luteal cell are at their highest levels in mid-luteal phase, the time when it would be least appropriate to undergo luteolysis. This potentiation may have an important biological function at this stage, and may involve the interaction of PGF$_{2\alpha}$ and other ovarian hormones such as PGE$_2$. Prostaglandin E$_2$ is at its highest concentrations during the mid-luteal phase [Patwardhan and Lanthier, 1980 and 1985] and is reported to have antagonistic actions against PGF$_{2\alpha}$-mediated luteolysis. Perhaps PGE$_2$-mediated antagonism of PGF$_{2\alpha}$-mediated luteolysis enhances the luteotrophic responsiveness of these cells.

**Summary**

Prostaglandin F$_{2\alpha}$ and GnRH appear to be bimodal regulators of steroidogenesis in the human ovarian cell. In addition to the antisteroidogenic abilities of PGF$_{2\alpha}$ and GnRH (10$^{-6}$ M; D$_1$ and D$_8$), these results suggest that PGF$_{2\alpha}$ (10$^{-9}$ M; D$_8$ and D$_{12-14}$) and GnRH (10$^{-9}$ M; D$_8$) may play a role in the maintenance of the corpus luteum through their potentiation of hCG-stimulated progesterone production (Fig. 61).
Figure 61. Dual (bimodal) actions of Prostaglandin F$_{2\alpha}$ on progesterone and estradiol production, in human luteal cells.
B. Interaction of PGF$_{2\alpha}$ with GnRH

Progesterone Response

This study examined the interactions of GnRH and PGF$_{2\alpha}$ in the presence and absence of hCG. In general, at the concentrations tested, neither GnRH nor PGF$_{2\alpha}$ altered progesterone or estradiol production in D$_1$ cultures. However, when GnRH and PGF$_{2\alpha}$ were co-applied, a significant stimulation of progesterone and estradiol production was seen. Furthermore, in D$_8$ cultures where a weak luteotrophic action was seen with PGF$_{2\alpha}$ treatment, GnRH potentiated PGF$_{2\alpha}$-stimulated progesterone production, but was again ineffective on its own. These results suggest that PGF$_{2\alpha}$ requires GnRH as a permissive factor in order for its luteotrophic action to be present, and that GnRH on its own is not a luteotrophin. The ability of PGF$_{2\alpha}$ to stimulate progesterone production in D$_8$ cultures suggests that endogenous GnRH may be present. Alternative explanations would be that a second permissive factor exists, or that PGF$_{2\alpha}$ does not require one in later cultures. Interestingly, the magnitude of the response is much greater in D$_8$ cultures. When viewed in three dimensions, the nature of the interactions between GnRH and PGF$_{2\alpha}$ are intriguing. At optimal PGF$_{2\alpha}$ concentrations, GnRH stimulated progesterone production appears linear and concentration dependent. At optimal GnRH conditions, PGF$_{2\alpha}$-stimulated progesterone production is bell curve-like. One remarkable characteristic of GnRH plus PGF$_{2\alpha}$-mediated progesterone production is the consistency of the response. In this laboratory, the luteotrophic response to PGF$_{2\alpha}$ alone is only present in cells from about 50\% of patients, while in these experiments, 100\% of patients demonstrated a luteotrophic response with co-application of these two hormones.

In D$_1$ cultures and at the concentrations tested, GnRH was not luteolytic, although it was in D$_8$ cultures. Gonadotrophin-releasing hormone potentiated PGF$_{2\alpha}$-mediated luteolysis in D$_1$ and D$_8$ pre-cultured human GLCs.

Estradiol Response

Estradiol production was also regulated in a remarkable fashion by the co-application of GnRH and PGF$_{2\alpha}$. In the presence of high concentrations of GnRH, PGF$_{2\alpha}$ linearly and concentration dependently stimulated estradiol production. These effects of GnRH appeared to
be permissive, as PGF$_{2\alpha}$-mediated stimulation of estradiol production was not present in the absence of GnRH. Moreover, the linear stimulation of estradiol production by PGF$_{2\alpha}$ (in the presence of high concentrations of GnRH) appeared to be similar to the response seen in eight-day cultures (in the absence of exogenous GnRH; not shown). Perhaps the levels of endogenous GnRH in the culture system increase with culture time, with the effect of modulating the response over culture-time. Gonadotrophin-releasing hormone alone, significantly stimulated estradiol production in a bell curve-like manner. However, in the presence of high concentrations of PGF$_{2\alpha}$, GnRH-mediated stimulation of estradiol production shifted from being bell curve-like to linear. The effects of PGF$_{2\alpha}$ on the response to GnRH were modulatory rather than permissive.

Implications

The striking difference in the progesterone and estradiol responses to PGF$_{2\alpha}$ and/or GnRH, may play an important role in the regulation of the luteal phase or even the menstrual cycle. If one superimposes the three dimensional plots of these two hormones, one can see that as the concentrations of PGF$_{2\alpha}$ and/or GnRH change, so does the ratio of progesterone to estradiol production. For example, at high concentrations of GnRH and middle concentrations of PGF$_{2\alpha}$ estradiol production is low relative to progesterone production, which is at its highest. However, at high concentrations of GnRH and PGF$_{2\alpha}$ estradiol production is at its highest, while progesterone production is at its lowest level. Thus, due to the co-operative nature of PGF$_{2\alpha}$ and GnRH, subtle changes in the concentrations of these hormones can have profound effects on the ratio of progesterone to estradiol production. With this in mind, it is interesting to note that under conditions conducive to a luteotrophic response (ie. high progesterone production; PGF$_{2\alpha}$ 10$^{-9}$ and GnRH 10$^{-5}$ M) estradiol production is relatively low, while under conditions optimal for luteolysis (ie. low progesterone production; PGF 10$^{-6}$ and GnRH 10$^{-5}$ M) estradiol production is high.

