NOVEL ACTION OF THE GONADOTROPIN SURGE IN RHESUS MONKEYS: EXPRESSION OF PROGESTERONE RECEPTORS IN GRANULOSA CELLS

by

Yasmin Aladin Chandrasekher

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ABSTRACT

Immunocytochemical techniques using specific antibodies to the human estrogen (E) and progestin (P) receptors (R) recently provided the first detailed evidence on possible target cells for these steroids in the primate (e.g., rhesus monkey) ovary. The observation that PR staining first appeared in the granulosum of the dominant follicle during the periovulatory interval led to the proposal of a novel action of the midcycle gonadotropin surge to induce these receptors in luteinizing granulosa cells. The current study was designed to test this hypothesis. The specific aims were to determine: 1) whether administration of an ovulatory dose of hCG after follicular stimulation induces PR in luteinizing granulosa cells; 2) whether gonadotropin (LH/CG) surges of varying duration stimulate PR expression in granulosa cells; 3) if LH surge action to induce PR is associated with the expression of PR mRNA in granulosa cells; and 4) if LH acts directly on granulosa cells, or indirectly via gonadotropin-triggered mediators (e.g., prostaglandins) to stimulate PR expression.

Specific immunocytochemical staining for PR was undetectable in granulosa cells from monkeys following follicular stimulation, but prior to any ovulatory stimulus. In contrast, the majority of cells stained intensely for PR when collected 27 h after administration of an ovulatory hCG stimulus. LH surge durations of 4,8, and 14 h were typically unable to induce PR expression. However, LH surges of 18-24 h induced PR expression in granulosa cells from the majority of animals. Increasing the duration of the LH surge to 48 h consistently resulted in granulosa cells from all animals expressing PR similar to that observed with hCG treatment.

Using reverse transcription-polymerase chain reaction assays, PR mRNA was detected in germinal epithelium-enriched cortical sections of the ovary and in

granulosa cells of periovulatory follicles. In contrast, ER mRNA was detected in cortical sections of the ovary, but not in granulosa cells of periovulatory follicles. Granulosa cells were also cultured *in vitro* to determine whether LH or other factors directly stimulated PR expression. Nonluteinized granulosa cells cultured for 1 day were typically devoid of PR staining in the presence or absence of hCG, FSH, PGE_2, and db-cAMP. By day 4, treatment with these agonists resulted in PR staining in some cells, whereas control cultures remained PR negative. PGE_2 treatment consistently yielded the greatest response.

Thus, new information is provided for the presence of PR in granulosa cells from periovulatory, but not preovulatory follicles, whereas ER is absent in these cells. The current study provides direct evidence for the hypothesis that the gonadotropin surge induces PR expression in luteinizing granulosa cells of mature follicles in primates. PR expression is dependent on the duration of the LH surge; intervals (<14 h) comparable to surges in nonprimate species have no effect, whereas 18-27 h induces PR expression. *In vitro* data suggest that LH may directly induce PR in granulosa cells, but a similar action of PGE_2 suggests that local intrafollicular factors could play a role in PR expression.

CHAPTER 1

INTRODUCTION

The growth and maturation of a species-specific number of follicles, followed by ovulation and transformation of the follicles into corpora lutea which function for a limited life span comprise the sequential events within the ovarian cycle in mammals. Cyclic ovarian function is dependent upon the complex interaction between secreted hormones and cells within the hypothalamic-pituitary-ovarian axis. The hypothalamic gonadotropin releasing hormone (GnRH) stimulates the release of the pituitary hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH), which regulate the growth and maturation of the follicle, including follicular synthesis of the steroid hormone, estradiol. Estradiol priming of the hypothalamic-pituitary axis is required for the midcycle surge of gonadotropin that stimulates the resumption of oocyte meiosis with breakdown of the germinal vesicle and ovulation or rupture of the follicle leading to extrusion of the fertilizable oocyte. In addition, the gonadotropin surge is instrumental in the process of luteinization resulting in the conversion of follicular cells from estradiol- to progesterone-secreting cells that are important components of the developing corpus luteum. The LH-dependent corpus luteum secretes progesterone, required for uterine development that can accomodate implantation of fertilized oocytes. In the nonfertile cycle, the corpus luteum regresses and progesterone levels decrease prior to the onset of menses.

It is well-known that an important action of ovarian steroid hormones is to stimulate the growth and differentiation of reproductive organs such as

the uterus, vagina, and mammary glands. Considerable research on the mechanism of action of steroid hormones in classical target tissues has led to a general model of steroid action. The initial event involves the interaction of hormones with their specific receptors. This leads to formation of a hormone-receptor complex that can bind to steroid-responsive genes and regulate their transcription. There is also mounting evidence that steroid hormones may serve as local modulators of ovarian function. However, comparable studies on steroid action in the ovary are compromised or difficult to interpret due to high levels of endogenous steroids in the tissues. Recent immunocytochemical studies of steroid receptors provided the first detailed evidence on possible target cells in the mammalian ovary, including those in primate species such as monkeys and humans (Hild-Petito *et al.*, 1988; Iwai *et al.*, 1990; Press and Greene, 1988). An unexpected distribution of estrogen receptor was observed in the monkey ovary, localizing this receptor to the germinal (i.e., surface) epithelium, but not to follicles or corpora lutea. In contrast, progestin receptors were detected in various monkey ovarian structures including the germinal epithelium, follicle, and corpus luteum (Hild-Petito *et al.*, 1988).

This project was designed to further investigate the presence and regulation of steroid receptors, particularly those for progesterone, within the primate ovary during the menstrual cycle. The specific hypothesis tested was that the midcycle surge of gonadotropin in the rhesus monkey induces the expression of progesterone receptors in luteinizing granulosa cells. The following literature review is meant to provide an overview of ovarian function and its regulation by gonadotropic hormones and local factors, particularly steroid hormones, and to provide the basis for the rationale behind the hypothesis.

Follicular Development - Role of Gonadotropins

Follicle formation in the ovary begins during embryonic development. The oocyte progresses through the stages of meiotic prophase, and becomes arrested in the diplotene stage of the first meiotic division. Ovarian somatic cells proliferate during the embryonic period and eventually envelop single oocytes. A primordial follicle is made up of a small oocyte surrounded by flattened somatic cells, and a complete basement membrane around each follicle. There is species variation in the time of appearance of the first primordial follicles. The first such follicles were detected day 14 postpartum in the rabbit (Peters, 1978), and 4.5 months postconception in humans (Gillman, 1948). A complete description of the mechanisms regulating follicle formation awaits further inquiry.

A large number of primordial follicles are formed ready to proceed with follicular development with some follicles starting to grow when they are formed (Fig. 1). However, a period of quiescence follows for most primordial follicles that may last for months or years. The factors that initiate follicular development have not been determined, but evidence indicates that it is independent of gonadotropin stimulation. Initially, nuclear and cytoplasmic volume increases occur in the oocyte. The zona pellucida is also laid down during the early stages of follicle growth. Zona pellucida proteins are first detected in follicles with squamous granulosa cells (Kang, 1974; Wolgemuth *et al.*, 1984). This early growth process is seen in the juvenile, prepubertal, as well as reproductive years. Once growth beyond the primordial stage commences, the follicle either develops to maturity or undergoes atresia.

The capability to complete follicular development appears with

puberty and the onset of cyclicity. Growth and maturation beyond the early stages of follicular growth are dependent on gonadotropins, especially FSH. The next stage of follicular growth (i.e., preantral stage) involves mitosis of granulosa cells due to FSH action. Up to six or eight layers of granulosa cells are detected in the larger preantral follicles. FSH receptors can be detected in granulosa cells at this stage, with cells responding to FSH *in vivo* and *in vitro* (Richards, 1980; Erickson and Hsueh, 1978; Hillier *et al.*, 1980; Zeleznik and Hillier, 1984). The theca becomes detectable as a morphologically distinct layer, differentiating from surrounding stromal cells, but the time of appearance may be species-dependent. The first detection of theca cells in the mouse occurs in follicles with a fully-grown oocyte and with granulosa cells consisting of two or three layers (Peters, 1969). Theca cells in the hamster are detected in follicles with seven to eight layers of granulosa cells (Roy and Greenwald, 1985). Preantral follicles are relatively undifferentiated and have a limited potential for steroidogenesis. This information is based mainly on observations using the rodent as a model.

Differentiation of follicles to the preovulatory stage is characterized by important morphological and functional changes that are gonadotropin-dependent. Morphologically, the appearance of fluid-filled spaces among the granulosa cells gives rise to a single antral cavity. The appearance of the antrum signals a reduction in cell proliferation and the beginning of differentiated functions in the follicle. The theca layer consisting of the inner theca interna and the outer theca externa is also morphologically differentiated. The functional change in the differentiating antral follicle is its ability to produce increasing amounts of estrogen. Follicular estrogen biosynthesis, involving both granulosa and theca cells, is under the influence

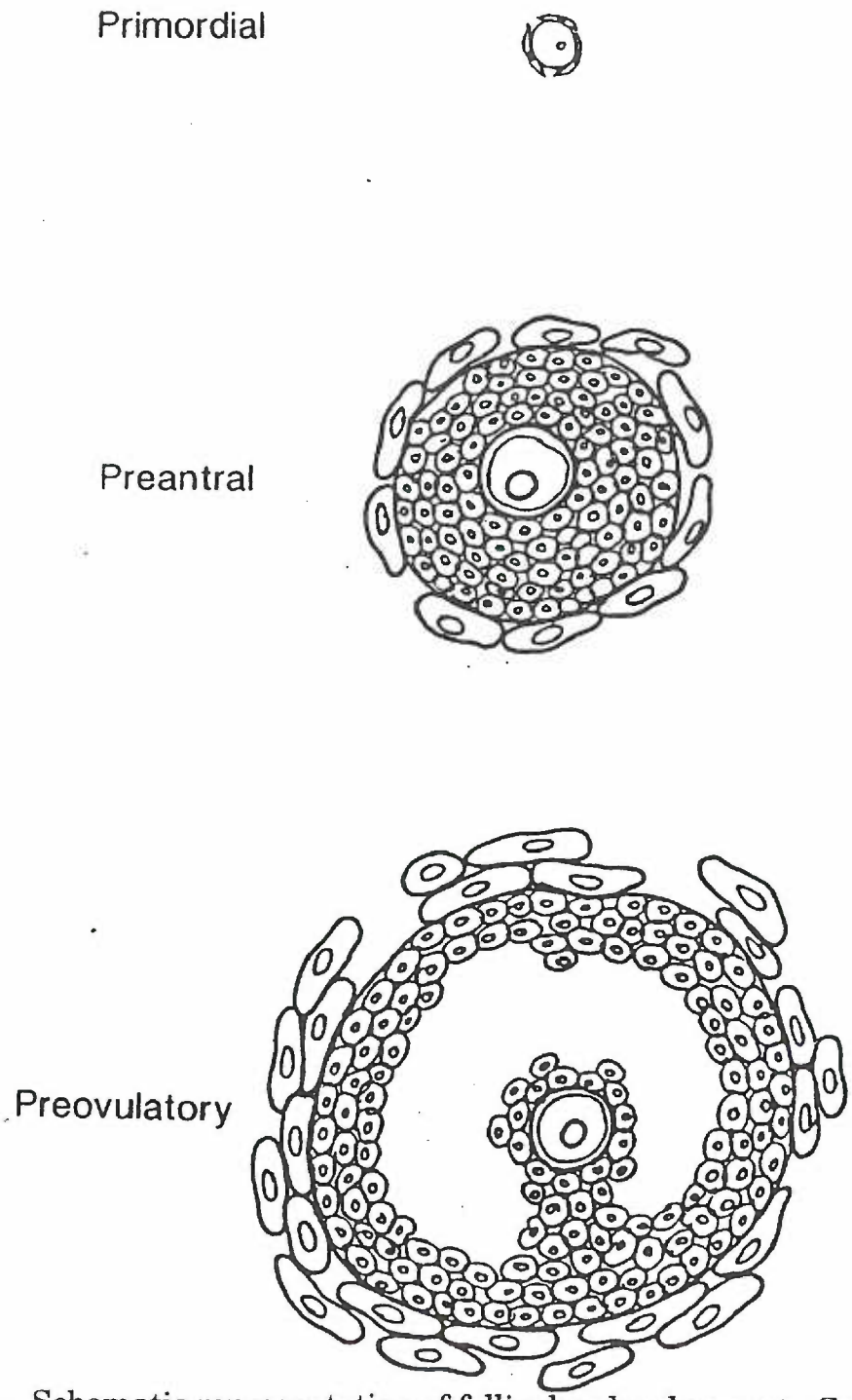


Fig. 1. Schematic representation of follicular development. Growth and maturation beyond the early stages of follicular growth are dependent on gonadotropins, especially FSH. Growth to the preantral stage involves mitosis of granulosa cells. Differentiation of follicles to the preovulatory stage is characterized by important changes that includes the appearance of a single antral cavity.

of gonadotropins, and is known as the "two cell-two gonadotropin" theory. In response to LH, the theca cells secrete androgens. The androgens diffuse across the basal lamina and in response to FSH are converted to estrogens by granulosa cells. The estrogen is subsequently released into both the follicular fluid and the peripheral circulation. The importance of FSH at this stage of follicular growth is apparent, since FSH withdrawal is followed by the loss of FSH and LH receptors and aromatase enzyme (Richards, 1980; Uilenbroek *et al.*, 1980).

Initially, the growth of the oocyte occurs concommitantly with that of the follicle. The oocyte completes its growth early in follicular development, but is maintained in meiotic arrest. Oocytes removed from follicles undergo spontaneous meiotic maturation *in vitro* (Pincus and Enzmann, 1935). It is the intrafollicular environment that inhibits the resumption of meiosis in oocytes that have become meiotically competent. Meiotic maturation can be inhibited in cultured oocytes by the addition of follicular fluid (Chang, 1952) and in cocultures of oocytes and granulosa cells (Tsafriri and Channing, 1975). It has been postulated that secretion of an inhibitory substance by granulosa cells called oocyte maturation inhibitor (OMI) is responsible for the prevention of meiotic maturation. Thus, the concept has emerged that a substance present in the follicle prevents meiosis from resuming.

Current investigations involve a search for potential regulators of meiosis resumption. Eppig and coworkers have focused on identifying OMI in follicular fluid. Their investigation using cumulus-oocyte preparations from PMSG-stimulated mice led to the identification of hypoxanthine (Downs *et al.*, 1985). Subsequent observations indicated that a decrease in hypoxanthine and adenosine levels occurred in the follicular fluid of mouse follicles following germinal vesicle breakdown (GVBD) (Eppig *et al.*, 1985).

Their conclusion was that the two purines are OMI. Furthermore, in PMSG-primed mice injected intraperitoneally with inosine monophosphate inhibitors, GVBD was observed (Downs and Eppig, 1987). Recently, Mullerian inhibiting substance has also been shown to act on rat cumulus-enclosed oocytes as an OMI (Takahashi *et al.*, 1986). Thus active research in the identification for OMI is ongoing.

Cyclic adenosine monophosphate (cAMP) has been shown to inhibit meiosis. Meiotic arrest *in vitro* can be maintained with membrane-permeable analogs of cAMP, as well as with cAMP phosphodiesterase inhibitors (Cho *et al.*, 1974; Wassarman *et al.*, 1976; Magnusson and Hillensjo, 1977; Dekel and Beers, 1978). Maturation can be induced in such oocytes by treatment with an inhibitor of the catalytic subunit of cAMP-dependent protein kinase (Bornslaeger *et al.*, 1986). The levels of cAMP in mouse and rat oocytes before GVBD are higher than those measured following breakdown (Schultz *et al.*, 1983; Vivarelli *et al.*, 1983; Racowsky, 1984). There are two possible sources of cAMP present in oocytes. One source is the granulosa cell from which cAMP could be transferred to the oocyte through gap junctions (Dekel and Beers, 1978). The other is the oocyte itself. However, it is not clear whether an increase in oocyte cAMP is via transfer from granulosa cells or the result of the transfer of factors that stimulate the oocyte to generate cAMP. Indeed, oocytes of several species have been shown to produce cAMP. The question remains whether these quantities are high enough for maintenance of meiotic arrest in oocytes.

It is known that the majority of follicles in the ovary that start to grow will not reach maturation before degenerating by a process called atresia (Richards, 1980; Ryan, 1981; Byskov, 1979). Atresia results in the death of the granulosa cells and the oocyte. Recently, atresia has been characterized

as a form of physiological cell death (Hughes and Gorospe, 1991) with the accompanying morphological features termed apoptosis or programmed cell death (Kerr, 1971). In contrast to granulosa cells and the oocyte, theca cells survive following a process of extensive hypertrophy and continue to synthesize androstenedione in response to LH (Erickson *et al.*, 1985). Two types of atresia (Erickson, 1986) are distinguishable based on morphology. In type A atresia, initial changes occur in the oocyte and are most often seen in preantral follicles. Type B atresia follows initial changes in granulosa cells and is often observed in antral follicles. The cellular and molecular events of atresia remain to be defined.

