AUTOCRINE/PARACRINE REGULATION OF STEROIDOGENESIS IN HUMAN OVARIAN CELLS

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to the required standard.

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ABSTRACT

This study investigated the hypothesis that local ovarian substances regulate gonadotropin-dependent steroidogenesis in the human ovary and form a paracrine and autocrine network for granulosa cell responses to endocrine stimulation. The specific substances investigated were inhibin, activin, follistatin, angiotensin II and III (A II and A III), prostaglandin F$_2$α (PGF$_{2α}$) and GnRH. Inhibin-related peptides were isolated in follicular fluid and were stimulated by FSH. Inhibin-A had no effect on basal and gonadotropin-stimulated progesterone production in human granulosa-luteal cells. Activin-A inhibited hCG/FSH-stimulated but not basal progesterone production, suggesting its role as an inhibitor of luteinization. Follistatin stimulated basal progesterone production, suggesting its antagonistic role for activin-A. Intracellular cAMP levels were inhibited by activin and stimulated by follistatin. Follistatin may induce the preovulatory progesterone peak which is necessary to trigger the LH surge. Activin-A and follistatin form a balanced autocrine network to regulate proper progesterone production from granulosa cells around the ovulatory phase. Inhibin βA-subunit mRNA expression was increased by hCG treatment in cultured human granulosa cells, suggesting inhibin and activin production are subject to endocrine regulation. For comparison with another steroidogenic tissue, inhibin subunit mRNA expression in human trophoblast cells was also studied. GnRH and cAMP stimulated inhibin α and βB mRNA expression in human term placental cells. cAMP also stimulated inhibin βA mRNA expression. These results provide evidence for the interaction of local regulators and gonadotropins within the human ovary and placenta.
A renin-angiotensin system exists in human ovary and was stimulated by LH. Effects of A II and A III on steroidogenesis of granulosa-luteal cells were examined. A II and A III bound to the granulosa-luteal cell surface. A III but not A II inhibited hCG-stimulated progesterone production. A III had an additive effect on PMA, a diacylglycerol analog, inhibited progesterone production response to hCG and PMA-pretreatment did not block the inhibitory action of A III. These suggested that inhibitory effect of A III was not modulated by protein kinase C pathway. A III may play a luteolytic role during the luteal phase.

Effects of GnRH and PGF$_{2\alpha}$ on hCG-stimulated progesterone production were studied. GnRH had no effect on basal and hCG-stimulated progesterone production. PGF$_{2\alpha}$ failed to have consistent effects on basal and hCG-stimulated progesterone production. These results are different from those observed in the rat ovary and may be due to species difference in the cellular response to PGF$_{2\alpha}$.

Taken together, the results of this study showed that ovarian local regulators play key roles in the control of steroidogenesis by granulosa-luteal cells. The physiological significance of the actions of these local regulators might be that they interact with each other to fine-tune the endocrine response of the ovary to the robust actions of the gonadotropins.


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LIST OF ABBREVIATIONS

adenosine triphosphate (ATP)
angiotensin I (A I), II (A II) and III (A III)
arachidonic acid (AA)
basic fibroblast growth factor (bFGF)
cAMP-responsive element (CRE)
8-bromo-cyclic AMP (8-Br-cAMP)
cyclic adenosine monophosphate (cAMP)
diacylglycerol (DAG)
dihydrotestosterone (DHT)
EDTA (ethylenediaminetetraacetate)
epidermal growth factor (EGF)
equine chorionic gonadotropin (ECG)
fluorescein isothiocyanate (FITC)
follicle-stimulating hormone (FSH)
germinial vesicle breakdown (GVBD)
gonadotropin releasing hormone (GnRH)
human chorionic gonadotropin (hCG)
human menopause gonadotropin (hMG)
hydroperoxycicosatetra-enoic acids (HPETEs)
hydroxyeicosatetraenoic acids (HETEs)
20α-hydroxyprogesterone (20α-OH-P)
3β-hydroxysteroid dehydrogenase (3β-HSD)
inositol(1,4,5)trisphosphate (Ins(1,4,5)P₃)
iso-butyl-methylxanthine (IBMX)
insulin-like growth factor I (IGF-I)
LB medium (Luria-Bertani medium
leukotrienes (LTs)
low-density lipoprotein (LDL)
luteinizing hormone (LH)
messenger ribonucleic acid (mRNA)
3-(N-morpholino) propanesulfonic acid (MOPS)
nordihydroguaiaretic acid (NDGA)
phorbal 12-myristate 13-acetate (PMA)
phospholipase C (PLC)
polyethylene glycol (PEG)
prolactin (PRL)
prostaglandins (PGs)
prostaglandin E₂ (PGE₂)
prostaglandin F₂α (PGF₂α)
prostaglandin I₂ (PGL₂)
protein kinase C (PKC)
radioimmunoassay (RIA)
reverse transcription-PCR (RT-PCR)
SDS (sodium didecyl sulfate)

somatomedin C (SmC)

SSC (sodium chloride/sodium citrate buffer)

SSPE (sodium chloride/sodium phosphate and EDTA buffer)

TE buffer (tris-EDTA)

thromboxane A$_2$ (TXA$_2$)

thyroid-stimulating hormone (TSH)

thyrotropin releasing hormone (TRH)

transforming growth factor B (TGF-B)

trichloroacetic acid (TCA)
Much of the material contained in this thesis has been previously published by the author, or is in preparation for submission to be published. Full details of authorship follow;


free calcium increased by prostaglandin F2α (PGF2α), gonadotropin-releasing hormone, and angiotensin II in rat granulosa cells and PGF2α in human granulosa cells. Endocrinology 1992. 130:1837-1843


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1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Postpubertal human ovaries are endocrine tissues in which many compartments interact to produce a fertilizable ovum and secrete sex steroids during the menstrual cycle. Ovarian functions are regulated by gonadotropins secreted by the anterior pituitary. However, changes in the blood level of gonadotropins cannot account for some phenomena occurring during the menstrual cycle. An example would be the occurrence of ovulation in only one of the two ovaries despite perfusion by identical circulating gonadotropin concentrations. Not all follicles mature to the same extent during a given cycle within the ovary from which ovulation occurs. After ovulation, only the dominant follicle is thought to form the corpus luteum. The mechanisms of maintenance and luteolysis of corpus luteum remain unclear. Many substances identified in the ovaries of laboratory mammals have local regulatory actions. These substances may form an elaborate network of highly integrated paracrine and autocrine systems, although this remains to be proven. Current information suggests that the ovary may play an active role in the initiation and maintenance of reproductive cyclicity. The hypothalamus and pituitary play a permissive and tonic role in this connection. Intra-follicular substances may respond to gonadotropins to integrate a proper biological response by granulosa cells. Therefore, intraovarian substances may act locally to modify gonadotropin functions in the ovary, controlling follicular development and steroidogenesis. The studies of this thesis addressed this hypothesis using human granulosa-lutein cell cultures to study the regulation of sex steroid secretion and transcription of messenger ribonucleic acid
(mRNA) coding for some local substances.

1.2 OVARIAN HISTOLOGY

The human ovary is divided into a peripheral cortex and an inner medulla. The cortex contains three parts: 1. The germinal epithelium, which is the outermost cellular layer of the cortex; 2. the stroma, containing connective tissue and interstitial cells; 3. the follicular complex. The medulla contains the loose connective tissue, nerves, lymph vessels, blood vessels and interstitial cells. The follicular complex contains follicles and corpora lutea in various stages of development (Fig. 1).

Figure 1. The structure of human ovary: showing the follicular development and corpus luteum formation. (Modified from Yen S S C & Jaffe R B, Reproductive Endocrinology, 3rd ed. Saunders W B Company 1990).
1.2.1 Follicular growth and atresia

The follicle is a basic unit of the ovary. An ovarian follicle is composed of the oocyte associated with a layer or layers of granulosa cells. The oocyte and granulosa cells are separated from the surrounding stroma by a basement membrane, the "basal lamina." Blood vessels and theca cells are outside the basal membrane. The theca cells are spindle-shaped cells transformed from interstitial cells. Granulosa cell proliferation and differentiation, theca cell hypertrophy and oocyte growth all combine to increase follicular diameter (McNatty, 1981). Localized fluid appears among the granulosa cells when the follicular diameter reaches 100-200 microns. This process eventually transforms the primary follicle into an antral follicle, then a preovulatory follicle. This process only occurs during the reproductive lifespan (Harrison 1962; Lintern-Moore et al., 1974). Antral fluid contains protein-bound and free sex steroids, local substances, plasma proteins, mucopolysaccharides and electrolytes (Edwards 1974). These components are secreted by granulosa and theca cells or diffuse from vascular spaces outside the basal membrane. One follicle ovulates and gives rise to a corpus luteum during each menstrual cycle. Other follicles undergo atresia. More than 99 per cent of follicles are lost by atresia (Ingram, 1962). The functional lifespan of the human corpus luteum is approximately 14 ± 2 days. The granulosa cells undergo morphological changes (called luteinization) with capillary and theca cell penetration to form the corpus luteum (Harrison, 1962, Fig. 1). Follicular development is separated into three phases:

1) The preantral growth phase concerns the conversion of primordial follicles to primary follicles. This phase is continuously operational from the fifth month of
fetal life to the end of adult life and occurs independent of gonadotropic support. The mechanisms responsible for recruiting some primordial follicles remain a mystery.

2) The tonic growth phase concerns the conversion of primary follicles to antral follicles. This phase may be follicle-stimulating hormone (FSH)-dependent.

3) The gonadotropin-dependent growth phase concerns follicular growth through the antral to preovulatory stage and ovulation. This phase is gonadotropin-dependent (Fig. 2).

Figure 2. The complete follicular growth trajectory. M, menses; Gn, gonadotropin. Ovul, ovulation.
1.2.2 *Morphology of granulosa cells*

The granulosa cells are from the intraovarian rete ovarii (Byskov & Rasmussen, 1973; Byskov, 1978). The differentiation of granulosa cells is not uniform in a given follicle. As the antral follicle develops, these cells become organized into morphologically distinguishable regions with specialized functions. The mural granulosa cells are closer to the basal membrane, while the cumulus cells surround the oocyte. Cumulus cells physically support the oocyte and provide nutrients for oocyte growth. Cytoplasmic extensions from the cumulus cells penetrate the zona pellucida, allowing signal exchange for coordinated follicular and oocyte maturation. The majority of granulosa cells are mural, lining the follicular cavity. The mural cells are interconnected by extensive intercellular gap junctions, resulting in cellular electrical coupling to yield an integrated and functional syncytium (Adashi, 1991).

1.3 **OVARIAN PRODUCTS**

1.3.1 *Steroid biosynthesis*

Ovaries have the capacity to synthesize all three classes of sex steroids (progestin, estrogen and androgen) from their common precursor, cholesterol (Fig. 3). Cholesterol from both low-density lipoprotein and high-density lipoprotein has been demonstrated to serve as a precursor for steroidogenesis in the ovarian follicle (Gwynne & Strauss, 1982). Lipoprotein uptake from plasma is regulated by the availability of serum lipoproteins and the lipoprotein receptor-dependent uptake system. Cholesterol synthesis by granulosa cell
has also been reported (Schuler et al., 1979; Turek et al., 1982). Estrone and estradiol
are the major steroid products of the follicle. Progesterone and 17α-hydroxyprogesterone
are the major products of the corpus luteum. Studies using (labelled or unlabelled C\textsubscript{21} and
C\textsubscript{19}) precursors showed that isolated granulosa cells produce progesterone and estrogens
as well as 17α-hydroxyprogesterone (Fowler et al., 1978; Channing 1969; Tsang et al.,
1980). Isolated theca cells produce progesterone, 17α-hydroxyprogesterone, and
androstenedione (Tsang et al., 1980; McNatty et al., 1979).

1.3.1.1  \textit{Estrogen biosynthesis}
Granulosa cells are the cellular source of the two most important ovarian steroids, estradiol and progesterone. Progesterone synthesis requires only granulosa cells but estrogen synthesis requires cooperation between granulosa and theca cells. Androgens (mainly androstenedione) produced by luteinizing hormone (LH)-stimulated theca cells are the main substrate for estrogen biosynthesis by FSH-stimulated granulosa cells. FSH induces granulosa cell aromatase activity, which is absent in theca cells. Both FSH and LH action on steroidogenesis require membrane-associated adenyl cyclase (Knecht et al., 1983). Adenyl cyclase converts intracellular adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP binds to the regulatory subunit of protein kinase A. The catalytic subunit of protein kinase A is activated by cAMP and phosphorylates intracellular proteins. However, the exact proteins involved in the phosphorylation remain unknown.

1.3.1.2 Androgen biosynthesis

The rate-limiting step in androgen biosynthesis by theca cells is catalyzation of pregnenolone or progesterone by 17α-hydroxylase/C17,20-lyase enzyme complex. The enzyme complex is located in the microsomal fraction. Comparatively speaking, granulosa cells contain almost no 17α-hydroxylase/C17,20-lyase (Short, 1962; Bjersing & Carstensen, 1967). The lack of these enzymes in granulosa cells indicates that both granulosa and theca cells participate in androgen and estrogen biosynthesis. The 17α-hydroxylase/C17,20-lyase complex hydrolyses at the C17 position before the side chain is cleaved from the C21 steroids (progestins) to form C19 steroids (androgens). This is a key
step in hormonal control of follicular steroid secretion. Androgens synthesized by theca/interstitial cells are transported to the follicular fluid and to the blood circulation as a precursor of estrogen synthesis.

1.3.1.3 Progestin biosynthesis

Granulosa cells synthesize progestins using cholesterol derived from circulating serum lipoproteins (Brown & Goldstein, 1976). Low-density lipoprotein (LDL) particles bind to specific membrane receptors. LDL-receptor complexes enter the cell by receptor-mediated endocytosis (Goldstein et al., 1979; Anderson et al., 1977). The endocytotic vesicles fuse to lysosomes and yield free cholesterol (Brown et al., 1975). The free cholesterol is either re-esterified and stored in lipid droplets or is transported to mitochondria for steroidogenesis. Human follicular fluid contains little LDL but cholesterol can be generated endogenously and used to synthesize progesterone (Simpson et al., 1980; Schuler et al., 1979; Turek & Strauss, 1982). The rate-limiting step in progestin synthesis is side-chain cleavage of cholesterol to pregnenolone, a C₂₁ compound, by side-chain cleavage cytochrome P-450 in the inner mitochondrial membrane. Pregnenolone is the key steroidogenic intermediate common to all steroids produced by follicles. Both granulosa and theca cells convert pregnenolone to progesterone by two enzymes, 3β-hydroxysteroid dehydrogenase (3β-HSD) and \(^5\), \(^4\)-isomerase (Samuels et al., 1951; Cheatum & Warren, 1966). Isomerase activity appears excessive (Philpott & Peron, 1971; Dimino & Campbell, 1976), such that the production of progesterone from pregnenolone is mainly regulated by 3β-HSD. The secretion of progesterone by granulosa
cells may also be modulated by conversion of progesterone to 20α-hydroxyprogesterone (20α-OH-P) by the 20α-hydroxysteroid dehydrogenase. The concentration of 20α-hydroxysteroid dehydrogenase is high in corpora lutea undergoing luteolysis (Biersing, 1977; Lahav, 1977; Eckstein, 1977). 20α-OH-P is less active as a progestational agent than the precursor progesterone. As 20α-reduced steroids are poor substrates for C17,20-lyase, 20α-hydroxysteroid dehydrogenase may play a significant role in determining the available amount of C21-substrates for androgen conversion. Granulosa and theca cells provide precursors for androgen synthesis by theca/interstitial cells. Granulosa cells are the main source of progesterone during the early follicular phase, when few LH receptors are on the membrane of theca/interstitial cells.

1.3.2 Biosynthesis of Local nonsteroidal substance

Granulosa cells secrete regulatory proteins, prostaglandins and catecholamines. Table 1 lists some locally produced ovarian substances.

Among intraovarian substances, inhibin-related peptides, prostaglandins and neuropeptides such as gonadotropin releasing hormone (GnRH) have been the subject of intense investigation. Most of these agents do not act in the traditional endocrine fashion. They may act locally to modulate the growth and function of somatic and germ cells. Potential intraovarian communication may take on one of the following configurations:

1. Paracrine communication which involves actions of substances secreted from producer cells on neighbour target cells.
Table 1. LOCAL REGULATORY OVARIAN SUBSTANCES

<table>
<thead>
<tr>
<th>PEPTIDES</th>
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<th>OTHERS</th>
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<tbody>
<tr>
<td>Inhibin</td>
<td>Angiotensins</td>
<td>Prostaglandins</td>
</tr>
<tr>
<td>Activin</td>
<td>Interleukins</td>
<td>Estradiol</td>
</tr>
<tr>
<td>Fibroblast growth factor</td>
<td>Follistatin</td>
<td>Progesterone</td>
</tr>
<tr>
<td>Transforming growth factor α/β</td>
<td>Insulin-like growth factor</td>
<td>Catecholamines</td>
</tr>
<tr>
<td>Mullerian Inhibiting Factor</td>
<td>Epidermal growth factor</td>
<td></td>
</tr>
<tr>
<td>Gonadotropin-releasing hormone</td>
<td>Tumor necrosis factor α</td>
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</tbody>
</table>

2. Autocrine communication which involves the actions of a substance on the cells of origin.

Although ovarian cells produce a variety of nonsteroidal products, relatively little attention has been given to this issue. The function of most of these local substances remains a mystery. A selected few are discussed below.

1.3.2.1 Biosynthesis of inhibin-related peptides

1.3.2.1.1 Isolation and characterization of inhibin-related peptides

Inhibin, activin and follistatin have been isolated from follicular fluid of several mammalian species. There are two forms of inhibin (A and B), inhibin A composed of a common α subunit (MW. 18000) and one of two distinct β subunits, βA (MW. 14000); inhibin B composed of α and βB subunit (MW. 14000). Activin also has two forms, one
consisting of a homodimer of βA subunit (Activin A) and another a heterodimer β subunit, βA and βB subunits (Activin AB). Activin B, the inhibin βB subunit homodimer, has not yet been isolated from natural sources. The protein family includes transforming growth factor β (TGF-β), mullerian inhibiting factor and several other members (Ling et al., 1986; Vale et al., 1986). Inhibin subunit structures show a high degree of inter-species homology, with the α-subunits being 80% homologous among human, bovine, porcine, murine and ovine inhibin. The βA-subunit is 100% homologous, aside from one amino acid substitution in the ovine βA subunit, and the βB subunit is more than 95% homologous (Forage et al., 1987). The α, βA and βB subunit genes have been cloned (Mason et al., 1985) and recombinant inhibin A and activin A recently became available for investigation (Broxmeyer et al., 1988).

Follistatin is a single-chain, glycosylated protein with at least three isoforms (molecular weights ≈ 32000, 35000 and 39000) which are structurally different from the inhibins (Robertson et al., 1983; Ueno et al., 1987). Follistatin was first isolated from porcine and bovine follicular fluid (Robertson et al., 1987; Vale et al., 1986). Natural follistatin suppresses FSH release in vitro with only 5-30% potency in comparison with inhibin (Robertson et al., 1990). Follistatin, inhibins and activins are products of granulosa cells.

1.3.2.1.2 Regulation of biosynthesis of inhibin-related peptides

The major source of ovarian inhibin is the granulosa cell (Bicsak et al., 1986). Inhibin concentration was found to increase with follicular size and estradiol
concentrations (Saito et al., 1991). Inhibin is also produced by the corpus luteum (Davis et al., 1987; Schwall et al., 1990). Cultured human luteinizing cells produced inhibin (Adashi, 1985). Inhibin α- and βA-subunit mRNAs were identified in rat and human corpora lutea (Mock et al., 1983). FSH is the regulator of inhibin production. Low doses of LH following FSH exposure cause inhibin release from granulosa cells (Bicsak et al., 1986; Zhiwen et al., 1988a). Higher doses of LH/human chorionic gonadotropin (hCG) inhibit FSH-induced inhibin production in vitro (Zhiwen et al., 1987) and in vivo (Lee, 1983). Several local ovarian substances also affect inhibin production by granulosa cells in vitro. Insulin-like growth factor I (IGF-I) stimulated inhibin production by rat granulosa cells in a time- and dose-dependent manner (Zhiwen, 1987). TGFβ, an activin homolog, dose-dependently increased basal and FSH-stimulated inhibin production (Zhiwen, 1988b). 8-bromo-cAMP (8-Br-cAMP) and prostaglandin E₂ (which increases cellular adenyl cyclase activity) dose-dependently stimulated inhibin production, as did phosphodiesterase inhibitor and forskolin (Klein et al., 1991).

Epidermal growth factor (EGF) inhibits both basal and FSH-stimulated inhibin production (Klein et al., 1991; Kim et al., 1989; Flanders et al., 1989). EGF also inhibits the 8-Br-cAMP- and prostaglandin E₂-stimulated inhibin production (Flanders et al., 1989). TGFα from theca/interstitial cells has an EGF-like activity in porcine granulosa cells (Hsu et al.). Inhibin production by granulosa cells can be regulated at a local level by other members of the inhibin/TGFβ family. Activin-stimulated and follistatin-suppressed inhibin production by rat granulosa cells in vitro have been reported (Sugino, 1988a; Xiao et al., 1990).
Activin production likely occurs during latter stages of folliculogenesis. Schwall (1990) reported β subunit expression (without α subunit expression) in small antral follicles in the primate ovary. However, little is known about regulation of activin production.

The follistatin gene sequence has been deduced from porcine (Esch 1987) and murine (Shimasaki, 1989) cDNA clones. Northern blot analyses showed that ovarian follistatin mRNA transcription was stimulated by equine chorionic gonadotropin (ECG) treatment of immature rats but not affected by an ovulatory hCG dose (10 IU/rat) after ECG (Shimasaki, 1989). In situ hybridization studies showed that follistatin mRNA was low in primordial follicles but dramatically increased in granulosa cells of antral and preovulatory follicles. Follistatin mRNA was not detected in theca, stroma or interstitial cells and some corpora lutea showed slight hybridization. Granulosa cells, however, gave a strong positive signal indicative of the origin of follicular follistatin. There are no reports on the regulation of follistatin production in vitro. Follistatin production by differentiated granulosa cells from preovulatory follicles is FSH- but not LH-dependent (Klein et al., 1991). Follistatin is an activin binding protein in the rat ovary (Nakamura et al., 1990; Saito et al., 1991).

1.3.2.2 Biosynthesis of intraovarian growth factors

1.3.2.2.1 Insulin-like growth factor-I (IGF-I)

IGF-I is a 70-amino acid polypeptide believed to play many metabolic roles. The
ovary is a site of IGF-I production, reception and action (Adashi et al., 1985). The granulosa cell is the only cellular site of IGF-I gene expression. Granulosa and theca-interstitial cells possess specific receptors for IGF-I (Adashi et al., 1985).

1.3.2.2.2  Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α)

EGF is a single polypeptide chain of 53 amino acids. In situ hybridization of tissue sections indicates that RNA complementary to cloned EGF probes may be present in many tissues. EGF membrane receptors exists on bovine, ovine and murine granulosa cells (Mock & Niswender, 1983). TGF-α, an EGF structural analog, is a single-chain, 50 amino acid polypeptide binding to a common EGF/TGF-α receptor. TGF-α is structurally the same as EGF (St-Arnaud et al., 1983), raising the possibility that theca-interstitial cell-derived TGF-α may exert paracrine effects on adjacent granulosa cells.