The present results further support the hypothesis that PGF$_{2\alpha}$ and GnRH have very similar and/or complementary roles in the ovary. Teleologically speaking, the advantages of a dual hormone system over a single hormone system seem obvious. Should one system be deficient or fail, the second system would provide a backup. Conversely, when both systems are
working amplification and fine tuning of the signal are improved. Interestingly, a positive feedback loop may exist in this system, as GnRH has been shown to stimulate arachidonic acid and PGE$_2$ from the luteal cell, and PGF production in the placenta [Kawai and Clark, 1985 and 1986; Hillensjo et al., 1982; Siler et al., 1986]. It is unknown if PGF$_{2\alpha}$ stimulates GnRH production in these cells. The bell curve-like response to PGF$_{2\alpha}$ could act as a fine tuning or switching mechanism, allowing a luteotrophic response to turn into a luteolytic one should PGF$_{2\alpha}$ levels increase much beyond nM concentrations. This bimodal response to prostaglandins is not unique to the corpus luteum [Cohen and Rimon, 1992; Sano and Shichi, 1993; Puschel et al., 1993; Hargrove et al., 1975]. For example, in the rat and rabbit testicular tissues, bimodal responses to PGE$_2$ and PGE$_1$ have been reported, respectively [Hillensjo et al., 1982; Sano and Shichi, 1993]. Rat epididymal adipocytes displayed a PGE$_2$-mediated inhibition and stimulation of cAMP at concentrations of 10 mM and >10 mM, respectively. In the rabbit, testicular contractions were stimulated and inhibited by PGE$_1$, at concentrations of 1-10 nM and 100 nM, respectively. Non-reproductive tissues also have been shown to exhibit bimodal responses to prostaglandins [Siler et al., 1986; Cohen and Rimon, 1992]. In rat hepatocytes, PGE$_2$ can act in a glycogenolytic and in a antiglycogenolytic fashion at concentrations of 10 $\mu$M and 1 nM, respectively. As mentioned previously, these glycogenolytic and antiglycogenolytic actions are likely mediated through the inositol triphosphate and cAMP pathways, respectively.

Controversy over which effect (luteotrophic or luteolytic) is physiological are bound to arise from these results and those of others. The reported Kd for the PGF$_{2\alpha}$ receptor is in the nano-molar range [Lake et al., 1994], close to the concentrations at which the luteotrophic response is present. It is likely that both responses are physiological and that their temporal relationship to luteolysis and early pregnancy is important. As mentioned above, studies have demonstrated the predominance of the luteotrophic response in the mid-luteal phase [Khan et al., 1989; Richardson and Masson, 1980; Michael and Webley, 1993], corresponding well with a potential role in the promotion of early pregnancy. In support of this notion, there have been reports of enhanced progesterone production in the presence of hCG plus PGF$_{2\alpha}$ compared with hCG alone [Suginami et al., 1976], as well as studies that have demonstrated the abrogation of PGF$_{2\alpha}$-mediated luteolysis when cellular exposure to hCG or prolactin preceeds PGF$_{2\alpha}$ exposure [Harris and Murphy, 1981; Suginami et al., 1976; Michael and Webley, 1991b].
Two models by which GnRH may be permissive or potentiatory with respect to PGF$_{2\alpha}$-mediated steroidogenesis are depicted in Figures 62 and 63. In short, GnRH may provide a missing component in a PGF$_{2\alpha}$-affected signal transduction system, or it may promote the production of *de novo* PGF$_{2\alpha}$. Further treatment of this subject may be found below in the signal transduction section (p. 132).

With enhanced magnitude and consistency of both the luteotrophic and luteolytic actions of PGF$_{2\alpha}$ and GnRH when co-applied, the potential for improving the clinical applications of these two hormones exists. Currently, potent, long-lasting GnRH analogues have been applied to numerous therapeutic applications in the female including: contraception; treatment of central precocious puberty; and sex steroid-dependent benign and malignant diseases of the reproductive organs [Molcho et al., 1984; Bhasin et al., 1984; Nillius, 1985]. One of the most common uses of GnRH analogues is the down-regulation of pituitary function in preparation for super-ovulation as part of an *in vitro* fertilization protocol. Conversely, attempts to inhibit luteal function and induce luteolysis or early abortion with GnRH have not been very successful [Nillius, 1985]. The contraceptive effects of GnRH appear to be safe, reliable and reversible in women [Nillius, 1985].

*Experimental Model*

When examining the interactions of two or more hormones, the author has found multi-concentration experiments to be much more revealing than single concentration studies. The difficulties with these experimental models lie in visualization of the results. Plotting data in three dimensions with the two interacting agents on the horizontal axes and the response on the vertical axis simplifies interpretation of the results. Further enhancement of visualization of the results with a contour plot of the data was achieved. This design and the improved speed of modern computers in plotting such data has revealed a more complex interaction between these two hormones than would be visible with a standard two dimensional experimental design. Certainly numerous other hormones are interacting in similar or even more complex manners.