Rat follicles in the early antral stage undergo atresia unless FSH levels above baseline are present (Hirshfield, 1982). If the FSH surge is blocked in cycling rats with antibodies (Welschen and Dullaart, 1976), barbiturates (Hirshfield and Midgley, 1978) or follicular fluid (Hoak and Schwartz, 1980) continued growth of antral follicles to the preovulatory stage does not occur. In the monkey, the initiation of follicular growth and the selection of the dominant follicle are also dependent on the appropriate levels of FSH. During the luteal phase, serum levels of FSH remain below 10 mIU/ml. This level of FSH is not enough for stimulation of folliculogenesis further than the early antral stage. Following luteal regression, serum levels of FSH increase to 15-20 mIU/ml. This level seems to be sufficient for stimulation of the final stages of follicular growth (Zeleznik, 1990). The stimulated follicle produces increasing amounts of estrogen which suppress FSH secretion, thus preventing other follicles from entering the final growth pool. In contrast to insufficient amounts of FSH resulting in atresia, excessive levels of LH are proposed to induce this process (Hirshfield, 1991).

In summary, the earliest changes that occur as primordial follicles

start development do not depend on gonadotropin stimulation. Subsequent follicular growth becomes dependent on gonadotropins. Insufficient FSH or excessive LH results in atresia. Under the influence of gonadotropins, a group of follicles is stimulated to grow and each has the potential to proceed to ovulation. Both granulosa and theca cells undergo morphological and functional changes as only a species-specific number of follicles proceeds to the preovulatory stage. With gonadotropin stimulation, theca cells synthesize androgens that are aromatized to estrogen by granulosa cells, resulting in the increasing level of estrogen that is the hallmark of a preovulatory follicle. In contrast, the destiny of the remaining growing follicles is atresia, resulting in the death of the granulosa cells and the oocyte.

Follicular Development - Role of Local Factors, Particularly Steroid Hormones

A variety of nonsteroidal substances are proposed to play a role in follicular development (Ackland *et al.*, 1992). Many growth factors (insulin-like growth factors, epidermal growth factor, transforming growth factors, etc.) have been identified that affect the functions and the growth and differentiation of ovarian cells *in vitro*. A second group encompasses the inhibin family of hormones, including inhibin and activin. Evidence supports a role for inhibin in FSH secretion as well as a potential local role in steroidogenesis. Another protein, activin, is proposed to act as stimulator of FSH secretion. The physiological role of gonadal activin on gonadotropin secretion awaits further study. Other partially defined substances such as follicle regulatory peptide (FRP) may also be involved in the regulation of follicular development. FRP is suggested to inhibit follicular growth by

causing atresia. Thus, a critical area of research is the definition of a physiologic role for these substances in follicular development and their mechanisms of action in follicle cell types.

It has also been proposed that steroids act locally to control follicular development. Both *in vivo* and *in vitro* studies on rodents support a stimulatory role for estrogens in follicular development. Pharmacological doses of estrogens stimulate preantral follicular growth and prevent atresia in hypophysectomized prepubertal rats (Hsueh *et al.*, 1984; Goldenberg *et al.*, 1983). In contrast, hypophysectomized rats treated with gonadotropins and anti-estrogen antisera show decreased follicular growth and induction of atresia (Hsueh *et al.*, 1984; Reiter *et al.*, 1972). Estrogen acts as a mitogen in the rat ovary (Richards, 1980) and in synergism with FSH stimulates granulosa cell proliferation. Several actions of FSH in the follicle are promoted by estrogen. Estrogen acts to facilitate FSH-stimulated cAMP formation and cAMP-dependent protein kinase activity (Richards, 1980; Richards *et al.*, 1983; Darbon *et al.*, 1984). FSH-stimulated aromatase activity in rat granulosa cells is augmented by estradiol (Richards *et al.*, 1979; Adashi and Hsueh, 1982). Estradiol is reported to augment FSH-induced formation of LH receptors (Richards *et al.*, 1976; Richards *et al.*, 1979). The positive action of estrogen is important for follicle development in that it allows low levels of FSH in the follicular fluid to result in a maximal response by granulosa cells (McNatty *et al.*, 1975).

The stimulatory effects of estrogen on follicular development in rodents do not necessarily apply to other species (Hutz, 1989). Stimulatory effects of estrogen on follicular development in primates are questionable. Koering (1987) showed that diethylstilbestrol (DES) treatment of juvenile rhesus monkeys did not increase the number or size of growing preantral or

antral follicles. Similarly, estrogen enhancement of FSH action on aromatase activity was not observed in cultured marmoset granulosa cells (Hillier *et al.*, 1987). Inhibitory actions of estrogen on follicular development have been observed. Treatment of rhesus monkeys with exogenous estrogen resulted in atresia of the preovulatory follicle (Clark *et al.*, 1981), but it is unclear whether this is a direct effect or an indirect effect due to inhibition of pituitary gonadotropin secretion. Thus, the role of estrogen in primate follicular development does not parallel that in rodents and needs to be further clarified.

In contrast to estrogen action in rodents, progesterone suppresses follicular development by inhibition of FSH actions. FSH-stimulated estrogen production (Schreiber *et al.*, 1980; Fortune and Vincent, 1983) and LH receptor induction (Schreiber *et al.*, 1982) are inhibited by progestins in cultured rat granulosa cells. Progestin inhibition of aromatase activity was not the result of direct competitive inhibition of the enzyme nor of a suppression of FSH-stimulated production of cAMP (Schreiber *et al.*, 1980). Setty and Mills (1987) reported a progesterone-induced decrease in follicular size and estradiol content in the rabbit. This inhibitory action of progesterone was proposed to be exerted directly on the ovary.

Luteal progesterone is also proposed to inhibit follicular growth in primates, but it is unclear if its action is due to a local or pituitary site of action. Progesterone treatment following lutectomy in women resulted in decreased levels of serum FSH for 24 hours (Baird *et al.*, 1984). In rhesus monkeys, progesterone treatment decreased serum FSH levels for 40 hours during the follicular phase (Resko *et al.*, 1981). The conclusion from these studies was that progesterone suppression of follicular growth occurred through the inhibition of FSH release. Other studies have concluded that the

inhibitory effect of progesterone is via direct action on the ovaries. Systemic progesterone treatment after lutectomy inhibited follicle growth in monkeys, but did not decrease serum FSH levels (Goodman and Hodgen, 1977; Goodman and Hodgen, 1982). Treatment with exogenous FSH and LH (Zeleznik and Resko, 1980) stimulated follicular maturation despite elevated levels of progesterone, suggesting that the inhibitory action of progesterone can be overcome by supraphysiological doses of gonadotropins.

Both stimulatory and inhibitory effects of androgens have been reported on follicular growth and granulosa cell function. In some species, androgen administration results in atresia. That testosterone can induce type A atresia in preantral follicles is shown by studies in which endogenous or exogenous testosterone resulted in the death of the oocyte and granulosa cells (Payne *et al.*, 1956; Hillier and Ross, 1979; Schwall and Erickson, 1981). It has been proposed that theca cells secrete increased levels of testosterone in response to low levels of LH (Louvet *et al.*, 1975). The effect of testosterone has been shown to involve a decrease in estrogen receptor numbers in granulosa cells of preantral follicles (Saiduddin and Zassenhaus, 1978) leading to a reduced ability to respond to estrogen. Stimulatory observations of androgens both *in vivo* and *in vitro* have also been reported. Androgen augmentation of FSH responses in granulosa cells has been observed (Hsueh *et al.*, 1984; Hillier, 1991). Thus, the effect of androgen in rats seems to vary depending on the absence or presence of FSH. Androgens induce atresia in the absence of FSH, whereas in its presence androgens act as tropic hormones.

The Periovulatory Interval - Role of Gonadotropins

In all species studied, a rise in serum estradiol precedes the preovulatory LH surge (Abraham *et al.*, 1972; Moghissi *et al.*, 1972; Korenman and Sherman, 1973; Knobil, 1974; Thorneycroft *et al.*, 1974; Leyendecker *et al.*, 1975; Laborde *et al.*, 1976; Pauerstein *et al.*, 1978). In addition, exogenous estrogens trigger LH surges that are similar in shape and duration to spontaneous LH surges (Vande Wiele *et al.*, 1970; Nillius and Wide, 1971; Tsai and Yen, 1971; Leyendecker *et al.*, 1972; Monroe *et al.*, 1972 a, b; Yen and Tsai, 1972; March *et al.*, 1979). A threshold level of estradiol is thought to be required for induction of the preovulatory LH surge (Leyendecker *et al.*, 1990). Estradiol action at the level of the pituitary to initiate the gonadotropin surge was proposed following studies on rhesus monkeys with acutely-transected pituitary stalks. In these animals, estrogen treatment initiated gonadotropin surges (Ferin *et al.*, 1979). Alternatively, the rising levels of estradiol secreted by the preovulatory follicle may interact with steroid-responsive neurons in the central nervous system and result in the midcycle release of GnRH (Levine *et al.*, 1985) that in turn stimulates the release of LH from the pituitary. A GnRH surge has been observed in association with the LH surge in non-primate species (Conn *et al.*, 1987). Similar results have not been shown experimentally in primates. The midcycle surge of LH is the trigger for ovulatory events in the follicle, including resumption of oocyte meiosis, follicular rupture, and luteinization.

One major action of the LH surge is to stimulate the resumption of meiosis in oocytes arrested in the diplotene stage (of prophase) of the first meiotic division. LH action results in the completion of the first meiotic division evidenced by the breakdown of the germinal vesicle and extrusion of

the first polar body. The process then becomes arrested at metaphase of the second meiotic division. In most mammals, completion of meiosis occurs following fertilization.

The events between the LH surge and that of resumption of oocyte meiosis remain to be elucidated. There are several observations which address this issue. Since LH action on steroidogenesis is through the second messenger cAMP and because cAMP can induce meiosis in follicle-enclosed oocytes, a role for this substance in the cascade of events leading to resumption of oocyte meiosis has been proposed. However, cAMP has also been shown to prevent meiosis in isolated oocyte-cumulus complexes. The explanation for this contradictory action has been suggested to be the following: LH effects are thought to be mediated by cAMP in granulosa cells. In contrast, the action of cAMP within the oocyte is to inhibit meiosis (Tsafriri, 1988; Eppig, 1987). However, confirmation of this hypothesis is lacking. Preovulatory gonadotropin action to initiate oocyte maturation may be through a decrease in maturation-arresting factors available to the oocyte via gap junctions. Other observations suggest that gonadotropins induce the production of a positive signal by cumulus cells that results in oocyte maturation (Eppig, 1987). Positive maturation-inducing substances have been identified in amphibians (Schuetz, 1967; Masui, 1967) and fish (Nagahama *et al.*, 1983; Kanatani *et al.*, 1969).

A second major action of the preovulatory surge of LH is to trigger a series of biochemical and morphological steps that result in ovulation (LeMaire *et al.*, 1987). Enzymatic activity, arachidonic acid metabolites, and steroids have been identified as intermediaries leading to follicle wall dissociation and ovulation. The role of arachidonic acid metabolites and steroids is discussed in a later section. This cascade of events results in the

rupture of the follicle with the subsequent release of the oocyte. Expulsion of the oocyte following follicular rupture is aided by the process of cumulus expansion. LH acts to stimulate the production of hyaluronic acid which becomes deposited around the oocyte and results in the dispersion of the cumulus (Eppig, 1979). *In vitro*, cumulus expansion can also be stimulated by FSH (Eppig, 1979). Substances produced by the oocyte are thought to mediate cumulus expansion (Buccione *et al.*, 1990; Salustri *et al.*, 1990).

The role of proteolytic enzymes in gonadotropin-induced follicular rupture is an important one. Prior to follicular rupture, the follicle wall becomes thin at the apex followed by a breakthrough at the point of the stigma. Follicular wall thinning occurs concommitantly with fragmentation of collagen fibrils (Espey, 1962; Bjersing and Cajander, 1974a; Bjersing and Cajander, 1974b; Downs and Longo, 1983; Martin and Miller-Walker, 1983). The proteolytic enzymes plasminogen activator (PA), plasmin, and interstitial collagenase are thought to be essential for rupture of the follicle (Tsafriri *et al.*, 1990). Follicular fluid and granulosa cell PA levels increase following the gonadotropin surge (Deutinger *et al.*, 1988; Reich *et al.*, 1985; Strickland and Beer, 1987). In contrast to granulosa cells which mainly produce tissue PA (tPA), theca cells have been reported to produce mainly urokinase-type (u) PA (uPA) (Canipari and Strickland, 1986). Increases in tPA levels result in an elevation of intrafollicular plasmin levels which in turn act to weaken the follicular wall. Gonadotropins stimulate an increase in ovarian collagenolysis (Reich *et al.*, 1985) and collagenase activity (Curry *et al.*, 1986) in the preovulatory interval. Gonadotropins have also been shown to stimulate the expression of PA (Liu *et al.*, 1991) and interstitial collagenase mRNA (Reich *et al.*, 1991) in rat ovaries. Local regulation of proteolytic enzyme activity is under the influence of inhibitors including PA

inhibitors, α_2-antiplasmin, tissue inhibitor of metalloproteinase (TIMP), and α_2-macroglobulin. A recent study by Chun and colleagues (1992), observed cell-specific expression of the mRNAs for PAI-1 and TIMP-1 in the rat ovary following LH/hCG treatment. Thus studies confirm that regulation of both proteolytic enzymes and their inhibitors occurs in the preovulatory interval.

Since LH is known to efficiently induce ovulation, it has been generally accepted as the ovulatory hormone despite the occurrance of preovulatory surges of both LH and FSH in many mammals. Initial studies provided some information about the role of FSH in ovulation. In rats, treatment with FSH antisera did not block ovulation (Schwartz *et al.*, 1973). However, another study showed that when FSH antibodies in a gonadotropin antiserum preparation were neutralized, the preparation was less effective in preventing ovulation in hamsters (Goldman and Mahesh, 1969). These were followed by studies in rodents showing that highly purified FSH induced ovulation *in vivo* and *in vitro* (Nuti *et al.*, 1974; Tsafriri *et al.*, 1976; Greenwald and Papkoff, 1980; Armstrong and Opavsky, 1988; Lipner *et al.*, 1974; Yang and Papkoff, 1973; Shaykh *et al.*, 1985). A recent study re-addressed this issue by using recombinant FSH preparations that were completely free of LH contamination for induction of ovulation (Galway *et al.*, 1990). Hypophysectomized rats implanted with a minipump received recombinant FSH (4 IU/day) to stimulate follicular growth. This was followed by a surge dose of recombinant FSH. The results clearly showed the ability of FSH alone to induce ovulation. Overall, the above observations suggest that exogenous FSH can induce ovulation, however, the role of endogenous FSH is unclear.

Some controversy exists about the intracellular signal transduction pathways stimulated by the gonadotropin surge that result in follicular

rupture. The cAMP-adenylate cyclase pathway has received considerable attention in this matter. A direct ovulation-inducing action of cAMP has been demonstrated in the rabbit and the rat (Holmes *et al.*, 1986; Brännstrom *et al.*, 1987). Holmes and coworkers (1986), using *in vitro* perfused rabbit ovaries, showed that forskolin (a nonreceptor mediated stimulator of adenylate cyclase) induced ovulation. Similarly, *in vitro* perfusion of ovaries from immature PMSG-treated rats with forskolin or db-cAMP plus 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor) resulted in ovulation (Brännstrom *et al.*, 1987). In contrast, other studies do not support a cAMP-mediated action of gonadotropins on ovulation. Ovulation was not induced with db-cAMP in the absence of gonadotropins (Hosoi *et al.*, 1989). Furthermore, cAMP perfused *in vitro* was reported to inhibit hCG-induced ovulation in rabbit ovaries (Yoshimura *et al.*, 1986).

Recently, a role for the calcium/phospholipid-dependent protein kinase-C (PKC) pathway in gonadotropin-induced follicular rupture was suggested. This pathway involves the hydrolysis of phosphatidylinositol 4,5-biphosphate by phospholipase C, resulting in the formation of diacylglycerol (DG) and inositol triphosphate. Upon activation by DG, PKC phosphorylates specific intracellular proteins. LH action via the PKC pathway has been reported. In the rat, administration of staurosporine, an inhibitor of PKC, has been shown to inhibit PMSG-induced ovulation (Guerre *et al.*, 1991). Further implication of a role for the PKC pathway in mediation of gonadotropin-induced follicular rupture comes from studies by Kaufman and colleagues (1992) on the *in vitro* perfused rabbit ovary. Treatment with phorbol 12,13-dibutyrate (PdBU), a PKC stimulator, resulted in a dose-related induction of ovulation in the absence of gonadotropins. Inhibition in a

dose-related manner of hCG-induced ovulation was observed following treatment with staurosporine. Thus, these studies provide intriguing insight into involvement of the PKC pathway in mediation of gonadotropin action in ovulation. However, it is unclear whether the PKC-mediated follicular rupture occurs directly through hCG activation or indirectly via hCG activation of other intermediaries that utilize PKC as a second messenger.