1.3.2.2.3  Transforming growth factor-β1 (TGF-β1)

TGF-β1, a homodimeric polypeptide comprised of two identical 112 amino acid chains, is produced in the ovary (Kudlow et al., 1987; Skinner et al., 1987). Ovarian theca-interstitial and granulosa cells may be sites of TGF-β1 production and action (Magoffin et al., 1989).

1.3.2.2.4  Basic fibroblast growth factor (bFGF)

bFGF, a 146-amino acid polypeptide, is a mitogen for a wide variety of tissues.
An N-terminal-truncated form lacking the first 15 residues has been identified in the corpus luteum (Gospodarowicz et al., 1985). Recent studies indicate that theca-interstitial cells are also a site of bFGF reception and action (Adashi 1991).

1.3.2.3 **Biosynthesis of renin-angiotensins**

The wide variety of tissues identified to have a renin-angiotensin system include brain, adrenal gland, arterial wall, mesenteric vasculature, heart, testis, uterus and ovary (Weishaar et al., 1991; Sramek et al., 1988). Prorenin, renin-like activity and angiotensin II (A II) have been found in human follicular fluid at concentrations greater than in blood, suggesting that the ovary produces these substances (Itskovitz et al., 1988; Glorioso et al., 1986; Fernandez et al., 1985; Do et al., 1988). Circulating prorenin and renin concentrations were increased 10-fold in gonadotropin-stimulated women before ovulation (Culler et al., 1986). Renin is an aspartyl protease, cleaving its hepatic substrate angiotensinogen to form angiotensin I (A I). A I is converted to A II by angiotensin-converting enzymes. The A II, in turn, is converted to angiotensin III (A III) by the same enzyme (Adashi, 1991).

1.3.2.4 **Biosynthesis of prostaglandins and leukotrienes**

Prostaglandins (PGs) were discovered by Von Euler in the 1930s as active components of human seminal fluid. They are important products of the ovarian cells and their secretion is under hormonal control (Triebwasser et al., 1978; Clark et al., 1978). The PG precursor is arachidonic acid (AA), which is a C\textsubscript{20.4} polyunsaturated fatty acid.
Esterification of cell membrane phospholipids in the 2-acyl position allows glycerol and AA to be released. The free AA concentration in cells is less than $10^6 \text{M}$. The free acid concentration in tissue represents a balance between acid release by hydrolysis and re-esterification. Free AA undergoes two metabolic pathways as outlined in Figure 3. The cyclooxygenase pathway forms the endoperoxide intermediate prostaglandin $\text{H}_2$ (PGH$_2$), which is then converted by isomerase to biologically active molecules, prostaglandin $\text{E}_2$ (PGE$_2$), prostaglandin $\text{D}_2$ (PGD$_2$), prostaglandin $\text{F}_{2\alpha}$ (PGF$_{2\alpha}$), prostaglandin $\text{I}_2$ (PGI$_2$) and thromboxane $\text{A}_2$ (TXA$_2$). The letters following the abbreviation PG indicate the position of the oxygen-containing substitutes present in the cyclonptane ring. The 2-series PGs are formed from AA. The 1-series and 3-series PGs are formed from 8,11,14-eicosatrienoic and 5,8,11,14,17-eicosapentaenoic acid, respectively. Another metabolic pathway of AA is provided by lipoxygenase enzymes. The products of the lipoxygenase enzymes are hydroperoxyeicosatetra-enoic acids (HPETEs) which can then be converted into hydroxyeicosatetraenoic acids (HETEs), leukotrienes (LTs) and lipoxins (Fig. 4).

hCG-induced PG production occurs in granulosa and theca cells of preovulatory follicles (Hedin et al., 1987). Cyclooxygenase, the enzyme which forms PGs from AA, is inhibited by nonsteroidal anti-inflammatory drugs such as aspirin and indomethacin (Flower & Vane, 1974). Lipoxygenase activity is inhibited by compounds such as nordihydroguaiaretic acid (NDGA) (Salari et al., 1984).

1.3.2.5 **Biosynthesis of GnRH-like peptides**

Peripheral circulating GnRH concentrations are insufficient to bind to ovarian
GnRH receptors (Aten, 1986). Direct physiological actions of GnRH at the ovary may depend upon production of GnRH-like substances by the ovary. Murine, bovine and

Figure 4. Prostaglandins and arachidonic acid metabolic cascade.

depend upon production of GnRH-like substances by the ovary. Murine, bovine and

1.4 Ovarian regulation

The menstrual cycle is a repetitive operation of the hypothalamic-pituitary-ovarian axis associated with target tissue changes. The menstrual cycle is divided into follicular, ovulatory and luteal phases (Fig. 2). The menstrual cycle is controlled by GnRH secretion from the hypothalamus. GnRH is transported to the anterior pituitary cells through the hypothalamic-hypophyseal portal system and stimulates gonadotropin secretion from the pituitary. Gonadotropins regulate the production of sex steroids and some local substances by the ovary. However, current information suggests that ovarian products play an active role in the regulation of ovarian function. The hypothalamus and pituitary may play a permissive role.

1.4.1 Neuroendocrine regulation of the ovary
GnRH and prolactin (PRL) regulating factors are secreted from the neurons in the anterior hypothalamus and arcuate nucleus. GnRH and PRL regulating factors are axonally transported to the median eminence. GnRH and PRL regulating factors are secreted in the median eminence, diffuse into the hypothalamic-pituitary portal vasculature and are delivered to the adenohypophyseal gonadotropes and lactotropes.

1.4.1.1 **GnRH**

GnRH is a decapeptide secreted in a pulsatile manner into the hypothalamic-pituitary portal circulation. Several neurotransmitters are involved in GnRH secretion. The amplitude and frequency of the pulses are regulated by catecholamines and neuropeptides. These inputs to the GnRH neurons are affected by the feedback of estradiol and progesterone (Yen, 1982). Estradiol and dihydrotestosterone (DHT) are concentrated in the nuclei of catecholaminergic neurons; the target neurons for these two steroids are surrounded by catecholaminergic terminals (Heritage et al., 1980). GnRH binds to specific receptors on gonadotropes and stimulates LH and FSH secretion. Gonadotropin responses to GnRH are maximal when GnRH receptor numbers are highest. Increased GnRH concentration and pulse frequency increases GnRH receptor numbers, a process known as upregulation. GnRH receptors will be downregulated if continuous GnRH administration is substituted for pulsatile stimulation (Clayton & Catt 1981).

1.4.1.2 **Prolactin (PRL) regulating factors**

Human pituitary lactotropes are tonically suppressed by hypothalamic factors.
Dopamine, a neurohormone produced in the arcuate nucleus, is an important inhibitor. Dopamine is secreted into the portal circulation in concentrations (0.7 ng/ml) sufficient to inhibit PRL release (Neill J et al., 1961). Dopamine binds to specific membrane receptors on lactotropes. Hypothalamic thyrotropin releasing hormone (TRH) has both PRL and thyroid-stimulating hormone (TSH) releasing function.

1.4.2 Gonadotropic hormone regulation of the ovary

The control of gonadal function and reproduction in primates and other mammalian species is exerted predominantly through three gonadotropin hormones secreted by the pituitary, FSH, LH and PRL.

1.4.2.1 Chemistry of gonadotropic hormones

FSH and LH have many chemical similarities and differ from PRL. FSH and LH have two polypeptide subunits bound with a noncovalent, high affinity bond. One subunit, designated α, is common to the glycoproteins LH, FSH, hCG and TSH (Ward, 1978; Jutisz & Tertrin-clary, 1974). The other subunit, designated β, differs in its amino acid sequence in each glycoprotein and carries the specific hormone activity. The molecular weight of LH and hCG is about 28,000 while that of FSH is 33,000. The genes encoding human LH and hCG β-subunits have relatively little genetic drift and differ mainly in the expression of the C-terminal translated region. This sequence codes for an additional SER-rich region in hCG that attaches O-linked carbohydrate chains that are not present in the LH molecule. The molecular weight of PRL is
approximately 22,000. PRL is a single polypeptide chain with no carbohydrate moieties.

Three internal disulphide bridges are incorporated into the secondary structure of PRL.

<table>
<thead>
<tr>
<th>TABLE 2. FSH-STIMULATED FUNCTIONAL PARAMETERS IN CULTURED GRANULOSA CELLS</th>
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<tbody>
<tr>
<td><strong>1. Enhancement of steroidogenesis</strong></td>
</tr>
<tr>
<td>a. Estrogen biosynthesis</td>
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<tr>
<td>Induction of aromatases</td>
</tr>
<tr>
<td>b. Progesterone and 20α-OH-P biosynthesis</td>
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<tr>
<td>Induction of cholesterol side-chain cleavage enzymes and</td>
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<tr>
<td>mitochondrial cytochrome P450 activity</td>
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<tr>
<td>Induction of 3β-hydroxysteroid dehydrogenase</td>
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<tr>
<td>Induction of 20α-hydroxysteroid dehydrogenase</td>
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<tr>
<td><strong>2. Induction of specific plasma membrane receptors</strong></td>
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<td>a. LH receptor formation</td>
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<td>b. Prolactin receptor formation</td>
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<tr>
<td>c. β2-adrenergic receptor formation (coupling)</td>
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<tr>
<td>d. Lipoprotein receptor formation</td>
</tr>
<tr>
<td>e. FSH receptor formation</td>
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<tr>
<td>f. EGF receptor formation</td>
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<tr>
<td><strong>3. Secretion of nonsteroidal cell products</strong></td>
</tr>
<tr>
<td>a. Inhibin</td>
</tr>
<tr>
<td>b. Plasminogen activator</td>
</tr>
<tr>
<td>c. Prostaglandins</td>
</tr>
<tr>
<td>d. Proteoglycans (mucopolysaccharides)</td>
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<tr>
<td><strong>4. Stimulation of general functions</strong></td>
</tr>
<tr>
<td>a. DNA synthesis</td>
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<tr>
<td>b. Protein synthesis</td>
</tr>
<tr>
<td>c. Glucose uptake and lactate formation</td>
</tr>
<tr>
<td>d. Cell roundup and aggregation</td>
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<tr>
<td>e. Gap junction formation</td>
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<tr>
<td>f. Microvilli formation</td>
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<tr>
<td><strong>5. Plasma membrane-related processes</strong></td>
</tr>
<tr>
<td>a. Adenyl cyclase activation and cAMP formation</td>
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<tr>
<td>b. Formation of cAMP binding protein</td>
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<tr>
<td>c. Phosphodiesterase activation</td>
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1.4.2.2 \textit{Role of gonadotropin hormones}

1.4.2.2.1 \textit{Role of FSH}

FSH induces ovarian follicular maturation and granulosa cell development. FSH stimulates the granulosa cells to produce estrogens, progestins and various nonsteroidal substances. The action of these granulosa cell products ensures optimal folliculogenesis and oocyte maturation. FSH receptors are present in the granulosa cells. The FSH-regulated granulosa cell functions are summarized in Table 2.

Based on rat studies, a two-cell, two-gonadotropin hypothesis for ovarian estrogen synthesis is summarized (Fig. 5). According to this model, LH stimulates androgen synthesis from cholesterol in theca-interna cells (Tsang et al., 1980; Makris & Ryan, 1975). Androgens diffuse across the lamina basalis and are converted to estrogen by granulosa cells. FSH increases the granulosa cell aromatase activity \textit{in vitro} and \textit{in vivo}. FSH also stimulates progesterone production in cultured granulosa cells (Channing 1974; Veldhuis et al., 1980). FSH regulates granulosa cell progestin synthesis by increasing the activities of cholesterol side-chain cleavage enzymes and 3β-hydroxysteroid dehydrogenase (Jones et al., 1982).

FSH increases LH, PRL and EGF receptor content in granulosa cell membrane. FSH increases the cholesterol utilization by increasing LDL receptor numbers in the cell
membrane. FSH may increase its own receptor numbers in granulosa cells (Amsterdam et al., 1981; White & Ojeda, 1981).

FSH stimulates granulosa cell division in vivo and increases gap junction formation (Amsterdam et al., 1981). cAMP is the second messenger of FSH action in granulosa cells. FSH stimulates cAMP formation, which activates cAMP-dependent protein kinase A in granulosa cells (Richards et al., 1983; Conti et al., 1984).

Figure 5. The two-cell/two-gonadotropin hypothesis of follicular estrogen production.

1.4.2.2.2 Role of LH

LH stimulates preovulatory follicular growth, induces ovulation and regulates
corpus luteum function. hCG has the same effects as LH because LH and hCG interact with the same receptor in luteal, granulosa and theca interna cells. LH effects on cultured granulosa-luteal cells are listed in Table 3. LH increases aromatase activity in cultured granulosa cells exposed to FSH (Wang et al., 1981; Zhuang et al., 1982). LH stimulates progesterone production from the corpus luteum in vivo, dissociated luteal cells and FSH-primed granulosa cells in vitro (Zhuang et al., 1982; Schomberg et al., 1967; Mason et al., 1961). LH stimulates progesterone production by increasing cholesterol esterase activity and lipoprotein receptors, resulting in increased free intracellular cholesterol (Behrman & Armstrong, 1969; Caffrey et al., 1979). LH also increases the activities of side-chain cleavage and 3β-hydroxysteroid dehydrogenase enzymes to convert cholesterol to pregnenolone (Madej, 1980; Caffrey et al., 1979). LH does not affect the activity of 20α-OH-steroid dehydrogenase. LH increases PRL receptors in granulosa cells in vivo and in vitro. LH may increase granulosa cell responsiveness to α-adrenergic agents (Sheela Rani et al., 1983). Granulosa and luteal cells secrete many nonsteroidal substances. Prostaglandin synthesis is stimulated by LH (Marsh et al., 1981; Jones & Hsueh, 1982). LH also induces oocyte maturation and the release of plasminogen activator, proteoglycans and relaxin (Yanagashita et al., 1981; Gebauer et al., 1978). LH alters granulosa cell morphology and cell function. LH reduces the size of granulosa cell gap junctions. This may serve to signal the resumption of meiotic maturation and to allow granulosa cell desegregation. LH stimulates transcription and translation of steroidogenic enzymes and the enzymes for PG production. LH inhibits DNA synthesis by luteinized bovine and murine granulosa cells (Rao et al., 1978; Clark et al., 1976;
LH stimulates cAMP production (Marsh, 1976). LH does not affect phosphodiesterase activity (Marsh, 1970). Microfilament and Ca\textsuperscript{2+} may be involved in adenyl cyclase activation.

**TABLE 3. LH-MODULATED FUNCTIONAL PARAMETERS IN CULTURED RAT GRANULOSA CELLS**

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<td>Induction of 3β-hydroxysteroid dehydrogenase</td>
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<tr>
<th>2. Modulation of plasma membrane receptors</th>
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<tr>
<td>a. Increase in PRL receptor formation</td>
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<tr>
<td>b. Increase in β\textsubscript{2} adrenergic receptor formation</td>
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<td>c. Down-regulation or stimulation of LH receptors</td>
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<td>d. Increases in lipoprotein receptor formation</td>
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<th>3. Secretion of non-steroidal local regulators</th>
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<tbody>
<tr>
<td>a. Plasminogen activator</td>
</tr>
<tr>
<td>b. Mucopolysaccharides</td>
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<tr>
<td>c. Prostaglandins</td>
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<td>d. Relaxin</td>
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<th>4. Modulation of general cell functions</th>
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<tr>
<td>a. Increase in protein synthesis</td>
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<td>b. Increase in glycolysis</td>
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<tr>
<td>c. Alteration of cell shape</td>
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<td>d. Reduction in size of gap junctions</td>
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<td>e. Inhibition of DNA synthesis</td>
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<th>5. Plasma membrane-related processes</th>
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<tr>
<td>a. Adenyl cyclase activation and cAMP formation</td>
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<tr>
<td>b. Stimulation of cAMP-dependent protein kinase activity</td>
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<td>c. Stimulation of phospholipid turnover</td>
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1.4.2.2.3 Role of prolactin (PRL)

PRL has little or no luteotropic effect on human ovarian function, although PRL acts as a luteotropic hormone in rodents and several other mammalian species. However, PRL does have important regulatory effects on pituitary-ovarian function in women. Hyperprolactinemia decreases gonadotropin secretion by impairing GnRH release.

1.4.3 Role of steroid Hormones

1.4.3.1 Role of estrogen

Estrogens maintain secondary sexual characteristics and exert feedback action on the hypothalamic-pituitary unit to affect synchronized preovulatory gonadotropin release. Table 4 describes various granulosa cell functions regulated locally by intraovarian estrogens.


Estrogen increases intercellular gap junction formation in granulosa cells (Merk et al., 1972). Estrogen also enhances gonadotropin-initiated and maintained follicular development (Payne & Runser, 1958). Estrogens also regulate granulosa cell estrogen production. Various native and synthetic estrogens augment FSH- and LH-induced
aromatase activity (Adashi & Hsueh, 1982; Zhuang et al., 1982). This may partially explain maintenance of the dominant follicle in the ovary. Once a chosen follicle is producing a significant amount of estrogen, this follicle has the capacity to produce more estrogens than neighbouring follicles. However, it is not known how the selected follicle continues to secrete elevated estrogens in the face of declining FSH concentrations (Zeleznik, 1981). Receptors of EGF and IGF-1 localized on granulosa cells suggest that these growth factors have a role on follicular development (Jones et al., 1982; St-Arnaud et al., 1983; Adashi et al., 1985) and steroidal hormone production of granulosa cells (May et al, 1985). Other local substances such as inhibin related peptides have not been studied in this point. It is possible local substances involve in the continuously elevated estrogen concentration within selected follicles.

Estrogens secreted by antral follicles during the early follicular phase

<table>
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<tr>
<th>TABLE 4. DIRECT EFFECTS OF ESTROGENS ON FOLLICULAR GRANULOSA CELLS</th>
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<tr>
<td>1. Granulosa cell proliferation (growth of follicles)</td>
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<td>2. Antiatretic action</td>
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<td>3. Increases in coupling between granulosa cells through intercellular gap junctions</td>
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<td>4. Increases in FSH induction of antrum formation</td>
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<td>5. Increases in granulosa cell estrogen receptor content</td>
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<td>6. Increases in FSH stimulation of granulosa cell LH and FSH receptors</td>
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<td>7. Increases in FSH-induced cAMP formation</td>
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<td>8. Increases in FSH-induced aromatase</td>
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<td>9. Increases in FSH-induced progesterone production</td>
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suppress pituitary FSH and LH secretion. During the late follicular phase, increased estrogens are essential for the generation of the preovulatory estrogen surge. This elevated estrogen exerts positive feedback to increase LH and FSH release and may increase gonadotropin-stimulated estrogen biosynthesis.

Estrogens are essential to gonadotropin-mediated maintenance of corpus luteum function by enhancing gonadotropin-stimulated progesterone synthesis. This suggests that estrogens may act locally to enhance the sensitivity of luteal cells to pituitary gonadotropins.

1.4.3.2 Role of progestins

Although the granulosa cells produce large quantities of progesterone, its local action in modulating follicular growth and granulosa cell functions is not clear. Unilateral ovarian progesterone implants in monkeys inhibit follicular growth without affecting the contralateral ovary (Goodman & Hodgen, 1979). Further, follicular growth begins immediately following lutectomy, suggesting that locally elevated progesterone concentrations inhibit folliculogenesis. The progesterone receptor has been identified in the cytoplasm of rodent, bovine, lapin and human granulosa cells (Schreiber & Erickson, 1979; Philibert et al., 1977; Jacobs & Smith, 1980; Jacobs et al., 1980; Pasqualini et al., 1980). Progesterone receptors translocate to the nucleus (Naess 1981). The presence of ovarian progesterone receptors and high progesterone concentrations (4x10^6M) in follicular fluid (McNatty, 1975) suggest an intrafollicular regulatory role for progesterone.

Progesterone regulates its own production by granulosa cells. Pretreatment with
progesterone for 2 days enhanced FSH- and LH-stimulated progesterone and 20α-OH-P production in cultured granulosa cells (Fanjul et al., 1975). These findings may relate to the autonomy of luteal cell progesterone production and represent another example of an autocrine control mechanism.

Sustained progesterone secretion during the preovulatory stage may exert a local action at the ovarian level to induce ovulation. Progesterone treatment enhanced LH-stimulated ovulation in hypophysectomized rats in vivo (Fanjul et al., 1975). This steroid may also contribute to ovulation in hamster ovaries obtained during proestrus and incubated with progesterone in vitro (Baranczuk & Fainstat, 1976).

Progestin inhibits FSH-stimulated estrogen production and LH receptor formation in cultured rat granulosa cells (Schreiber et al., 1981; Schreiber et al., 1982).

1.4.3.3 Role of androgen

Androgens are produced by theca and interstitial cells of the ovary and play an important role in follicular development. Specific androgen binding sites have been identified in the cytoplasm of granulosa cells and translocate to the nucleus after androgen binding (Schreiber et al., 1976).

Androgens stimulate follicular atresia and antagonize estrogen-induced ovarian weight gain. This is accompanied by necrosis of granulosa cells and ovum death (Payne et al., 1958, 1959). Pretreatment of intact rats with dihydrotestosterone (DHT) prevents FSH induction of LH receptors in granulosa cells (Farookhi, 1980). This inhibitory effect of DHT is antagonized by estrogen treatment. Androgens may counteract estrogen actions
by decreasing ovarian estrogen receptor content (Saiduddin et al., 1978). Androgen and estrogen concentrations in follicular fluid vary with the stage of follicular development. Elevation of the androgen to estrogen ratio is invariably associated with atresia (McNatty et al., 1975; Kemeter et al., 1975). Although androgen treatment induces atresia, androgen stimulates ovarian aromatase activity. Decreased androgen levels are accompanied by decreased aromatase activity (Katz et al., 1976). Aromatase activity increases with restoration of androgen levels (Katz et al., 1979).

Androgens also stimulate progestin biosynthesis by granulosa cells. Administration of androgens to intact rats enhances the ability of FSH and LH to increase progesterone production by subsequently isolated granulosa cells (Leung et al., 1979). Thus, concomitant treatment with FSH and androgen increases the responsiveness of rat granulosa cells to LH, as measured by progesterone and cAMP production (Goff et al., 1979). Androgens act synergistically with FSH to stimulate progesterone production in cultured rat granulosa cells (Welsh et al., 1982). The mechanism of the stimulatory effect of androgens on progestin synthesis has been examined. Androgen and FSH act synergistically to stimulate lipoprotein utilization by cultured rat granulosa cells (Schreiber et al., 1982). Androgens have no effect on FSH binding, cAMP production or phosphodiesterase activity (Nimrod, 1977). Thus, androgens exert both stimulatory and inhibitory effects in granulosa cells.

The effect of androgens in vivo may vary according to the stage of follicle development. Androgens in the presence of high FSH concentrations promote estrogen and progestin synthesis and the development of preovulatory follicles. High androgen
concentrations in the presence of low FSH concentrations may lead to atresia.

1.4.4 Role of nonsteroidal local regulators

1.4.4.1 Role of gonadotropin-releasing hormone (GnRH)

Hypothalamic GnRH acts on the pituitary gland to increase gonadotropin release. Direct GnRH actions have also been demonstrated in rat granulosa cells (Hsueh et al., 1981a).