*Summary*

These studies reveal the complex luteolytic and luteotrophic actions of GnRH and PGF$_{2\alpha}$. 
Figure 62. Gonadotrophin-releasing hormone (GnRH) acts as a permissive or potentiatory factor for prostaglandin F2α (PGF2α)-mediated luteotrophic and luteolytic effects, respectively. This model suggests that GnRH may provide a missing signal transduction factor, thus completing or enhancing the signal transduction pathways by which PGF2α functions.
Figure 63. Gonadotrophin-releasing hormone (GnRH) acts as a permissive or potentiatory factor for prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$)-mediated luteotrophic and effects luteolytic, respectively. This model suggests that GnRH may stimulate *de novo* synthesis of PGF$_{2\alpha}$ through a PKC-dependent stimulation of arachidonic acid (AA) production, as is seen in some other systems [Smith and Borgeat 1988].
C. Interaction of PGF$_{2\alpha}$ with PGE$_2$.

In eight-day precultured GLCs, both PGF$_{2\alpha}$ and PGE$_2$ stimulated progesterone production in a bell curve-like manner. As with previous experiments PGF$_{2\alpha}$ exerted its maximal luteotrophic effect at a concentration of 1 nM. Interestingly, the bell curve-like stimulation mediated by PGE$_2$ was shifted to the right (when compared to that of PGF$_{2\alpha}$) such that maximal stimulation of progesterone production was seen at concentrations of 10 to 100 nM. In both cases, maximal luteotrophic effects were present at or near the Kds of their receptors [Abramovitz et al., 1994; Abramovitz et al., 1994b; Lake et al., 1994].

The combination of these hormones resulted in an elaborate regulation of progesterone production whereby the bell curve was inverted. There are a number of possible explanations for the complexity of this regulation, including: 1) the non-standard saturation kinetics of PLA$_2$; 2) product inhibition of PGE$_2$ production by PGF$_{2\alpha}$; 3) receptor cross reactivity; 4) the opposing actions of these hormones on common signal transduction pathways; and 5) the combination PGF$_{2\alpha}$-mediated luteolytic and luteotrophic effects in the presence of PGE$_2$. It is likely that all of these factors play a role in these findings.

The physiological significance of these findings is not obvious at first glance. However, these results provide a potential mechanism for the changing functional role of the corpus luteum as luteinization progresses. Changing the ratio of PGF$_{2\alpha}$ to PGE$_2$ can profoundly alter progesterone production, a result seen in other reports which compared luteal cells in early, mid and late luteal stages [Pathwardhan and Lanthier, 1985]. Although complex in nature, these results should not be ignored as the ratio of PGF$_{2\alpha}$ to PGE$_2$ is known to change in vitro. Taken with the confounding effects of PGF$_{2\alpha}$ and GnRH, there exists more than adequate room for explaining the changing responses of granulosa and luteal cells with differentiation. Further, these results suggest that PGF$_{2\alpha}$ interactions with PGE$_2$ form a sophisticated means of regulating progesterone production in human granulosa-luteal cells.
D. Signal Transduction of PGF\textsubscript{2\alpha}-Mediated Luteolysis

This study has examined the signal transduction pathways utilized in the anti-gonadotrophic (or luteolytic) actions of PGF\textsubscript{2\alpha} in the human granulosa-luteal cell. Specifically, the ability of PGF\textsubscript{2\alpha} to inhibit both hCG- and isoproterenol-stimulated progesterone production was examined, as well as hCG-stimulated estradiol production. Furthermore, the signal transduction pathways involved in this effect were examined with pertussis- and cholera-toxin, as well as with forskolin and db-cAMP.

As seen above, the basal responses to PGF\textsubscript{2\alpha} were quite variable (Fig. 47, 49, and 51-53). This may be due to differences in the endogenous levels of PGF\textsubscript{2\alpha} within the culture media, differences in cellular differentiation state or differences in endogenous levels of interacting hormone levels. Further studies are underway to determine the exact nature of this phenomenon.

Pertussis Toxin Sensitive G-Protein

It was found that PGF\textsubscript{2\alpha} is exerting its anti-gonadotrophic (specifically anti-hCG) actions through a pertussis toxin sensitive G-protein. These data were supported by the ability of PGF\textsubscript{2\alpha} to inhibit CTX-stimulated progesterone and estradiol production, and by the reversal of this inhibition by the addition of PTX. These data also suggest that PGF\textsubscript{2\alpha} may exert its anti-gonadotrophic actions at an early step in the signal transduction cascade.

Reports in the literature support the potential role of G-proteins in the signal transduction of PGF\textsubscript{2\alpha}. Firstly, human granulosa-luteal cells have been examined immunocytochemically to reveal a number of G-protein alpha subunits, including, $G_{\alpha s}$, $G_{\alpha i3}$, $G_{\alpha i1,2}$ and $G_{\alpha o}$ (namely, $G_{\alpha q}$ and $G_{\alpha i1}$), while $G_{\alpha o}$ was undetectable by three different antibodies [Lopez et al., 1995]. Intracellular cAMP levels in human granulosa cells appear to be regulated by the ratio of $G_{\alpha s}$ and $G_{\alpha i}$-subunits, while $G_{\alpha q,11}$ and $G_{\alpha i}$ levels regulate the accumulation of inositol phosphates [Lopez et al., 1995]. Coupling of one or both of these identified $G_{\alpha i}$-subunits to the PGF\textsubscript{2\alpha}-receptor could explain the PTX sensitivity of the anti-gonadotrophic action of PGF\textsubscript{2\alpha}, as well as the regulation of both cAMP and inositol phosphates by PGF\textsubscript{2\alpha}. 
Further supporting a role for G-proteins in the signal transduction of PGF$_{2\alpha}$ are the sequences and predicted structure of the cloned prostanoid receptors. All of these receptors possess the seven-transmembrane domain structure characteristic of G-protein coupled receptors [Lake et al., 1994; Abramovitz et al., 1994; Adam et al., 1994; Boie et al., 1994 and 1995; Funk et al., 1993]. Additionally, the human EP$_3$-family of receptors is capable of inhibiting cAMP production through a pertussis toxin-sensitive G-protein [An et al., 1994]. It is not known if PGF$_{2\alpha}$ is acting through single or multiple G-proteins, as is seen in the actions of gonadotrophin-releasing hormone (GnRH) in the gonadotrope [Hawes et al., 1993].