Luteinization is the third major process triggered by the midcycle gonadotropin surge. To effectively describe luteinization, it is essential to focus on both morphological and functional changes of steroidogenic cells in the preovulatory follicle as well as in the postovulatory developing corpus luteum. Granulosa cell luteinization commences in the basal layer and proceeds towards the antral layer. Following follicular rupture, granulosa lutein cells divide into small groups. Around this time, hyperplasia of granulosa cells stops, while hypertrophy of lutein cells is detected. The cellular response to LH is seen in various compartments including the mitochondria and the smooth endoplasmic reticulum (SER). In mitochondria, an increase in the complexity of the cristae with lammelliform-villiform cristae is seen (Rigby *et al.*, 1986; Crisp *et al.*, 1970; Crisp and Channing, 1972). Mitochondria clustering to the perinuclear area is also detected. Furthermore, an increase in the number of lipid inclusions was reported. Luteinization involves changes in the SER observed both *in vivo* and *in vitro*. Maximum development of the SER occurs concommitantly with peak progesterone production (Amsterdam and Rotmensch, 1987).

The pattern of steroidogenesis by ovarian cells changes from predominantly estrogen to progesterone biosynthesis following exposure to the LH surge and beginning before follicle rupture. LH stimulation of progesterone production involves changes in cholesterol metabolism and in

steroidogenic enzyme activities. A major source of cholesterol for steroidogenesis is blood-borne lipoprotein. There are species differences in the primary source of steroidogenic cholesterol: for example, primate ovarian cells use low-density lipoproteins (LDL), whereas rat cells utilize high-density lipoproteins (HDL). LDL promoted steroidogenesis begins with the interaction of lipoproteins with cell surface receptors (Paavola *et al.*, 1985). Internalization of the receptor-bound LDL is followed by the separation of LDL from its receptor. The LDL protein is degraded in lysosomes. Acid lipase cleaves the cholesterol esters and results in the release of free sterol. The LH surge has been proposed to induce the expression of LDL receptors in luteinizing granulosa cells (Carr, 1990). Bramley and colleagues (1987) reported a small number of LDL binding sites on human granulosa cells from preovulatory follicles and increased numbers in developing and mature corpora lutea. Exposure *in vitro* to hCG, has been shown to induce the expression of LDL receptors and to stimulate LDL uptake by human luteinizing granulosa cells (Golos *et al.*, 1985; Golos *et al.*, 1986; Golos and Strauss, 1987). These results support a direct action of LH, but do not provide information for a role of the LH surge in this action. In monkeys undergoing controlled ovarian stimulation, an ovulatory injection of hCG promoted the uptake of DiI-LDL in granulosa cells over that seen in nonluteinized granulosa cells. This study suggested that the ovulatory surge of gonadotropin induces LDL receptor expression in granulosa cells (Brannian *et al.*, 1992). Thus, an increase in the expression of LDL receptors results in an increase in the binding of ^{125}I-LDL followed by catabolism of the internalized LDL (Golos *et al.*, 1985).

Gonadotropins also regulate the activities of steroidogenic enzymes. LH action on progesterone biosynthesis during luteinization occurs mainly to

promote the conversion of cholesterol to pregnenolone (Hsueh *et al.*, 1984). Cytochrome $P450_{SCC}$, as well as adrenodoxin and adrenodoxin reductase (associated electron-transport chain) levels are increased by gonadotropins via an increase in mRNAs encoding these proteins (Strauss *et al.*, 1988). Expression of bovine $P450_{scc}$ is low in follicles, but levels greatly increase with luteinization (Waterman *et al.*, 1986). High levels of P450scc are detected via immunocytochemistry in the majority of granulosa lutein cells.

The conversion of the granulosa layer from an avascular to a vascular tissue also begins around the time of ovulation. This conversion is essential since it permits delivery of substrates to luteal cells. Luteinized granulosa cells and the corpus luteum are reported to secrete an angiogenesis-inducing substance (Gospodarowicz and Thakral, 1978). Several possible substances have been identified. Angiotensin II has been shown to have angiogenic activity (Fernandez *et al.*, 1985a). Follicular fluid has also been shown to contain high levels of renin and proteases, which are known to cleave angiotensinogen. Renin-like activity is elevated in follicular fluid compared to that in plasma only in women receiving gonadotropin stimulation and not in non-stimulated women (Fernandez *et al.*, 1985b; Brameld *et al.*, 1990). Interleukin-1 (IL-1) is also thought to have a role in angiogenesis. A high level of IL-1 has been detected in follicular fluid (Loukides *et al.*, 1990). IL-1 can stimulate latent collagenase, gelatinase, and PA. Degradation of tissues by proteases permits endothelial cells to migrate and form new vessels (Masure and Opdenakker, 1989). Vascular endothelial growth factor has been detected in rat luteal cells, but not in granulosa cells. And finally, transforming growth factor β, present in follicular fluid, has been shown to be angiogenic (Brown *et al.*, 1990; Merwin *et al.*, 1990; Pepper *et al.*, 1990). Though potential angiogenic substances have been identified, this area of

research remains largely undeveloped.

The Periovulatory Interval - Role of Local Factors, Particularly Steroid Hormones

It is generally believed that prostaglandins (PGs) play a role in the ovulatory process. An ovulatory dose of LH stimulates an increase in the levels of PGE and PGF in developing rabbit ovarian follicles (LeMaire *et al.*, 1987). *In vivo* administration of indomethacin, a cyclooxygenase inhibitor, inhibits ovulation in rabbits as well as other species and results in unruptured, luteinized follicles. Exogenous administration of prostaglandins relieves this inhibition. Recent studies question the role of PGs in ovulation. Doses of indomethacin that dramatically decreased ovarian PG synthesis did not inhibit ovulation in the rabbit (Espey *et al.*, 1986) or the rat (Espey *et al.*, 1988; Espey *et al.*, 1989; Tanaka *et al.*, 1989). Tanaka and colleagues (1991) determined the effect of indomethacin on ovarian levels of 5-, 12-, and 15-hydroxyeicosatetraenoic acid methyl esters (HETES), compounds formed in the lipoxygenase pathway of arachidonic acid metabolism. The dose of indomethacin that reduced ovulation rate also reduced 15-HETE levels, but not 5-HETE or 12-HETE. In contrast, inhibition of PGE and PGF levels occurred with even lower doses of indomethacin that did not inhibit ovulation rate. In addition, administration of an epostane dose that inhibited ovulation also decreased levels of 15-HETE and progesterone. These results suggest that 15-HETE may play a more important role in ovulation than PGs. In addition, there may be an interaction of the lipoxygenase pathway with progesterone resulting in an effect on

ovulation. The remaining section focuses on the role of steroids in the periovulatory interval.

The LH surge is followed by a dramatic change in steroidogenesis, leading to increased production of progesterone. The change in steroidogenesis led to the hypothesis that progesterone may play a role in ovulation. Earlier studies on the role of progesterone in follicular rupture used inhibitors of steroid biosynthesis. These studies resulted in conflicting observations about progesterone action in ovulation. Treatment of rats with aminoglutethimide or cyanoketone resulted in the observation that steroidogenesis and ovulation were not related (Bullock and Kappauf, 1973). Similarly, aminoglutethimide and cyanoketone inhibited steroidogenesis, but not ovulation in rabbit ovaries perfused *in vitro* (Yoshimura *et al.*, 1986; Yoshimura *et al.*, 1987). Other studies, using inhibitors of steroidogenesis or progesterone antiserum, have shown a role for progesterone in ovulation (Lipner and Greep, 1971; Lipner and Wendelken, 1971; Snyder *et al.*, 1984; Tsafriri *et al.*, 1987; Brannstrom and Janson, 1989; Mori *et al.*, 1977). One drawback of these earlier studies was the nonspecificity of the inhibitors used. Another reason for varying results could be due to time of inhibitor administration. Espey and colleauges (1990, 1991) have shown that epostane, a 3β-hydroxysteroid dehydrogenase inhibitor, given at 3, 6, or 7 hours following hCG results in differing patterns of steroid inhibition.

Studies using RU486, a progesterone and glucocorticoid receptor antagonist, demonstrate that this compound blocks ovulation in rats and mice (Tsafriri *et al.*, 1987; Loutradis *et al.*, 1991). Recently, Iwamasa and colleagues (1992) measured ovulation rate and proteolytic enzyme activities in immature rats treated with PMSG/hCG, RU486, or RU486 plus progesterone. Their studies confirm earlier observations that RU486 inhibits

ovulation and showed that this compound was effective when given between 2 hours before and 4 hours after hCG. Furthermore, this effect could be reversed by the administration of exogenous progesterone. Their results demonstrated that progesterone acted to regulate proteolytic enzyme activity. Thus, recent studies support a role for progesterone as a part mediator in the ovulatory process.

Another role for progesterone has been hypothesized during the period of the developing corpus luteum. Rothchild (1981) suggested that progesterone stimulates its own secretion in a positive feedback manner based on several observations. First, a direct relationship between luteotropins and the pattern of progesterone secretion is not observed during luteal development. Second, the amount of progesterone in the corpus luteum is proportional to the rate of progesterone secretion. Finally, the detection of progesterone receptors in the corpus luteum support an autocrine action of progesterone. However, detailed experiments to support this are lacking.

Corpus Luteum Function and Life Span - Role of Gonadotropins

The luteinization process initiated in the follicle wall during the periovulatory interval culminates in the formation of the mammalian corpus luteum. In general, the ability of the corpus luteum to secrete progesterone is dependent on tropic hormones from the pituitary. However, the corpus luteum has a finite life span resulting in cessation of progesterone secretion even in the presence of luteotropic hormones. The pattern of progesterone secretion reflects the life span of the corpus luteum. Secretion is divided into a rising phase as the corpus luteum develops, followed by a plateau in levels

due to an active corpus luteum, and then a declining phase as the tissue regresses in the ovarian cycle.

The importance of LH as a regulator of the corpus luteum has been the focus of many investigations using varied techniques such as hypophysectomy, treatment with specific anti-LH antibodies or GnRH antagonists to decrease circulatory or bioactive LH levels. The observations of Vande Wiele and colleagues (1970) on hypophysectomized women were the first to suggest that low levels of LH during the luteal phase were required for normal functioning of the primate corpus luteum. Hypophysectomized women were given human menopausal gonadotropins (a combination of FSH and LH) to stimulate follicular development followed by the administration of a bolus of LH to induce ovulation and corpus luteum formation (Vande Wiele *et al.*, 1970). These women exhibited low progesterone levels and premature menses. However, normal levels of progesterone secretion and luteal phase length resulted if LH supplementation occurred after ovulation.

Further support for continual LH requirement for normal function of the primate corpus luteum comes from studies in which LH/CG immunoneutralization with LH or CG antisera in monkeys resulted in a decrease in progesterone levels in the luteal phase (Moudgal *et al.*, 1972; Groff *et al.*, 1984). Treatment with antisera for two days resulted in a transient decline in progesterone levels; at the end of treatment progesterone levels increased to normal values. In contrast, administration of antiserum for four days resulted in continued suppression of progesterone levels followed by luteolysis.

Studies using GnRH antagonists generally support a role for LH in controlling normal luteal progesterone production and life span in monkeys (Fraser *et al.*, 1986; Fraser *et al.*, 1987) and women (Mais *et al.*, 1986). When

Fraser and colleagues (1987) administered a GnRH antagonist for three consecutive days in the early luteal phase in monkeys, a complete inhibition of progesterone production and a shortened luteal phase was observed. If exogenous gonadotropin (CG) was given within three days of initiation of antagonist treatment, progesterone secretion resumed. Similarly, Mais and coworkers (1986) reported that women treated with a GnRH antagonist in the mid luteal phase recovered corpus luteum function following hCG administration.

There have been a few studies which failed to confirm a necessary role for LH in corpus luteum function. Asch and associates (1982) hypophysectomized monkeys following ovulation. They measured normal amounts of progesterone in the monkeys, and concluded that LH was not needed for normal luteal function. However, low levels of LH were detectable in several animals which may have been sufficient to support the corpus luteum. Balmaceda and colleagues (1983) reported that treatment of monkeys with a GnRH antagonist in the luteal phase did not inhibit estradiol or progesterone levels or cause luteolysis. Perhaps the doses of GnRH antagonist used in this study were not sufficient for complete suppression of LH levels. The amount of LH required for normal luteal function is not well defined.

A series of studies by Hutchison and Zeleznik using rhesus monkeys with hypothalamic lesions provided strong evidence for the importance of LH in supporting the corpus luteum. Lesions of the medial basal hypothalamus terminate the endogenous secretion of GnRH, which when replaced with exogenous pulsatile GnRH restores LH secretion and normal menstrual cycles in monkeys. In the first study (Hutchison and Zeleznik, 1984), interruption of pulsatile GnRH in the early or mid-luteal phase of the cycle,

resulted within two to five days in a decrease in LH and progesterone levels and the premature onset of menses. In a second study (Hutchison and Zeleznik, 1985), cessation of GnRH pulses in the early or mid-luteal phase followed by resumption of pulses after three days, resulted in a rapid restoration in LH and progesterone levels. In addition, these animals exhibited luteal phase lengths that were similar to those in control animals. However, corpus luteum function was not restored in monkeys in which pulsatile GnRH was stopped in the late luteal phase and then followed by resumption of pulses in three days. These studies support a requirement for LH in the function of the primate corpus luteum, but suggest a permissive role for this gonadotropin in controlling its life span.

Various studies in the 1970-1980s determined that LH had a direct effect on corpus luteum function (Marsh and LeMaire, 1974; Stouffer *et al.*, 1977a; Stouffer *et al.*, 1980; Dennefors *et al.*, 1982). Primate luteal cells were incubated in the presence or absence of LH or CG, and progesterone production was determined. *In vitro*, LH and CG acutely stimulated progesterone production by human and monkey luteal cells, whereas FSH did not. When luteal cells were cultured in the presence or absence of hCG, stimulation of progesterone production over controls occurred for up to eight days (Stouffer *et al.*, 1980). Thus, a direct action of LH on luteal function is supported by these studies.

The current model of LH action in the corpus luteum involves LH/CG binding to receptors at the cell surface. This results in activation of adenylate cyclase which is followed by the production of cyclic adenosine monophospate (cAMP). Coupling occurs through G proteins located in the plasma membrane which interact with the hormone receptor and the catalytic unit of adenylate cyclase. This is followed by the activation of

cAMP-dependent protein kinase (protein kinase A), and the resultant phosphorylation of intracellular proteins and cell activity changes. Specific, high-affinity receptors for LH/CG have been detected in the primate corpus luteum (Rao *et al.*, 1976; Cameron and Stouffer, 1982). That LH/CG acts via cAMP to stimulate progesterone production (Marsh, 1970) is supported by the following studies. LH/CG have been shown to stimulate adenylate cyclase in the primate corpus luteum (Marsh and LeMaire, 1974; Eyster and Stouffer, 1985). In intact human luteal cells, LH/CG increase cAMP and progesterone production (Marsh and LeMaire, 1974; Dennefors *et al.*, 1982). Dibutyryl cAMP (db-cAMP) was shown to enhance progesterone production by monkey luteal cells *in vitro* to a similar extent as that resulting from gonadotropin (LH/CG) exposure (Stouffer *et al.*, 1979).

GnRH pulses are translated into the LH pulses of the ovarian cycle. The pulsatile secretion of LH varies in primates across the menstrual cycle (Filicori *et al.*, 1984; Filicori *et al.*, 1986; Ellinwood *et al.*, 1984). That progesterone secretion is also pulsatile in women and nonhuman primates has been reported by several labs (Filicori *et al.*, 1984; Soules *et al.*, 1988; Healy *et al.*, 1984; Ellinwood *et al.*, 1984; Fraser *et al.*, 1986). Pulsatile progesterone secretion can be detected in the mid and late luteal phase, but cannot be identified in the early phase of the luteal cycle (Filicori *et al.*, 1984; Ellinwood *et al.*, 1984). The higher levels of progesterone act on the hypothalamus resulting in lower frequency of pulsatile LH secretion (Goodman and Karsch, 1980; Soules *et al.*, 1984). Thus, pulsatile LH secretion can induce progesterone pulses from the corpus luteum at least in the mid and late luteal phases.

It has been hypothesized that the decreasing frequency of LH pulses results in reduced luteotropic support followed by luteolysis. However, LH

pulse frequency does not appear to play a role in initiating luteolysis. In one study on hypothalamic-lesioned monkeys, Hutchison and Zeleznik (1986) began low frequency GnRH pulses similar to those observed during the late luteal phase after ovulation. In these animals, serum progesterone levels rose to normal midluteal concentrations with the majority of monkeys having luteal phases of normal length. Soules and colleagues (1987) observed that when women received high frequency pulsatile GnRH in the luteal phase, the length of the luteal phase was not increased. Thus, luteal regression occurs regardless of the pattern of pulsatile gonadotropin secretion presented to the corpus luteum.

Another pituitary hormone, prolactin, functions as a luteotropic hormone in some species. Prolactin is considered the major luteotropin in the rat (Rothchild, 1965; Greenwald and Rothchild, 1968; Hilliard, 1973), the ferret (Donovan, 1963; Murphy, 1979) and in the mink (Papke *et al.*, 1980). However, a role for prolactin in the primate corpus luteum is controversial. One reason for this has been the difficulty in distinguishing direct effects of prolactin on the corpus luteum from effects on the hypothalamus/pituitary leading to disturbances in LH and FSH secretion. Richardson and colleagues (1985) used hypothalamic-lesioned monkeys that were hyperprolactinemic, euprolactinemic, and hypoprolactinemic. Monkeys treated with GnRH had menstrual cycles with normal patterns of progesterone and a timely onset of luteolysis despite high to nondetectable prolactin levels. Thus prolactin may not be essential for primate luteal function. However, prolactin binding sites (Bramley *et al.*, 1987) as well as immunocytochemical staining for prolactin (Khan-Dawood, 1988) have been reported in human luteal tissue. These studies suggest a direct action of prolactin on the corpus luteum, although acute effects of prolactin on luteal cells *in vitro* have not been reported. The

physiologic role of prolactin in primate luteal function remains to be determined.