Treatment with GnRH or GnRH agonists inhibits FSH-stimulated estrogen and progestin production (Hsueh et al., 1979a, b). Concomitant treatment with GnRH or GnRH agonists inhibits FSH-stimulated LH and PRL receptor formation in cultured rat granulosa cells (Hsueh et al., 1980, 1983). The inhibitory effect of GnRH on LH and PRL receptor formation and FSH-stimulated steroidogenesis is blocked by concomitant treatment with GnRH antagonists (Jones et al., 1981; Navickis et al., 1982; Clark et al., 1980; Massicotte et al., 1980). These results suggest that GnRH effects are mediated by GnRH binding sites in granulosa cells. GnRH modulates various other FSH-stimulated responses in granulosa cells, such as inhibition of FSH-stimulated cAMP formation (Clark et al., 1980; Massicotte et al., 1980; Knecht et al., 1981) and augments FSH-stimulated prostaglandin production (Clark et al., 1980). GnRH also inhibits FSH-stimulated ovarian EGF receptor content (St-Arnaud et al., 1983). The mechanism by which GnRH inhibits various FSH-dependent processes is unclear; however, GnRH decreases FSH receptors in granulosa cells (Renta et al., 1983). This effect of GnRH may partially account for its
inhibitory effects.

GnRH and GnRH agonists also inhibit the stimulation of steroidogenesis by cholera toxin, PGE\textsubscript{2} and cAMP analogs (Hsueh et al., 1980; Jones et al., 1981, 1983; Knecht et al., 1981a; Hiller et al., 1981). The response of granulosa cells to GnRH (ie. stimulation or inhibition of steroidogenesis) varies with the maturational stage of these cells as well as with the duration of exposure to GnRH (Popkin et al., 1983; Rani et al., 1983).

Specific high affinity GnRH binding sites have been demonstrated in granulosa (Pieper et al., 1980; Jones et al., 1981) and luteal (Clayton et al., 1980) cells. Autoradiographic studies demonstrate that these ovarian GnRH receptors are present in luteal, thecal and granulosa cells at all stages of cellular differentiation (Pelletier et al., 1982). Photoaffinity labelling of ovarian GnRH receptors has identified two specific components with apparent molecular weights of 60,000 and 54,000 (Hazum et al., 1982). Since only one form of these GnRH receptors is found in the pituitary, the extra component of the ovarian receptor may be related to the different functions of GnRH in the gonad. Treatment with GnRH in vivo increases or decreases GnRH receptor content in the granulosa cells, depending on the dose and time of treatment (Pieper et al., 1980). FSH maintains GnRH receptor content in cultured granulosa cells (Ranta et al., 1982).

GnRH-induced decreases in progesterone production may result from the inhibition of 3\textbeta-hydroxysteroid dehydrogenase activity and enhancement of 20\alpha-hydroxysteroid dehydrogenase activity (Jones et al., 1982a, 1981). The major effects of GnRH may be decreased pregnenolone production, coupled with increased conversion of progesterone
to 20α-OH-P. Modulation of these steroidogenic enzymes by GnRH is blocked by concomitant treatment with GnRH antagonists. An inhibitory effect of GnRH on FSH-induced cAMP production has been observed in cultured porcine and murine granulosa cells (Knecht et al., 1981b). These effects may be due to inhibition of adenyl cyclase activity and stimulation of phosphodiesterase activity (Knecht et al., 1981c). GnRH-inhibited cAMP production by rat granulosa cells may be Ca²⁺-dependent (Ranta et al., 1983). GnRH and GnRH agonists inhibit ovarian luteal functions in pregnant and non-pregnant rats (Hsueh et al., 1980). GnRH inhibits LH-, PRL- and β₂ adrenergic-stimulated estrogen and progesterone production in rat granulosa cells (Jones et al., 1982b). GnRH also inhibits PRL-induced LH receptor content in rat granulosa cells (Jones et al., 1982b).

Other studies have demonstrated direct stimulatory effects of GnRH on various ovarian functions (Table 4).

Treatment of granulosa cells with GnRH stimulates estrógen, progesterone and 20α-OH-P production (Dorrington et al., 1982; Clark 1982; Davis et al., 1983). GnRH stimulates phospholipid labelling in granulosa cells (Davis et al., 1983; Naor et al., 1982) as well as luteal cells (Leung et al., 1983). The GnRH effect on phospholipid turnover may serve as a plasma membrane transducing event to initiate steroidogenesis and other events. Additionally, cAMP may be involved in GnRH action in ovarian cells (Knecht et al., 1981b; Naor et al., 1982). GnRH also stimulates maturation of follicle-enclosed rat oocytes in vitro (Leung et al., 1983) and induces ovulation in hypophysectomized rats (Hillensjo et al., 1980, 1982; Corbin et al., 1981).

**TABLE 5. STIMULATORY EFFECT OF GNRH ON GRANULOSA CELL FUNCTIONS AND REPRODUCTIVE PROCESSES**

<table>
<thead>
<tr>
<th>1.</th>
<th>Stimulation of estrogen biosynthesis by increasing aromatase activity</th>
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<tr>
<td>2.</td>
<td>Stimulation of progesterone and 20α-OH-P synthesis by increasing 3β-hydroxysteroid dehydrogenase activity</td>
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<td>3.</td>
<td>Increases in protein synthesis</td>
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<td>4.</td>
<td>Increases in follicular lactate formation</td>
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<td>5.</td>
<td>Increases in plasma membrane phospholipid turnover</td>
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<tr>
<td>6.</td>
<td>Induction of Oocyte maturation and ovulation in hypophysectomized rats</td>
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<tr>
<td>7.</td>
<td>Stimulation of prostaglandin production</td>
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<td>8.</td>
<td>Stimulation of plasminogen activator activity</td>
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1.4.4.2 \textit{Role of inhibin-related peptides}

Activin inhibits and inhibin/follistatin stimulate the secretion of FSH by pituitary gonadotropes (Robertson et al., 1985; Ling et al., 1985; Ling et al., 1986; Vale et al., 1986).

1.4.4.2.1 \textit{Local action of inhibin}

There is increasing evidence of local actions of inhibin on ovarian and testicular function (Tsafriri et al., 1989; Sum et al., 1989; Ying et al., 1986; Shukovski, 1990).
Porcine (Ying et al., 1986, 1987) but not bovine (Hutchinson et al., 1987; Hillier et al., 1990) inhibin may suppress FSH-induced estrogen production by rat granulosa cells in vitro. Using purified bovine inhibin A (MW=32000, >200ng ml⁻¹), Sugino et al. reported (Hillier et al., 1991a, b; Hsueh et al., 1987). Although granulosa cells possess inhibin only weak suppression of progesterone production by rat granulosa cells in vitro. Inhibin increased LH-stimulated androgen production by human theca and rat testicular cells receptors (Sugino et al., 1988; Woodruff et al., 1990), effects of inhibin on these cells are unclear. However, inhibin may alter the steroidogenic response of murine and bovine granulosa cells to gonadotropins (Xiao et al., 1990).

Two paracrine actions of inhibin have been reported. Inhibin has been shown to enhance LH-induced androgen production by rat theca cell preparations. This effect of inhibin can be attenuated by activin (Hsueh et al., 1987). Inhibin A has been found to inhibit spontaneous maturational divisions of rat oocytes (O et al., 1989). This inhibition was dose-dependent with an IC₅₀ of 4 Nm, similar to concentrations found in bovine follicular fluid (Burger et al., 1988). These paracrine actions of inhibin are yet to be confirmed in other species.

### 1.4.4.2.2 Local action of activin

Activin A increased basal and FSH-stimulated aromatase activity, progesterone and inhibin production in undifferentiated murine granulosa cells (Xiao et al., 1990). Erythroid differentiation factor (EDF), which was shown to be activin (Sugino et al., 1988a), also induced FSH and LH receptors on rat granulosa cells in vitro (Sugino et al.,
1988b,180). Binding sites for EDF have been demonstrated on granulosa cells and EDF receptor numbers are increased by FSH (Sugino et al., 1988a). The report from the same laboratory provided controversial results. It suggested that activin inhibited FSH-stimulated progesterone production by rat granulosa cells (Hutchinson et al., 1987). However, activin A has a time- and dose-dependent inhibitory effect on progesterone and oxytocin production in differentiated bovine granulosa cells (Shukovski et al., 1990).

Activin may act in a paracrine fashion to suppress LH-induced androstenedione production in rat theca cells and to antagonize the action of inhibin in the presence of LH. These paracrine effects of the granulosa cells would indirectly regulate the supply of androgen substrate required by granulosa cells to produce estrogen.

1.4.4.2.3 Local action of follistatin

Follistatin acts as an activin-binding protein (Kogawa et al, 1991). The detection of follistatin and inhibin-β mRNA and protein in the rat ovary suggests a local regulatory action involving follistatin and activin interactions (Shimasaki et al., 1989). The effects of follistatin on basal and FSH-stimulated hormone production by rat granulosa cells have been investigated (Xiao et al., 1990). Follistatin decreased FSH-stimulated aromatase activity and inhibin secretion and increased progesterone production. However, follistatin had no effects on basal aromatase activity or progesterone and inhibin production (Xiao et al., 1990). Follistatin enhanced LH-stimulated progesterone production in undifferentiated but not differentiated bovine granulosa cells (Shukovski et al., 1991).

Follistatin accelerated the rate of meiotic maturation of rat oocytes in culture (O
et al., 1989). This is opposite to reported effects of inhibin on oocyte maturation. Yet follistatin and inhibin have similar actions on the pituitary gland. The effects of follistatin on the human ovary are unknown. Like activins and inhibins (Li et al., 1992; Hildj 1979; Hiller et al., 1991b), follistatin may well affect steroid hormone production in the human ovary.

1.4.4.3 Role of intraovarian growth factors

The ovarian follicle undergoes cell proliferation during growth. Examination of endocrine growth factors has centred on their growth-promoting actions in the ovary. However, these growth factors may also modulate cell differentiation. This action may be uncoupled from effects on cellular proliferation (Hildj 1979; Chait et al., 1980; Johnson et al., 1980; Tashijian et al., 1982; Turo et al., 1982; Schmid et al., 1984; Baird et al., 1985). The following growth factors or growth factor families have been examined relative to their involvement in ovarian physiology.

1.4.4.3.1 Role of EGF and TGF α

Studies of EGF and ovarian function have been performed largely in vitro using granulosa cells. EGF has been reported to attenuate FSH-induced LH/hCG receptor formation in rat granulosa cells (Mondschein et al., 1981). EGF not only decreased LH/hCG receptor numbers but also affected granulosa steroidogenesis. EGF inhibited FSH-, dibutryl cAMP-, cholera toxin-, and PGE₂-stimulated estrogen production in rat granulosa cells (Hsueh et al., 1981b). The inhibitory action of EGF does not exclusively
reside within granulosa cells. LH-stimulated androgen secretion by rat theca-interstitial cells is also inhibited by EGF (Erickson et al., 1983). Although EGF attenuates FSH action, EGF has been shown to stimulate FSH receptor content of porcine granulosa cells (Osterman et al., 1979). Therefore, it has been difficult to formulate a consensus as to the role of EGF in folliculogenesis.

The effect of EGF on progestin production is less clear. EGF has been reported to have no effect or to inhibit basal and FSH-stimulated progesterone production by granulosa cells after two days of culture (Jones et al., 1982c; Knecht et al., 1983). EGF stimulated progestin production and enhanced FSH-stimulated 20α-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase activities in estrogen primed immature, hypophysectomized rats (Schomberg et al., 1984). The reason underlying the differences in cell responsiveness to EGF in terms of progestin secretion are unknown but may involve the endocrine status of the animal and culture conditions of granulosa cells. Based on the above information, EGF plays an inhibitory role with respect to granulosa cell function. However, this inhibitory role may be related only to specific periods of cell differentiation or follicular development.

EGF is a mitogenic factor in the ovary and is a major growth stimulating factor required for elevated granulosa cell proliferation. Despite the presence of other growth factors, such as IGF-I, insulin, and PDGF, granulosa cell proliferation is impaired in the absence of EGF (Gospodarowicz D et al., 1979).

TGFα has an amino acid sequence similar to EGF. TGFα binds to EGF receptors. Since TGFα acts via EGF receptors, the actions of TGFα mimic those of EGF. Recent
studies, however, suggested that the two growth factors may have different potencies, if not divergent actions. TGFα was found to be more potent than EGF in stimulating angiogenesis in the hamster cheek pouch assay. Since the formation of the corpus luteum requires massive blood vessel formation, TGFα may have an effect during the early stages of corpus luteum formation.

1.4.4.3.2 Role of insulin and IGFs

The use of insulin to supplement culture media for ovarian cells and the presence of ovarian receptors and insulin action on granulosa cell metabolism and steroidogenesis have been reviewed (Veidhuis et al., 1983; May et al., 1981). As there appears to be no report of local insulin production, the action of IGFs on ovarian function will be addressed.

Addition of somatomedin C (SmC)/IGF-I to granulosa cells from small (4-6 mm) bovine follicles caused mitogenic responses in serum-free cultures (Savion et al., 1981). Likewise, IGF-I and IGF-II or insulin enhanced [H³]-thymidine incorporation into DNA of porcine granulosa cells (Baranao et al., 1984). Addition of SmC/IGF-I augmented FSH-stimulated progesterone accumulation but had limited effects on basal progesterone production (Adashi et al., 1985a,b). Similarly, SmC/IGF-I stimulated progesterone production by porcine granulosa cell cultures (Baranao et al., 1984). IGF-II synergized with FSH in stimulating progesterone production (Veldhuis et al., 1983). Similarly, SmC/IGF-I synergized with estradiol-17β in enhancing porcine granulosa progesterone and pregnenolone synthesis (Veldhuis, 1985). SmC/IGF-I synergized with
FSH in enhancing aromatase activity of rat granulosa cells but had no effect on basal aromatase activity (Adashi et al., 1985c). Finally, SmC/IGF-I synergized with FSH in inducing LH receptors (Adashi et al., 1985d). Therefore, IGFs may be involved in the regulation of follicular growth and differentiation.

1.4.4.3.3 **Role of bFGF and TGFβ**

bFGF, a 146-amino acid polypeptide, is a mitogen for a wide variety of mesoderm-derived and neuroectoderm-derived cells. Although the physiological function of bFGF in the ovary remains unclear, evidence suggests that bFGF may play a central role in growth and development of granulosa-lutein cells (Gospodarowicz 1989). BFGF stimulates the replicative lifespan of cultured bovine, porcine, lapin, rodent and human granulosa cells (Gospodarowicz et al., 1977, 1978, 1979). More recent studies indicated that theca-interstitial cells are also a site of bFGF reception and action. bFGF is a potent attenuator of gonadotropin-supported androgen synthesis by theca-interstitial cells.

TGFβ is a polyfunctional regulator in the ovary. TGFβ has been shown to alter the proliferation and differentiation of rat granulosa cells (Dorrington et al., 1988, Knecht et al., 1989). Evidence suggests that ovarian theca-interstitial cells, as well as granulosa cells, may be sites of TGFβ production and action (Magoffin et al., 1989). The potential significance of TGF-β to ovarian physiology remains unknown (Knecht et al., 1989).

1.4.4.4 **Role of ovarian renin-angiotensin system**

A II may be involved in ovarian neovascularization. A II stimulated bFGF
production in bovine luteal cells, bFGF being a potent angiogenic factor (Sterling et al., 1990). A II has also been shown to regulate steroidogenesis. In vitro studies on rat ovarian fragments from ECG-stimulated rats showed a stimulatory effect of A II on estradiol secretion (Pucell et al., 1987; Bumpus et al., 1988). However, studies with granulosa cells from diethylstilbestrol-treated rats suggested no effect of A II on aromatase activity (Pucell et al., 1988). A II increased gonadotropin-stimulated progesterone, testosterone and estradiol production by cultured human granulosa cells (Palumbo et al., 1988a). A II had no effect on basal progesterone secretion by cultured bovine luteal cells and inhibited LH-stimulated progesterone production (Sterling et al., 1990). It appears that there may be species-dependent and cellular differentiation-dependent differences in effects of A II on ovarian steroidogenesis. Recent studies indicated the involvement of A II in ovulation and oocyte maturation (Pellicer et al., 1988, Kuo et al., 1991). Studies with perfused rat and rabbit ovaries demonstrated that A II induced ovulation in the presence or absence of gonadotropins. An A II antagonist, saralasin, reversed the effect of A II (Kuo et al., 1991, Peterson et al., 1993, Yoshimura et al., 1992). A II also induces oocyte maturation in rats. Saralasin dose-dependently inhibited hCG-stimulated germinal vesicle breakdown (GVBD) (Palumbo et al., 1988a).

Prorenin concentration correlates with follicular development, oocyte-cumulus-complex maturity and oocyte viability in humans (Ward 1978). Studies of other species have shown that active renin content of the ovary is not correlated with oocyte maturity (Daud et al., 1988). Angiotensins may play a role in follicular development through modulation of steroidogenesis. Since estrogens predominate in the follicular fluid with
meiotically arrested oocytes, 17β-estradiol has been reported to inhibit GVBD in porcine oocytes (McGaughey, 1977). Progesterone and testosterone can also inhibit GVBD in mouse oocytes (Smith et al., 1980). Seibel et al. reported that estradiol concentrations were lower in follicular fluid containing degenerated oocytes than in that containing healthy oocytes (Seibel et al., 1989). Thus, angiotensin concentrations in dominant follicles regulate estradiol concentrations of follicular fluid, which could effect oocyte maturation.

Although many factors, including inhibin-related peptides, growth factors, prostaglandins and other substances affect steroidogenesis, growing evidence indicates that angiotensins play a significant role in this process.

1.4.4.5 Role of intraovarian prostaglandins

Prostaglandins play an important role in follicular rupture in a variety of mammalian species. PG synthesis inhibitors block ovulation. Antiserum against PGF$_{2\alpha}$ inhibits ovulation. Elevated PG concentrations occur in preovulatory follicles (Behrman, 1979). PGE$_2$ and PGF$_{2\alpha}$ are increased in follicular fluid but it has not been established whether PGE$_2$, PGF$_{2\alpha}$ or both induce follicular rupture. Plasminogen activator from granulosa cells is secreted into the follicular fluid in response to gonadotropins, inducing digestion of the follicular wall by plasmin (Beers et al., 1975). The role of PGs in this process is unclear (Strickland et al., 1976). The role of PGs in human luteal regression is controversial, whereas in non-primate mammalian species PGF$_{2\alpha}$ is the major physiological luteolysin (Behrman, 1979). PGF$_{2\alpha}$ treatment is directly luteolytic,
immunization against PGF$_{2\alpha}$ prolongs the life span of the corpus luteum (as does treatment with cyclooxygenase inhibitors) and increased circulating PGF$_{2\alpha}$ of uterine origin occurs at the approximate time of luteal regression in non-primate mammals. PGF$_{2\alpha}$ induces functional regression of the corpus luteum by a receptor-mediated process in rats, independent of changes in ovarian or luteal blood flow (Pang et al., 1981). PGF$_{2\alpha}$ uncouples the occupied LH receptor from adenylate cyclase, which decreases LH-dependent cAMP accumulation and steroidogenesis (Thomas et al., 1978). The mediators of PGF$_{2\alpha}$ effects are unknown but do not appear to involve Ca$^{2+}$ or protein kinase C. Yet these specific mediators produce effects similar to that of PGF$_{2\alpha}$ (Pepperell et al., 1989; Musicki et al., 1990). PGF$_{2\alpha}$ may be luteolytic in humans but this conclusion must be tempered by the relative insensitivity of the human corpus luteum to PGF$_{2\alpha}$. The human uterus plays no role in the luteolytic process. However, PGF$_{2\alpha}$ is produced by the human corpus luteum and PGF$_{2\alpha}$ receptors are present in human luteal tissue (Powell et al., 1974).

Estrogens induce premature luteal regression in humans (Johansson et al., 1971) and monkeys (Karsch et al., 1973). Based on studies in monkeys, this action of estrogen appears to be directly within the ovary (Karsch et al., 1976). The luteolytic action of estrogen in monkeys is linked to PG production; estrogen increases PGF$_{2\alpha}$ concentrations in ovarian blood and indomethacin blocks estrogen-induced luteolysis (Auletta et al., 1976). Nonprimate mammals are refractory to PGF$_{2\alpha}$ during the early luteal phase in vivo and in vitro (Hall et al., 1981). An endogenous PGF$_{2\alpha}$ antagonist or inadequate PGF$_{2\alpha}$ receptor complement may protect the corpus luteum from regression in the early luteal
1.4.4.6 Intracellular signalling in the ovary

Ovarian cellular functions are regulated by peptide hormones, neurotransmitters, other nonsteroidal substances and steroids. These hormones regulate ovarian cells via intracellular second messengers after the binding of hormone and receptor. Hormone receptors in the ovary are separated into two categories: membrane receptors and intracellular receptors. Most peptide hormones interact with membrane receptors while most steroid hormones interact with intracellular receptors. The actions of hormones may be mediated by one or more of several intracellular signalling pathways. Although the mechanism of action of many ovarian hormones is unclear, various signalling pathways which may be involved will be described. Fig. 6 illustrates the general pathways and second messengers generated by hormone-receptor interaction.
Two major pathways appear to be involved in the peptide hormone actions. G proteins (GP) regulate adenylate cyclase, latter hydrolyzes ATP to cAMP. cAMP activates protein kinase A (PKA), PKA phosphorylates substrate proteins and stimulates steroidogenesis. Other hormones such as GnRH-receptor complex activates phospholipase A₂ and phospholipase C and produce AA and inositol triphosphate (IP3)/DAG by hydrolysis of polyphosphoinositides (PIP2). DAG activates protein kinase C (PKC), IP3 releases intracellular Ca²⁺, and AA metabolizes to prostaglandins. PKC, Ca²⁺ and prostaglandin F₂₀ inhibit the enzymes involved in steroidogenesis.

1.4.4.6.1 Mechanism of transduction by steroid hormone receptors

Steroid hormones are hydrophobic and can diffuse through cellular membranes to bind with specific intracellular receptors. Steroid hormone-receptor complexes induce gene expression by binding to specific DNA sequences which act as transcriptional enhancers. Direct induction of transcription of a few specific genes is known as the primary response. The products of these genes may then activate or inactivate other genes to produce a secondary response. Thus, a steroid hormone can cause complex
changes of ovarian cells. The same receptor protein regulates different genes in different target cells, as DNA-binding proteins required for specific gene transcription differ between cell types.

1.4.4.6.2 Adenylate cyclase-cAMP pathway

A large number of hormones exert their effects by increasing intracellular cAMP concentration. Membrane receptors may be associated with two classes of G proteins; Gs protein activates adenylate cyclase, while Gi protein inhibits this enzyme. The G proteins are heterotrimers consisting of α-, β- and γ-subunits (Neer et al., 1990). Activation of G proteins occurs when agonists bind to receptors, releasing GDP from G protein and allowing GTP to bind in its place. Activation triggers the α-subunit to dissociate from the β-γ-subunits, rendering the α-subunit free from the receptor. The α-subunit exhibits intrinsic GTPase activity which hydrolyzes GTP to GDP. Released α-subunit binds to and activates the catalytic subunit of adenylate cyclase with energy generated from the hydrolysis of GTP. Activated adenylate cyclase hydrolyzes ATP to cAMP, which subsequently activates protein kinase A in the cytoplasm. The activation of protein kinase A phosphorylates proteins involved in steroidogenesis. Alternatively, receptor-mediated inhibition of adenylate cyclase may occur by activation of Gi proteins. Two bacterial toxins which stimulate Gs and inhibit Gi (cholera toxin and pertussis toxin, respectively) are useful tools for the study of the adenylate cyclase-cAMP pathway and its involvement in intracellular signalling. Intracellular cAMP is degraded to 5'-AMP by the action of phosphodiesterase. cAMP may be the second messenger for LH, FSH and hCG in
ovarian cells (Kolena et al., 1992; Goff et al.; 1977, Marsh et al., 1966; Tsang et al., 1979). PGE₂ also increases cAMP in cultured granulosa cell differentiation (Behrman, 1979; Kolena et al., 1992; Goff et al., 1977).