Thus far, PGF$_{2\alpha}$ has been demonstrated to lower gonadotrophin- and prostaglandin E$_2$-stimulated rises in cAMP, as well as increase intracellular calcium and inositol phosphates [Davis et al., 1989; Currie et al., 1992; Pepperell et al., 1989; Lahav et al., 1987]. The DP, IP and EP$_3$-family of receptors are all coupled to cAMP regulation [Adam et al., 1994; Boie et al., 1994 and 1995; An et al., 1994], while the DP, FP, EP$_1$ and EP$_3$-family of receptors are coupled to rises in intracellular calcium [Abramovitz et al., 1994; Adam et al., 1994; Boie et al., 1995; Funk et al., 1993; An et al., 1994]. It is unknown if the actions of PGF$_{2\alpha}$ are exerted through single or multiple receptors. With PGF$_{2\alpha}$ and PGE$_2$ both being present and active in the human granulosa and luteal cells [Grinwich et al., 1976; Richardson and Masson, 1980; Pathwardhan and Lanthier, 1985; Satoh et al., 1981; Watson et al., 1979], it is probable that multiple prostanoid receptors exist in these cells. Furthermore, the currently cloned prostanoid receptors all possess varying degrees of cross-reactivity with PGE$_2$ and PGF$_{2\alpha}$ [Lake et al., 1994; Abramovitz et al., 1994; Adam et al., 1994; Boie et al., 1994 and 1995; Funk et al., 1993; An et al., 1994].

Although the present results indicate that the anti-gonadotrophic effects of PGF$_{2\alpha}$ are due to a pertussis toxin sensitive G-protein, it is unclear if they are mediated through G$_{\alpha_i}$, G$_{\alpha_p}$ or both. The G-protein alpha-subunit designated G$_{\alpha_p}$ is not a single G-protein but is, in fact, a family of G-proteins capable of activating phosphoinositide phosphodiesterase [Cockcroft and Stutchfield, 1988]. More than one G$_p$ pathway often exists within a single cell type, with distinct proteins coupling different receptors to phosphatidyl inositol hydrolysis selectively, thus allowing for regulation of the magnitude of phosphatidyl inositol hydrolysis [Ashkenazi et al., 1989]. Within the Gp family of G-proteins there exists two sub-families, a pertussis toxin-sensitive and a pertussis toxin-insensitive G$_p$, both of which are involved in PLC regulation [Martin et al., 1991]. For example, bovine adrenal fasciculata cells possess angiotensin-II...
receptors which are coupled to the phosphoinositide pathway through pertussis toxin-sensitive and insensitive G\textsubscript{p} proteins [Langois et al., 1990]. This example demonstrates the ability of a single receptor to be coupled to multiple-forms of G\textsubscript{p}, providing for a complex regulation of the phosphoinositide pathway.

If the anti-gonadotrophic effects of PGF\textsubscript{2\alpha} are mediated by G\textsubscript{ol}, this would probably be through a direct effect of G\textsubscript{ol} on adenylate cyclase and/or through the ‘mopping-up’ of G\textsubscript{os}-subunits by free beta/gamma-subunits freed when G\textsubscript{ol} was released. Alternatively, if the anti-gonadotrophic effects of PGF\textsubscript{2\alpha} are mediated through a pertussis toxin sensitive G\textsubscript{op}, it is likely that inhibition of the cAMP pathway would be through elevated levels of inositol phosphates, calcium, diacylglyceride and PKC activity and through the actions of these messengers on the cAMP pathway. In the sheep, it has been demonstrated that elevated levels of phospholipase-C activity involves a pertussis toxin-sensitive protein [McCann and Flint, 1993]. It is also possible that the PGF\textsubscript{2\alpha}-receptor is coupled to more than one G-protein. Finally it has been suggested that a single G\textsubscript{ol}-like G\textsubscript{op} protein may be capable of multiple actions [Magnaldo et al., 1988]. In hamster fibroblasts, thrombin is capable of inhibiting adenylate cyclase via a G-protein, while the G-protein mediated-activation of PKC causes a stimulatory effect on adenylate cyclase. This indirect stimulatory effect is exerted by PKC action directly on an element of the adenylate cyclase-G\textsubscript{os} complex [Magnaldo et al., 1988]. If a similar mechanism existed in the human granulosa-luteal cell, this might help to explain the transient stimulatory effects of PGF\textsubscript{2\alpha} on basal progesterone production.