Although not considered a classical target for FSH action, it has been suggested that the primate corpus luteum is directly influenced by FSH. FSH binding sites were detected in ten of thirty-two human corpora lutea (McNeilly *et al.*, 1980) with tissues from the early luteal phase demonstrating the most binding. *In vitro* studies also showed that luteal tissues from the early and mid-luteal phase of the cycle produce estrogen, but not progesterone, in response to FSH (Hunter and Baker, 1981). Recently, Ohara and colleagues (1987) using isolated subpopulations of luteal cells from the human corpus luteum, reported that FSH acts mainly on large luteal cells to stimulate estrogen production. FSH, however, did not affect progesterone secretion by large luteal cells. Possible *in vivo* effects of FSH on luteal production of estrogen were also reported. In women, intravenous injections of purified FSH when administered during the early to mid-luteal phase of the cycle, resulted in an elevation of peripheral estradiol (Ohara *et al.*, 1989). Thus a previously unrecognized role for FSH in the regulation of estradiol production by the corpus luteum deserves consideration and further experimentation.

Corpus Luteum Function and Life Span - Role of Local Factors, Particularly Steroid Hormones

The role of local factors synthesized in the corpus luteum or the ovary has also been the focus of investigations concerned with regulation of luteal function and life span. This review focuses on the role of steroids in the regulation of the corpus luteum, though some attention is given to

prostaglandin involvement in this process.

Uterine prostaglandin (PG)$F_{2\alpha}$ induces luteolysis in many species, including the sheep (Horton and Poyser, 1976; Goldberg and Ramwell, 1975; McCracken *et al.*, 1972; Ginther, 1974; Hansel, 1975; Ramwell *et al.*, 1977), rats, mice, rabbits, and cows (reviewed in Rothchild, 1981). In primates, uterine prostaglandins are not essential for luteal life span, since hysterectomy did not affect the luteal phase (Beling *et al.*, 1970; Neill *et al.*, 1969). The proposed luteolytic actions of $PGF_{2\alpha}$ in primates could occur through autocrine or paracrine mechanisms in corpora lutea. A local action is supported by several observations. $PGF_{2\alpha}$ production and receptors have been detected in primate luteal tissues (Liedtke and Seifert, 1978). In nonhuman primates (Johnson *et al.*, 1988) *in vitro* production of luteal $PGF_{2\alpha}$ is the greatest in the early stage of the luteal phase. There is a drop in $PGF_{2\alpha}$ levels in the midluteal phase followed by a rise in the late luteal phase. $PGF_{2\alpha}$ also inhibits gonadotropin-stimulated progesterone production by primate luteal tissue from mid- and late luteal stages *in vitro*. *In vivo*, direct intraluteal treatment of $PGF_{2\alpha}$ into monkeys in the midluteal phase of the cycle results in a decline in progesterone secretion and in premature luteolysis (Auletta *et al.*, 1984; Zelinski-Wooten and Stouffer, 1990). However, studies with prostaglandin synthesis inhibitors are consistent with a more complex role for PG action in the corpus luteum (Manaugh and Novy, 1976; Sargent *et al.*, 1988). With the discovery of partially tropic PGs produced by the corpus luteum, the complex interaction of these compounds became essential to understand. Intraluteal infusion of PGE_2, D_2, or I_2 in the presence of $PGF_{2\alpha}$ in monkeys is able to prevent early luteolysis by exogenous $PGF_{2\alpha}$ administration (Zelinski-Wooten and Stouffer, 1990). Thus, a direct role for PGs in the regulation of the primate

corpus luteum is likely. However, a physiological role for PGs in luteal function remains to be determined.

An important role for estrogen as the primary luteotropic factor has been established in the rabbit corpus luteum (Greenwald and Rothchild, 1968; Hilliard, 1973). The rabbit corpus luteum becomes dependent on estrogens after four or five days (Miller and Keyes, 1975). This is consistent with a study where growth of the corpus luteum and progesterone secretion were similar for a few days after ovulation in rabbits hypophysectomized directly after ovulation and in intact rabbits (Yuh, 1980). Another study showed that LH antiserum treatment induced luteolysis in rabbits only if given when corpora lutea were older than three days. This effect could be blocked with estrogen treatment (Spies and Quadri, 1967).

In contrast, in other species such as monkeys and women, estrogen produced by the corpus luteum has been proposed to induce luteolysis. Rising concentrations of serum or tissue estrogen in close proximation to the onset of luteal regression (Hotchkiss *et al.*, 1971; Butler *et al.*, 1975), makes estrogen's role as a luteolysin possible. Premature luteolysis is observed following administration of exogenous estrogen or diethylstilbestrol to human and nonhuman primates during the luteal phase (Karsch *et al.*, 1973; Gore *et al.*, 1973). However, other studies suggest that endogenous estrogen is not an important regulator of luteolysis. Treatment with an aromatase inhibitor (Ellinwood and Resko, 1983) or estrogen antagonists (Albrecht *et al.*, 1981; Westfahl and Resko, 1983) does not result in lengthening the life span of the macaque or baboon corpus luteum during the menstrual cycle. Thus these studies suggest a disparity between the actions of exogenous and endogenous estrogen in inducing luteolysis.

The site of action of exogenous estrogen-induced luteolysis was

initially unclear (Karsch and Sutton, 1976; Schoonmaker *et al.*, 1982). Recent *in vivo* studies, however, indicate that estrogen treatment results in luteolysis only if serum LH levels are inhibited (Schoonmaker *et al.*, 1982; Westfahl and Kling, 1982). Hutchison and colleagues (1987) used hypothalamic-lesioned rhesus monkeys in which gonadotropin secretion was regulated by pulsatile GnRH administration to determine estrogen's site of action. Insertion of estrogen-containing capsules failed to induce luteal regression when LH levels were maintained with GnRH. The major site of estrogen action according to the authors was the hypothalamus and not the corpus luteum.

Androgens have recently received attention as potential regulators of corpus luteum function. This is supported by immunocytochemical evidence for androgen receptors in monkey and human corpora lutea (Hild-Petito *et al.*, 1991; Horie *et al.*, 1992). Hild-Petito and colleagues (1991) immunocytochemically examined the distribution of androgen receptors in primate tissue from early, mid-, and late luteal phases of the menstrual cycle. Intense positive staining for the androgen receptor was detected in luteal cells from the early and midluteal phases. Corpora lutea of the late luteal phase also stained for the receptor. In contrast, androgen receptors were not detected in fully regressed corpora lutea in the early follicular phase of the next cycle. Interestingly, luteal cells that stained positively for the androgen receptor also expressed 3ß-hydroxysteroid dehydrogenase thus suggesting a role for androgen in cells involved in the production of progesterone. Hild-Petito and colleagues (1991) also observed a similar distribution of androgen receptors in the ovaries of estrus and pseudopregnant rabbits. Another study using human luteinized granulosa cells reported that testosterone and dihydrotestosterone inhibited gonadotropin-stimulated progesterone

production as well as LH/hCG expression (Polan *et al.*, 1986). Inhibin production by human luteinized granulosa cells is stimulated by testosterone (Tsonis *et al.*, 1987). Though a limited number of studies have addressed androgen action in the corpus luteum, further studies are required to elucidate androgen function in primate and nonprimate luteal tissue.

Mechanisms of Steroid Hormone Action - Steroid Receptor Structure and Function

The binding of individual steroids to specific receptors in target organs is transduced into intracellular events that lead to a biological response. Little is known about the mechanisms of steroid hormone action in the ovary. In contrast, considerable research on the mechanism of action of steroid hormones in classical target tissues led to an evolving model of steroid action. The concept that specific receptors for steroids exist was first supported by Glasscock and Hoekstra (1959) and Jensen and Jacobson (1962) who observed that target organs accumulate and retain [^{3}H]estrogens. The existence of specific steroid receptors was confirmed when Talwar and colleagues (1964) and Toft and Gorski (1966) observed a macromolecule in cytosol fractions of the rat uterus that exhibited characteristics predicted for an estradiol receptor. The initial model of steroid action envisioned hormone interaction with a receptor in the cytoplasm. This was thought to result in the activation of the hormone receptor complex followed by its translocation into the nucleus. Interaction of the receptor with steroid-responsive genes led to an alteration in the pattern of gene expression (Gorski and Gannon, 1976; Yamamoto and Alberts, 1976). However, the initial cytoplasmic localization of progestin and estrogen receptors using steroid-binding properties, is now

thought to be an artifact resulting from harsh experimental conditions.

The proposal by Williams and Gorski (1971) that the majority of unoccupied estrogen receptor is nuclear initiated a reevaluation of the cellular localization of steroid receptors. Initial immunocytochemical studies by King and Greene (1984) using monoclonal antibodies to the estrogen receptor confirmed the nuclear localization of unoccupied receptors in the human and rabbit endometrium. In addition, recent studies showed that the intracellular location of steroid receptor proteins is dependent upon nuclear localization signals. In contrast to the glucocorticoid receptor which requires hormone binding for nuclear translocation, both progestin and estrogen receptors are found in the nucleus due to constitutively active translocation signals (Guiochon-Mantel *et al.*, 1989; Picard and Yamamoto, 1987; Picard *et al.*, 1990a; Gronemeyer, 1992). Thus the current model of steroid hormone action involves progestin and estrogen receptors localized in the nucleus. Hormone binding converts the steroid receptor into a transcriptionally competent factor that can bind to specific hormone response elements (HREs) of target genes to regulate transcription (Gronemeyer, 1992). The following review emphasizes information regarding progestin and estrogen receptors.

The progestin receptor, unlike other steroid hormone receptors, is detectable as two proteins of dissimilar molecular weights designated as A and B. The molecular weights of these receptor proteins are 86 and 72 K in the chicken (Gronemeyer *et al.*, 1986; Conneely *et al.*, 1987), and 98 and 86 K in humans (Misrahi *et al.*, 1987). In the rabbit, only the B form of the progestin receptor was detected (Logeat *et al.*, 1985). When complementary DNAs which included the coding region of the human or rabbit progestin receptor were transcribed/translated in vitro, the B protein was detected as the predominant product (Misrahi *et al.*, 1988). This agrees with earlier

studies in the rabbit showing that A formation was the result of post-translational proteolysis (Logeat *et al.*, 1985). However, this does not explain the detection of both A and B forms in the human (Horwitz *et al.*, 1985; Feil *et al.*, 1988). Recent studies continue to focus on this issue. The two receptor proteins in the chicken are hypothesized to originate from one mRNA via alternate initiation from two AUG codons (Conneely *et al.*, 1987; Conneely *et al.*, 1989: Tora *et al.*, 1988). Similarly, the two progestin receptor forms in the human are thought to originate from two promoters with translation initiation occurring at two inframe ATGs (Kastner *et al.*, 1990). However, there are also reports of multiple chicken progestin receptor mRNAs with one of the transcripts reported to selectively code for form A (Jeltsch *et al.*, 1990). Thus, whether the A form of the progestin receptor is physiologically produced remains to be determined.

Progestin and estrogen receptors belong to a gene superfamily of trans-acting transcriptional factors that includes receptors for steroids, thyroid hormone, and retinoic acid. Progestin and estrogen receptors have been cloned from several species (Loosfelt *et al.*, 1986; White *et al.*, 1987; Koike *et al.*, 1987; Green *et al.*, 1986; Maxwell *et al.*, 1987). The analysis of deduced receptor sequences has led to the description of three functional domains that include an amino-terminal domain which is separated from the steroid-binding, carboxy-terminal region by a central cysteine-rich DNA binding domain. The steroid receptors can also be described in terms of regions of high sequence homology designated as C1-C3 (Carson-Jurica *et al.*, 1990). The highest homology among different steroid receptors is found in the C1 region. The C1 region in the DNA-binding domain is made up of 66-68 amino acids which include nine completely conserved cysteines. The two zinc fingers involved in DNA-binding are formed by this domain. The four cysteines that

are part of each finger are associated with one zinc molecule, and a linker region of 15-17 amino acids is found between the two fingers (Umesono and Evans, 1989). The other two homologous regions, C2 and C3, are located in the C terminal steroid-binding domain of receptors. The hydrophobic nature of these regions makes their involvement in hormone binding likely. In contrast to these regions that are highly conserved, the amino-terminal region is hypervariable with low homology between receptors. This latter region spans 185 and 567 amino acids, respectively, for the human estrogen and progestin receptors. Thus, steroid receptor function has been divided in terms of hormone binding, DNA binding, and gene activation.

The role of the hormone binding domain in steroid receptor function has been the focus of many studies. Deletion of amino acids from the C-terminal of progestin and estrogen receptors results in loss of hormone binding activity (Carson *et al.*, 1987; Gronemeyer *et al.*, 1986; Dobson *et al.*, 1989; Kumar *et al.*, 1986). Interestingly, deletion of the C-terminal portion of the progestin receptor leads to gene activation that is constitutive in vivo (Carson *et al.*, 1987). Such studies led to the concept that the function of the hormone binding domain is to repress receptor action. This could occur as follows: the hormone binding domain prevents DNA binding by keeping the receptor in an inactive form. Alternatively, the interaction of the hormone binding domain with other proteins could maintain an inactive receptor. A 90 kd heat shock protein (hsp90) has been detected in both estrogen and progestin receptor complexes (Catelli *et al.*, 1985; Redeuilh *et al.*, 1987, Renoir *et al.*, 1986; Joab *et al.*, 1984) and there is some evidence suggesting that the C-terminal portion of the receptor interacts with hsp90 (Carson-Jurica *et al.*, 1990). Steroid receptors exist in complexes (7-10 S) when isolated from cells prior to hormone activation and under hypotonic

conditions (Baulieu, 1987; Sherman and Stevens, 1984). *In vitro* treatment of such complexes with hormone or salt leads to dissociation that enables the receptor to become activated. Ligand-dependent dissociation of receptor-hsp90 complexes may be involved in receptor activation (Pratt, 1990; Baulieu *et al.*, 1990). However, other studies shed doubt on this hypothesis. Yeast cells depleted of hsp90 do not exhibit constitutively active receptors (Picard *et al.*, 1990b). Moreover, Pekki (1991) failed to detect nuclear hsp90 progestin receptor complexes in chick oviduct epithelial cells. Thus, the mechanism whereby the hormone activates the receptor *in vivo* is unknown.

Activated steroid hormone/receptor complexes regulate transcription via interaction with gene sequences called hormone responsive elements (HRE). These response elements occur as palindromes or direct repeats of two core motifs. The spacing and orientation of the motifs are thought to determine both the likelihood of receptor binding as well as the type of response achieved (i.e., activation, repression) following receptor interaction (Gronemeyer, 1992). The estrogen response element (ERE) consensus sequence (5'-GGTCAnnnTGACC) is palindromic, containing a non-conserved three-nucleotide spacer (Beato, 1989; Klein-Hitpass *et al.*, 1988; Martinez *et al.*, 1987; Klein-Hitpass *et al.*, 1986; Klock *et al.*, 1987). Three amino acids (glu, gly, ala) in the estrogen receptor DNA binding domain are involved in recognition of the ERE (Mader *et al.*, 1989; Umesono and Evans, 1989). Using nuclear magnetic resonance spectroscopy of the estrogen receptor DNA binding domain (Schwabe *et al.*, 1990) it was shown that binding of the domains to the response element occurs as dimers that involves two receptors. The progestin receptor is known to activate the glucocorticoid response element in the mouse mammary tumor virus promoter (Ham *et al.*, 1988). However, the consensus response element for the progestin receptor

still needs to be clearly defined.

The transcriptional activation of target genes involves more than the DNA-binding domain of the receptor. Studies using deletion mutants without the amino terminal or the hormone binding region observed that transcription was activated less in these mutants than in the wild-type receptor. Interestingly, transcription activity was not completely stopped (Meyer *et al.*, 1990; Gronemeyer, 1992), suggesting the presence of more than one transcription activation function (TAF, TAF-1 and TAF-2). Meyer and colleagues (1990) reported that linking of either the amino terminal region or the hormone binding domain of the progestin receptor to the DNA binding domain of GAL4 resulted in transcription of GAL4-responsive genes. Similarly, two TAFs are located in the amino terminal and hormone binding domain, respectively, of the estrogen receptor (Webster *et al.*, 1988; Tora *et al.*, 1989). Steroid receptor activity can be affected by both the target cell type as well as gene promoter complexity. Estrogen receptor TAF-2 induced activity is greater than that by TAF-1 in HeLa cells (Kumar *et al.*, 1987). In contrast, both TAF-1 and TAF-2 activated transcription in chicken embryo fibroblasts (Tora *et al.*, 1989; Berry *et al.*, 1990). Progestin receptor TAF activity has also been suggested to vary depending on cell type (Bocquel *et al.*, 1989).

In summary, considerable progress has been made in the past two decades in elucidating the structure and function of steroid hormone receptors. Steroid receptor functions include hormone binding, DNA binding and gene activation. Though many steps in the mechanism of steroid action have been elucidated, further study is required especially to determine the *in vivo* relevance of results obtained from *in vitro* observations.