1.4.4.6.3 Calcium and protein kinase C pathway

Many hormone receptors are coupled to G proteins which promote activation of phospholipase C (PLC). PLC hydrolyzes membrane-bound polyphosphoinositides, the products of which act as second messengers in Ca²⁺ mobilization and activation of calmodulin-dependent enzymes and protein kinase C (PKC). The various second messengers derived from inositol lipids has been reviewed (Catt et al., 1991). Briefly, PLC activation results in rapid metabolism of phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P₂) in the membrane, releasing inositol(1,4,5)trisphosphate (Ins(1,4,5)P₃) and diacylglycerol (DAG) into the cytosol. Ins(1,4,5)P₃ releases Ca²⁺ from the endoplasmic reticulum (Furuichi et al., 1989; Ross et al., 1989) and calsequestrin-containing organelles (termed calciosomes) to stimulate Ca²⁺ release (Volpe et al., 1988). Receptors for Ins(1,4,5)P₃ bind with high specificity and affinity and have been estimated to release at least 20 Ca²⁺ ions (Joseph et al., 1984). The extent of the initial Ca²⁺ response may be partially due to positive feedback of Ca²⁺ on PLC activity. Additional steps in the degradative and synthetic pathways of inositol metabolism include generation of additional signal molecules, InsP₅ and InsP₆, prior to recycling of inositol into membrane phosphatidylinositols (Valejo et al., 1987).

A second branch of the PLC pathway originates from the generation of DAG.
DAG regulates the activity of the PKC family of calcium/phospholipid-dependent enzymes by controlling their affinity for \(Ca^{2+}\) and for phosphatidylserine (Nishizuka 1984). Therefore, DAG stimulates PKC activation in the absence of a change in \([Ca^{2+}]_i\). Further, simultaneous elevation of \([Ca^{2+}]_i\) and \(Ins(1,4,5)P_3\) enhances PKC activity if DAG concentrations are suboptimal (Catt et al., 1989). Phorbol esters, compounds which bear physical homology to DAG, activate PKC by binding to the regulatory region of the enzyme. Phorbol esters are useful tools for the study of hormones which act by stimulating the PLC pathway.

PKC may also exert negative feedback through inhibition of polyphosphoinositide hydrolysis and promotion of \(Ins(1,4,5)P_3\) metabolism (Catt et al., 1989). PKC may alter receptor affinity through phosphorylation of membrane receptors. Seven subspecies of PKC have been reported (Nishizuka, 1988).

GnRH is a peptide hormone and its effects are mediated by specific receptors. The mechanism of GnRH action on the ovary has been investigated. GnRH and GnRH agonists stimulate the breakdown of polyphosphoinositides into inositol phosphates and DAG in the ovary (Leung et al., 1983; Ma et al., 1985; Minegishi et al., 1985). \(Ca^{2+}\) is required for GnRH action in granulosa cells (Ranta et al., 1983) and protein kinase C has been characterized in the ovary (Noland et al., 1984).

DAG may be hydrolysed by DAG lipase resulting in the generation of arachidonic acid, another second messenger. It has been proposed that some subspecies of PKC are also activated by phospholipid metabolites, such as DAG, arachidonic acid and lipoxin A (Nishizuka, 1988; Hansson et al., 1986).
1.5 Localization, secretion and action of inhibin in human placenta

The placenta functions as an endocrine organ during pregnancy. It maintains the pregnancy after the luteo-placental shift. The synthesis of steroid and peptide hormones are regulated by local placental and fetal factors. hCG is a polypeptide hormone that regulates progesterone production from the corpus luteum and placenta (Simpson et al., 1981). A placental GnRH has been proposed as a local modulator of hCG secretion (Butzow, 1985; Kim et al., 1987; Petraglia et al., 1987). The presence of inhibin-like bioactivity and immunoreactivity in placenta of several mammalian species suggests a role for inhibin in the endocrinology of pregnancy (McLachlan et al., 1986; Petraglia et al., 1987). Further evidence that inhibin functions in pregnancy is the observation that inhibin was increased rather than declined during the late luteal phase of women who had conceived following in vitro fertilization (McLachlan et al., 1987a). Immunohistochemical staining showed that inhibin-like immunoactivity was localized in centrally situated cytотrophoblast cells; peripheral syncytiotrophoblast were unstained. However, fluorescent-labelleд antisera specific to each subunit indicated that inhibin α was localized to cytотrophoblast. Inhibin βB-subunit was identified in the syncytial layer of the villi. Inhibin βА-subunit was present in the cytотrophoblast and syncytial layer (Petraglia et al., 1991). Placental inhibin synthesis has been confirmed using molecular techniques. Inhibin α-subunit mRNA was cloned and sequenced from a human term placental cDNA library (Mayo et al., 1986). Inhibin βA- and βB subunits have been detected in the placenta by S1-nuclease analysis (Meunier et al., 1988a). Northern blot analysis of placenta inhibin mRNAs revealed that the βA subunit was present in the...
highest concentration, suggesting local synthesis of activin-A (Meunier et al., 1988a).

Inhibins and activins regulate hCG and GnRH synthesis and secretion in human placental cells (Petraglia et al., 1987). The ability of activin but not inhibin to stimulate hCG release was confirmed using placental cell perfusion (Currie et al., 1993).
GENERAL OBJECTIVES

This study examined (1) the effect of local regulators on progesterone production by human granulosa cells, (2) the regulation of synthesis of these local substances in granulosa and placental cells and (3) the mechanisms of action of these local substances.

BACKGROUND AND RATIONALE

The physiologic processes of the ovarian cycle have classically been attributed to changes in hormonal output of the hypothalamic-pituitary axis. The following phenomena suggest that local substances play key roles in the regulation of the microenvironment of the ovary or individual follicles which react to gonadotropins:

1. Ovulation occurs in one ovary, but gonadotropin concentrations are the same in the blood perfusing the two ovaries.

2. Generally, only one follicle responding to LH during a given cycle ovulates.

3. The hormonal profiles of follicular fluid differ among follicles and determine follicular development.

4. The maintenance and lysis of the corpus luteum are not explained by gonadotropin regulation.

Substances in ovarian tissues and follicular fluid act locally, either alone or by modulating the actions of the gonadotropins, to change the functions of ovarian cells. The inhibin-related peptides, as well as GnRH, PGF$_{2\alpha}$ and angiotensins are synthesized in the ovary. Their roles as local regulators in human ovaries are uncertain. As an experiment model, a granulosa-luteal cell culture system that is responsive to hCG and
FSH stimulation was used. As progesterone production is stimulated by FSH and LH in cultured granulosa cells, progesterone production served as a functional marker. The results of these studies will advance the understanding of the endocrine function of the hypothalamic-pituitary-ovarian axis.

HYPOTHESIS

This study tested the hypothesis that specific intraovarian substances (activin, inhibin, follistatin, A II & A III, PGF$_{2\alpha}$ and GnRH) are involved in gonadotropin-dependent processes in the human ovary and, more specifically, on production of ovarian steroidal hormones.

SPECIFIC OBJECTIVES

1. To examine the regulatory effect of inhibin and activin on basal and gonadotropin-stimulated progesterone production and the mechanisms underlying the effects of inhibin and activin on human granulosa cells.

2. To examine the regulatory effect of follistatin on basal and hCG-stimulated progesterone production and the mechanisms underlying the effects of follistatin on human granulosa cells.

3. To examine the regulatory effects of GnRH, PGF$_{2\alpha}$ and angiotensins on basal and hCG-stimulated progesterone production.

4. To examine inhibin-subunit gene expression in human granulosa and placental cells.
2.0 GENERAL MATERIALS AND METHODS

2.1 HUMAN GRANULOSA CELL PREPARATION

Human granulosa cells were harvested during oocyte collection in the University of British Columbia *In Vitro* Fertilization Program. Ovulation was induced using a combination of clomiphene citrate and exogenous gonadotropins. Clomiphene citrate (100 mg, Serono) was taken daily by mouth between days 3-7 of the menstrual cycle. Menotropins (75-150 IU im, Pergonal, Serono) were given from day 6 and rapid assay monitoring of circulating estradiol (Diagnostic Products, Los Angeles, CA) and luteinizing hormone (Serono) was begun. The follicle was tracked with ultrasonography and menotropin stimulation stopped when at least 2 leading follicles were 18 mm or greater in diameter. Human chorionic gonadotropin (10,000 IU im, Serono) was given approximately 32 h before aspiration of 10-20 follicles using a transvaginal approach. Granulosa cells were harvested from the follicular fluid contents after the oocyte was identified for *in vitro* fertilization. The use of human granulosa cells was approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects of the University of British Columbia.

Follicular contents were centrifuged at 1000 x g and supernatant was decanted. Cells were suspended in 1-2 ml Hank's balanced salt solution (Gibco, Burlington, ON) containing 0.5% bovine serum albumin (BSA, Sigma, St. Louis, MO), layered onto 40% Percoll solution (Sigma) and centrifuged at 1700 x g for 20 min at 20 C (Davis et al., 1989). The cells were then washed and resuspended at the density of $10^5$ cells/ml media.
199 supplemented with 10% fetal bovine serum (FBS), sodium penicillin (100 IU/ml) and streptomycin (100 μg/ml) (M199, Gibco). The cells were plated and cultured in 48-well plates in replicates of 8 wells per treatment. The incubation media were replaced with M199 containing 2% FBS at 48 h and subsequently with M199 containing 0.5% BSA and the various treatments at 96 h after plating. Cells were incubated at 37 C in humidified air with 5% CO₂. Incubation media were collected every 24 h during incubation for progesterone measurement. Progesterone concentration was highest in first 24 h incubation and gradually decreased until fifth day incubation. The basal progesterone concentration increased when cell concentration increased. The basal progesterone concentration at same cell concentration also varied with different patients. Incubation after 96h was chosen for treatment time and progesterone and 20α-OH-P were measured by radioimmunoassay (RIA) after 24 h treatment.

The cultured human granulosa cells in this study have been exposed to the superovulated dose of human menopause gonadotropin (hMG) (Serono) and hCG, most of these cells will be luteinized during the culture. The rat granulosa cells mentioned during discussions in this thesis were collected by superstimulating immature rats with ECG and hCG.

### 2.2 HUMAN PLACENTA CELL PREPARATION AND HORMONE TREATMENT

Use of human placental cells was approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects of the University of British
Columbia. Term placentae were from elective cesarean section. Membranes and connective tissues were removed by dissociating term trophoblast in Media 199 (0.5% BSA, 100 IU penicillin G sodium and 100 μg streptomycin/ml supplemented M199, Gibco, Burlington, Ontario) through a 150 μ-screen (Sigma, St. Louis, MO). Hematocytes were removed by centrifuging (1700 x g for 20 min) the suspensions on 40% Percoll in M199 as above. Cell viability exceeded 90% as determined by Trypan Blue exclusion test and cell concentration was adjusted to 10^8/ml. The purified cell suspension (20-30 ml) was cultured in 50 ml culture tubes (Corning Inc., Corning, New York) in a 37 C shaking water bath (30 cycles/min) for 6 h with or without 8-Br-cAMP (150 μM) and GnRH (100 nM) (Sigma).

2.3 HORMONE ANALYSIS

Concentrations of progesterone and 20α-OH-P in media were determined by validated RIA with specific antisera provided by Dr. D. T. Armstrong of the University of Western Ontario.

The cells were resuspended in 6% trichloroacetic acid (TCA) and sonicated 3 times at 90 watts for 10 seconds for intracellular cAMP assay. cAMP concentrations were assayed using a commercial kit (Rianen cAMP [^{125}I] RIA kit, DuPont, Sarnia, ON).

2.3.1 Radioimmunoassay (RIA) for steroids

Reagents and buffer:
1) The assay buffer (PBSG) was 0.1 M phosphate buffered saline (pH 7.4):

\[\text{NaCl 80 gm, KCl 2 gm, Na}_2\text{HPO}_4 \text{ 11.5 gm, KH}_2\text{PO}_4 \text{ 2 gm, 0.1\% (w/v) thimerosal,}
\] 
0.1\% (w/v) gelatin, stored at 4C.

2) The progesterone (Sigma) standards were serially diluted in glass distilled ethanol from 0.32 mM stock solution. A standard curve was set up with 8 reference concentrations ranging from 0.5 nM to 64 nM.

3) The 20\alpha-OH-P (Sigma) standards were serially diluted in glass distilled ethanol from 0.32 mM stock solution. A standard curve was set up with 8 reference concentrations ranging from 0.5 nM to 64 nM.

4) The antisera were rabbit anti-progesterone and 20\alpha-OH-P antisera powders provided by D. T. Armstrong, raised against 4-pregnen-6\beta-ol-3,20-dione hemisuccinate:bovine serum albumin conjugate and 4-pregnen-20\alpha-ol-3-one:bovine serum albumin conjugate (Steraloids, Wilton, NH). These were used at a final dilution of 1:12500 w/v in PBSG and gave approximately 50\% binding of tracer.

5) The radio-tracers were \textsuperscript{3}H-progesterone (NET-724, NEN DuPont, Mississauga, ON) and \textsuperscript{3}H-20\alpha-OH-P.

6) The dextran charcoal solution was 0.025\% dextran and 0.25\% charcoal dissolved in PBSG.

7) Scintiverse (Fisher).

Intra-assay coefficients of variation were 6.8\% for progesterone and 7.8\% for 20\alpha-OH-P, respectively. Sensitivity of assays was determined by the detectably lowest hormone concentration plus 2 x standard deviation (SD). The sensitivity of both assays
was 0.5nM.

**Protocol:**

1) 100 μl progesterone standard or sample solution was added to each tube, each standard in triplicate and each sample in duplicate.

2) 100 μl diluted antibody solution was added to each tube.

3) 100 μl \(^3\text{H}\)-progesterone (10000 cpm, 10 nM) was added in each tube.

4) 100 μl PBSG was added in all tubes to make final volume 400 μl.

5) Incubation at 4 C for 16-24 h.

6) 500 μl dextran charcoal solution was added to each tube.

7) Incubation for 10 min at 4 C.

8) Centrifugation 2500 x g for 10 min at 4 C.

9) Supernatant was transferred to vials.

10) Scintiverse (3 ml) was added to each vial which was counted by LKB β-counter.

11) The progesterone concentrations of samples were calculated from the standard curve.

### 2.3.2 Radioimmunoassay (RIA) for cAMP

**Reagents and buffer:**

Reagents and buffer were provided in the kit.

**Protocol:**

1) 300 μl TCA was added to each cell culture well and cells were sonicated at 30 watts for 30 sec.

2) The cell suspension was collected and centrifuged at 10000 x g for 5 min.

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3) The supernatant was transferred to 10 ml glass tube for ether extraction.
4) Ether (3 ml) was added in the tube and vortex for 1 min.
5) Ether phase was removed and this procedure was repeated 2 more times.
6) The supernatant (sample) was used for intracellular cAMP assay.
7) The sample was acetylated by adding 5 µl acetylation reagent to each sample and then immediately vortexed. The sample was incubated for at least 3 min at room temperature, then 900 µl of assay buffer was added to each tube.
8) 100 µl of each standard solution and sample was added to test tubes.
9) 100 µl working tracer solution was added to all tubes.
10) 100 µl antiserum complex was added to all tubes.
11) The tubes were incubated overnight (16-18 h) at 2-8 C.
12) 0.5 ml cold cAMP precipitator was added to all tubes except total count tubes.
13) The tubes were centrifuged for 15 min at 1200 x g after vortexing.
14) The supernatant were decanted into a radioactive waste container by gently inverting all tubes once. The tube was kept inverted for 1 min to facilitate removal of remaining droplets.
15) All tubes were counted on LKB gamma counter and the cAMP concentrations were calculated from the standard curve.

2.3.3 Statistical analysis

The experiments were performed with eight wells per treatment. The human granulosa cells were from one patient in each experiment. The experiments were
repeated by using the cells from different patients. Statistical significance of the data were determined by analysis of variance followed by Sheffe’s test.
3.0 EFFECT OF INHIBIN AND ACTIVIN ON PROGESTERONE AND 20-α-OH-P PRODUCTION IN HUMAN GRANULOSA CELLS

3.1 INTRODUCTION

There is increasing evidence of local actions of inhibin and activin on ovarian and testicular function in addition to their known actions on FSH secretion at the pituitary (Tsafriri et al., 1989; Sum et al., 1989; Ying et al., 1986; Shukovski, 1990). Inhibin and activin increased and decreased, respectively, LH-stimulated androgen production by human theca and rat testicular cells (Hillier et al., 1991a&b; Hsueh et al., 1987). Although granulosa cells possess inhibin and activin receptors (Sugino et al., 1988a; Woodruff et al., 1990), their effects on these cells are unclear. However, inhibin and activin may alter the steroidogenic response to gonadotropins in murine and bovine granulosa cells (Shukovski et al., 1990; Woodruff et al., 1990; Xiao et al., 1990). The genes encoding inhibin subunit alpha, beta A and beta B have been cloned and recombinant inhibin A and activin A recently became available for investigation (Mason et al., 1985; Broxmeyer et al., 1988). Autocrine regulation of human ovarian function by inhibin and activin has not been elucidated. This study investigated effects of recombinant inhibin A and activin A on progesterone and 20α-OH-P and cAMP production in human granulosa cells.
3.2 TREATMENTS

3.2.1 Activin A-, inhibin A- and gonadotropin-regulated steroidogenesis

The effects of activin A and inhibin A on the steroidogenic response of cultured granulosa cells to gonadotropins were investigated by adding to the culture medium hCG (1 IU/ml) (Sigma) or FSH (0.5 μg/ml ovine FSH, NIADDK-oFSH-16, NIH, Bethesda, MD) with or without 100 ng/ml recombinant human activin A or inhibin A (Genentech, San Francisco, CA).

3.2.2 Dose-dependency and time-course study of activin A on hCG stimulated steroidogenesis

Increasing doses of activin A (1 to 100 ng/ml) were added with hCG (1 IU/ml) to the cultured cells and the cells were incubated for 16 h. The incubation media were collected for measurement of progesterone and 20α-OH-P. A time-course analysis was performed by adding hCG (1 IU/ml), with or without activin A (100 ng/ml) to the cells and harvesting the media at 6, 12, 18 and 24 h of incubation for progesterone analysis.

3.2.3 Effects of activin A on cAMP-stimulated progesterone production

8-bromo-cAMP (8-Br-cAMP, 1.5 mM, Sigma) and forskolin (50×10⁻⁶M, Sigma), with or without activin A (100 ng/ml), were added to cells. The incubation media were collected 16 h later and analyzed for progesterone.

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3.2.4 Effects of activin A on cellular cAMP accumulation

Activin A (100 ng/ml) was added to the cells, with or without hCG (1 IU/ml) and in the presence or absence of iso-butyl-methylxanthine (IBMX, 0.5 mM). Medium progesterone and intracellular cAMP were measured after 24 h incubation.

3.3 RESULTS

3.3.1 Effect of activin A and inhibin A on gonadotropin-stimulated progesterone and 20α-OH-P accumulation

Recombinant human activin A and inhibin A at a concentration of 100 ng/ml did not affect basal progesterone synthesis (P>0.05). Progesterone concentrations were increased 2-6 fold by hCG or FSH (P<0.01). Activin A inhibited hCG-stimulated progesterone production (P<0.01) and FSH-stimulated progesterone production (P<0.01) (Fig. 7). Inhibin A had no effect (P>0.05). Activin A suppressed hCG-stimulated 20α-OH-P accumulation (P<0.01, Fig. 8).

3.3.2 Dose- and time-dependent inhibition of progesterone and 20α-OH-P production by activin A

The effect of activin A on progesterone and 20α-OH-P production in response to hCG was dose-dependent. The effect of activin A was significant at a dose of 3.7 ng/ml (Fig. 9). Time-course experiments revealed that progesterone production was decreased by activin A at 18 and 24 h of treatment (Fig. 10).
Figure 7. Effect of 100 ng/ml human recombinant inhibin-A and activin-A on 1 IU/ml hCG- or 0.5 μg/ml ovine-FSH-stimulated progesterone production by human granulosa cells. Cells were incubated for 24 h. Values are mean ± sem (n=8). Similar results were obtained in other 5 experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.01).
Figure 8. Effect of 100 ng/ml human recombinant inhibin-A and activin-A on 1 IU/ml hCG-, 0.5 µg/ml ovine-FSH- and 1.5 mM 8-Br-cAMP-stimulated 20α-OH-P production by human granulosa cells. Cells were incubated for 24 h. Values are mean ± sem (n=8). Similar results were obtained in other 2 experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.01).
Figure 9. Effect of increasing concentrations of recombinant human activin A (3-100 ng/ml) on hCG (1 IU/ml)-stimulated progesterone production. Cells were incubated for 24 h. Values are mean ± sem (n=8). Similar results were obtained in other 2 experiments.

* P<0.05, ** P<0.01
Figure 10. Time-course effect of recombinant human activin A (100ng/ml) on hCG (1 IU/ml)-stimulated progesterone production by human granulosa cells. Cells were incubated for 24 h. Values are mean ± sem (n=8).

* P<0.05, ** P<0.01
3.3.3 Activin A- and cAMP-induced progesterone and 20α-OH-P in accumulation

Progesterone stimulation by 8-Br-cAMP (1.5 mM) was not affected by 100 ng/ml activin A (P>0.05, Fig. 11). Activin A did not affect forskolin-stimulated progesterone accumulation (Fig. 11; P>0.05). The accumulation of 20α-OH-P in response to 8-Br-cAMP was not affected by activin A (Fig. 8; P>0.05).