In the gonadotrope, it has been demonstrated that GnRH may exert its actions through as many as three G-proteins [Hawes et al., 1993]. Briefly, GnRH has been demonstrated to stimulate IP production through a PTX-sensitive G-protein (Gp), while a distinct CTX-sensitive G protein can sensitize the gonadotrope to luteinizing hormone (LH) release through cAMP. Finally, a third CTX/PTX-insensitive G-protein can mediate LH release. Furthermore, there has been the suggestion of cross-talk between the CTX-sensitive G-protein and the PKC pathway [Barnes and Conn, 1993]. In view of the remarkable similarities (signal transduction and steroidogenic effects) between GnRH and PGF\textsubscript{2\alpha} [Quirk et al., 1995; Leung and Steele, 1992; Stoljelkovics et al., 1994] and their receptors [Lake et al., 1994; Abramovitz et al., 1994; Stoljelkovics et al., 1994] in the ovary, similar complexities may play a role in the signal transduction of the actions of PGF\textsubscript{2\alpha} in the human granulosa-luteal cells.
**Adenylate Cyclase and cAMP**

In these studies, PGF$_{2\alpha}$ inhibited forskolin- but not db-cAMP-stimulated progesterone production from human granulosa-luteal cells, suggesting that it is exerting its actions at or above the level of gonadotrophin-dependent adenylate cyclase. In previous studies, forskolin-stimulated progesterone production from large luteal cells (bovine CL) was inhibited by PGF$_{2\alpha}$ only in the presence of endothelial cells [Girsh et al., 1995]. This action was attributed to the secretion of PGI$_2$ by endothelial cells. In contrast to the present results, several other studies in rat and bovine luteal cells, have demonstrated the inhibition of progesterone production by PGF$_{2\alpha}$ at sites distal to adenylate cyclase [Rajkumar et al., 1988; Benhaim et al., 1987; Dorflinger et al., 1983]. This inhibition may be mediated by a reduction in the sensitivity of the cells to cAMP.

In light of the irreversibility of forskolin-activation of adenylate cyclase it is unlikely that PGF$_{2\alpha}$ is exerting it's inhibitory actions solely on adenylate cyclase. These actions may be exerted in part through a phosphodiesterase family member (Michael and Webley 1991a).

The β-adrenergic receptor, one of the most studied and understood of receptors, has been well established as a seven transmembrane, G-protein coupled receptor which activates the production of cAMP from adenylate cyclase [Briggs 1982; Barak et al., 1995; O'Dowd et al., 1988]. Several studies have demonstrated that isoproterenol (a β-adrenergic agonist) is capable of stimulating cAMP and progesterone production from granulosa and luteal cells in non-primate species [Leung, 1985]. However, thus far there have been conflicting reports regarding the effects of isoproterenol on progesterone production and adenylate cyclase activity in the granulosa-luteal cells of the human [Fohr et al., 1993; Casper and Cotterell, 1984; Hellensjo et al., 1985]. This study demonstrates that isoproterenol is capable of stimulating progesterone production in the human granulosa-luteal cell, and that this stimulation may be inhibited by PGF$_{2\alpha}$. The ability of PGF$_{2\alpha}$ to inhibit isoproterenol-stimulated estradiol production was not examined in these studies, although isoproterenol was found to stimulate estradiol production (not shown).

**Protein Kinase C**

As mentioned above, previous studies have demonstrated the ability of PKC inhibitors to partially inhibit PGF$_{2\alpha}$-mediated luteolysis. These studies have confirmed this result. The
inhibitory actions of PKC inhibitors are not as complete as those of PTX, suggesting that PKC inhibitors are blocking the effects of PGF$_{2\alpha}$ through an indirect means. Based on the known pathways by which prostaglandin production is stimulated in other models [Smith and Borgeat 1988], the author would like to suggest that endogenous PGF$_{2\alpha}$ production may be stimulated by the calcium-diglyceride-PKC pathway in these cells. This would help to explain the partiality of the PKC inhibitory effect on PGF$_{2\alpha}$-mediated luteolysis, as de novo prostaglandin synthesis would be blocked, although existing PGF$_{2\alpha}$ could still be effective until degraded. Moreover, it would provide a mechanistic explanation for the ability of GnRH to potentiate or permit the effects of PGF$_{2\alpha}$, as GnRH is known to stimulate the calcium-diglyceride-PKC pathway as well (see Fig. 64). Additionally, the known stimulation of this signal transduction pathway by PGF$_{2\alpha}$ could provide a positive feedback mechanism. In support of this idea is the ability of indomethacin to block the luteotrophic effects of PGF$_{2\alpha}$. As indomethacin is a blocker of de novo prostaglandin production, this effect suggests that in order for PGF$_{2\alpha}$ to exert its actions in these cells a feedback loop is necessary. Given the short half-life of PGF$_{2\alpha}$, this would make sense.

*De Novo Protein Synthesis*

Interestingly, the effects of PGF$_{2\alpha}$ are reported to be dependent on de novo protein synthesis, as actinomycin-D blocks them [Fitz et al., 1993]. It would not be surprising if this de novo protein is either an eicosogenic enzyme or GnRH.