Steroid Receptor Regulation

Tissue sensitivity to hormones can be influenced by regulating the levels of the respective receptor molecules. There are many reports of hormonal regulation of progestin and estrogen receptor levels in reproductive tissues. One common mechanism involves estrogen stimulation of the synthesis of both estrogen and progestin receptors, with progestin antagonism of these effects. However, a variety of recent studies suggest that this mechanism is not universal and that cell- and tissue-specific differences exist in the regulation of progestin and estrogen receptors.

Estrogen regulates the level of estrogen receptor in target tissues such as the uterus, vagina, and mammary gland. Estrogen target cells generally maintain a constitutive level of the receptor and estrogen acts to increase these receptor levels. Several investigations suggest that this is due to an increase in receptor protein synthesis (Cidlowski and Muldoon, 1974; Cidlowski and Muldoon, 1976; Jensen *et al.*, 1969; Mester and Baulieu, 1975; Mester *et al.*, 1974; Sarff and Gorski, 1971). In naturally cycling macaques, estrogen receptor immunocytochemical staining is increased in the endometrium at the end of the follicular phase under the influence of estrogen (Brenner *et al.*, 1990). Most stromal fibroblasts as well as glandular epithelial cells stain positively for the estrogen receptor. Stimulatory effects of estrogen on the mRNA level of its receptor are also reported in T47-D cells and in the rat liver and pituitary (Read *et al.*, 1989; Shupnik *et al.*, 1989).

Several studies have shown that estrogen treatment also increases progestin receptor levels in target tissues such as the uterus (Freifeld *et al.*, 1974; Hsueh *et al.*, 1975; Hsueh *et al.*, 1976; Leavitt *et al.*, 1974; Luu *et al.*, 1975; Milgrom *et al.*, 1973; Rae *et al.*, 1973; Reel and Shih, 1975; Toft and

O'Malley, 1972). Similarly, in the monkey uterus, estrogen acts on the endometrium to increase progestin receptor levels (Brenner *et al.*, 1990). Estrogen also increases progestin receptor levels in hypothalamic neurons (Kato and Onouchi, 1977; McEwen, 1978; Sar and Stumpf, 1973). In addition, estrogen regulation of progestin receptor mRNA was shown in MCF-7 cells and in neurons of the hypothalamus (Nardulli *et al.*, 1988; Romano *et al.*, 1989).

Progestin regulation of estrogen and progestin receptor levels occurs in target tissues such as the uterus. Progestin action is reported to decrease the levels of the estrogen receptor (Brenner *et al.*, 1974; Clark *et al.*, 1977; Hsueh *et al.*, 1975; Mester and Baulieu, 1975; Pavlik and Coulson, 1976; Okulicz *et al.*, 1981a; Okulicz *et al.*, 1981b; West *et al.*, 1978). In the macaque, suppression of estrogen receptor levels in the endometrium occurs during the luteal phase under the influence of progestins. This pattern of estrogen receptor staining in the macaque endometrium is also observed in spayed monkeys that are hormone-treated to induce artificial cycles. Treatment for two weeks with estradiol followed by a two week treatment with a progestin implant resulted in a substantial decrease in immunocytochemical staining for the estrogen receptor (Brenner *et al.*, 1990). Similarly, the action of progestins to downregulate the levels of its own receptor was shown by several groups. In the guinea pig uterus, Milgrom *et al.* (1973) and Freifeld *et al.* (1974) have shown this effect of progestins. In the monkey, a decrease in progestin receptor staining was observed in the glandular epithelium of the endometrium following ovulation (Brenner *et al.*, 1990).

It is, however, becoming increasingly apparent that the ability of hormones to regulate steroid receptor levels is dependent on the cell type within tissues, including the uterus. In the macaque, estrogen receptor

levels are generally suppressed throughout the endometrium by the end of the luteal phase. Zone IV of the basalis, however, is an exception and exhibits positive staining for estrogen receptor up to the end of the luteal phase (Brenner *et al.*, 1990). Similar regional differences in the effect of progestins on progestin receptor levels in human uterine cells were observed. In the proliferative phase of the menstrual cycle, immunoreactive levels of the progestin receptor increased in the endometrium, myometrium, and stroma (Clarke *et al.*, 1987; Garcia *et al.*, 1988; Press and Greene, 1988; Press *et al.*, 1988; Zaino *et al.*, 1989). The levels of progestin receptor decrease dramatically in epithelial cells following ovulation, but not in some glands in the endometrial basalis (Press *et al.*, 1988). Thus, there seems to be differential regulation of progestin receptor levels in various groups of epithelial cells. Lack of progestin receptor down regulation in the presence of high serum progesterone has also been reported in pregnancy. Khan-Dawood and Dawood (1984) reported the maintenance of progestin receptors in the decidua and myometrium of uterine biopsies from eight patients undergoing cesarian sections.

Recent observations suggest that some of the actions of estrogens and progestins on target organs may be mediated by local paracrine factors. During the luteal-follicular transition between menstrual cycles, stromal but not glandular epithelial cells of the monkey endometrium express estrogen and progestin receptors (Brenner *et al.*, 1990). Nevertheless, the receptor-negative glandular epithelial cells exhibit estrogen-dependent DNA synthesis and mitosis. These effects can be inhibited by progestin administration. Similarly, ciliated cells of the monkey oviduct do not express estrogen or progestin receptors, but estrogen-dependent ciliogenesis is observed that can be inhibited by progestin treatment (Brenner *et al.*, 1990). Thus the

involvement of intercellular mediators in the actions of estrogens and progestins seems likely. Increasing attention is focusing on growth factors and growth factor binding proteins as possible candidates. Ovarian steroids reportedly stimulate the synthesis of specific growth factors in the uterus (Fay and Grudzinskas, 1991). A role for insulin-like growth factor (IGF) and epidermal growth factor (EGF) as potential mediators for steroid action has been proposed.

In summary, direct estrogen and progestin action through receptor-mediated pathways occurs in specific cells of reproductive target organs to regulate estrogen and progestin receptor levels. However, it is apparent that the ability of hormones to regulate steroid receptor levels is dependent on the cell type within a tissue. In addition, estrogen and progestin-dependent intercellular actions are proposed that might be mediated by growth factors.

CHAPTER 2

RATIONALE FOR THE PRESENT STUDY

Evidence is accumulating that estrogens and progestins may act as local regulators of ovarian function in various species. However, studies on steroid action in the ovary are difficult to interpret due to high levels of endogenous steroids in the tissues. Classical radioligand binding techniques suggested the presence of estrogen and progestin receptors in the ovaries of several species (Lee *et al.*, 1971; Richards, 1975; Glass *et al.*, 1984; Schreiber and Hsueh, 1979; Jacobs *et al.*, 1980). Recent immunocytochemical techniques using specific antibodies to the human estrogen and progestin receptors provided the first detailed evidence on possible target cells in some species, particularly monkeys and humans (Hild-Petito *et al.*, 1988; Iwai *et al.*, 1990; Press and Greene, 1988). An unexpected distribution of estrogen receptor was observed in the monkey ovary, localizing this receptor to only the germinal epithelium, not to follicles or corpora lutea. In contrast, progestin receptors were detected in various monkey ovarian structures including the germinal epithelium, follicle, and corpus luteum (Hild-Petito *et al.*, 1988). The general absence of estrogen receptors in various structures of the macaque ovary suggested that the regulation of progestin receptors may differ from that in classical target tissues. Based on their observation that progestin receptors in the granulosum of the dominant follicle appeared during the periovulatory interval, Hild-Petito and colleagues (1988) proposed a novel role for the midcycle gonadotropin surge to induce progestin receptor expression in luteinizing granulosa cells. Whether this is a direct action of the gonadotropin surge or an indirect action via other mediators (e.g., prostaglandins) of periovulatory changes is unknown. This project was

designed to further investigate the presence and regulation of steroid receptors, particularly those for progestin, within the primate ovary during the menstrual cycle. The specific hypothesis tested was that the midcycle surge of gonadotropin in the rhesus monkey induces the expression of progestin receptors in luteinizing granulosa cells.

Studies in various species confirm that the gonadotropin surge elicited by estrogen from the mature follicle, is the major stimulus for periovulatory events, including resumption of oocyte meiosis, ovulation, as well as luteinization of the follicle wall and its conversion into the corpus luteum. The LH surge duration in the natural menstrual cycle of monkeys and women is 48-50 hours. However, the amplitude and duration of the LH surge required for ovulatory events in primate follicles is ill-defined. In the rat and rabbit, LH surges of four to eight hours are sufficient for oocyte maturation, ovulation and formation of the corpus luteum in normal cycles (Fink, 1988). Induction of various events in rat and rabbit follicles during the ovulatory process require differing thresholds of gonadotropin exposure with resumption of oocyte meiosis initiated by less gonadotropin than ovulation (Peluso, 1990; Bomsel-Helmreich *et al.*, 1989). Similar studies have not been performed in primate species. Thus, this study was also designed to investigate the requirement of varying LH surge durations for periovulatory events in primate follicles, particularly for progestin receptor expression and progesterone production by macaque granulosa cells.

To effectively study periovulatory events initiated by the gonadotropin surge an ovarian stimulation model can be used (Wolf *et al.*, 1990). This model is routinely used in rats which receive PMSG for follicular development, followed by an ovulatory dose of hCG (Espey *et al.*, 1991). Similar models that use LH and FSH for follicular devlopment followed by

hCG as an ovulatory stimulus are now available in primates (Wolf *et al.*, 1990). The ovarian stimulation protocol in rhesus monkeys yields follicles that are able to ovulate after hCG administration and results in the formation of functional corpora lutea (VandeVoort *et al.*, 1989). This model is useful for several reasons. First, it eliminates the variability in the follicular phase observed in the natural cycle and provides a method for precisely timing the onset, magnitude and duration of the LH surge. Secondly, since the protocol results in the development of multiple follicles, the number of granulosa cells available for study is vastly increased over that from natural cycles. Finally, granulosa cells from both nonluteinized preovulatory follicles as well as luteinizing periovulatory follicles can be obtained. Overall, the model enables the study of events immediately prior to and following the ovulatory gonadotropin surge as well as subsequent luteal function.

The current study used rhesus monkeys in ovarian stimulation protocols to test the hypothesis that the midcycle surge of gonadotropin induces the expression of progestin receptors in luteinizing granulosa cells. The specific aims of this study are the following: 1) whether administration of an ovulatory dose of hCG after follicular stimulation induces progestin receptors in luteinizing granulosa cells; 2) to determine whether gonadotropin (LH/CG) surges of varying duration stimulate progestin receptor expression in granulosa cells; 3) to determine if LH surge action to induce progestin receptor is associated with the expression of progestin receptor mRNA in granulosa cells; and 4) to investigate direct actions of LH and that of gonadotropin-triggered mediators in progestin receptor expression.

CHAPTER 3

TITRATING LUTEINIZING HORMONE (LH) SURGE REQUIREMENTS FOR OVULATORY CHANGES IN PRIMATE FOLLICLES. PROGESTERONE RECEPTOR EXPRESSION IN LUTEINIZING GRANULOSA CELLS

Abstract

The events in granulosa cells that are initiated by the midcycle LH surge during luteinization of the primate follicle are poorly defined. This study was designed 1) to determine whether an ovulatory dose of hCG can induce progesterone receptors (PR) in macaque granulosa cells, and if so, 2) to begin titrating gonadotropin requirements for PR expression and progesterone production by luteinizing granulosa cells. Rhesus monkeys were treated with human FSH and LH for up to 9 days to stimulate growth of multiple follicles. The next day, animals (n = 4-5/group) received: (a) no ovulatory stimulus; (b) 1000 IU hCG, im; (c) 1 injection of 100 μg GnRH, sc (GnRH-1); (d) 3 injections of GnRH (GnRH-3) at 3-h intervals (0800, 1100, 1400 h); or (e) 2 injections of 50 μg GnRH agonist (GnRHa), sc, 8 h apart (0800 and 1700 h). Granulosa cells obtained by follicle aspiration 27 h after the hCG or initial GnRH/GnRHa injection, or on days 8 or 10 from animals receiving no ovulatory stimulus, were processed for indirect immunocytochemistry using a monoclonal antibody to human PR (JZB39). Specific staining for PR, determined by comparing cells incubated with PR antibody versus a nonspecific antibody, was undetectable in granulosa cells from monkeys without an ovulatory stimulus. In contrast, the majority (64 ±

5%) of cells from hCG-treated animals stained intensely for PR. In the GnRH/GnRHa groups, granulosa cells from only one animal (i.e., one GnRH-3 monkey) showed positive staining for PR. During 24 h culture in Ham's F-10 medium containing 10% monkey serum, basal progesterone production by cells from the hCG-treated group (2163 nmol/L/8 x 10^4 cells) was higher than that by cells from the no ovulatory stimulus/GnRH-1/GnRH-3/GnRHa groups (60, 111, 194, and 332 nmol/L, respectively). Granulosa cells from the hCG-treated group were less responsive to hCG *in vitro*, in terms of enhanced progesterone production (2 times control levels), than cells from the other four groups (up to 30 times control levels). This study provides direct evidence that an ovulatory dose of hCG induces PR expression in granulosa cells of luteinizing follicles during stimulated cycles in rhesus monkeys. However, repeated injections of GnRH/GnRHa that produced surge levels (> 100 ng/mL) of endogenous LH for up to 14 h failed to induce PR expression or progesterone production by granulosa cells. Thus an extended LH surge more typical of that in the normal menstrual cycle (48 - 50 h) may be necessary for PR expression and luteinization of granulosa cells in primate follicles.

Introduction

The surge of luteinizing hormone (LH) in the normal menstrual cycle initiates many changes in the follicle destined to ovulate, including luteinization of granulosa cells. A novel role for the midcycle LH surge to induce progesterone receptors (PR) in luteinizing granulosa cells was proposed by Hild-Petito and colleagues (1988), after observing the appearance of PR in the granulosum of the dominant follicle in monkeys

during the periovulatory interval. Ovarian stimulation protocols that promote the growth of multiple preovulatory follicles in women (Jones *et al.*, 1982) and nonhuman primates (Wolf *et al.*, 1990) can be used as a model system for studying LH surge requirements for periovulatory changes in granulosa cells. A bolus of human chorionic gonadotropin (hCG) is routinely used as the stimulus for ovulatory changes in follicles after follicular stimulation (Jones *et al.*, 1982; Wolf *et al.*, 1990). However, the amplitude and duration of the gonadotropin (endogenous LH or exogenous CG) surge required for luteinization events in granulosa cells are ill-defined.

This laboratory recently reported that injections of GnRH or GnRH agonist (GnRHa) produce surge levels of endogenous LH after follicular stimulation in rhesus monkeys (Zelinski-Wooten *et al.*, 1991). Zelinski-Wooten and associates (1991) compared the ability of hCG and GnRH/GnRHa-elicited LH surges to reinitiate meiosis in oocytes and alter circulating levels of steroids in stimulated cycles. This tandem study was designed to determine 1) whether administration of an ovulatory dose of hCG after follicular stimulation induces PR in luteinizing granulosa cells, and if so, 2) whether GnRH/GnRHa-elicited LH surges of varying duration stimulate PR expression and progesterone production by granulosa cells in stimulated macaque follicles.

Materials and Methods

Animals

General husbandry and housing of rhesus monkeys at the Oregon Regional Primate Research Center (ORPRC) have been described previously (Molskness *et al.*, 1987). Adult, regularly cycling females were checked daily for menses (onset of menses = cycle day 1). From day 1 of the menstrual

cycle until the next onset of menses, daily blood samples were obtained at 0800 h from unanesthetized monkeys by saphenous venipuncture. Serum was stored at -20 C until measurement of progesterone and estradiol by radioimmunoassay (Hess *et al.*, 1981; Resko *et al.*, 1975) in the ORPRC Hormone Assay Core Laboratory.

Ovarian Stimulation

Ovarian stimulation protocols have been described previously (Zelinski-Wooten *et al.*, 1991). Beginning at menses, rhesus monkeys were given one of two regimens of human gonadotropins to stimulate the growth of multiple follicles (VandeVoort *et al.*, 1989): 60 IU hFSH (Metrodin, Serono Laboratories, Inc., Randolph, MA; Days 1-6) followed by 60 IU hFSH + 60 IU hLH (Pergonal, Serono; Days 7-9) or 60 IU hFSH + 60 IU hLH (Days 1 to 7 or 9). To induce ovulatory maturation, animals (n = 4-5/group) received on the next day: a) no ovulatory stimulus; b) 1000 IU hCG, im (Profasi, Serono); c) 1 injection at 0800 h of 100 µg GnRH, sc (GnRH-1; Relisorm, Serono); d) 3 injections of 100 µg GnRH (GnRH-3) at 3-h intervals (0800, 1100, 1400 h); or e) 2 injections of 50 µg GnRH agonist, leuprolide acetate (GnRHa; Lupron, TAP Pharmaceutical, North Chicago, IL), sc, 8 h apart (0800 and 1700 h). The levels and patterns of LH (or LH-like) bioactivity produced by each ovulatory treatment are detailed by Zelinski-Wooten and colleagues (1991).