3.3.4 Effect of activin A on cellular cAMP accumulation

Incubation of human granulosa cells with hCG, with or without activin A, increased intracellular cAMP concentrations after 16 h of treatment. There was no difference in the intracellular cAMP concentrations between hCG and hCG + activin A treated groups (Fig. 12). The level of cAMP stimulated by hCG was enhanced in the presence of IBMX (P<0.01). Activin A attenuated the cAMP response to hCG in the presence of IBMX (P<0.01) (Fig. 12). Progesterone concentrations were reduced by activin A, irrespective of the presence or absence of IBMX (P<0.01, Fig. 13).
Figure 11. Effect of recombinant human activin A (100 ng/ml) on 8-Br-cAMP (1.5 mM)- and forskolin (5 x10^{-5}M)-stimulated progesterone production by human granulosa-luteal cells. Cells were incubated for 24 h. Values are mean ± sem (n=8). Similar results were obtained in other 4 experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.01).
Figure 12. Effect of recombinant human activin A (100 ng/ml) on hCG (1 IU/ml)-stimulated cellular cAMP production, in the absence or presence of IBMX (0.5 mM). Cells were incubated for 24 h. Intracellular cAMP was extracted according to the text. Values are mean ± sem (n=8). Similar results were obtained in 3 other experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.01).
Figure 13. Effect of recombinant human activin A (100 ng/ml) on hCG (1 IU/ml)-stimulated progesterone production, in the absence or presence of IBMX (0.5 mM). Cells were incubated for 24 h. Values are mean ± sem (n=6). Similar results were obtained in 2 separate experiments. Different superscripts (above the sem bars) denote statistical differences (1 vs 2, P<0.01; 1 vs 3, 2 vs 3, P<0.05).
3.4 DISCUSSION

Peptides of the inhibin family, secreted from granulosa cells into the follicular fluid, regulate pituitary gonadotropin secretion and may also exert paracrine/autocrine functions within the ovary (Sum et al., 1989; Ying et al., 1986; Hsueh et al., 1987, Sugino et al., 1988a; Woodruff et al., 1990). Inhibin and activin were originally identified as an inhibitor and stimulator, respectively, of FSH secretion. Inhibin and activin were first isolated from ovarian follicular fluid and subsequently shown to be products of granulosa cells. Since then, inhibin has been shown to potentiate and activin to suppress androgen production in response to LH in human theca and rat testis cells (Hillier et al., 1991a; Hsueh et al., 1987). This provides evidence of a paracrine action of these gonadal peptides. Reports of autocrine effects of inhibin and activin on granulosa cells have been inconsistent. Purified bovine activin was shown to suppress FSH-stimulated progesterone production in rat granulosa cells, while inhibin had no effect (Hutchinson et al., 1987). Another report suggested that activin potentiated FSH-stimulated progesterone production in rat granulosa cells (Xiao et al., 1990; Sugino et al., 1988b). Here, it was found that recombinant activin A (100 ng/ml) inhibited FSH- and hCG- stimulated progesterone production by 90% and 70% respectively, compared with the gonadotropins alone. This supports the hypothesis that activin, but not inhibin, inhibits gonadotropin-induced ovarian progesterone production. Dose-dependency experiments revealed an ED50 value of about 3.7 ng/ml (approximately 10^-10 M). This may be within the range found in ovarian follicular fluid, even though the concentration of activin in follicular fluid is low (about 10%) compared to that of inhibin (Zhiwen et al., 1987; Sugino et al., 1988b; Robertson et
al., 1986, McLachlan et al., 1987b). Similar doses of recombinant inhibin A had no effects on basal or hCG-stimulated progesterone production. This observation was similar to that of a previous study of granulosa cell differentiation (Hutchinson et al., 1987). It was reported that higher levels (60 IU/ml) of inhibin were produced by rat granulosa cells when stimulated with FSH (Zhiwen et al., 1987). Thus, generation of endogenous inhibin by the cells may have affected the results from the present experiments where exogenous gonadotropins were added. Sugino et al. observed a suppressive effect of inhibin on progesterone secretion in rat granulosa cells (Sugino et al., 1988b).

The possible mechanisms underlying inhibitory effects of gonadotropin-stimulated progesterone production by activin A were investigated. Inhibition of hCG-stimulated progesterone accumulation by activin A takes several hours. A similar inhibition of 20α-hydroxy-progesterone accumulation by activin A suggests that activin attenuates progesterone production rather than promoting conversion of progesterone to its major 20α-hydroxylated metabolite. Possible mechanisms may involve inhibition of gene transcription and translation of rate-limiting enzymes in the steroidogenic pathway (such as cytochrome P450 scc). Alternately, activin may interfere with steps between the coupling of gonadotropin receptor and the adenylate cyclase-cAMP cascade. Experiments using 8-Br-cAMP and an adenylate cyclase activator, forskolin, with or without activin, suggest that inhibitory effects of activin occur prior to gonadotropin-induced cAMP accumulation. Using IBMX to block phosphodiesterase activity, cAMP levels in the hCG + IBMX group was higher than the hCG group, suggesting metabolic rate of cAMP is relatively high. hCG-induced cAMP, in the presence of IBMX, was reduced by activin
A. This suggests that activin A may have reduced the cAMP response to hCG by inhibiting cAMP synthesis rather than by enhancing phosphodiesterase-induced cAMP catabolism (Fig. 12). Although hCG-stimulated cAMP accumulation was enhanced by IBMX, progesterone accumulation was not affected (Fig. 8). This implies that hCG treatment resulted in a maximal progesterone response which cannot be increased by blockade of cAMP degradation, or hCG-stimulated progesterone production needs on slight increase of intracellular cAMP concentration. Progesterone accumulation in the activin A + hCG and activin A + hCG + IBMX groups were equally attenuated as compared to hCG alone and hCG + IBMX, respectively (Fig. 13). These observations questioned that activin inhibits gonadotropin-stimulated progesterone production by blocking cAMP synthesis, therefore inhibitory effect of activin on Gn-stimulated progesterone by other intracellular pathway should also be considered.

The present results suggest that activin A may serve as a negative regulator of gonadotropin-stimulated progesterone production in human granulosa cells. It is generally agreed that activin stimulates FSH secretion which, in turn, increases estrogen production in the ovary. Thus, it is plausible that the overall action of activin may be to promote follicular differentiation and delay the onset of luteinization. This could be achieved through the stimulation of FSH secretion at the pituitary and the suppression of gonadotropin-stimulated progesterone production at the ovary via an autocrine/paracrine mechanism.
4.0 STIMULATION OF PROGESTERONE SECRETION BY RECOMBINANT FOLLISTATIN-288 IN HUMAN GRANULOSA CELLS

4.1 INTRODUCTION

Follistatin production in the rat ovary occurs exclusively within granulosa cells (Nakatani et al., 1991). Follistatin production by differentiated bovine granulosa cells harvested from preovulatory follicles is FSH- but not LH-dependent (Klein et al., 1991).

Follistatin is an activin binding protein (Nakamura et al., 1990). Activin suppresses gonadotropin-stimulated progesterone production by human granulosa cells (Li et al., 1992 & Chapter 3.0). Follistatin may also locally regulate steroid hormone production in the human ovary. Follistatin augments FSH-stimulated progesterone production by rat granulosa cells (Xiao et al., 1990) and enhances LH-stimulated progesterone production in undifferentiated but not in differentiated bovine granulosa cells (Shukovski et al., 1991). Taken together with the above findings, the presence of follistatin (Shimasaki 1989; Nakatani et al., 1991) and inhibin-β (Ling et al., 1986; Vale et al., 1986; Woodruff et al., 1988; Meunier et al., 1988) mRNA within the ovary suggests local functions of inhibin, activin and follistatin (Xiao et al., 1990; DePaolo et al., 1991). A recombinant human follistatin (follistatin-288) has been developed (Inouye et al., 1991). The present experiments were designed to test the hypothesis of direct actions of inhibin-related peptides in human granulosa cells collected from superovulated
4.2 TREATMENTS

4.2.1 Recombinant expression of human follistatin-288

Recombinant human follistatin with 288 amino acids was expressed in Chinese hamster ovary cells under the control of the simian virus-40 promoter, as detailed in a previous report (Inouye et al., 1991).

4.2.2 Follistatin-induced steroidogenesis

Recombinant human follistatin-288 (3 nM) was added in M199 with 0.5% BSA, with or without hCG (1 IU/ml, Sigma). Progesterone and 20α-OH-P were measured.

4.2.3 Dose-dependency and time-course study of follistatin action

Increasing doses of follistatin (0.1 to 3 nM) were added to the cultured cells and the cells were incubated for 24 h. Time-course experiments examined follistatin (3 nM) effects. Progesterone concentrations were measured in incubation media after 6, 12, 24 and 36 h.

4.2.4 Effects of follistatin on cAMP-stimulated progesterone production

8-Br-cAMP (1.5 mM, Sigma), with or without follistatin (3 nM), was added to the cells. Progesterone and 20α-OH-P concentrations were measured in incubation media.
after 24 h.

Intracellular cAMP concentrations were measured.

4.2.5 Effects of follistatin on cellular cAMP accumulation

Follistatin (3 nM) and hCG (1 IU/ml) were added to the cells in the presence of 0.5 mM IBMX. Progesterone and intracellular cAMP were measured.

4.3 Results

4.3.1 Dose-dependent increase of progesterone secretion by follistatin

Treatment of human granulosa cells with follistatin stimulated progesterone secretion. The effect of follistatin on progesterone secretion was dose-dependent and was significant at 0.33 nM (P<0.01) and remained elevated at 1 and 3 nM (Fig. 14).

4.3.2 Effect of follistatin on basal and hCG-stimulated progesterone production

The effect of recombinant human follistatin was examined in the presence or absence of exogenous gonadotropin (Fig. 15). Follistatin (3 nM) increased (P<0.01) basal progesterone secretion by approximately 3-fold in the absence of exogenous gonadotropin (Fig. 15). Progesterone concentrations were increased 3.5 fold by hCG (1 IU/ml, P<0.01). Follistatin did not affect hCG-stimulated progesterone secretion (ie. in cells where follistatin and hCG were tested together, follistatin did not augment hCG-stimulated progesterone secretion (P>0.05).
Figure 14. Effect of recombinant human follistatin-288 (0.1-3 nM) on basal progesterone secretion. Cells were incubated for 24 h. Values are mean ± sem (n=8). Similar results were obtained in 3 separate experiments. ** P<0.01.
Figure 15. Effect of recombinant human follistatin-288 (3 nM) on basal and hCG (1 IU/ml)/8-Br-cAMP (1.5 mM)-stimulated progesterone secretion by human granulosa cells. Cells were incubated for 24 h. Values are mean ± sem (n=8). Similar results were obtained in other 4 experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.01).
4.3.3 *Dose-dependent increase in 20α-OH-P production by follistatin*

To investigate the possible effect of follistatin on progesterone metabolism, the concentration of 20α-OH-P in the culture medium following a 24 treatment period was determined. Similar to the effect on progesterone accumulation, basal 20α-OH-P secretion was markedly enhanced in the presence of increasing doses of follistatin (Fig. 16). The stimulation by follistatin became significant at a dose of 1 nM.

4.3.4 *Effect of follistatin on hCG-stimulated 20α-OH-P production*

Treatment of the cells of hCG (1 IU/ml) for 24 h significantly enhanced the accumulation of 20α-OH-P in the culture medium (Fig. 17). A similar stimulation was observed with follistatin (3 nM). In cells that were treated with follistatin and hCG concomitantly, the accumulation of 20α-OH-P was not stimulated further when compared with either treatment alone (P>0.05).

4.4.5 *Time-dependent increase of progesterone production by follistatin*

Follistatin stimulated progesterone secretion at 24 h in time-course experiments (Fig. 18, P<0.01).

4.4.6 *Effect of follistatin on cAMP-stimulated progesterone and 20-α-OH-P production*

8-br-cAMP-stimulated progesterone and 20α-OH-P secretion were not affected by follistatin (3 nM) (Figs. 15 and 17, P>0.05).
Figure 16. Effect of recombinant human follistatin-288 (0.1-3 nM) on basal 20α-OH-
P production. Cells were incubated for 24 h. Values are mean ± sem (n=8). * P<0.05.
Figure 17. Effect of recombinant human follistatin-288 (3 nM) on basal and hCG (1 IU/ml)/8-Br-cAMP (1.5 mM)-stimulated 20α-OH-P production by human granulosa cells. Cells were incubated for 24 h. Values are mean ± sem (n=8). Similar results were obtained in other 4 experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.05).
(n=8). Similar results were obtained in other 4 experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.05).

4.4.7 Effect of Follistatin on cellular cAMP accumulation

Incubation of human granulosa cells with follistatin or hCG, in the presence of IBMX, resulted in increased cellular cAMP concentrations after 24 h incubation (Fig. 19, P<0.01). Cellular cAMP concentrations between hCG and follistatin treated groups were similar (P>0.05). In these experiments, progesterone concentrations in the incubation media were increased by follistatin or hCG in the presence of IBMX (P<0.01). The effects of follistatin and hCG on the accumulation of cAMP and progesterone were not additive (P>0.05).
Figure 18. Time-course effect of recombinant human follistatin-288 (3 nM) on basal progesterone production. Cells were incubated for 24 h. Values are mean ± sem (n=8). ** P<0.01.
Figure 19. Effect of recombinant human follistatin-288 (3 nM) and hCG (1 IU/ml) on progesterone secretion (upper panel) and cellular cAMP accumulation (lower panel) in the presence of iso-butyl-methylyxanthine (IBMX, 0.5 mM). Cells were incubated for 24 h. Intracellular cAMP was extracted according to the text. Values are mean ± sem (n=8). Similar results were obtained in other 3 experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.01).
4.5 Discussion

Follistatin-288 is 8-10 times more potent than the native protein and similar to the potency of inhibin-A in vitro, but more potent and longer acting than inhibin-A in vivo (Xiao et al., 1990). The physiological role of follistatin in the human ovary has not been reported. Although follistatin has thus far been measured only in human ovarian follicular fluid (Schneyer et al., 1992, Robertson et al., 1990), a number of studies have localized follistatin message and/or protein in the granulosa cells of ovaries from other species (Shimasaki, 1989; Nakatani et al., 1991; Ying 1988; Findley et al., 1990). The present observation that follistatin stimulates basal progesterone and 20α-OH-P accumulation in human granulosa cells provides the first evidence for a direct action of follistatin in the human ovary and suggests the potential for an autocrine role. It has been demonstrated that activin-A decreased hCG-stimulated progesterone and 20α-OH-P production in human granulosa cells (Saito et al., 1991). Follistatin is a binding protein of activin (Robertson et al., 1990; Nakamura et al., 1990). Thus, it can be postulated that a balance between follistatin and activin-A activity contributes to the regulation of the granulosa cell response to gonadotropins.

Follistatin increased basal progesterone concentrations with equal potency to hCG, suggesting that follistatin may be an important regulator in the ovary. Follistatins are produced from differentiated granulosa cells in an FSH-dependent manner (Klein et al., 1991). Increased FSH is necessary for development of antral follicles to the preovulatory stage (Meunier et al., 1988c), a phase in follicular maturation when synthesis of follistatins is stimulated (Klein et al., 1991). Increased basal progesterone
secretion from human preovulatory granulosa cells by follistatin suggests that follistatin could in part contribute to the rise in progesterone before the preovulatory LH surge. Higher progesterone concentrations in larger follicles than in smaller follicles before the LH surge (Schwall et al., 1990), as well as an elevated circulating progesterone concentration, may facilitate estrogen stimulation of the LH surge (Woodruff et al., 1988). The ED_{50} for the increase in progesterone secretion by follistatin was about 10^{-10}M, supporting the hypothesis that the effects of follistatin were physiological. The mechanism of action of follistatin is not known. Our results show that increased basal progesterone is accompanied by an increase in cAMP accumulation. In the presence of IBMX, which blocks phosphodiesterase activity, cellular cAMP concentrations in the follistatin group were higher than in the control group (Nakatani et al., 1991). Although the receptor of follistatin has not been identified, follistatin-induced cAMP accumulation suggests that the putative follistatin receptor could be coupled with a G_j/adenylate cyclase complex. Whether or not cAMP serves as a second messenger for follistatin-stimulated progestin accumulation cannot be determined from the present experiments. It is interesting to note that in the presence of hCG or exogenous cAMP, follistatin fails to affect progestin accumulation. This may have resulted from the already maximal progesterone production in the presence of hCG (1 IU/ml) or cAMP (1.5 mM). The time-course studies demonstrated that the follistatin-induced increase in progesterone secretion was observed at 24 h, which was delayed compared to hCG-stimulated progesterone production. The slower time-course of action of follistatin may implicate an indirect action such as through the binding of activin (Robertson et al., 1990; Nakamura et al.,
1990) and disinhibition of activin suppression of progesterone synthesis (Saito et al., 1991). This potential mechanism of action remains to be investigated. The other possibility that follistatin might have blocked progesterone metabolism was examined by measuring 20α-OH-P concentrations in the follistatin-treated cells. The observation of parallel stimulatory effects of follistatin on progesterone and 20α-OH-P concentrations argues against a suppression of the 20α hydroxylase step.

The results of this study differ from report of the effect of follistatin on bovine granulosa cells (Hillier et al., 1991b, Hsueh et al., 1987). Follistatins had no effect on differentiated bovine granulosa cells but augmented LH-stimulated progesterone production in the undifferentiated stage of such cells (Hillier et al., 1991b). In rat granulosa cells, follistatin augments FSH-stimulated but not basal progesterone production (Xiao et al., 1990). The cause for this apparent species difference in the granulosa cell response to the direct action of follistatin is not known. It might be due to different degrees of the granulosa cell differentiation in different studies. In summary, follistatin increased basal but not hCG- or cAMP- stimulated progesterone secretion by differentiated human granulosa cells. Increased basal progesterone concentrations before the LH surge may partly result from increased follistatin production within preovulatory follicles. Although the mechanism of follistatin is unknown, the increase of basal progesterone secretion by follistatin is accompanied by an elevated intracellular cAMP formation. The stimulatory effects of follistatin on progesterone and 20α-OH-P secretion suggest that follistatin may be a potential regulator of ovarian steroidogenesis.
5.0 EFFECT OF ANGIOTENSIN III ON PROGESTERONE PRODUCTION BY HUMAN GRANULOSA CELLS

5.1 INTRODUCTION

Prorenin, renin and A II were found within the stromal, thecal and luteal cells of human and rat ovaries using immunochemical methods. Granulosa cells of preovulatory follicles and atretic follicles stain intensely for both renin and A II (Palumbo et al., 1989). The principal function of the renin-angiotensin system may involve regulation of cardiovascular homeostasis. However, increasing evidence suggests other roles for this regulatory system in endocrine tissues. Specifically, components of the renin-angiotensin system appear to alter ovarian steroidogenesis in several mammalian species (Bumpus et al., 1988; Fernandez et al., 1985; Culler et al., 1986; Jerry et al., 1988). A II has no effect on basal progesterone production by cultured bovine granulosa cells and inhibits LH-stimulated progesterone production (Sterling et al., 1990). A II has a brief half life, being rapidly cleaved by angiotensinase to the active septapeptide, A III. It appears that there are no reports about the effect of A III on steroidogenesis in the ovary from different species.

The angiotensin-converting enzyme which converts decapeptide A I to the actapeptide A II and the angiotensinase which converts the hepapeptide A II to A III have been reported to exist in rat ovarian follicles (Daud et al., 1990; Speth et al., 1988). This suggests that ovary may secrete its own A III. The presence of
angiotensin receptors in granulosa and thecal cells of different species supports the postulate of a biological role for angiotensins in the ovary (Pucell et al., 1987; Speth et al., 1988; Husian et al., 1987; Miyazaki et al., 1988). In addition, both A II and A III have been reported to bind to the similar receptors (Goodfriend, 1991). However, the function(s) of A II and A III in the human ovary are unclear. This study examined the role of angiotensins in progesterone production by human granulosa-lutein cells. These cells are presumably programmed to undergo atresia or luteinization and are uniquely suited to the study of the autocrine and paracrine role of angiotensins in human ovary.

5.2 TREATMENTS

5.2.1 A II and A III conjugation and binding

Fluroisothiocyamate labelling of A II and A III was performed as reported by Woodruff 1991 with the following modifications. A II and A III (1 mg, Peninsula, Belmont, CA) were mixed with 100 μg fluorescein isothiocyanate (FITC, Sigma) in 1 ml 1 M sodium carbonate, pH 9.5. The mixture was stirred for 1 h and pH balanced to 9.0-9.5 at 20 C. Conjugated and unconjugated FITC were separated using a G-25 sephadex column eluted with 0.01 M sodium phosphate buffer, pH 7.4. The FITC-conjugated A II and A III were determined by measuring the final protein concentration and fluorescence with a spectrometer (280 nm) and fluorometer (excitation 488 nm, emission 525 nm). Binding activity was measured by incubating
granulosa cells (in M199 with 0.5% BSA) with FITC-conjugated A II and A III for 30 min at 37 C. Cells were washed 3 times with M199 containing 0.5% BSA. Nonspecific binding was determined in the presence of 100-fold excess of unlabelled A II and A III. Binding is reported as fluorescent intensity versus nonspecific binding using a Nikon fluorescent microscope (Nikon type 108).

5.2.2 Effects of A II and A III on hCG-stimulated steroidogenesis

Effects of A II and A III on steroidogenic response to hCG were examined by adding 1 IU hCG/ml (Sigma) with or without 10^{-5} M A II and A III. Progesterone and 20\alpha-OH-P were measured by RIA.

5.2.3 Dose-dependency and time-course study of A III on hCG-stimulated steroidogenesis

Increasing doses of A III (10^{-9}-10^{-5} M) were added with hCG (1 IU/ml) to the cultured cells and incubated for 24 h. The incubation media were collected for progesterone and 20\alpha-OH-P RIA. hCG (1 IU/ml) was added to cells with or without A III (10^{-5} M) and incubation media were collected at 6, 12, 24 and 36 h for progesterone time-course analysis.

5.2.4 Effects of A III on cAMP-stimulated progesterone production

8-Br-cAMP (1.5 mM, Sigma) was added to cells with or without A III (10^{-5} M). Progesterone was determined.
5.2.5 Effects of PMA on hCG- and cAMP-stimulated progesterone production

Phorbol 12-myristate 13-acetate (PMA, 10^{-7}M, Sigma) was added to the cells with or without hCG (1 IU/ml) and cAMP (1.5 mM). Progesterone was measured.

5.2.6 Dose-dependent effect of PMA on hCG-stimulated progesterone production

PMA (10^{-10}-10^{-6}M) was added to cell culture media with hCG (1 IU/ml). Progesterone was measured.

5.2.7 Effect of A III on hCG-stimulated progesterone production in cells pre-treated with PMA

PMA (10^{-7}M) was added to cell culture media. Cells were rinsed 3 times in M199 with 0.5% BSA at 24 h. A III (10^{-5}M) with or without hCG (1 IU/ml) was added to the cells. Media were collected at 24 h for progesterone determination.

5.2.8 Additive effect of PMA and A III on hCG-stimulated progesterone production

hCG (1 IU/ml), hCG with PMA (10^{-7}M), with or without A III (10^{-6} and 10^{-5}M) were added to the cells. Progesterone were measured.

5.3 RESULTS

5.3.1 A II and A III binding to cultured human granulosa cells

The binding activity of A II and A III was examined using UV light after
incubation of FITC-labelled A II and A III with human granulosa cells. No autofluorescence was observed for unlabelled proteins. The binding specificity was determined by incubation with 100 fold higher concentrations of unconjugated A II and A III. Results showed that A II and A III specifically bound to human granulosa cells (Fig. 20, 21).

5.3.2 Effect of A III on hCG-stimulated progestin accumulation

Treatment of the cells with A II or A III (10^{-5} M) did not affect basal progesterone synthesis (P>0.05, Fig. 22). Progesterone concentrations were increased 2-3 fold by hCG (P<0.01). A III suppressed hCG-stimulated progesterone production (P<0.01). A II was not effective in this regard (P>0.05). A III also had a trend to suppress hCG-stimulated 20\alpha-OH-P accumulation (Fig. 23).