*Summary*

The anti-gonadotrophic actions of PGF$_{2\alpha}$ are mediated through a pertussis-toxin sensitive G-protein (possibly G$_i$, G$_p$ or both; Fig. 65). Prostaglandin F$_{2\alpha}$ is capable of inhibiting progesterone production in response to hCG (Fig. 66), isoproternol (Fig. 66), CTX (Fig. 67A) and forskolin (Fig. 67B), but not db-cAMP (Fig. 67), strongly suggesting that PGF$_{2\alpha}$ is exerting its anti-gonadotrophic actions at or above the level of adenylate-cyclase. Further supporting this conclusion is the ability of PTX to reinstate the stimulatory actions of CTX following PGF$_{2\alpha}$ administration. There exists the potential that these actions are exerted through a member of the phosphodiesterase family of enzymes.
Figure 64. Proposed positive feedback loop for prostaglandin F$_{2\alpha}$ synthesis (PGF$_{2\alpha}$). Gonadotrophin-releasing hormone (GnRH) and PGF$_{2\alpha}$ are both known to stimulate the calcium-diglyceride-protein kinase C (PKC) pathway. Moreover, PKC pathway is reported to stimulate de novo prostaglandin synthesis in some systems. Thus, there exists the possibility that PGF$_{2\alpha}$ and GnRH may provide positive feedback on de novo PGF$_{2\alpha}$ synthesis in the human granulosa-luteal cell.
Figure 65. Pertussis toxin blocks prostaglandin $F_2\alpha$ ($\text{PGF}_{2\alpha}$)-mediated inhibition of human chorionic gonadotrophin (hCG)-stimulated steroidogenesis in the human luteal cell. $G$ - stimulatory G-protein; $G$ - pertussis toxin sensitive G-protein; $\text{AC}$ - adenylate cyclase (AC); cAMP - cyclic adenosine monophosphate; and PKA - protein kinase A
Figure 66. Prostaglandin F2α (PGF2α)-mediated inhibition of human chorionic gonadotrophin (hCG)- and isoproterenol-stimulated steroidogenesis, in the human granulosa-luteal cell. G - stimulatory G-protein; AC - adenylate cyclase (AC); cAMP - cyclic adenosine monophosphate; and PKA - protein kinase A
Figure 67. Prostaglandin F2α (PGF2α)-mediated inhibition of cholera toxin (A) and forskolin (B) stimulated progesterone and estradiol production in human granulosa-luteal cells. G - stimulatory G-protein; AC - adenylate cyclase (AC); cAMP - cyclic adenosine monophosphate; and PKA - protein kinase A
Figure 68. The inability of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) to inhibit dibutyl cyclic adenosine monophosphate (cAMP) stimulated progesterone and estradiol production in human granulosa-luteal cells. PKA - protein kinase A
An inverse relationship between progesterone production and PGF$_{2\alpha}$-R mRNA levels was revealed in the present studies. Human chorionic gonadotrophin and PGF$_{2\alpha}$ both inhibited PGF$_{2\alpha}$-R mRNA levels. Maximal inhibition of PGF$_{2\alpha}$-R mRNA levels was seen at 1 nM PGF$_{2\alpha}$ in the presence of hCG. As this receptor is only recently cloned in the human and rat [Abramovitz et al., 1994; Lake et al., 1994], there exists only one other report of PGF$_{2\alpha}$-R mRNA regulation in the literature. Moreover, the effects of PGF$_{2\alpha}$ on PGF$_{2\alpha}$-R mRNA levels have not been examined. However, the effects of hCG have been examined [Ristimaki et al., 1997]. This report demonstrated an hCG-mediated upregulation of PGF$_{2\alpha}$-R mRNA levels—a result differing from the present results. The difference between these two reports may be explained by the fact that these experiments were performed on cells of different culture periods.

Inhibition of PGF$_{2\alpha}$-R mRNA and presumably PGF$_{2\alpha}$-R would reduce the effectiveness of PGF$_{2\alpha}$-mediated luteolytic effects. Thus, the inverse bell curve-like autoregulation of PGF$_{2\alpha}$-R mRNA by PGF$_{2\alpha}$ may explain its bell curve-like effects on progesterone production. Notably, maximal stimulation of progesterone production in the presence of hCG and PGF$_{2\alpha}$ (1 nM) occurred when PGF$_{2\alpha}$-R mRNA levels were at their lowest. Thus, rather than potentiating hCG in a true sense, PGF$_{2\alpha}$ is inhibiting its own luteolytic effects allowing more effective stimulation by gonadotrophins. The mechanism by which PGF$_{2\alpha}$ autoregulates its receptor mRNA needs to be studied further.

In summary, PGF$_{2\alpha}$ negatively autoregulates its receptor mRNA. Moreover, PGF$_{2\alpha}$ feeds back on its steroidogenic effects through this autoregulation.
VIII – SYNOPSIS

The aforementioned studies examined the effects of prostaglandin-F$_{2a}$ (PGF$_{2a}$) on progesterone and estradiol production, as well as DNA and PGF$_{2a}$-R mRNA levels in the human granulosa-luteal cell (GLC). Additionally, the interactions of PGF$_{2a}$ with human chorionic gonadotrophin (hCG), gonadotrophin-releasing hormone (GnRH) and prostaglandin E$_2$ (PGE$_2$) were examined with respect to progesterone and estradiol production. In one study, cells were collected from small (<12 mm) and large (>12 mm) follicles separately, permitting the examination of follicle size-dependent alterations in steroidogenesis. Pharmacological techniques were utilized to elucidate the signal transduction pathways involved in the anti-gonadotrophic effects of PGF$_{2a}$. Moreover, these experiments were performed on one-(D$_1$), eight-(D$_8$) and/or twelve- to fourteen-day (D$_{12-14}$) cultured GLCs in order to reveal culture time-dependent alterations in cellular response.

Briefly, GLCs collected from patients undergoing in vitro fertilization (IVF) were cultured for the time periods described above, followed by a 24 h treatment period, after which media were collected and assayed for progesterone and estradiol while cells were extracted for DNA or total RNA.

A. Basic Physiological Responses to PGF$_{2a}$

Human GLCs undergo morphological luteinization and then luteolysis with increasing time in culture. Culture-time, concentration and/or follicle-size dependent alterations in PGF$_{2a}$- and GnRH-mediated regulation of human GLC steroidogenesis in the presence and/or absence of human chorionic gonadotrophin (hCG) were investigated. This study clearly demonstrated functional differentiation of human granulosa-luteal cells in culture.