Granulosa Cell Isolation

Granulosa cells were obtained by follicle aspiration (Wolf *et al.*, 1989) at laparotomy 27 h after the hCG or initial GnRH/GnRHa injection, and on day 8 or 10 in animals receiving no ovulatory stimulus. After removal of

oocytes (Zelinski-Wooten *et al.*, 1991), follicular aspirates were pooled and centrifuged. The cell pellet was resuspended in Ham's F-10 medium (Gibco Laboratories, Grand Island, NY), supplemented with amphotericin B (2.5 mg/L), penicillin G (0.07 g/L), streptomycin (0.1 g/L), sodium bicarbonate (1.2 g/L), HEPES buffer (4.7 g/L; Sigma Chemical Co., St. Louis, MO), and 0.1% bovine serum albumin (BSA, Sigma) pH 7.35. The cell suspension was centrifuged at 470 x g for 30 minutes on a 40% Percoll (Sigma)-60% Hanks Balanced Salt Solution containing 0.1% BSA to collect granulosa cells with reduced blood cell contamination (Greenberg *et al.*, 1990). Granulosa cells were counted using a hemacytometer, and viability was assessed by trypan blue exclusion.

Immunocytochemistry for PR

Granulosa cells were plated on Lab-Tek Chamber Slides (160,000 cells/well; Nunc Inc., Naperville, IL) coated with Cell-Tak (15 µg/well; Collaborative Research, Inc., Bedford, MA). Cells were frozen in liquid propane and stored at -20 C until analysis of PR by indirect immunocytochemistry as described previously by Hild-Petito and colleagues (1988). Cells were incubated with either a monoclonal antibody against human PR (JZB39; 2.5 µg/mL) or a nonspecific antibody (AT, antibody to antigen B of timothy grass pollen; 10 µg/mL). The human PR antibody was prepared against PR obtained from the T47D human breast carcinoma cell line (Greene *et al.*, 1988), and recognizes the ligand-occupied as well as unoccupied cytosol and nuclear PR A and B forms. The antibody complex was visualized by incubation with avidin-DH and biotinylated horseradish peroxidase-H complex (ABC kit from Vector Laboratories, Burlingame, CA) followed by treatment with diaminobenzidine tetrahydrochloride (DAB) and

hydrogen peroxide. Photomicrographs were taken with planapochromatic lenses on a Zeiss light microscope (Zeiss, New York, NY) using Technical Pan Film (Eastman Kodak, Rochester, NY) and a Wratten 47 filter. The film was shot at ASA 20 and developed in Technidol. The percentage of nuclei staining for PR was determined in nonblinded observations by a random count of 500 cells.

Progesterone Production by Granulosa Cells

Granulosa cells (80,000 cells/well) from each of 4-5 monkeys per treatment group were cultured on glass chamber slides coated with Cell-Tak, in Ham's F-10 containing 10% monkey serum in the presence or absence of hCG (100 ng/mL, CR123; n = average of 4 replicates/monkey with or without hCG). Cell cultures were incubated at 37 C in humidified 95% air/5% CO_2. Media were collected after 24 h of culture and frozen for measurement of progesterone content by radioimmunoassay (Hess *et al.*, 1981) at a later date.

Statistical Analyses

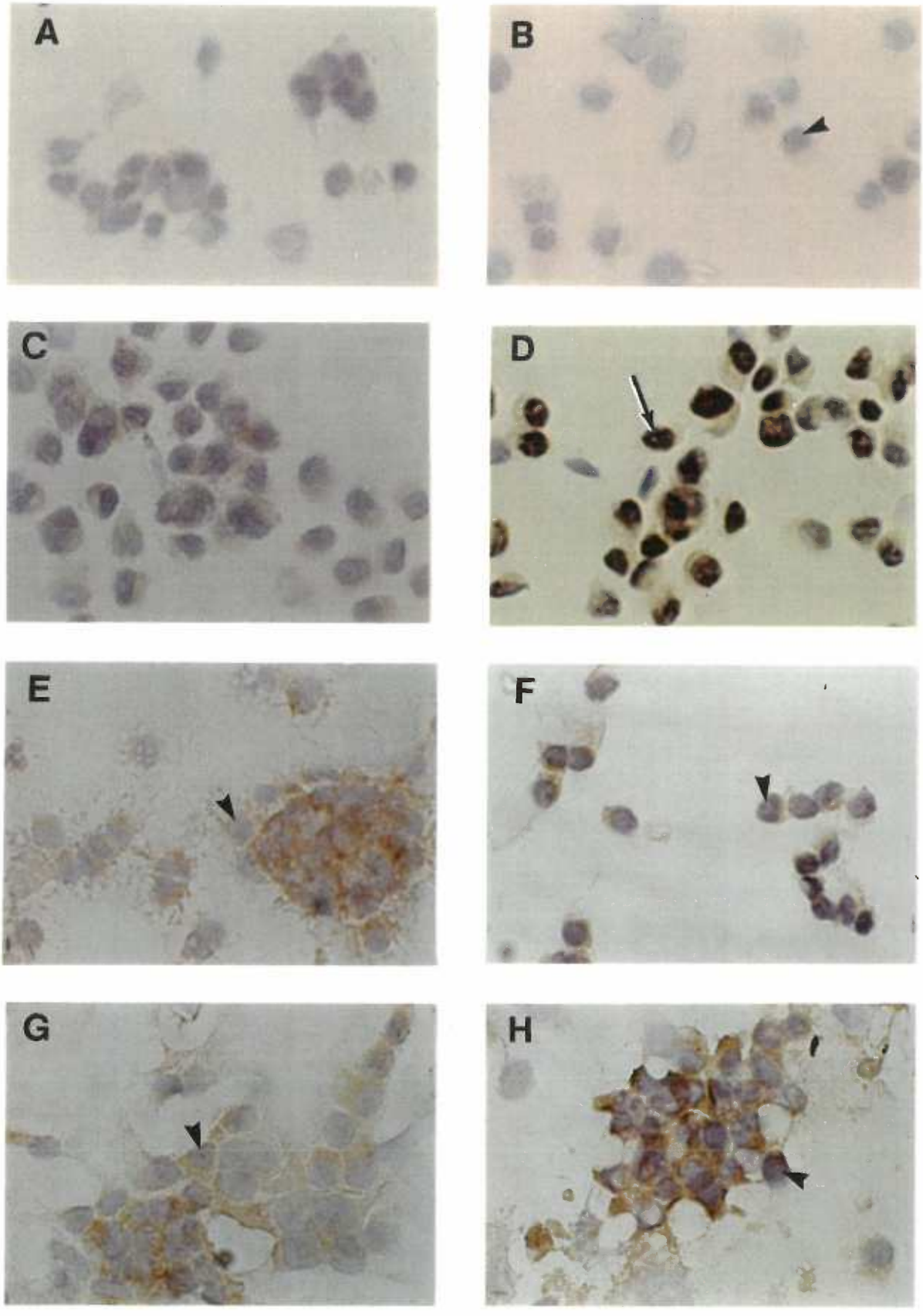
Steroid data were transformed before statistical analyses when heterogeneity of variance was present. Significant ($p<0.05$) differences in progesterone content in media cultured with or without hCG were analyzed by a one-way ANOVA, followed by the Duncan's Multiple Range or *t* test for comparison of means. The correlation between the interval of *in vivo* exposure to surge levels of gonadotropin (LH or CG) and control levels of progesterone production *in vitro* was determined using linear regression.

Results

PR Expression

No nuclear staining was observed when granulosa cells collected from monkeys receiving either no ovulatory stimulus or hCG were treated with the control antibody, antitimothy grass pollen (Fig. 1, A and C). Specific nuclear staining was also undetectable after cells from five animals receiving no ovulatory stimulus were incubated with PR antibody (Fig. 1B). The percentage of nuclei staining for PR in a random count of 500 cells was 0 ± 0% (± SEM, n = 5). Cytoplasmic staining was considered nonspecific since it occurred when cells were treated with either antibody. In contrast, granulosa cells from five monkeys administered an ovulatory dose of hCG stained intensely positive for PR (Fig. 1D). Most granulosa cells (64 ± 5%, n = 5) in this group exhibited nuclear PR staining appearing as a dark precipitate.

Granulosa cells collected from the GnRH/GnRHa groups described by Zelinski-Wooten and colleagues (1991) were also immunocytochemically examined for the presence of PR. As in granulosa cells from monkeys receiving no ovulatory stimulus, there was no specific nuclear staining for PR in cells from monkeys receiving one injection of GnRH that elicited surge levels of endogenous LH for 4 h (Fig. 1E; 0 ± 0% PR-positive cells, n = 4). Increasing exposure to surge levels for 8 h by giving three injections of GnRH (Zelinski-Wooten *et al.*, 1991), resulted in intense positive staining for PR in 44 % of the cells from one animal (data not shown). However, granulosa cells from the other GnRH-3 monkeys (n = 3) demonstrated no specific staining for PR (Fig. 1F). When animals (n = 4) were treated with GnRH agonist to prolong LH surge levels for > 14 h (Zelinski-Wooten *et al.*, 1991), granulosa cells showed no specific staining for PR (Fig. 1, G and H).


A
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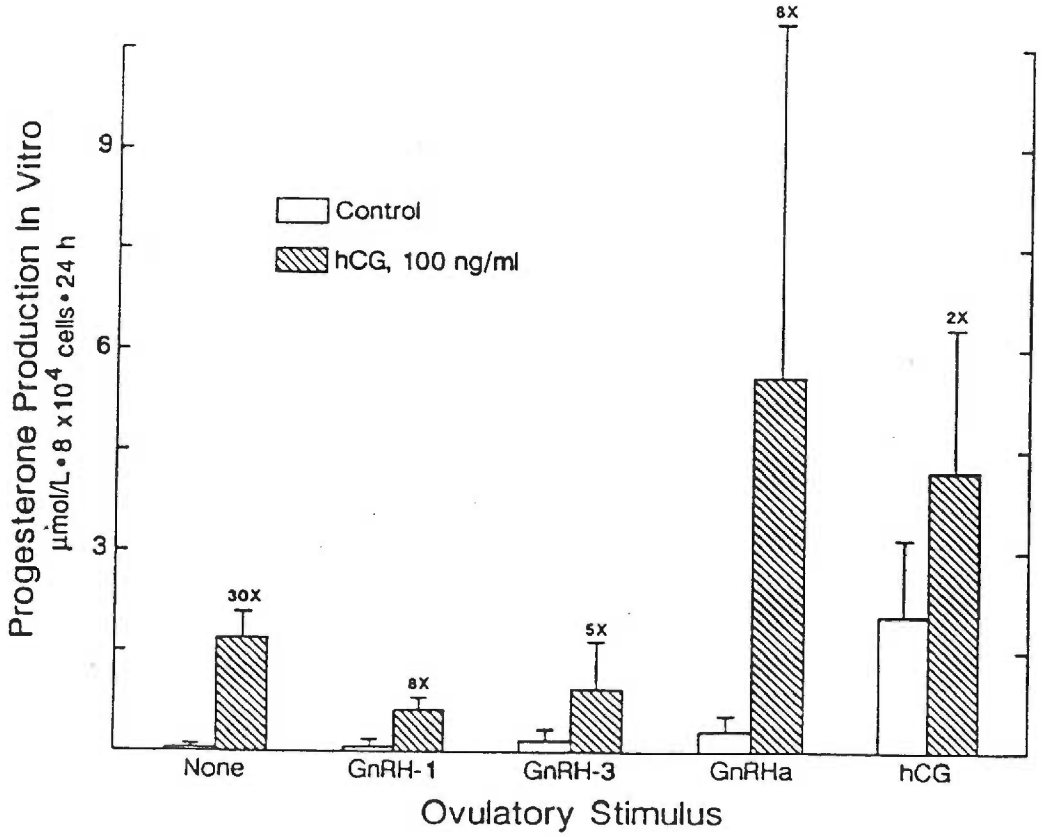
Progesterone Production *in vitro*

Progesterone production by granulosa cells from each of the five groups receiving different ovulatory stimuli is summarized in Fig. 2. Progesterone production by granulosa cells from monkeys receiving no ovulatory stimulus was low during 24 h culture under control conditions. Granulosa cells from monkeys receiving hCG as an ovulatory stimulus produced significantly greater amounts of progesterone under control conditions than did cells from the group receiving no ovulatory stimulus ($p < 0.05$). The presence of hCG *in vitro* increased progesterone production over controls, but granulosa cells from hCG-treated animals were less responsive ($p < 0.01$) than those from the no ovulatory stimulus group. *In vitro*, hCG enhanced progesterone production 30-fold by cells from monkeys receiving no ovulatory stimulus, but only 2-fold by cells from hCG-treated animals that already exhibited elevated progesterone production.

Granulosa cells from the GnRH/GnRHa groups produced low amounts of progesterone during 24 h of culture under control conditions (Fig. 2). In the GnRH-3 group, cells from one monkey produced higher amounts of progesterone *in vitro* than cells from the other three monkeys (709 versus 22 ± 10 nmol/L). These cells also stained positively for PR. There was no difference in control progesterone production when each GnRH/GnRHa group was compared to the group receiving no ovulatory stimulus ($p > 0.05$). However, there was a positive correlation ($r = 0.55$; $p < 0.01$) between the interval of *in vivo* exposure to surge levels of gonadotropin (LH or CG) and control levels of progesterone production *in vitro* (0, 4, 8, 14, 27 h LH/CG surge versus 60, 111, 194, 332, 2163 nmol/L, respectively). Progesterone production by granulosa cells in the GnRH/GnRHa groups was stimulated up


Progesterone Production In Vitro
µmol/L • 8 x10⁴ cells • 24 h
9
6
3
Control
hCG, 100 ng/ml
30X
8X
5X
8X
2X
None
GnRH-1
GnRH-3
GnRHa
hCG
Ovulatory Stimulus

to 8-fold over control values by hCG treatment *in vitro*. In the presence of hCG, progesterone production by cells from GnRHa (but not GnRH-1 or GnRH-3)-treated animals was similar to that by cells from hCG-treated monkeys.

Discussion

This study provides direct evidence that an ovulatory dose of hCG in stimulated cycles (VandeVoort *et al.*, 1989) induces PR expression in granulosa cells during early luteinization in rhesus monkeys. PR staining was not detected in granulosa cells isolated from monkeys receiving no ovulatory stimulus after ovarian stimulation. In contrast, granulosa cells from animals receiving hCG as an ovulatory stimulus showed intense PR staining within 27 hours. Earlier studies in this laboratory (Hild-Petito *et al.*, 1988) indicated that PR first appeared in granulosa cells of the dominant follicle in monkeys during the periovulatory interval in spontaneous menstrual cycles. Specific nuclear staining for PR was also detected immunocytochemically in human granulosa cells obtained from *in vitro* fertilization protocols (Greenberg *et al.*, 1990). Taken together, these observations are consistent with a previously unrecognized role for the gonadotropin surge (i.e., endogenous LH or exogenous CG) in primates to induce PR in luteinizing granulosa cells.

Control of PR expression in the primate ovary appears different from that in classical target tissues, such as the uterus, where estrogen induces PR formation through a receptor-mediated pathway (Katzenellenbogen, 1980). It is unlikely that PR expression in luteinizing granulosa cells involves an indirect action of gonadotropin mediated by LH-dependent

estrogen production. First, all treatment groups had similar serum estradiol patterns showing continuously rising levels during the follicular phase until the time of follicle aspiration (Zelinski-Wooten *et al.*, 1991). Second, estrogen receptors (ER) were not detected in granulosa cells of the primate follicle at any time before or after the LH surge (Hild-Petito *et al.*, 1988). Only the germinal epithelium in the macaque ovary contained ER detectable by immunocytochemistry (Hild-Petito *et al.*, 1988). Estrogen-dependent intercellular interactions have been proposed in the female monkey genital tract where ER is present only in stromal cells while the adjacent epithelium shows estrogen-dependent effects (Brenner *et al.*, 1990). Such cell-cell interactions seem unlikely between the theca and granulosa cells in the macaque follicle since ER has not been detected in either cell type (Hild-Petito *et al.*, 1988). Thus, gonadotropin induction of PR in granulosa cells does not seem to involve an estrogen-mediated mechanism. However, it remains to be determined whether PR expression in luteinizing granulosa cells is a direct action of LH/hCG or the result of other gonadotropin-triggered events in the periovulatory follicle (LeMaire *et al.*, 1987).

LH actions on ovarian target cells via cAMP-mediated pathways result in both acute and long-term increases in steroidogenesis. Acute actions include promotion of cholesterol transport into mitochondria, while long-term actions include stimulation of mRNA encoding components of the steroidogenic machinery (Strauss *et al.*, 1988). Our data support the concept that progesterone synthesis by primate granulosa cells is stimulated by the LH surge (Channing, 1980). Granulosa cells isolated from monkeys receiving no ovulatory stimulus after ovarian stimulation regimens produced little progesterone during 24 h of culture under control conditions, whereas granulosa cells from monkeys receiving hCG as an ovulatory stimulus

produced large amounts of progesterone *in vitro*. In addition, there was a positive relationship between increased progesterone production and PR expression in granulosa cells by 27 h after administration of an ovulatory dose of hCG. These findings suggest an autocrine action of progesterone in luteinizing cells prior to ovulation and during the differentiation of the primate corpus luteum. Thus, the LH surge may have a dual role in developing luteal tissue to stimulate progesterone production for endocrine actions outside the ovary and to sensitize luteal cells to local receptor-mediated actions of progesterone. Determination of the physiological role of PR in luteinizing granulosa cells remains an important goal.