5.3.3 Dose- and time-dependent effects of A III

A III suppression of hCG-stimulate progesterone production was dose-dependent. Effect of A III on hCG-stimulated P_4 production was observed at 10^{-7} M (Fig. 24). The ED_{50} for the effect of A III on hCG-stimulated progesterone production was 3.2 ± 0.38 x 10^{-8} M (based on 2 separate experiments). Time-course experiments revealed that progesterone production was inhibited by A III at 24 and 36 h of treatment (P<0.01, Fig. 25).
Figure 20. The binding sites of FITC-labeled A II on human granulosa-luteal cells. The cultured granulosa cells were incubated with (1,2) or without (3,4) 100 x concentration of A II for 30 min at 37 C before incubation with FITC-labeled A II. The cells were washed with 0.5% BSA M199 for three times. The cells were photographed using Nikon microscope (x 400) by normal light (1,3) or ultraviolet dark fields (2,4).
Figure 21. The binding sites of FITC-labeled A III on human granulosa-luteal cells. The cultured granulosa cells were incubated with (1,2) or without (3,4) 100 x concentration of A III for 30 min at 37 C before incubation with FITC-labeled A III. The cells were washed with 0.5% BSA M199 for three times. The cells were washed with 0.5% BSA M199 for three times. The cells were photographed using Nikon microscope (x 400) by normal light (1,3) or ultraviolet dark fields (2,4).
Figure 22. Effects of 10^{-5}M A II/III on basal and hCG (1 IU/ml)-stimulated progesterone production by human granulosa cells. Cells were incubated for 24 h. Values are mean ± sem (n=8). Similar results were obtained in other 4 experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.01).
5.3.4 Effect of PMA on progesterone production

PMA (10^{-7}M) did not affect basal progesterone synthesis (P>0.05, Fig. 26). Progesterone concentrations were increased 2-3 fold by hCG (P<0.01). PMA inhibited hCG-stimulated progesterone production (P<0.01). The effect of PMA on progesterone production in response to hCG was dose-dependent (Fig. 27). The minimum effective concentration of PMA was 10^{-8}M (P<0.01). The ED_{50} for the effect of PMA on hCG-stimulated progesterone production was 8.7 ± 0.6 nM (based on 2 separate trials. The inhibitory effects of A III and PMA was additive (P<0.01), (Fig. 28).

5.3.5 cAMP-stimulated progesterone production

8-Br-cAMP-stimulated progesterone production (1.5 mM) was not affected by A III 10^{-4}M or PMA 10^{-7}M (P<0.05, Fig. 29).

5.3.6 Effect of A III in cells pre-treated with PMA

A III suppressed hCG-stimulated progesterone production after 24 h of PMA pre-treatment (P<0.01, Fig. 30).
Figure 23. Effects of A III (10^{-5}M) on hCG (1 IU/ml)-stimulated 20α-OH-P production. Cells were incubated 24 h. Values are mean ± sem (n=8).

** p<0.01.
Figure 24. Effects of Angiotensin III (10^-10^5 M) on hCG (1 IU/ml)-stimulated progesterone production. Cells were incubated for 24 h. Values are mean ± sem (n=8).

Similar results were obtained in other 2 experiments. ** p<0.01.
Figure 25. Temporal profile of the effects of A III (10^{-5} M) on hCG (1 IU/ml)-stimulated progesterone production by human granulosa cells. Cells were incubated for 24 h. Values are mean ± sem (n=8). Similar results were obtained in other 2 experiments. ** p<0.01.
Figure 26. Effects of PMA (10^-7 M) on hCG (1 IU/ml)-stimulated progesterone production by human granulosa cells. Cells were incubated for 24 h. Values are mean ± sem (n=8). Similar results were obtained in other 3 experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.01).
Figure 27. Effects of PMA (10^{-10} - 10^{-6} M) on basal progesterone production. Values are mean ± sem (n=8). Similar results were obtained from other 2 experiments. ** p<0.01.
Figure 28. Effects of A III (10^{-6}-10^{-5} M) + PMA (10^{-7} M) on hCG (1 IU/ml)-stimulated progesterone production. Values are mean ± sem (n=8). Similar results were obtained in other 3 experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.01).
5.4 DISCUSSION

Result with fluorescent-labelled A II and A III indicated that human granulosa cells are sites of both A II and A III action. This result agrees with the previous finding that the A II receptors existed in murine and bovine ovary or follicles (Pucell et al., 1987; Daud et al., 1990; Speth et al., 1988; Husian et al., 1987; Miyazaki et al., 1988) and A II and A III may share the same receptors (Goodfriend, 1991). Dose-dependent suppression of hCG-stimulated progesterone production by A III suggested that A III, but not A II, is an active inhibitor of progesterone production in the human ovary. The possibility that the des-aspartic acid derivative of A II (A III) is an agonist with potency exceeding its progenitors in certain specific sites of action has been proposed (Goodfriend, 1991). Whereas A III is a less potent vasoconstrictor than A II, A III is as potent as A II in the adrenal glomerulosa (Cook et al., 1990). A III may also be the active angiotensin in the brain (Wright et al., 1990; Einspanier et al., 1991). In the adrenal gland, A III is a powerful stimulator of aldosterone secretion, and stimulates prostaglandin formation. The biological effects of A II in ovarian cells are obscure. A II secretion may be episodic in pre- and post-ovulated porcine follicles but the episodes appear to have no effect on steroidogenesis (Lacy et al., 1989). The effects of A II in this study showed inconsistent results. Other studies have shown that A II interferes with steroidogenesis in the ovary of other mammals and amphibians (Kuo et al., 1991; Lightman et al., 1987). This suggests species differences or different rates of degradation of the angiotensins in different species. Alternatively, A III may be formed by the sequential action of the aminopeptidase and converting enzyme on the decapeptide angiotensin I.
Effects of A III (10^{-5}M) and PMA (10^{-7}M) on cAMP (1.5 mM)-stimulated progesterone production by human granulosa cells. Cells were incubated for 24 h. Values are mean ± sem (n=8). Similar results were obtained in other 5 experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.05).
Figure 30. Effects of A III (10^{-5} M) on hCG-stimulated progesterone production by PMA (10^{-7}M) pre-treated cells. Cells were pretreated with PMA for 24 h. After pretreatment, cells were washed three times, and treated with A III and hCG for 24 h. Values are mean+sem (n=8). Similar results were obtained in other 3 experiments. Different superscripts (above the sem bars) denote statistical differences (P<0.01).
The angiotensin converting enzyme has been reported to exist in the rat ovary (Sterling et al., 1990; Bumpus et al., 1988). The ED$_{50}$ for A III suppression of hCG-stimulated progesterone production was about 10 fold of the KDa (3 nM) for A III binding (Auletta et al., 1988), which suggested the physiological significance of the action of A III. It is possible that A II is secreted into the follicle and then converted to A III by an angiotensinase and A III but not A II, suppresses hCG-stimulated progesterone production by the human ovary. LH treatment increased renin-like activity and A II immunoactivity in human follicular fluid, suggesting that LH is a major factor in the regulation of the ovarian renin-angiotensin system (Carrithers et al., 1990). Therefore, there may be feedback loop between renin-angiotensin system and LH, which regulates their actions in human ovary.

Intracellular mechanisms underlying the inhibitory actions of A III on hCG-stimulated progesterone production are unknown. The A II receptor has been reported to couple with adenylate cyclase and PLC and A II and A III increased inositol polyphosphates in a dose-dependent manner (Audinot et al., 1991; Leung et al., 1988; Davis et al., 1989). DAG is generated by the activation of PLC. The phorbol ester PMA, like DAG, is an activator of PKC. PMA has been reported to inhibit FSH-stimulated progesterone production in the rat (Spat, 1988) and hCG-stimulated cAMP production (Kojima et al., 1985) in cultured human granulosa cells. This study examined the effect of PMA on hCG-stimulated progesterone production in cultured human granulosa cells. The data suggested that PMA treatment of granulosa cells mimicked the effect of A III in human granulosa cells.
A II has been shown to stimulate polyphosphoinositide turnover and increase formation of DAG and inositol trisphosphate in several tissues (Kojima et al., 1985; Wang et al., 1985). The possibility that the inhibition of hCG-stimulated progesterone production by A III is mediated via the activation of PKC was examined. The results of this study suggested that suppression of hCG-stimulated progesterone production by A III and PMA were additive. Further, the inhibitory effects of A III on hCG-stimulated progesterone production were observed in cells pre-treated with PMA. This suggested that the inhibitory action of A III may not involve PKC activation, but may occur via other signal pathways such as the adenylate cyclase system and calcium and calcium-binding protein system. Indeed, A II has been reported to have some effect on [Ca$^{2+}$]$_i$ in rat granulosa and luteal cells (Wang et al., 1989; Currie et al., 1992). Further studies are necessary to elucidate the intracellular signalling mechanisms of A III.

In summary, A III inhibits hCG-stimulated progesterone production in human granulosa cells. A II at similar concentrations does not produce a consistent effect. This suggested that A III may be the dominant effector on steroidogenesis in the human ovary. Binding sites of A II and A III, as detected by fluorescent-labelling, provided further evidence that human granulosa cells are targets of angiotensin action. Although PMA inhibited hCG-stimulated progesterone production in human granulosa cells, the action of A III may not involve the activation of PKC.
6.0 EFFECT OF GnRH ON PROGESTERONE PRODUCTION BY HUMAN GRANULOSA CELLS

6.1 INTRODUCTION

Circulating GnRH concentrations in non-pregnant animals are considered too low to interact with extrapituitary tissues (Marchetti et al., 1988; Eskay et al., 1977; Nett et al., 1974). However, membrane-bound GnRH receptors and GnRH-like peptides appear to be present in the ovary of some mammalian species, including the human. Such locally produced GnRH potentially modulates ovarian activity via paracrine and autocrine pathways (Hsueh et al., 1981a; Pieper et al., 1981; Jones et al., 1980; Hillensjo et al., 1980; Latouche et al., 1989). GnRH activity within the ovary may be inhibitory or stimulatory. Notably, GnRH is thought to suppress gonadotropin-induced steroidogenesis (Latouche et al., 1989) but stimulates oocyte maturation and ovulation (Hsueh et al., 1981a; Latouche et al., 1989). A GnRH agonist suppressed basal, LH- and FSH-stimulated cAMP accumulation and progesterone secretion in porcine granulosa cells (Hsueh et al., 1988; Harwood et al., 1980).

LH/hCG exposure may induce FSH-sensitized granulosa cells to produce a GnRH-like peptide (Hsueh et al., 1988). Sufficient, perhaps continuous, exposure to such a factor could act at pituitary GnRH receptors to desensitize gonadotropes, explaining the absence of an endogenous LH surge after treatment with a GnRH agonist (Hsueh et al., 1988).

GnRH may directly suppress FSH-induced adenylate cyclase activity,
steroidogenesis and gonadotropin receptor expression in rat, pig, hamster, rabbit and chicken granulosa and luteal cells (Latouche et al., 1989; Demoulin et al., 1991). GnRH agonists also suppressed progesterone production from spontaneously luteinized human granulosa cells in vitro (Knecht et al., 1983a) and granulosa-lutein cells from preovulatory follicles aspirated after hCG administration to in vitro fertilization patients (Parinaud et al., 1988). Conflicting studies suggest that GnRH does not act at the ovary to alter gonadotropin-stimulated steroidogenesis in vitro (Knecht et al., 1981a; Bramley et al., 1985), and undifferentiated granulosa cells from small human follicles were not susceptible to GnRH-suppression (Casper et al., 1982).

6.2 TREATMENTS

6.2.1 GnRH, GnRH-agonist, and gonadotropin-regulated steroidogenesis

Effects of GnRH (Sigma) and GnRH-agonist ([D-Ala^6]-LHRH, Sigma) on steroidogenic response to gonadotropins were investigated by adding hCG (1 IU/ml), with or without GnRH (10^{-7}M), or GnRH-agonist. Human granulosa-luteal cells were incubated with GnRH for 24 or 48 h. Progesterone was measured by RIA.

6.3 RESULTS

6.3.1 Effect of GnRH on hCG- and cAMP-stimulated progesterone accumulation
Treatment of the cells with GnRH (10^7M) for 24 h did not affect basal progesterone synthesis (P>0.05, Fig. 31). Progesterone concentrations were increased 2-3 fold by hCG (P<0.01). GnRH had no effect on hCG-stimulated progesterone production (P>0.05, Fig. 31). Progesterone concentrations were increased 2-3 fold by a cAMP analog, 8-Br-cAMP. GnRH had no effect on cAMP-stimulated progesterone production (P>0.05, Fig. 31). Increased incubation time to 48 h had same result as that of 24 h incubation.

6.3.2 Effect of GnRH-agonist on hCG- and cAMP-stimulated progestin accumulation

Treatment of the cells with GnRH-agonist (10^7M) did not affect basal progesterone synthesis (P>0.05, Fig. 32). Progesterone concentrations were increased 2-3 fold by hCG (P<0.01). GnRH-agonist had no effect on hCG-stimulated progesterone production (P>0.05, Fig. 32). Progesterone concentrations were increased 2-3 fold by a cAMP analog, 8-Br-cAMP. GnRH-agonist had no effect on cAMP-stimulated progesterone production (P>0.05, Fig. 32).
Figure 31. Effect of GnRH (10^{-7}M) on basal and hCG (1 IU/ml)-stimulated progesterone production by human granulosa cells. Cells were incubated for 24 and 48 h. Values are mean ± sem (n=24). Different superscripts (above the sem bars) denote statistical differences (P<0.01).
Figure 32. Effect of GnRH agonist (10^7M) on basal and hCG (1 IU/ml)/cAMP (1.5 mM)-stimulated progesterone production by human granulosa cells. Values are mean ± sem (n=6). Different superscripts (above the sem bars) denote statistical differences (P<0.01).
6.4 DISCUSSION

LH/hCG treatment induced GnRH-like peptide secretion from FSH-sensitized granulosa cells (Hsuch et al., 1981a). The effect of GnRH on ovarian steroidogenesis is inconsistent. The results from murine, porcine, hamster, rabbit, chicken and human granulosa and luteal cells showed that GnRH suppress FSH-induced steroidogenesis and gonadotropin receptor expression (Massicotte et al., 1980; Hillensjo et al., 1980; Demoulin et al., 1991; Wickings et al., 1990; Behrman et al., 1980). Other studies showed that GnRH did not have effects on ovarian steroidogenesis (Bramley et al., 1985; Casper et al., 1982).

Results obtained here support the contention that GnRH does not affect progesterone production by human granulosa cells. The lack of effect of a GnRH agonist suggested that the lack of effects of GnRH was not because of its short half life. Incubation for 48 h with GnRH did not alter the results. This suggested that GnRH does not act at the ovary to alter gonadotropin-stimulated steroidogenesis. GnRH also lacked the ability to affect intracellular Ca\(^{2+}\) in human granulosa cells (Currie et al., 1992). Ca\(^{2+}\) is required for GnRH-suppression of cAMP and steroid production during prolonged incubation of rat ovarian cells (Sharpe et al., 1990). The reason for the conflicting results is unknown. Individual variation of granulosa cells from different patients and different pretreatment could be the explanation.
7.0 EFFECTS OF PROSTAGLANDIN F\textsubscript{2\alpha} ON PROGESTERONE PRODUCTION BY HUMAN GRANULOSA CELLS

7.1 INTRODUCTION

Studies using dispersed luteal cells from a variety of non-primate mammals have revealed that PGF\textsubscript{2\alpha} is a potent antagonist of LH/hCG action (Niswender et al., 1985; Lahav et al., 1976; Behrman et al., 1979; Richardson et al., 1980; Kenny et al., 1986; Harrison et al., 1987; Rogers et al., 1983, 1985). Evaluation of data in primates shows that the model used (dispersed vs. whole tissue), the concentration of PGF\textsubscript{2\alpha} used and the age of the corpus luteum are important variables in establishing conclusions. Studies using granulosa cells from preovulatory follicles and minced luteal tissue from monkeys and humans (Channing et al., 1975; Rogers et al., 1985; McNatty et al., 1975; Henderson et al., 1977; Auletta et al., 1983; Dennefors et al., 1982; Hamburger et al., 1979; Patwardhan et al., 1974) have shown that PGF\textsubscript{2\alpha} inhibits basal and LH-stimulated progesterone production. Incubations of dispersed luteal cells produced conflicting results. Using dispersed human luteal cells and lower concentrations of PGF\textsubscript{2\alpha}, PGF\textsubscript{2\alpha} was found to promote hCG-stimulated progesterone production during early and mid-luteal phases (Richardson et al., 1980). This stimulatory effect of PGF\textsubscript{2\alpha} was lost in the late luteal phase. Thus, a clear-cut effect of PGF\textsubscript{2\alpha} in the human ovary is lacking. The studies here were designed to examine effects of PGF\textsubscript{2\alpha} on steroidogenesis in human granulosa cells.
7.2 TREATMENTS

7.2.1 PGF$_{2\alpha}$, PGF$_{2\alpha}$-agonist, and gonadotropin-regulated steroidogenesis

Effects of PGF$_{2\alpha}$ (Sigma) and the PGF$_{2\alpha}$-agonists, cloprostenol and lutalyse (Gifts from Dr. Rajamahendran, Univ. of British Columbia) on steroidogenic response to gonadotropins were investigated by adding hCG (1 IU/ml) with or without PGF$_{2\alpha}$ (10E7M) or agonist. Progesterone were measured.

7.2.2 Dose-dependency of PGF$_{2\alpha}$ on hCG-stimulated steroidogenesis

Increasing doses of PGF$_{2\alpha}$ (10E10-10E6M) were added with hCG (1 IU/ml) to the cultured cells and the cells were incubated for 24 h. The incubation media were collected for progesterone RIA.

7.3 Results

7.3.1 Effects of PGF$_{2\alpha}$ and PGF$_{2\alpha}$-agonists on gonadotropin-stimulated progesterone accumulation

PGF$_{2\alpha}$ (10E7M) did not affect basal progesterone synthesis (P>0.05). Progesterone concentrations were increased 2-6 fold by hCG (P<0.01). PGF$_{2\alpha}$ did not inhibit hCG-stimulated progesterone production (Fig. 33). Cloprostenol inhibited hCG-stimulated progesterone production after 6 h incubation (P>0.05, Fig. 34). Lutalyse increased basal and hCG-stimulated progesterone production after 24 h incubation (Fig. 34, P<0.05).
7.3.2 *Dose-dependent inhibition of progesterone production by PGF$_{2\alpha}$*

PGF$_{2\alpha}$ at different doses had variable effects on progesterone production in response to hCG. PGF$_{2\alpha}$ numerically decreased hCG-stimulated progesterone production in 6 trials, the decrease being significant in only 2 trials at PGF$_{2\alpha}$ ($10^9$) and ($10^6$), respectively (Fig. 35).
Figure 33. Effect of PGF$_{2\alpha}$ (10$^{-7}$M) on hCG (1 IU/ml)/cAMP (1.5 mM)-stimulated progesterone production. Values are mean±sem (n=8). Results from four experiments are presented. Different superscripts (above the sem bars) denote statistical differences (P<0.01).
Figure 34. Effect of Cloprostenol and lutalyse on basal and hCG/cAMP-stimulated progesterone production. Human granulosa cells were incubated with cloprostenol for 6 and 24 h. Values are mean+sem (n=8). Different superscripts (above the sem bars) denote statistical differences (P<0.01).
Figure 35. Effect of PGF$_{2\alpha}$ (10$^{-9}$-10$^{-5}$ M) on hCG (1 IU/ml)-stimulated progesterone production. Cells were incubated for 24 h. Values are mean±sem (n=8). Results from six experiments are presented. ** P<0.01.
7.4 Discussion

Examination of the effect of PGF$_{2\alpha}$ on steroidogenesis in human granulosa cells provided inconsistent results. PGF$_{2\alpha}$ agonists, having longer metabolic half-lives, were used to prolong PGF$_{2\alpha}$ effects in culture. The same inconsistent results with cloprostenol and lutalyse suggested that metabolism of natural PGF$_{2\alpha}$ was not the reason for the inconsistencies. Again, patient variability may partially explain the variability in PGF$_{2\alpha}$ effects observed. The results suggested that the effects of PGF$_{2\alpha}$ on steroidogenesis depend on unknown local factors but not on gonadotropin concentrations. The concentration of the unknown factor(s) might alter the reaction of human granulosa to PGF$_{2\alpha}$. This provides evidence that effects of gonadotropins on steroidogenesis can be influenced by the cellular environment. The inconsistent results may also be caused by cell conditions related to the early luteal phase. An additive effect of PGF$_{2\alpha}$ on hCG-stimulated progesterone production during the early and mid-luteal phases was reported (Mason et al., 1986) in human luteal cells. PGF$_{2\alpha}$ ($10^{-5}-10^{-9}$M) increased free cytosolic Ca$^{2+}$ in individual human granulosa cells. This suggests that changes in intracellular Ca$^{2+}$ concentration can not be translated to changes in steroidogenesis by human granulosa cells.
8.0 HORMONAL REGULATION OF INHIBIN MESSENGER RNA IN HUMAN PLACENTAL CELLS

8.1 INTRODUCTION

Inhibins and activins were first isolated from gonads of both sexes and various species (Ling et al., 1986; Vale et al., 1986; Mason et al., 1985). Their functions in gonads have been studied in many species (Tsafriri et al., 1989; Sum et al., 1989; Ying et al., 1986; Shukovski 1990). Inhibin α, βA and βB subunit mRNA from human ovary have been sequenced (Mason et al., 1986). The study of inhibin gene regulation in human granulosa cells is difficult as there are not enough cells available for total RNA extraction. The regulation of inhibin gene expression in human term placental cells was investigated. The possibility that inhibin plays a role in pregnancy emerged from several studies. Bioactive and immunoactive inhibin levels have been reported in human placental extracts (McLachlan et al., 1986; Hochberg et al., 1981). These results were supported by the cloning and sequencing of inhibin cDNA from human term placenta (Mayo et al., 1986). Petraglia and Merchenthaler localized inhibin to the cytotrophoblast layer of the human placental villi by immunofluorescent staining method and showed that placental cultures can produce inhibin (Petraglia et al., 1987; Merchenthaler et al., 1987). Activin A may be synthesized by human term placenta as suggested by the presence of high levels of inhibin βA subunit mRNA (Meunier et al., 1988). In vivo studies showed that inhibin levels rose in first trimester with a further increase in the third trimester.
Inhibins and activins have been reported to regulate hCG and GnRH synthesis and secretion in human placental cells (Petraglia et al., 1987). Stimulation of hCG release by activin but not inhibin was confirmed using a placental cell perfusion system (Steele et al., 1993). However, little is known about the regulation of inhibin and activin secretion in human placenta. The inhibin α-subunit gene in rat contains a cAMP-responsive element (CRE) in the promoter region. Such a CRE has not been identified in rat βA- and βB-subunit genes (Chen et al., 1992). However, three CREs have been found in the human inhibin βB subunit gene (Mason et al., 1986). Inhibin βA mRNA expression appeared to be stimulated by 8-Br-cAMP in human fibrosarcoma cells (Tanimoto et al., 1992). Inhibin α-subunit increased during incubation of rat Sertoli cells in the presence of follicle-stimulating hormone (FSH) (Klaij et al., 1990). hCG, 8-Br-cAMP and adenylate cyclase activators (forskolin and cholera toxin) increased the release of inhibin from cultured human placental cells (Petraglia et al., 1987).

Placental GnRH may stimulate hCG secretion (Sailer-Cheater et al., 1981, 1984). GnRH stimulation of hCG secretion suggests a possible role for GnRH in the regulation of inhibin synthesis during pregnancy. This study investigated regulation of inhibin α, βA and βB subunit mRNA expression by GnRH and cAMP. A sensitive assay, reverse transcription-polymerase chain reaction (RT-PCR) for detection of inhibin subunit mRNA was employed to measure inhibin mRNA expression.
8.2 MATERIALS AND METHODS

8.2.1 Total RNA extractions

Trophoblast cells harvested after 6 h culture were centrifuged at 1000 x g for 5 min and cell pellets were resuspended in 4 M guanidinium isothiocyanate and homogenized using a polytron for 30 sec. Total RNA was prepared using the method of Chomezynski et al., 1987 and Glisin et al., 1974. RNA concentration was determined by UV-spectrophotometry.