Progesterone production in response to PGF$_{2a}$ changed with culture-time from inhibition to stimulation in D$_1$ and D$_{12-14}$ cultured GLCs, respectively. Cells at D$_8$ of culture were in a state of transition, with inhibition, stimulation or intermediate responses being possible. Similarly, estradiol responses changed from no response to a stimulatory response in D$_1$ and D$_8$ cultured GLCs, respectively. DNA levels were unaltered by PGF$_{2a}$ treatment.
In the presence of hCG similar culture-time dependent changes were observed. PGF$_{2\alpha}$ (10$^{-6}$ M) inhibited hCG-stimulated progesterone production in D$_1$ and D$_8$, but not in D$_{12-14}$ cultured GLCs. In contrast, PGF$_{2\alpha}$ (10$^{-6}$ M) potentiated hCG-stimulated progesterone production in D$_8$ and D$_{12-14}$, but not in D$_1$ cultured GLCs. A similar trend was seen with estradiol production. Human CG significantly stimulated progesterone and estradiol production from D$_1$ cultured GLCs collected from large follicles (> 12 mm), while it did not in cells collected from small follicles (< 12 mm). Consequently, PGF$_{2\alpha}$ significantly inhibited hCG-stimulated progesterone and estradiol production in GLCs collected from large, but not small follicles. Human CG stimulated progesterone production was inhibited by high concentrations of GnRH (10$^{-6}$ M) in D$_1$ and D$_8$ cultured GLCs. Human CG-stimulated progesterone production was inhibited by high concentrations of GnRH (10$^{-6}$ M) in D$_1$ and D$_8$ cultured GLCs.

In a fashion similar to PGF$_{2\alpha}$, GnRH (10$^{-9}$) was capable of potentiating hCG-stimulated progesterone production in D$_8$ human GLCs. Similar results were seen for estradiol production in D$_8$ GLCs. DNA levels were unaltered by these treatments.

**B. Confounding Interactions of PGF$_{2\alpha}$ with GnRH**

A second study examined the effects of PGF$_{2\alpha}$ and GnRH and their interactions on progesterone- and estradiol-production from D$_1$ and D$_8$ cultured human GLCs. In a preliminary experiment, GLCs were treated with vehicle, PGF$_{2\alpha}$ (10$^{-9}$ M), GnRH (10$^{-6}$ M) or PGF$_{2\alpha}$ plus GnRH in the absence or presence of hCG. It was demonstrated that PGF$_{2\alpha}$ and GnRH alone had no significant effect on progesterone or estradiol production in D$_1$ GLCs. However, the combination of PGF$_{2\alpha}$ plus GnRH caused a significant stimulation of progesterone and estradiol production. PGF$_{2\alpha}$ partially inhibited hCG-stimulated progesterone- and estradiol-production. Conversely, GnRH did not inhibit hCG-stimulated progesterone- or estradiol-production, although it did potentiate PGF$_{2\alpha}$-mediated inhibition of hCG-stimulated steroidogenesis.

In a second experiment (n=7 patients), vehicle, PGF$_{2\alpha}$ (10$^{-11}$ to 10$^{-6}$ M) and GnRH (10$^{-10}$ to 10$^{-5}$ M) concentration-response curves were crossed into a matrix of 49 separate treatments. Steroidogenic responses were plotted in three dimensions and as a contour map. Moreover, 'slices' of the three dimensional matrix were plotted in two dimenstions and analyzed statistically. Maximal stimulation of progesterone-production (2-3-fold) was seen when medium concentrations of PGF$_{2\alpha}$ interacted with high concentrations of GnRH (10$^{-6}$ to 10$^{-5}$ M). In the
presence of high concentrations of GnRH (10^{-6} M), PGF_{2\alpha} stimulated progesterone production in a bell curve-like fashion, as middle concentrations significantly stimulated while low and high concentrations did not. In the presence of middle concentrations of PGF_{2\alpha} (10^{-9} M), GnRH significantly stimulated progesterone production in a linear concentration-dependent manner. Prostaglandin F_{2\alpha} alone elicited no estradiol response. However in the presence of high concentrations of GnRH (10^{-5} M), a significant concentration-dependent stimulation was seen. Maximal stimulation of estradiol production was seen when high concentrations of PGF_{2\alpha} (10^{-6} M) and GnRH (10^{-5} M) were co-applied. Gonadotrophin-releasing hormone alone stimulated estradiol production in a bell curve-like manner, although in the presence of high concentrations of PGF_{2\alpha} (10^{-6} M), estradiol was stimulated in a linear concentration-dependent manner. Inhibition of cyclooxygenase-I (by indomethacin) prevented the luteotrophic effects of PGF_{2\alpha} in the presence and absence of GnRH in D_1 and D_8 cultured human GLCs.

**C. Confounding Interactions of PGF_{2\alpha} with PGE_2**

In D_8 cultured GLCs, PGF_{2\alpha} and PGE_2 concentration response curves were crossed and treated as with GnRH. Briefly, progesterone was significantly stimulated in a bell curve-like manner by PGF_{2\alpha} with maximal stimulation at 1 nM. A similar response to PGE_2 was seen, although the bell curve was shifted right. Maximal PGE_2-mediated stimulation of progesterone production was seen at 10 to 100 nM. However, in the presence of PGE_2 (10^{-7} M), PGF_{2\alpha} significantly inhibited progesterone production in an inverse bell curve-like manner with maximal inhibition at (10^{-10} to 10^{-8} M, PGF_{2\alpha}).