This is the first attempt to titrate LH surge requirements for PR expression and luteinization of granulosa cells in primate follicles. GnRH-1 treatment after follicular stimulation elicited endogenous LH levels of greater than 400 ng/mL within 1-2 h, and maintained surge levels (> 100 ng/mL) for 4 h (Zelinski-Wooten *et al.*, 1991). GnRH-3 and GnRHa treatment extended the duration of LH surge levels to 8 and 14 h, respectively (Zelinski-Wooten *et al.*, 1991). Our data show that, in contrast to hCG administration, GnRH/GnRHa injections were typically unable to induce PR expression. Using PR expression as an index of periovulatory change, cells from the GnRH/GnRHa group responded similarly to cells from the group receiving no ovulatory stimulus. Similarly, *in vitro* progesterone production by granulosa cells from the GnRH/GnRHa groups was not comparable to that by cells from monkeys receiving hCG as an ovulatory stimulus. An exception was granulosa cells from one GnRH-3 animal, which expressed PR and produced more progesterone than cells from the other three GnRH-3 monkeys. This GnRH-3 monkey also exhibited a functional luteal phase (Zelinski-Wooten *et al.*, 1991). Interestingly, the increasing interval of

exposure to gonadotropin (LH or CG) surge levels was correlated positively with the gradual rise in progesterone production by granulosa cells *in vitro*. Since a similar relationship was not apparent in the immunocytochemical studies, PR expression may require an abrupt threshold level/interval of LH exposure. LH surges of 4-8 h and 10-16 h are sufficient for corpus luteum formation and progesterone production in normal estrous cycles of rodents (Fink, 1988) and domestic animals (Baird *et al.*, 1981), respectively. However, LH surges of similar duration in the current study on monkeys failed to stimulate periovulatory events in granulosa cells, including PR expression, or lead to a functional luteal phase (Zelinski-Wooten *et al.*, 1991).

Although GnRH/GnRHa was administered to stimulate LH release by the pituitary, GnRH could also act in the follicle to counteract or synergize with the effects of LH. There are reports that GnRH and its agonists directly inhibit granulosa cell steroidogenesis in nonprimate species (Hsueh and Jones, 1981). In contrast, reports of direct GnRH actions on human granulosa cells have been inconsistent, ranging from no effect (Casper *et al.*, 1982) to either stimulatory (Olsson *et al.*, 1990) or inhibitory (Olsson *et al.*, 1990; Tureck *et al.*, 1982) effects on steroidogenesis *in vitro*. Although nonprimate ovaries contain GnRH receptors comparable to those in the pituitary (Knecht *et al.*, 1985), whether similar receptors exist in primate ovaries has not been resolved (Latouche *et al.*, 1989; Wickings *et al.*, 1990). The lack of blood cells and occasional absence of an oocyte in follicular aspirates of GnRHa-treated animals (Zelinski-Wooten *et al.*, 1991), suggests that the agonist had some effects on macaque follicles. In humans, the use of GnRH agonists in conjunction with gonadotropins for better management of ovarian stimulation does not appear to have detrimental effects (Loumaye, 1990). Reports that GnRH adjunctive therapy increases the exogenous

gonadotropin requirements for follicular development may reflect the loss of endogenous gonadotropin secretion (due to pituitary desensitization by GnRH), rather than ovarian effects of GnRH (Dodson, 1990). Although direct ovarian effects of potent, long-acting GnRHa deserve consideration, the data from monkeys experiencing GnRH-induced LH surges suggest that an extended (> 14 h) LH surge more typical of that in the normal menstrual cycle is necessary for PR expression and granulosa cell luteinization in primate follicles.

CHAPTER 4

INITIATION OF PERIOVULATORY EVENTS IN PRIMATE FOLLICLES USING RECOMBINANT AND NATIVE HUMAN LUTEINIZING HORMONE TO MIMIC THE MIDCYCLE GONADOTROPIN SURGE

Abstract

The amplitude and duration of the midcycle LH surge required for periovulatory changes in the primate follicle are incompletely defined. We reported previously that short (4-14 h) LH surges elicited by GnRH or GnRH agonist following stimulation of multiple follicular development were insufficient to induce periovulatory events in macaques. In contrast, a single injection of urinary hLH resulting in a 18-24 h surge one-half the duration of that in the spontaneous cycle, induced oocyte maturation plus luteinization and progesterone (P) receptor expression of granulosa cells, but did not support corpus luteum function. In this study, the periovulatory changes following LH surges of 48 h elicited using pituitary (pit) or recombinant (rec) hLH were compared to those after 24 h LH surge durations or after hCG treatment. Beginning at menses, rhesus monkeys were treated with human gonadotropins for 9 days to stimulate follicular growth. On day 10, animals (n=3-5/group) received either: a) hCG (1000 IU, im); b) two injections of pituitary LH (2500 IU pitLH x 2; im); c) one injection of recombinant LH (2500 IU x 1; im); d) two injections of recombinant LH (2500 IU recLH x 2). Oocytes and granulosa cells were obtained via follicle aspiration 27 h after the initial LH or hCG injection. In all groups, serum estradiol rose to similar peak levels by day 10. Circulating LH-like bioactivity was elevated for > 48 h

after hCG. Two injections of either recLH or pitLH elicited surge levels of bioactive LH for 36-48 h, with peak levels in the pitLH x 2 group (1914.2 ± 308.8 ng/ml) comparable to that after hCG treatment (1771 ± 226 ng/ml) and greater than that in the recLH x 2 treatment group (950.6 ± 205 ng/ml). One injection of recLH elicited peak values (987.6 ± 168.4 ng/ml), comparable to those in the recLH x 2 group, but surge levels above 100 ng/ml were sustained for only 18-24 h. The proportion of oocytes resuming meiotic maturation (68-76%) was similar in all groups. *In vitro* progesterone production by granulosa cells (GC) in all LH-treated groups was comparable to that produced by GC from the hCG-treated group and much greater than that by GC from animals receiving no ovulatory stimulus after follicular development. P receptor was detected immunocytochemically in GC from all animals regardless of the ovulatory stimulus received. Peak levels of progesterone in the luteal phase were comparable in pitLH x 2- and recLH x 2-treated monkeys (18.5 ± 10.4 vs. 8.1 ± 1.5 ng/ml) and approached that in hCG-treated monkeys (39.5 ± 18.0 ng/ml). However, progesterone levels in recLH x 1-animals (3.4 ± 1.5 ng/ml) were less ($p < 0.05$) than in hCG-treated monkeys. The duration of the luteal phase ranged from 8.5 ± 0.3 days in recLH x 1 monkeys to 12.4 ± 0.8 days in hCG-treated animals. Thus, LH exposure comparable to the interval of the spontaneous LH surge (48-50 h) induces periovulatory events similar to those elicited by an hCG bolus. Whereas attenuated LH surges of 18-24 h reinitiate oocyte meiosis and promote granulosa cell luteinization, longer surges of up to 48 h further promote corpus luteum development and function.

Introduction

The LH surge in the normal menstrual cycle initiates changes in the preovulatory follicle including resumption of oocyte meiotic maturation, follicle wall luteinization, and ovulation. However, the amplitude and duration of the gonadotropin surge required for ovulatory events are poorly defined. Studies in laboratory rats (Peluso, 1990; Ishikawa, 1992) and rabbits (Bomsel-Helmreich *et al.*, 1989) suggest that the threshold of gonadotropin exposure varies for the processes of oocyte maturation, luteinization and ovulation. Similar studies have not been performed in primate species where the duration of the LH surge is prolonged to 48-50 h.

Recent studies from this laboratory used follicular stimulation protocols in rhesus macaques, in which a spontaneous LH surge is typically absent, to titrate gonadotropin surge requirements for periovulatory changes in the follicle. A bolus of hCG, which is routinely used as an ovulatory stimulus, results in reinitiation of oocyte meiosis, luteinization and progesterone receptor (PR) expression in granulosa cells, and formation of functional corpora lutea (Zelinski-Wooten *et al.*, 1991; Chandrasekher *et al.*, 1991). In contrast, endogenous LH surges of $\leq$ 14 h duration elicited by GnRH or GnRH agonist were incapable of initiating periovulatory events in oocytes and granulosa cells, nor of supporting corpus luteum development and function (Zelinski-Wooten *et al.*, 1991; Chandrasekher *et al.*, 1991). However, a surge one-half the normal duration (up to 24 h) elicited by a single injection of human LH following ovarian stimulation induced early ovulatory changes in primate follicles including reinitiation of oocyte meiosis, but failed to support corpus luteum function (Zelinski-Wooten *et al.*, 1992). There are reports (Gonen *et al.*, 1990; Lanzone *et al.*, 1989) that longer 34-48

h surges induced by GnRH agonist treatment following follicular stimulation protocols in women yielded mature oocytes as well as functional luteal phases. This study was designed 1) to achieve LH surge durations typical of spontaneous cycles (48-50 h) using pituitary or recombinant hLH and 2) to compare the ovulatory changes elicited by 24 h and 48 h LH surge durations to those elicited by hCG in macaques following follicular development.

Materials and Methods

Animals and follicular development

General husbandry and housing of rhesus monkeys at the Oregon Regional Primate Research Center (ORPRC) have been described previously (Molskness *et al.*, 1987). Adult, regularly cycling females were checked daily for menses (onset of menses = cycle day 1). From day 1 of the menstrual cycle until the next onset of menses, daily blood samples were obtained at 0800 h from unanesthetized monkeys by saphenous venipuncture. Serum was stored at -20 C until measurement of progesterone and estradiol by radioimmunoassay (Hess *et al.*, 1981; Resko *et al.*, 1975) in the ORPRC Hormone Assay Core Laboratory.

The follicular stimulation protocol has been described previously (VandeVoort *et al.*, 1989). Beginning at menses, rhesus monkeys were given 60 IU human follicle stimulating hormone (hFSH; Metrodin, Serono Laboratories Inc., Norwell, MA) for six days, followed by 60 IU hFSH + 60 IU hLH (Pergonal, Serono) for 3 days. Half of the gonadotropin dose was injected im twice daily at 0800 and 1600 h. To induce ovulatory maturation, animals (n=3-5/group) received on the next day: a) hCG (1000 USP units, im, Profasi, Serono); b) 2 injections of 2500 IU human pituitary LH (pitLH x 2,

Serono) im, 18 h apart (0800 and 0200 h); c) 1 injection at 0800 h of 2500 IU human recombinant LH, im (recLH x 1, Serono); d) 2 injections of 2500 IU human recombinant LH (recLH x 2), 18 h apart (0800 and 0200 h). Multiple blood samples were collected for up to 72 h after the onset of hLH or hCG injection and serum levels of LH or LH-like (CG) bioactivity were determined using the mouse Leydig cell bioassay with rhesus LH RP-1 as the standard (Ellinwood *et al.*, 1984). LH surge levels were defined as _ 100 ng/ml. None of the animals included in the data analyses exhibited endogenous LH surges during human gonadotropin treatment.

Approximately equivalent amounts of bioactive LH from a pituitary preparation (2741 IU/vial; 95 % confidence limits 2192 - 3288 IU) and a recombinant preparation (3280 IU/vial; 95 % confidence limits 2374 -4532 IU) were to be administered in this protocol. Initial determinations of hormone activity were determined by Serono Laboratories, Inc. via standard *in vivo* bioassays. During *in vitro* bioassays at ORPRC to estimate circulating levels of LH, serial dilutions of the vial contents were analyzed for bioactivity (Fig. 1). Testosterone production elicited by a range of concentrations of hCG, pitLH, and recLH was parallel to that produced by the rhesus standard, LH RP-1. Average variance for the low, middle, and high levels of hormones is illustrated in Fig. 1. The *in vitro* assay suggested that similar amounts of bioactive hCG (79 ± 3 µg RP-1 equiv.) and pitLH (91 ±4 µg RP-1 equiv.) were administered per injection, but less recLH (21 ±1 µg RP-1 equiv.) was injected. The differences between the *in vitro* bioactivity of the vial preparations encompasses the range in precision of the various bioassays; however, the lower serum levels achieved in recLH versus pitLH-treated monkeys (see Results section) are consistent with apparent differences in the *in vitro*, and hence injected, bioactivity.

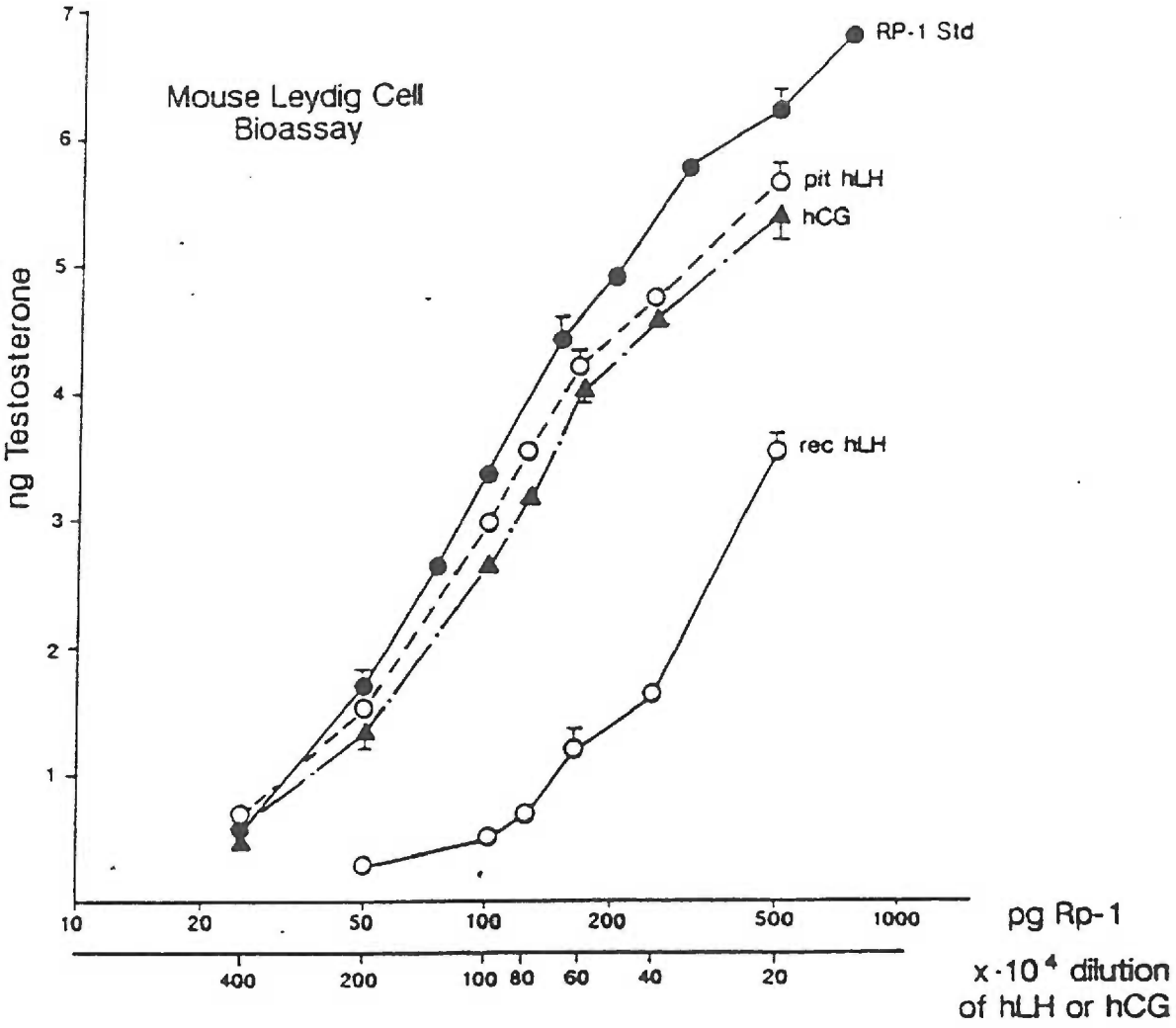


Fig. 1. Testosterone levels produced by a range of concentrations of rhesus LH RP-1 (standard curve) and serial dilutions of preparations of pitLH, recLH and hCG, in the mouse Leydig cell bioassay. Rhesus LH was assayed in triplicate, whereas dilutions of human gonadotropins were assayed in quadruplicate; standard errors are included for three points spanning the range of hormone levels.

Oocyte evaluation and *in vitro* fertilization

Follicles were aspirated at laparotomy 27 h after the hCG or initial hLH injection (Wolf *et al.*, 1989). After recovery of oocytes, cumulus cells were dispersed with hyaluronidase (Lanzendorf *et al.*, 1990). Nuclear status within 1-2 h of collection was used to determine oocyte maturity as follows: prophase I (germinal vesicle [GV] intact); metaphase I (MI; no GV, no polar body); or metaphase II (MII; one polar body).