8.2.2 Northern blot analysis

8.2.2.1 Preparation of plasmid vector DNA

8.2.2.1.1 Transformation

Ligated recombinant plasmid (10 µl, 1-5 ng/µl) was mixed with 200 µl of competent cells. After 30 min incubation on ice, the mixture was incubated at 42 C for 1 min, then 0.5 ml LB medium was added and incubated at 37 C for 1 h to allow expression of the antibiotic resistant gene. The cell mixture (0.2 ml) was spread over an LB-agar plate containing ampicillin (50 mg/L) for overnight culture. The positive colony was selected by small scale DNA preparation.

8.2.2.1.2 Small scale DNA preparation
The plasmid vectors (PGEM 4 containing inhibin Hα4, HβA-4 and GβB-5, kindly offered by Dr. Meunier H) was propagated in *E. coli* strain DH5α in 5 ml LB. The *E. coli* cells transformed with plasmid vector were incubated at 37°C until the exponential phase. The cells were collected by centrifugation and resuspended in 150 μl solution I (50 mM glucose, 25 mM Tris.Cl (pH8.0), and 10 mM EDTA (ethylenediaminetetraacetate, pH 8.0) and chilled on ice. Five min later, 200 μl of freshly prepared solution II (0.2 N NaOH and 1 % SDS (sodium didecyl sulfate) was added and mixed by inversion. Another 300 μl of ice-cold solution III (5 M potassium acetate 60 ml, glacial acetic acid 115 ml, H₂O 28.5 ml) was added and mixed for 5 min on ice. The mixture was extracted with equal volumes of phenol/chloroform. The supernatant was aspirated and dispensed into a fresh tube. Two volumes of 99% ethanol was added into supernatant for 5 min incubation at -20°C. The supernatant was centrifuged at 10000 x g for 10 min. The supernatant was discarded. The pellet was washed with 70% ethanol, then dissolved in 10 μl TE buffer (tris-EDTA, 10 mM Tris.Cl, 1 mM EDTA, pH 8.0). This plasmid "mini-prep" method was used for a rapid analytical restriction digest analysis for specific cDNA insert before large scale plasmid production.

8.2.2.1.3 Large scale plasmid preparation

*E. coli* cells transformed with plasmid vector were incubated with 250 ml LB medium (Luria-Bertani medium, bacto-tryptone, 10 g; bacto-yeast extract, 5 g; NaCl 10 g in 1 liter deionized H₂O) containing 12.5 mg ampicillin overnight at 37°C. Cells
were harvested at 5000 x g at 4 C for 15 min and resuspended in 6 ml freshly prepared ice-cold lysis buffer (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 15 % sucrose and 2 mg/ml lysozyme) at 4 C for 20 min. Twelve ml freshly prepared 0.2 N NaOH, 1 % SDS were added, mixed by inversion and kept on ice for 10 min. 7.5 ml of 3 M sodium acetate (pH4.6) was added for 20 min incubation on ice. The solution was centrifuged at 10000 x g for 15 min to allow separation of the DNA layer. The supernatant was removed and incubated with 50 µl of RNAse A (stock 1 mg/ml) for 20 min at 37 C. One volume of phenol:chloroform(1:1) saturated with TE buffer (pH7-8) was added and vortexed for 5 min, then centrifuged at 10000 x g for 10 min. This step was performed twice. The upper phase containing DNA was transferred into a fresh tube and 1 volume of chloroform:isoamyl alcohol (24:1) was added and vortexed. The phases were separated at 10000 x g for 10 min. The upper phase was transferred to a fresh tube containing 2 volumes of 95% ethanol. DNA was precipitated at 10000 x g for 20 min after 30 min incubation at -20 C. Sterile water (1.6 ml) was added to dissolve the pellet. 4 M NaCl (0.4 ml) and 2 ml of 13% polyethylene glycol (PEG, MW 6,000) were added for separating plasmid and bacterial DNA. Following 1 h incubation at 4 C, plasmid DNA was precipitated at 10000 x g for 10 min, washed with 70% ethanol and dissolved with distilled water to about 1mg DNA/ml.

8.2.2.2 Preparation of specific hybridization probe

The recombinant cDNA insert was isolated by restriction enzyme digestion,
followed by electrophoresis in 1% agarose gel. cDNA (25 ng) insert was labelled with 

\[^{32}P\]deoxycytidine triphosphate by a random primer labelling technique according to 

the procedure describe in the BRL random primer labelling system (BRL, Burlington, 

ON). DNA (25 ng) dissolved in distilled water was boiled at 95 C for 5 min, 

followed by rapid chilling on ice to denature the DNA. Two µl of 0.5 mM dATP, 

dTTP and dGTP, 15 µl of random primer buffer, 50 µCi of [α-\(^{32}P\)]dCTP and 1 µl of 

Klenow fragment (3U) were added and the mixture was incubated at 25 C for 1 h. 

The solution was heated at 100 C for 5 min followed by rapid chilling at 4 C. The 

probe was labelled to a specific activity of 10^9 dpm/µg DNA.

8.2.2.3 Northern analysis using specific probe 

Total RNA (40 µg) was denatured at 70 C for 15 min. The samples were 

electrophoresed through 1.0% (w/v) agarose, 2.2 M formaldehyde denaturing gel using 

20 mM 3-(N-morpholino) propanesulfonic acid (MOPS) buffer containing 8 mM 

sodium acetate and 1 mM EDTA (pH 8.0). Ribosomal RNAs (28S and 18S) were 

visualized under UV light to ensure the integrity of RNA samples. RNA was 

transferred to Hybond-N membranes (Amersham, Oakville, ON) by capillary transfer 

using 3 x SSC (sodium chloride/sodium citrate buffer, 1 x, 8.8 g NaCl; 4.4 g sodium 

citrate in 1 liter H₂O, pH 7). Following transfer, RNA was fixed to the membrane by 

UV light for 2 min.

8.2.2.4 Hybridization/Washing

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Prior to hybridization, the nylon membrane was soaked in a solution of prehybridization solution (50% formamide, 5 x SSPE (sodium chloride/sodium phosphate and EDTA buffer, pH7.4), 5 x Denhardt’s reagent, 0.1% SDS, 100 µg/ml denatured salmon sperm in 0.1% diethyl pyrocarbonate-treated H₂O) at 42 °C for at least 12 h. The filter was subsequently hybridized with a [³²P]dCTP labelled DNA probe overnight at 42 °C. The filter was washed 3 times at 42 °C for 20 min with about 100 ml each of 1x SSC containing 0.1% SDS and rinsed once with 0.1x SSC containing 0.1% SDS at 50 °C for 20 min. The blot was then air dried and autoradiographed by using intensifying screens at -70 °C.

8.2.3 Quantitative RT-PCR

8.2.3.1 Quantitation of Inhibin α, βA and βB subunit mRNA

Total RNA (5 µg) was electrophoresed on formaldehyde agarose gels. Ethidium bromide (1 µg) was added to each sample and the gel was photographed under UV-light following electrophoresis. Appearance of ribosomal RNA was used to assess the integrity and to confirm equal loading of RNA.

8.2.3.2 Synthesis of first strand cDNA from mRNA

Total RNA (5 µg) was subjected to first strand cDNA synthesis using a cDNA synthesis kit (Pharmacia, Baie d’urfe, QUE). Avian Myeloblastosis Virus (AMV) Reverse Transcriptase and 1.0 µg oligo d(T)₁₂₋₁₈ were added. The reaction buffer for
cDNA synthesis contained 10 mM Tris-HCl (pH 8.3), 6 mM Mg, 40 mM KCl, 50 mM DTT, 1mM dNTPs and 25 U RNase inhibitor. The reaction mixtures (15 µl) were incubated at 32 C for 1 h, then 95 C for 10 min and then chilled on ice. Synthesized cDNA (1 µl from total 15 µl) was used for PCR at standard conditions.

8.2.3.3 Primers of inhibin subunits

Primers derived from human inhibin α, βA and βB cDNA sequences (Mason et al., 1986) were used for amplification. The α primers correspond to nucleotides 807-828 (5'-CCCAGTTTTCATCTCCTCACTAC) and 1169-1188 (5'-TTCACCTCTGTGCTGG) of α subunit cDNA. The βA primers correspond to nucleotides 1186-1206 (5'-AGGTCAACATCTGCTGTAAG) and 1544-1564 (5'-TTCTCTGGACAACTGCT) of βA subunit cDNA. The βB primers correspond to nucleotides 748-768 (5'-TGTTGCAGGCAACAGTTTCTT) and 1150-1170 (5'-GAATGACTGTACTTAGCCCAC) of βB subunit cDNA. The identity of the amplified products was determined by sizing and Southern blot hybridization with cDNA probes (Fig. 36).

8.2.3.4 Quantitative PCR

Amplification was performed in the presence of 0.04 µCi [α<sup>32</sup>P] dCTP. The maximal incorporation of [α<sup>32</sup>P] dCTP into specific PCR products was about 20% of total counts. Amplification of β-actin mRNA served as an internal control. Optimal conditions of PCR were chosen by determining the ranges of exponential increase of product formation with the numbers of amplification cycles. Optimal cycle number
Figure 36. PCR amplification of inhibin α-, βA-, and βB-subunit mRNA from cultured placental cells. (A) Ethidium bromide stained PCR products separated in 1% agarose gel. Lanes: MW, molecular weight marker (1 kb DNA ladder, BRL, Toronto, ON); α, inhibin α-subunit PCR product; βA, inhibin βA-subunit PCR product; BB, inhibin βB-subunit PCR product. 5 μg of total RNA from human placental cells was used to synthesize cDNA. cDNA was amplified for 32 cycles. (B) Southern blot analysis. PCR products were blotted to Hybond nylon filter and were hybridized with [α^{32}P]-labeled specific inhibin subunit cDNA probes.
was determined for each batch of cDNA synthesized. Products were electrophoresed on 6% polyacrylamide gels. Following staining with ethidium bromide, products were excised from the gel and the radioactivity was counted by Cherenkow counting.

8.2.3.5 Statistical Analysis

The ratio of radioactivity (cpm) of inhibin subunit and β-actin was calculated for each sample. Each treatment group contained three samples in cell culture, and the experiment was repeated four times. Data were pooled from four experiments. mRNA values of inhibin subunits in treatment groups are reported as mean ± sem compared to control group. The n values, referring to the number of cell preparation, was 12. Area under the inhibin subunit mRNA expression response was compared by One-Way Analysis of Variance. Student-Newman-Keuls Multiple Comparisons Test was used to differentiate among means following a significant F-test.

8.3 RESULTS

8.3.1 Expression of human inhibin subunit mRNA

The yield of total RNA from human term placental cell culture is low (about 1 μg/10⁸ placental cells comparing with about 1 μg/10⁶ granulosa cells). Northern analysis of RNA was performed using the inhibin α, βA, and βB clones as probes. Inhibin βA mRNA were detectable with 40 μg total RNA in term placenta, but inhibin α and βB mRNA were not detectable (Fig. 37). Fig. 37 shows the autoradiography of
a Northern blot using 40 µg of total RNA from term placental cells treated with or without 8-Br-cAMP (1.5 mM) and GnRH (10^{-7}M). The Inhibin βA cDNA probe hybridized specifically to 7, 4.6, and 2.3 kb RNA species in human RNA. The level of inhibin βA mRNA in 8-Br-cAMP treated cells was higher than that in control cells (equal RNA loading was confirmed by hybridization of the blot with β-actin probe and autoradiography) (Fig. 37). When the same blot was probed with inhibin α and βB cDNA, no signal was detected (Fig. 37).
Figure 37. Northern analysis of inhibin α, βA and βB mRNA from term placental cells treated with or without 8-Br-cAMP (1.5 mM) and GnRH (10^{-7}M). Figure 37A shows ethidium bromide staining of 40 μg total RNA in RNA denature gel. C, control group; cAMP, 8-Br-cAMP (1.5 mM)-treated group; GnRH, GnRH (10^{-7}M)-treated group; rat uterus, 20 μg total RNA from rat uterus. Human term placentas were treated for 6 h at 37 C. Figure 37B shows the autoradiography of a Northern blot. The RNA denature gel was transferred to the Hybond nylon membrane which was hybridized with β-actin, inhibin α, βA, and βB [^{32}P]dCTP-labelled cDNA probe overnight at 42 C. After hybridization, the membrane was washed 3 times at 42 C for 20 min with about 100 ml each of 1x SSC containing 0.1% SDS and rinsed once with 0.1X SSC containing 0.1% SDS at 50 C for 20 min. The blot was then air dried and autoradiographed by using intensifying screens at -70 C.
A

C  cAMP  GnRH  uterus

--- 28s
--- 18s

B

β-actin

α

βA

βB

4.8kb
1.9kb
8.3.2 *Inhibin α, βA and βB mRNA expression in human placental cells*

The levels of α and βB mRNA are too low for Northern blot analysis using cDNA probes. Therefore, a more sensitive assay, quantitative RT-PCR for the detection of inhibin subunit mRNAs has been employed for measuring the levels of inhibin mRNA expression. Oligonucleotide primers were selected to 5' regions of α, βA and βB subunits mRNAs where their sequences are not homologous.

Fig. 36 shows the product formation for each subunit amplification. The size corresponded to the predicted fragment and authenticity was confirmed by Southern blot with the respective cDNA probes.

Fig. 38 shows the inhibin α, βA and βB PCR products from a pool of reverse transcribed cDNA samples at different cycles. The amount of PCR products of the three subunits increased logarithmically with cycle number until 40 cycles (Fig. 38). The results suggested that the levels of βA and α subunits mRNA expression were higher than that βB subunit.
Figure 38. Influence of PCR cycle number on product formation. (A) Ethidium bromide stained PCR products separated on 1% agarose gel. α, PCR products of inhibin α-subunit; βA, PCR products of inhibin βA-subunit; βB, PCR products of inhibin βB-subunit. The cycle number are shown on the x-axis. (B) PCR products were quantitated by counting the radioactivity of the excised bands. The counting number (cpm) were plotted against the number of cycles.
(A) 

(B) 

10^4

10^3

10^2

0 10 20 30 40 cycles

α

βA

βB

0 10 20 30 40 50 cycles

Cycle number of PCR

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Figure 39. Quantitative analysis of inhibin α-, βA-, and βB-subunit mRNA levels in 8-bromo cyclic AMP (cAMP) and GnRH treated placental cells. (A) Ethidium bromide stained PCR products separated on 6% polyacrylamide gel (data from 1 experiment). Lanes: MW, molecular weight marker; 1-3, inhibin subunit PCR products from control group; 4-6, inhibin subunit PCR products from cAMP treated group; 7-9, inhibin subunit PCR products from GnRH treated group; 10, control reaction without cDNA template. (B) 3 samples were pooled from 4 experiments in each treatment group, to a total n=12. The values on Y axis represent the ratio of treatment to control group. The data were analyzed by Student-Newman-Keuls Multiple Comparisons Test. * Significantly different from control group (P<0.05, n=12); ** Significantly different from control group (P<0.01, n=12).
8.3.3 Stimulation of inhibin subunit mRNA expression by 8-Br-cAMP and GnRH

Treatment with 8-Br-cAMP stimulated inhibin α-, βA- and βB-subunit mRNA expression (Fig. 39). GnRH stimulated inhibin α- and βB-subunit but not βA-subunit mRNA expression (Fig. 39). Each treatment group contained 12 samples, pooled from four experiments. As shown in Fig. 39, PCR products in β-actin groups did not change when 8-Br-cAMP or GnRH were added. The PCR products of inhibin α- and βB-subunit increased 2-3 fold by 8-Br-cAMP (150 μM) (P<0.01) and GnRH (100 nM) (p<0.01) (Fig. 39). The PCR product of inhibin βA-subunit slightly increased 1.3 fold by 8-Br-cAMP (150 μM) (P<0.05, Fig. 39).

8.4 DISCUSSION

Inhibins and activins are found in human placenta (Petraglia et al., 1987; Merchenthaler et al., 1987), but little is known about their regulation of synthesis. Quantitative RT-PCR is a sensitive technique for evaluation of mRNA comparing with Northern blot analysis. The high level of inhibin βA mRNA in human placenta is consistent with a previous report (Meunier et al., 1988a). The presence of inhibin βA precursors of 7.0, 4.6, and 2.3 kb in Northern blot analysis is also consistent with previous reports (LaPolt et al., 1989). The study of regulation of inhibin subunit mRNA expression showed that cAMP stimulated inhibin βA precursor mRNA expression. In order to study inhibin α and βB subunit mRNA expression, we used a more sensitive method, the quantitative RT-PCR. The quantification of mRNA levels by RT-PCR is affected by the efficiencies of both cDNA synthesis and PCR amplification. About 10-
30% variation was observed from cDNA synthesis and PCR. Each treatment was triplicated and the experiment was repeated four times for statistic analysis. Quantitative analysis (Fig. 38) showed that the amount of inhibin subunit mRNA expression was $\beta_A > \alpha > \beta_B$. This result is in agreement with Northern blot data (Meunier H, et al., 1988a).

8-Br-cAMP has been reported to stimulate inhibin production from human placental cells (Petraglia et al., 1987). The results of this study of inhibin $\alpha$-, $\beta_A$- and $\beta_B$-subunit at the mRNA level supports this observation. It suggests that cAMP may be a second messenger, inducing inhibin $\alpha$- and $\beta_B$-subunit expression from human placental cells. It has been shown that the inhibin $\alpha$-subunit gene contains a cAMP- and phorbol ester-inducible DNA fragment (Klaij et al., 1990; Feng et al., 1989). Inhibin $\alpha$-subunit mRNA levels increased when dibutylryl cAMP was administrated in rat Sertoli cells (Klaij et al., 1990), even though its promoter region does not appear to have a CRE (Feng et al., 1989). Inhibin $\beta_B$-subunit was also stimulated by 8-Br-cAMP in this study, which supports the finding that human inhibin $\beta_B$ subunit gene has three CRE (Mason et al., 1986). The expression of inhibin $\beta_B$-subunit mRNA has been reported to increase by treatment of rat Sertoli cells with cycloheximide, an activator of adenyl cyclase (Klaij et al., 1990). The increase of inhibin $\beta_B$ mRNA expression by cAMP may increase inhibin B or activin AB synthesis in human placenta. The higher mRNA level of inhibin $\beta_A$-subunit relative to that of inhibin $\alpha$- and $\beta_B$-subunit suggests that the expression of inhibin $\alpha$- and $\beta_B$-subunit mRNA, may be rate-limiting steps during inhibin synthesis in human placental cell. The findings that cAMP slightly increased and GnRH did not have any effect on inhibin $\beta_A$-subunit mRNA levels suggests that the mechanisms of regulation
of inhibin (αβA and αβB) and activin (βAβA and βAβB) synthesis may be different.

Previous reports have suggested a stimulating action of hCG on placental adenylate cyclase and cAMP formation (Davis et al., 1989; Bicsak et al., 1986) and a stimulating action of placental GnRH on hCG secretion (Sailer-Cheater et al., 1981; Currie et al., 1981; Seeburg et al., 1984). The possibility that the effect of GnRH involves stimulation of mRNA expression of inhibin subunits was investigated. Increases of mRNA expression of inhibin α- and βB-subunits by GnRH suggest a possible stimulating action of GnRH on placental inhibin production. The same effects of GnRH and cAMP on inhibin α- and βB-subunit mRNA expression suggest that the effects of GnRH may depend on an increase of intracellular cAMP level. GnRH-stimulated hCG production by human term placental cells also can be mimicked by cAMP (Currie et al., 1993). Therefore, it is possible that the intracellular pathway of GnRH in human placental cells is adenyl cyclase-cAMP-dependent.

Petraglia et al (1987) reported that anti-inhibin serum increased GnRH-like immunoreactivity (GnRH-LI) in human placental cell cultures (Petraglia et al., 1987). The presence of a feedback loop between inhibin and GnRH may have physiological significance. Maternal plasma inhibin levels are low during first trimester, increased in second trimester, peak in third trimester (Healy et al., 1990). The highest concentrations of GnRH-LI were seen in first half pregnancy, then were sustained in the second half (Siler-Khodr et al., 1984). It is possible that a low concentration of placental inhibin may contribute to an increased GnRH-LI production during early pregnancy, and high concentration of inhibin suppress the continuous increase of GnRH-LI production from
placenta during late pregnancy.

The high mRNA levels of inhibin βA subunit relative to that of inhibin α subunit suggest the formation of activin A in human placenta (Meunier et al., 1988a). Activin A may play a role in the control and regulations of growth and developmental functions according to its function in other tissues (DePaolo et al., 1991). Activin-A might be regulated by human placental lactogen (hPL) or other hormones in placental cells but not GnRH. Further experiments need to be done to investigate regulation of activin-A expression.

In summary, quantitative RT-PCR confirmed the existence of mRNA expression of inhibin and activin subunits in human placenta at term. cAMP and GnRH stimulated inhibin α- and βB-subunit mRNA expression. GnRH-stimulated inhibin mRNA expression may be cAMP-dependent. Activin-A may not be regulated by GnRH and cAMP.
9.0  HORMONAL REGULATION OF INHIBIN MESSENGER RNA IN HUMAN GRANULOSA CELLS

9.1  INTRODUCTION

Inhibin and activin have paracrine roles modulating ovarian thecal and granulosa cell gonadotropin-mediated steroidogenesis (Tsafriri et al., 1989; Sum et al., 1989; Ying et al., 1986; Shukovski et al., 1990; Sugino et al., 1988b). McLachlan et al. (1987) developed a RIA for inhibin and reported the pattern of inhibin secretion during the normal menstrual cycle and in gonadotropin-induced cycles. Additionally, inhibin was detected in follicular fluid and placenta (Robertson et al., 1985; McLachlan et al., 1986). Detection of human inhibin α- and βA-gene expression by standard Northern blotting techniques has been reported for placenta and corpus luteum (Petraglia et al., 1991; Meunier et al., 1988c). In situ hybridization localized inhibin βA mRNA to the granulosa cells of follicles; the α mRNA is expressed at low levels in other ovarian cell types (Woodruff et al., 1988; Meunier et al., 1988b). The regulation of inhibin secretion by steroid and polypeptide hormones has been examined in rat granulosa cells. The levels of secreted inhibin and inhibin mRNA are regulated by FSH in rat granulosa culture, consistent with in vivo data that FSH increases inhibin gene expression (Woodruff et al., 1988; Meunier et al., 1988). FSH increases inhibin α and βA mRNA transcription rates in rat primary granulosa cells (Woodruff et al., 1988). LH stimulates inhibin production only after induction of LH receptor with FSH (Bicsak et al., 1986; Zhiwen et al., 1988).
cAMP also stimulates inhibin production by granulosa cells, suggesting that effects of FSH and LH on inhibin production are cAMP-mediated. This study examined regulation of mRNA expression of 3 inhibin subunits by cultured human granulosa cells. This was accomplished using quantitative reverse transcription and polymerase chain reaction.

9.2 MATERIALS AND METHODS

9.2.1 Human granulosa cell treatment

hCG (1 IU/ml) or cAMP (1.5 mM) were added 96 h after initiation of culture. Each treatment group had 4 wells.

9.2.2 Total RNA extraction

Media were collected, following 24 h incubation with 0.5% BSA containing hormone treatment, for progesterone assay. The cells in each well were collected separately by adding 0.5 ml lysis buffer to each well. Total RNA was prepared using the RNaid kit method (Bio/Can Scientific). RNA concentrations were determined by UV-spectrophotometry.