**D. Signal Transduction of the Luteolytic Effects of PGF_{2\alpha}**

The third study utilized the G-protein effectors pertussis toxin (PTX) and cholera toxin (CTX), the \beta-adrenergic agonist (and known activator of the cAMP pathway) isoproterenol, forskolin, and the cAMP analogue dibutryl-cAMP (Db-cAMP) to examine the signal transduction pathways involved in the anti-gonadotrophic actions of PGF_{2\alpha} in D_8 GLCs. During the final 18 h of the pre-culture period, the cells were cultured in media or media containing PTX (50 ng/ml) and/or CTX (1 \mu g/ml). The cells were then treated with vehicle, PGF_{2\alpha} (10^{-6} M), hCG (1 IU/ml) or PGF_{2\alpha} plus hCG in the presence of vehicle, PTX, CTX or PTX plus CTX. In another
experiment, cells were treated with vehicle, PGF$_{2\alpha}$ (10$^{-6}$ M), IsoP (10$^{-5}$ M), or PGF$_{2\alpha}$ plus IsoP. It was demonstrated that PGF$_{2\alpha}$ caused a significant inhibition of hCG stimulated progesterone and estradiol production, and that this inhibition was abolished by PTX. Similarly, cholera toxin-stimulated progesterone and estradiol production was blocked by PGF$_{2\alpha}$, with PTX reversing this effect. Finally, PGF$_{2\alpha}$ also inhibited isoproterenol- and forskolin-stimulated, but not Db-cAMP stimulated progesterone production from eight day cultured human granulosa-luteal cells.

**E. Regulation of PGF$_{2\alpha}$-R mRNA**

Human GLCs (D$_1$) were exposed to culture media containing either vehicle, hCG (1 IU/ml) or hCG plus PGF$_{2\alpha}$ (10$^{-11}$ to 10$^{-6}$ M), for 24 h. Following the treatment period, cells were extracted for total RNA, which was confirmed to be intact by the presence of 18 and 28S bands revealed by RNA gel electrophoresis. A fixed quantity of mRNA (between 0.5 and 2 µg depending on yield) was reverse transcribed to cDNA and frozen (at -20 C) until used in semi-quantitative PCR. Transcripts for PGF$_{2\alpha}$-R were detected by PCR with two different sets of oligonucleotide primers based on the published human PGF$_{2\alpha}$-R sequence. PCR products were run on a 1.5% agarose gel, stained with ethidium bromide and/or autoradiographed when [³²P]dCTP was incorporated. PCR products were confirmed to be those of PGF$_{2\alpha}$-R by size and by Southern blot hybridization with an internal oligonucleotide probe. Photographs and/or autoradiograms of the gels or Southern blots were quantified by densitometry. These experiments were performed a minimum of three times on cells from a minimum of three separate patients. Similar results were seen in all experiments performed.

Prostaglandin F$_{2\alpha}$-R mRNA was significantly down-regulated by hCG when compared to the control. In contrast, progesterone and estradiol production were significantly stimulated by hCG. However, as described above, the addition of both low (10$^{-11}$ M) and high concentrations (10$^{-6}$ M) of PGF$_{2\alpha}$ restored PGF$_{2\alpha}$-R mRNA levels to those of the controls. A corresponding change in progesterone and estradiol levels was seen, such that hCG-stimulated steroidogenesis was significantly inhibited by these concentrations of PGF$_{2\alpha}$. Finally, the strongest effect of PGF$_{2\alpha}$ was seen at a concentration of 10$^{-9}$ M where PGF$_{2\alpha}$-R mRNA was barely detectable. As before, progesterone and estradiol production were inversely related to PGF$_{2\alpha}$-R levels, as hCG-stimulated progesterone and estradiol production were completely restored in the presence of 1 nM PGF$_{2\alpha}$. Messenger RNA levels for the β-actin gene were unaltered by these treatments.
IX – CONCLUSIONS

Human GLCs undergo morphological luteinization and luteolysis with culture-time. Steroidogenic responses to PGF$_{2\alpha}$ were culture-time and concentration dependent, with PGF$_{2\alpha}$ being either luteolytic or luteotrophic depending on these conditions.

Cyclooxygenase-I inhibition prevented the luteotrophic effects of PGF$_{2\alpha}$, suggesting that *de novo* prostaglandin synthesis is required for this effect. Furthermore, the luteotrophic effects of PGF$_{2\alpha}$ required GnRH as a permissive factor. Additionally, the luteotrophic effects of PGF$_{2\alpha}$ could also be regulated in a complex manner by PGE$_2$.

The luteolytic effects of PGF$_{2\alpha}$ were mediated through a pertussis-toxin sensitive G-protein (possibly G$_i$, G$_p$ or both). Prostaglandin F$_{2\alpha}$ inhibited cholera-toxin, isoproterenol and forskolin (but not db-cAMP) stimulated progesterone production, suggesting that this G-protein exerts its actions proximal to PKA (possibly at the level of adenylate cyclase or phosphodiesterase). The luteolytic actions of PGF$_{2\alpha}$ were potentiated by GnRH.

Finally, PGF$_{2\alpha}$ was capable of autoregulating its receptor mRNA levels and, thus, its ability to regulate steroidogenesis in the human GLC. An inverse relationship between PGF$_{2\alpha}$-R mRNA levels and steroidogenesis exists.
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