9.2.3 Quantification of Inhibin α, βA and βB subunit mRNA

Total RNA (0.5 µg) was electrophoresed on formaldehyde agarose gels. Ethidium bromide (1 µg) was added to each sample and the gel was photographed under UV-light following electrophoresis. The appearance of ribosomal RNA was used to assess the
integrity of the RNA and to confirm equal loading. Total RNA (0.5 μg) was subjected to first strand cDNA synthesis using a cDNA synthesis kit (Pharmacia, Baie D’Urfe, QUE). Synthesized cDNA (1 μl from total 15 μl) was used for PCR at standard conditions. Primers derived from human inhibin α, βA and βB cDNA sequences (Mason et al., 1986) were as described in section 7.2.4. The identity of the amplified products was determined by sizing and Southern blot hybridization with cDNA probes (Fig. 40). Amplification by PCR was performed as described in section 7.2.4.

9.2.4 Statistical Analysis

The data were analyzed as described in section 7.2.4.5.

9.3 RESULTS

9.3.1 Inhibin α, βA and βB mRNA expression in human granulosa cells

The yield of total RNA from human granulosa cell cultures is ≈ 1 μg/10⁶ cells. A more sensitive assay, RT-PCR, has been employed for measuring inhibin subunit mRNA expression using smaller cell numbers.

Fig. 40 shows the product formation for each subunit with PCR amplification. The size corresponded to the predicted fragment and authenticity was confirmed by Southern blot with the respective cDNA probes. Fig. 41 shows the inhibin α, βA and βB PCR products from a pool of reverse transcribed cDNA samples at different cycles. The amount of PCR products of the 3 subunits increased logarithmically with cycle number.
Figure 40. PCR assay of inhibin α-, βA-, and βB-subunit mRNA in cultured human granulosa cell RNA and Southern blot analysis of PCR products. Top: Ethidium bromide staining of PCR products separated in 1% agarose gel. Lanes: MW, molecular weight markers; α, inhibin α-subunit PCR product; βA, inhibin βA-subunit PCR product; βB, inhibin βB-subunit product. 500 ng of total RNA from 5 day cultured human granulosa-luteal cells was used to synthesize cDNA. cDNA was amplified for 30 cycles. Bottom: Southern blot analysis. PCR products were blotted to Hybond nylon filter and hybridized with [α-32P]-labeled specific inhibin subunit cDNA probes.
Figure 41. Different amplification cycles of PCR products of inhibin subunits using human granulosa-luteal cell total RNA. Top: Ethidium bromide staining of PCR products separated in 1% agarose gel. α, PCR products of inhibin α-subunit; βA, PCR products of inhibin βA-subunit; βB, PCR products of inhibin βB-subunit. Cycle numbers are shown. Bottom: The inhibin α-, βA-, and βB-subunit PCR products of different cycles were quantitated by counting the radioactivity of the excised gel bands. Counts (cpm) were plotted against the number of amplification cycles.
until 40 cycles (Fig. 41). The results suggested that \( \alpha \) and \( \beta B \) subunit mRNA expression were greater than for \( \beta A \) mRNA.

9.3.2 *Stimulation of inhibin subunit mRNA expression by 8-Br-cAMP and hCG*

Treatment with 8-Br-cAMP stimulated inhibin \( \alpha \)- and \( \beta B \)-subunit mRNA expression (Fig. 42). hCG stimulated inhibin \( \beta A \)-subunit but not \( \alpha \) and \( \beta B \)-subunit mRNA expression (Fig. 42). Each treatment group had 16 samples, pooled from 4 experiments. PCR products in \( \beta \)-actin groups did not change when 8-Br-cAMP or GnRH were added (Fig. 42). The PCR products of inhibin \( \alpha \)- and \( \beta B \)-subunit were increased 1.8-3 fold by 150 \( \mu \)M 8-Br-cAMP (P<0.01, Fig. 42). The PCR product of inhibin \( \beta A \)-subunit was increased 1.8 fold by 1 IU hCG (P<0.05, Fig. 42).
Figure 42. Quantitative analysis of inhibin α-, βA-, and βB-subunit mRNA levels in 8-Br-cAMP (0.15 mM) and hCG (1 IU/ml) treated granulosa-luteal cells.

Top: Ethidium bromide staining of PCR products separated in 1% agarose gel. Lanes: MW, molecular weight markers; hCG, hCG (1 IU/ml) treated group; cAMP, 8-Br-cAMP (1.5 mM) treated group; φ, control without cDNA template. Bottom: Quantitative analysis of inhibin α-, βA-, and βB-subunit mRNA levels in 8-Br-cAMP and hCG treated granulosa cells. Sixteen samples were pooled from 4 experiments in each treatment group and quantitated by counting the radioactivity of the excised gel bands. The values on the Y axis represent the ratio of treatment to control group. Data were analyzed by Student-Newman-Keuls Multiple Comparisons Test. Different superscripts (above the sem bars) denote statistical differences (P<0.01).
9.4 DISCUSSION

Inhibin α−, βA- and βB-subunit mRNA expressed in human granulosa-luteal cells provides evidence for the local activity of inhibins and activins. Inhibin α−-subunit mRNA is highly expressed in human granulosa-luteal cells in vitro, the βA- and βB-subunit mRNA are also expressed in the human granulosa-luteal cells but their expression levels are much lower. This result is consistent with reported findings in rat and human (Woodruff et al., 1987; Turner et al., 1989; LaPolt et al., 1989; Lapolt et al., 1990; Eramaa et al., 1993). Studies in monkey and human showed that α−-, βA-subunit mRNA, were detectable, but βB-subunit mRNA was weakly- or non-detectable in the granulosa-luteal cells (Erammaa et al., 1993; Schwall et al., 1990). The inhibin βB subunit immunoreactivity reported in human granulosa-luteal cells supports the finding in this study (Yamoto et al., 1991). Thus, the lower level of βB mRNA that could not be detected by Northern blot analysis but was detected by RT-PCR analysis is efficiently translated to protein. Although the significant biological differences of inhibin A and B, and activin A and AB has not been well understood, the finding of βB-subunit mRNA expression in human granulosa-luteal cells indicates that except inhibin A and activin A, inhibin B and activin AB also secrete from these cells and may play a role during ovulation and corpus luteum formation.

Stimulatory effects of FSH on inhibin secretion and subunit mRNA levels were mimicked by cAMP and cAMP-generating reagents, including forskolin and cholera toxin (Bicsak et al., 1986; Zhang et al., 1987; Lapolt et al., 1990). Inhibin α−- and βB-subunit mRNA expression in human granulosa cells were increased by 8-bromo cAMP but not
hCG. These results suggest that stimulation of α- and βB-subunit gene expression is through the cAMP-dependent protein kinase A pathway. The finding that cAMP increased inhibin α mRNA was consistent with reports that several potential cAMP-responsive elements (CRE) have been identified in the 5'-flanking regions of the rat and human inhibin α- and βB- subunit genes (Feng et al., 1989; Mason et al., 1986). Inhibin α-subunit mRNA expression also increased when dibutyryl cAMP was administrated in rat Sertoli cells (Klaij et al., 1990). The increase of inhibin βB mRNA expression by cAMP may increase inhibin B or activin AB synthesis in human granulosa-luteal cells. It is interesting that the effect of hCG on inhibin subunit gene expression differs with 8-bromo cAMP in this study. Although hCG increases the intracellular cAMP level and both hCG and FSH stimulate progesterone production in human granulosa-luteal cells (Li et al., 1992; Li et al., 1993), the effect of hCG on inhibin production may differ from FSH and cAMP. The mechanism causing different effects of hCG and cAMP on inhibin subunit gene expression is unknown. hCG may stimulate inhibin βA mRNA expression by intracellular pathways other than adenylate cyclase-cAMP, such as protein kinase C pathway, which may attenuate the intracellular cAMP effect on inhibin α- and βB-subunit gene expression and trigger inhibin βA-subunit gene expression. This result is supported by the previous studies that LH at low doses stimulated inhibin protein and mRNA from rat granulosa cells after incubation with FSH (Bicsak et al., 1986; Turner et al., 1989; Zhang et al., 1988), whereas high doses of LH/hCG inhibited PMSG/FSH-induced inhibin production in vitro (Zhang et al., 1988; Michel et al., 1991) and in vivo (Lee, 1983). hCG increased βA-subunit mRNA level when those of the α- and βB-subunit mRNA
unchanged. An increase in the βA/α mRNA ratio by hCG may reflect the increased inhibin A and activin-A synthesis. The physiological effects of inhibin A and activin A have been shown in rat and human follicles (Woodruff et al., 1990). Activin A promoted cell proliferation, inhibited progesterone production induced by gonadotropins, and stimulate basal oestradiol production in cultured human granulosa-luteal cells (Li et al., 1992; Khorasheh et al., unpublished data; Rabinovici et al., 1990). In order to elucidate the roles of different inhibin and activin dimers as regulators of human ovarian function, the study for the regulation of inhibin each subunit gene expression in human granulosa cells is the key step. This study shows that the granulosa-luteal cells of IVF patients can be used successfully in mRNA regulation studies of inhibin/activin subunits with highly sensitive quantitative RT-PCR analysis. The results suggest that inhibin α-, βA- and βB-subunit mRNA are expressed in cultured human granulosa-luteal cells and hCG and cAMP stimulate three subunit mRNA expression in the different mechanism.
10. GENERAL SUMMARY

Physiological Roles of Activin and Follistatin

The hypothesis that local substances mediate human granulosa-luteal cell responses to gonadotropin has been examined. Activin inhibited gonadotropin-stimulated progesterone production in these studies, inhibin had no effect on basal or hCG-stimulated progesterone production, while follistatin increased basal progesterone production. Activin, inhibin and follistatin have been isolated from mammalian follicular fluid (Ling et al., 1986; Ling et al., 1987, Robertson et al., 1987). Suppression of hCG/FSH-stimulated progesterone production by activin in cultured human granulosa-luteal cells suggests a role for activin in prevention of luteinization of preovulatory or large antral follicles in vivo. Higher levels (60 IU/ml) of inhibin were produced by rat granulosa cells when stimulated with FSH (Zhiwen et al., 1987). Thus, generation of endogenous inhibin by the cells may have affected the results from the present experiments where exogenous gonadotropins were added. The possible mechanisms underlying inhibitory effects of gonadotropin-stimulated progesterone production by activin A may involve in the interference with the coupling of gonadotropin receptor and adenylate cyclase-cAMP cascade. IBMX significantly increased intracellular cAMP accumulation, but did not change basal and hCG-stimulated progesterone production. This result suggested that blocking intracellular cAMP degradation to AMP had no effect on progesterone production. Although activin inhibited hCG-stimulated intracellular cAMP level, inhibition of gonadotropin-stimulated progesterone production by activin through
additional intracellular pathway has not been excluded. It is generally agreed that activin stimulates FSH secretion which, in turn, increases estrogen production in the ovary. Thus, it is plausible that the overall action of activin may be to promote follicular differentiation and delay the onset of luteinization.

The stimulatory effect of follistatin on progesterone production in cultured human granulosa-luteal cells suggests a regulatory role of follistatin in the human ovary and its potential for an autocrine role. Follistatin is a binding protein of activin (Robertson et al., 1990; Nakamura et al., 1990). Thus, it can be postulated that a balance between follistatin and activin-A activity contributes to the regulation of the granulosa-luteal cell response to gonadotropins. Increased FSH is necessary for development of antral follicles to the preovulatory stage (Meunier et al., 1988c), a phase in follicular maturation when synthesis of follistatin is stimulated (Klein et al., 1991). Increased basal progesterone secretion from human preovulatory granulosa cells mediated by follistatin suggests that follistatin could in part contribute to the rise in progesterone before the preovulatory LH surge. The results showed that increased basal progesterone is accompanied by an increase in cAMP accumulation. The action of follistatin may also implicate an indirect action such as through the binding of activin (Robertson et al., 1990; Nakamura et al., 1990) and disinhibition of activin suppression of progesterone synthesis (Saito et al., 1991). This potential mechanism of action remains to be investigated. The in vitro data indicate that follistatin, rather than inhibin, may function as an intra-ovarian antagonist of activin. Follistatin, as a binding protein of activin, may bind with activin to neutralize each others' action(s) on granulosa-luteal cells. The concentrations and/or
the ratio of follistatin and activin may play a key role in regulation of steroidogenesis by granulosa-luteal cells during different stages of cell differentiation.

Inhibin α-, βA-, and βB-subunit mRNA expression in human granulosa cells may be evidence for local inhibin and activin effects. This study showed that activin and follistatin act in an autocrine fashion. Inhibin α-subunit mRNA expression is greater than inhibin βA and βB mRNA expression. This is consistent with reported findings (Mayo et al., 1986; Mason et al., 1986; Davis et al., 1986). The results suggest that βA- and βB-subunit expression is the rate-limiting step for inhibin and activin synthesis. Furthermore, hCG may increase inhibin A and activin A synthesis by stimulating inhibin βA-subunit mRNA expression. Inhibin α- and βB-subunit mRNA levels were increased by 8-Br-cAMP but not by hCG. This suggests that effects of hCG and cAMP on inhibin subunit gene expression are not mediated through a common second messenger system. Hence, hCG may stimulate inhibin βA mRNA expression by intracellular pathways other than adenylate cyclase-cAMP pathway, while not increasing intracellular cAMP concentration enough to increase inhibin α and βB mRNA expression.

With this hCG-altered inhibin βA subunit expression, it is possible that hCG may increase inhibin A or activin A production from these cells. Activin A may act as a dominant follicle selector by maintaining high estrogen secretion (Khorasheh et al., unpublished data) and by inhibiting gonadotropin-stimulated progesterone production in given follicles. Furthermore, activin A and hCG/LH may feedback on activin-A and progesterone production from human granulosa-luteal cells. Since follistatin is an activin binding protein, the ratio of follistatin to activin may be critical to steroid production by
individual follicles. The hCG-stimulated intracellular cAMP concentration was decreased by activin, while the basal intracellular cAMP concentration was increased by follistatin. Thus follistatin may increase inhibin $\alpha$ and $\beta B$ subunit mRNA production by the increase of intracellular cAMP. This further suggested a balanced action between the opposing roles of activin-A and follistatin in other functions of granulosa-luteal cells regulated by intracellular cAMP (Fig. 43).

Figure 43. A model, suggested by the results, for the interaction of inhibin-related peptides and gonadotropins in the regulation of intracellular cAMP production, progestin synthesis and inhibin subunit mRNA transcription by human granulosa-luteal cells. AC, adenylate cyclase. Solid lines express
the established conclusion. Dash lines express the possible actions.

**Physiological Roles of A III**

A II and A III bind to, and are locally produced by human granulosa-luteal cells (Husain et al., 1987). LH, but not FSH, increases the concentration of renin-angiotensin in human follicular fluid (Carruthers et al., 1990). This suggests that angiotensins have local actions in the human ovary. A III may be induced by the LH surge during the early luteal phase, thus, the renin-angiotensin system may be involved in neovascularization and steroidogenesis during corpus luteum formation. In these studies A III inhibited hCG-stimulated progesterone production dose-dependently in cultured human granulosa-luteal cells. Thus, a negative feedback loop between the renin-angiotensin system and LH may regulate progesterone production by the human corpus luteum in the early phase. A III may also act as a luteolytic factor during the late luteal phase. This hypothesis would be supported by evidence of higher renin-angiotensin bioactivity in late luteal phase than that in early luteal phase. The intracellular mechanism of A III effect on steroidogenesis is unclear and the study did not support the involvement of the DAG-protein kinase C system (Fig. 44).
Figure 44. A model, suggested by the results, for the interaction of angiotensins, hCG and PKC in the regulation of progesterone production by human granulosa-luteal cells. AC, adenylate cyclase; PIP2, phosphatidylinositol 4,5-bisphosphate. DAG, Diacyl glycerol. Solid lines express the established conclusion. Dash lines express the possible actions.

Physiological Roles of GnRH and PGF$_{2\alpha}$

GnRH and PGF$_{2\alpha}$ are present in human follicular fluid. GnRH had no effect on
basal or hCG-stimulated progesterone production in this study. PGF$_{2\alpha}$ had inconsistent effects on basal and hCG-stimulated progesterone production. GnRH and PGF$_{2\alpha}$ had luteolytic effect on non-human mammalian granulosa-luteal cells, suggesting differences between species. The reason for the inconsistent effects of PGF$_{2\alpha}$ in this study may have been related to unknown factors secreted by granulosa cells or differences in cell pretreatment or differentiation. The effect of PGF$_{2\alpha}$ might be altered by these unknown factors. The different results reported in other studies in human and other mammals suggest that the effects of PGF$_{2\alpha}$ on progesterone production, by granulosa-luteal cells, depends on the species studied and the precise experimental conditions.

**Inhibin subunit mRNA expression in human term placenta**

Both inhibins and activins are found in human placenta (Petraglia et al., 1987; Merchenthaler et al., 1987), a finding confirmed in the present study, by the presence of specific mRNA for each subunit. However, little is known about the regulation of their synthesis. Using quantitative PCR, the relative amounts of inhibin subunit mRNA expression was shown to be $\beta A > \alpha > \beta B$. This finding agreed with the results obtained from Northern blot analysis (Meunier et al., 1988a). The high level of inhibin $\beta A$ mRNA and the presence of inhibin $\beta A$ precursors of 7.0, 4.6, and 2.3 kb in Northern blot analysis were consistent with previous reports (LaPolt et al., 1989; Meunier et al., 1988a), and suggested the preferential formation of activin A in the human placenta (Meunier et al., 1988a). Activin A may play a role in control of fetal growth and development, as suggested by the multiple functions of activin in diverse tissues (Depaolo et al., 1991).
The finding that 8-bromo cAMP increased inhibin α-, βA- and βB-subunit mRNA expression in cultured human placental cells was supported by a previous report (Petraglia et al., 1987). Furthermore, it has been shown that inhibin α- and βB-subunit gene contains the cAMP- and phorbol ester-inducible DNA fragment (Mason et al., 1986; Feng et al., 1989). The addition of 8-Br-cAMP led to higher levels of inhibin α- and βB-, than inhibin βA-subunit mRNA, suggesting that the expression of inhibin α- and βB-subunit mRNA may be a hormone-dependent and rate-limiting step during inhibin synthesis in human placental cell.

Previous reports have suggested a stimulatory action of hCG on placental adenylate cyclase and cAMP formation (Moore et al., 1992; Davis et al., 1986), and a stimulatory action of placental GnRH on hCG secretion (Sailer-Cheater et al., 1981; Currie et al., 1992; Currie et al., 1981; Seeburg et al., 1984). The present finding of GnRH-increased inhibin α- and βB-subunit mRNA expression, suggested a stimulatory action of GnRH on placental inhibin B production. The same effects of GnRH and cAMP on both inhibin α- and βB-subunit mRNA expression raises the possibility that the effect of GnRH is secondary to increased hCG synthesis and intracellular cAMP level. Alternately, it is possible that the action of GnRH in human placental cells is independent of the hCG-induced adenyl cyclase-cAMP pathway.

Treatment with anti-inhibin serum increased GnRH-like immunoactivity in human placental cell cultures (Petraglia et al., 1987), suggesting the presence of a regulatory feedback loop between inhibin and GnRH. Maternal plasma inhibin levels are low during the first trimester, increased in second trimester and peak in the third trimester (Healy et
al., 1990). The highest serum levels of immunoactive GnRH are seen in the first half pregnancy and become sustained in the second half (Siler-Khodr et al., 1984). It is possible that an increased concentration of placental inhibin may contribute to an increased GnRH production during pregnancy, and high concentration of inhibin may suppress the continuous increase of GnRH production from placenta during late pregnancy (Petraglia et al., 1987). The present finding that GnRH stimulates inhibin subunit mRNA expression at term further supports the notion of a complex interplay between GnRH and inhibin in the human placenta.
11. FUTURE EXPERIMENTS

11.1 ACTIVIN-A AND FOLLISTATIN

11.1.1 Interaction of activin-A and follistatin

Further experiments need to be done for the investigation of interaction of follistatin and activin-A. As they are binding proteins for each other, it can be postulated that a balance between follistatin and activin-A activity contributes to the regulation of the granulosa-luteal cell response to gonadotropins. The experiment with follistatin incubated with activin-A could show the neutralized action between follistatin and activin-A. The binding ratio of follistatin to activin should be about 4 to 1 when activin reach the maximum concentration (Takanori et al., 1990). In order to allow time for binding, a preincubation of follistatin with activin could also be attempted. These experiments should indicate if follistatin can antagonize the action of activin-A in progesterone production.

11.1.2 Regulation of inhibin subunit mRNA expression by FSH and follistatin

Inhibin α-, βA-, and βB-subunit mRNA is expressed in human granulosa cells and regulated by hCG and cAMP. Results in this thesis have shown that hCG increased inhibin βA-subunit mRNA expression and 8-Br-cAMP increased inhibin α and βB mRNA levels. These results suggest that the effects of hCG and cAMP on inhibin subunit gene expression are not related. FSH and hCG had different effects on inhibin subunit mRNA
expression and inhibin production (Bicsak et al., 1986; Turner et al., 1989; Zhang et al., 1988; Michel et al., 1991). Therefore, it is worthwhile to study FSH effect on inhibin subunit mRNA expression in these cells. Follistatin may increase inhibin α and βB subunit mRNA production by increasing intracellular cAMP.

The mechanism of such stimulatory effects of hCG on inhibin βA mRNA expression in human granulosa cells is unknown. Therefore it would be worthwhile to study the role of the PKC pathway on inhibin subunit gene expression in these cells in order to elucidate the mechanism of hCG stimulatory effect on inhibin βA mRNA expression.

11.2 EFFECT OF A II ON BASAL AND hCG-STIMULATED ESTRADIOL PRODUCTION AND MECHANISMS OF INHIBITORY EFFECT OF A III

11.2.1 Effects of A II on basal and hCG-stimulated estradiol production

Results within this thesis have shown that A II binds with human granulosa-luteal cells, but its effects on basal and hCG-stimulated progesterone production is not clear. Further experiments should focus on basal and hCG-stimulated estradiol production in these cells. By examining both progesterone and estrodiol production. Concurrently, it is expected that the autocrine/paracrine role of angiotensins in the ovary can be better defined.
11.2.2 *Mechanisms of inhibitory effect of A III on hCG-stimulated progesterone production*

The results within this thesis have shown that the inhibitory effect of A III is not through the DAG-PKC second messenger pathway. The possibility that A III inhibits hCG-stimulated intracellular cAMP, or increases intracellular free Ca^{2+} concentration should be examined.

11.3 *hCG-REGULATED INHIBIN SUBUNIT mRNA EXPRESSION IN HUMAN PLACENTAL CELLS*

The results within this thesis have shown that both cAMP and GnRH stimulated inhibin α and βB mRNA expression. Previous reports have suggested a stimulatory action of hCG on placental adenylate cyclase and cAMP formation (Moore et al., 1992; Davis et al., 1986) and a stimulatory action of placental GnRH on hCG secretion (Sailer-Cheater et al., 1981; Currie et al., 1992; Currie et al., 1981; Seeburg et al., 1984). It will be worthwhile to study the effect of hCG on inhibin subunit mRNA expression in these cells. To provide evidence for a stimulatory effect of GnRH on inhibin α and βB mRNA expression, intracellular cAMP levels after incubation with GnRH should be measured and compared with controls.
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