Rhesus monkey semen was collected by penile electroejaculation (Lanzendorf *et al.*, 1990) and prepared for *in vitro* fertilization (IVF) as described previously (Wolf *et al.*, 1989; Lanzendorf *et al.*, 1990) with some changes. Briefly, 0.1 ml semen was washed twice in 3 ml TALP-HEPES (pH 7.4) and the pellet resuspended in TALP. Sperm were incubated at 37 C for 4 h under 5% CO_2 and activated with dibutyryl cyclic AMP and caffeine for 1 h. Oocytes were cultured until reaching MII, and then inseminated with 50-100 x 10^3 sperm (Wolf *et al.*, 1989). Inseminated oocytes were examined 10-16 h later for fertilization by confirming the presence of the second polar body and two pronuclei. Culturing of zygotes for evidence of timely cleavage has been described previously (Lanzendorf *et al.*, 1990).

Granulosa cell isolation, progesterone production and progesterone receptor (PR) expression

After removal of oocytes, follicular aspirates were pooled and the cell suspension was centrifuged on a 40% Percoll (Sigma), 60% Hanks' Balanced Salt Solution containing 0.1% BSA to collect granulosa cells with reduced blood cell contamination (Chandrasekher *et al.*, 1991). Granulosa cells were counted using a hemacytometer and viability assessed by trypan blue

exclusion.

Granulosa cells were plated on Lab-Tek Chamber Slides (160,000 cells/well; Nunc Inc., Naperville, IL) coated with Cell-Tak (15 µg/well; Collaborative Research, Inc., Bedford, MA). Cells were frozen in liquid propane and stored at -20 C until analysis of PR by indirect immunocytochemistry as described previously (Chandrasekher *et al.*, 1991). Cells were incubated with either a monoclonal antibody against human PR (JZB39; 2.5 µg/ml) or a nonspecific antibody (AT, antibody to antigen B of timothy grass pollen; 10 µg/ml). The antibody complex was visualized by incubation with avidin-DH and biotinylated horseradish peroxidase-H complex (ABC kit from Vector Laboratories, Burlingame, CA) followed by treatment with diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide. The percentage of nuclei staining for PR was determined in nonblinded observations by a random count of 500 cells.

Granulosa cells (50,000 cells/well) were plated on 48-well plastic dishes (Costar, Cambridge, MA) and cultured in Ham's F-10 (Gibco Laboratories, Grand Island, NY) containing 10% monkey serum in the presence or absence of hCG (100 ng/ml, CR123; n= 4 replicates/monkey with or without hCG). Cell cultures were incubated at 37 C in humidified 95% air/5% CO_2. Media were collected after 24 h of culture and frozen for measurement of progesterone content by radioimmunoassay (Hess *et al.*, 1981) at a later date.

Statistical Analyses

Serum LH and progesterone levels were analyzed by ANOVA after transformation of values to logarithms. Comparison among means was accomplished using Newman-Keuls. Peak levels of estradiol and

progesterone were compared using ANOVA followed by Newman-Keuls. The proportion of oocytes at various stages of nuclear maturity were analyzed among groups using Fisher's exact test. Media levels of progesterone were transformed prior to statistical analyses when heterogeneity of variance was present. Differences in progesterone content in media cultured with or without hCG were analyzed by ANOVA, followed by *t* test for comparison of means. Differences at $p < 0.05$ were considered significant.

Results

Circulating steroid and LH-like bioactivity in monkeys during development of multiple preovulatory follicles followed by an ovulatory stimulus of hCG is shown in Fig. 2 and 6A, respectively. Serum estradiol increased continuously up to the day of hCG injection, and then decreased abruptly (Fig. 2). Following hCG injection, circulating LH-like bioactivity rose to surge levels (> 100 ng/ml) within 0.5 h with peak concentrations (1771 ± 226 ng/ml) detected within 6 h (Fig. 6A). Surge levels of LH-like bioactivity were maintained for > 48 h. Progesterone levels remained at baseline (< 1 ng/ml) during the follicular phase, and increased to 6.1 ± 2.6 ng/ml within 24 h after hCG injection. Peak levels of progesterone (39.5 ± 18.0 ng/ml) were achieved 5 days after hCG treatment followed by a decline to baseline 5-6 days later. A functional luteal phase of 12.4 ± 0.8 days followed hCG treatment.

The circulating estradiol pattern for monkeys receiving two injections of human pitLH following multiple follicular development (Fig. 3) was similar to that in the hCG group. As with hCG-treatment, > 100 ng/ml of LH was measured within 0.5 h of the first pitLH injection (Fig. 6B). The first

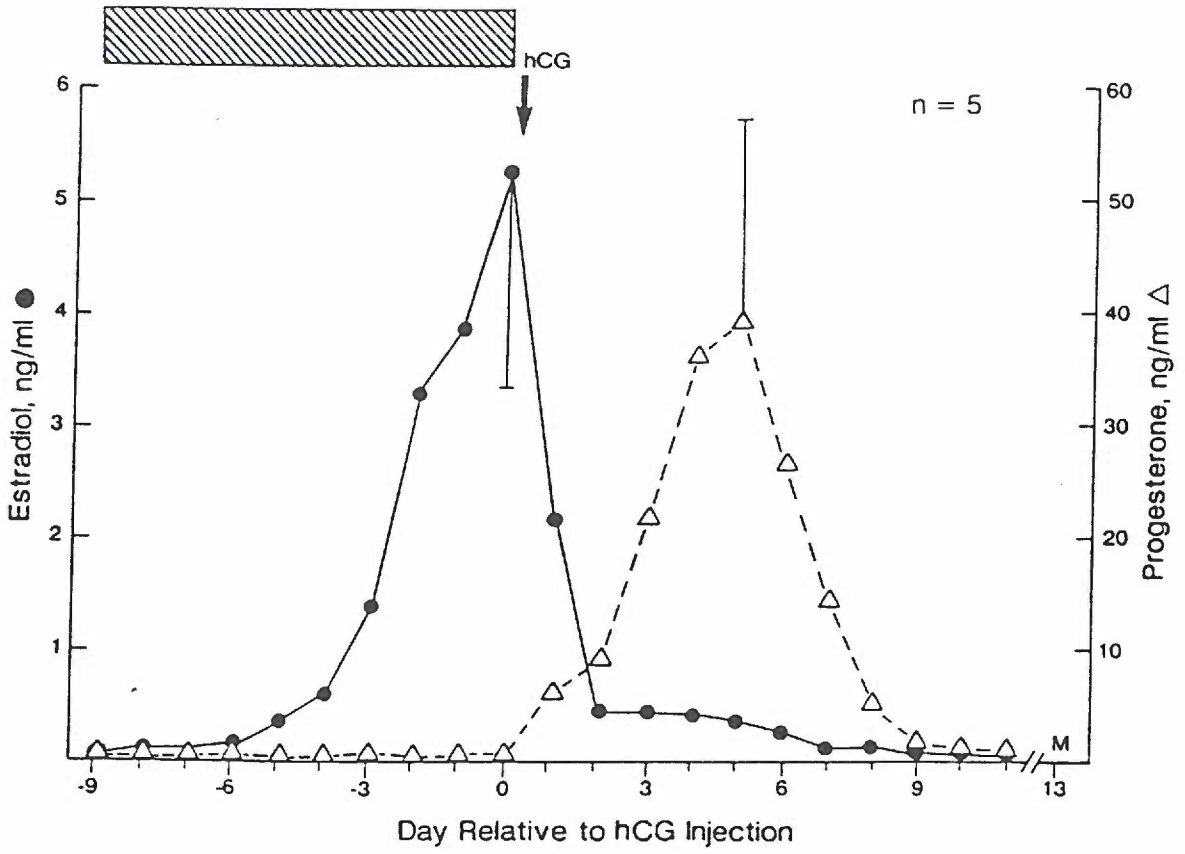


Fig. 2. Serum estradiol and progesterone levels in monkeys receiving human gonadotropins for nine days (hatched bar) to promote follicular development followed by hCG (arrow) to induce ovulatory maturation. Data are normalized to the day of hCG injection (Day 0). The SEM is included for peak steroid levels. An M designates the mean day of menses.

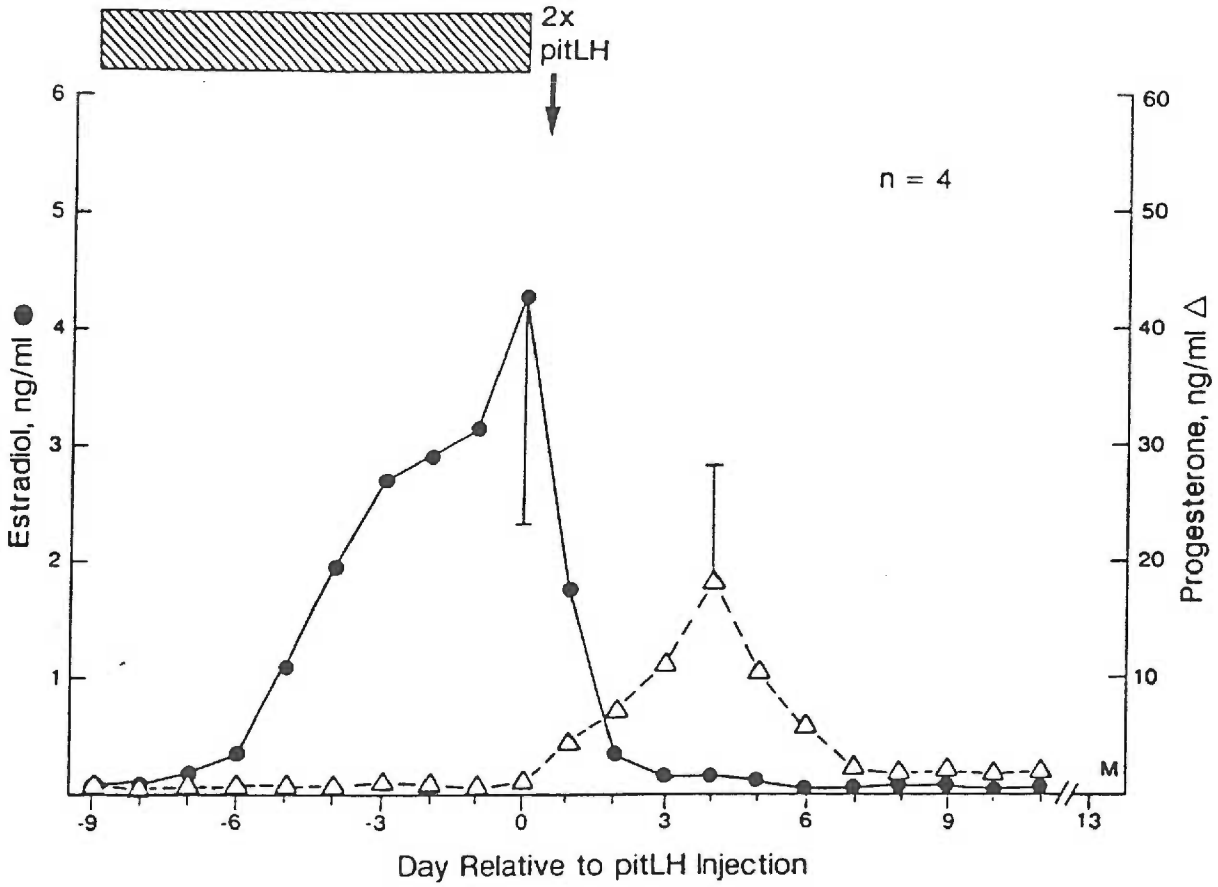


Fig. 3. Steroid levels in monkeys receiving 2 injections of pitLH for ovulatory maturation following 9 days of human gonadotropin treatment (hatched bar). Values are normalized to the day of pitLH injection (Day 0). The SEM is for peak steroid levels. An M marks the mean day of menses.

pitLH injection elicited an LH peak within 2 h followed by a decline in levels. The second pitLH injection caused another increase in LH levels giving rise to similar peak values as the first pitLH injection (1914 ± 309 vs. 1651 ± 216 ng/ml; $p > 0.05$). Peak levels of bioactive LH following pitLH injection were similar to those in hCG-treated animals. However, the duration of LH surge levels (> 100 ng/ml) was shortened to 36-48 h. Circulating progesterone levels at 24 h after the initial pitLH injection (4.4 ± 1.1 ng/ml) were similar to those in hCG-treated animals. Peak progesterone levels achieved four days after the pitLH injection (18.5 ± 10.4 ng/ml) tended to be lower than peak values in the hCG group, but this difference was not significant. Progesterone levels returned to baseline within 7-14 days of pitLH x 2 and the luteal phase of 12.5 ± 2.1 days was comparable to the hCG group.

Fig. 4 illustrates the steroid hormone concentrations in monkeys receiving a single injection of recombinant LH following multiple follicular development. Serum estradiol pattern and levels were similar to those of monkeys receiving hCG as an ovulatory stimulus (Fig. 2). Circulating levels of bioactive LH rose to > 100 ng/ml within 0.5 h of injection (Fig. 6C). Within 4 h following the recLH injection, bioactive LH levels were significantly lower ($p < 0.05$) than those seen after hCG or pitLH x 2 treatment and surge levels (> 100 ng/ml) were maintained for only 18-24 h. Serum progesterone levels increased within 24 h of hLH treatment, but tended to be lower than those seen after hCG injection (1.9 ± 0.8 vs. 6.1 ± 2.6 ng/ml). Peak levels of progesterone (3.4 ± 1.5 ng/ml) achieved 4 days after the recLH injection were also 12-fold lower than those achieved in the hCG group. Progesterone levels dropped to baseline 2-3 days later with a functional luteal phase of 8-9 days

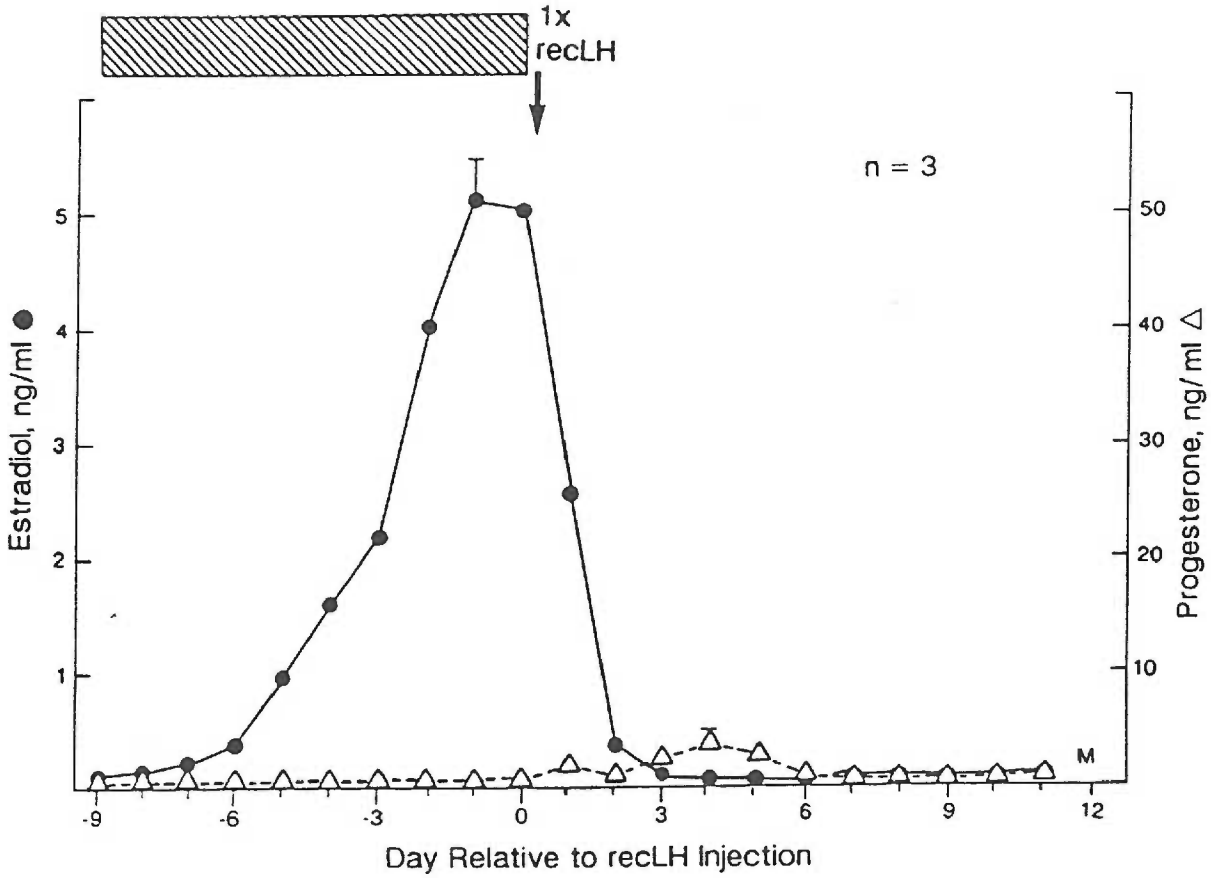


Fig. 4. Serum estradiol and progesterone levels in monkeys receiving 9 days of human gonadotropin treatment (hatched bar) followed by one recLH (1 x) injection for ovulatory maturation. The SEM is provided for peak steroid levels, and an M designates the mean day of menses.

seen in 2 of the 3 monkeys. The remaining monkey had a nonfunctional (< 1 ng progesterone/ml) luteal phase.

Steroid concentrations in animals treated with two injections of recLH following multiple follicular development are shown in Fig. 5. Serum estradiol increased to peak levels (3.9 ± 1.4 ng/ml) similar to those seen in other treatment groups, and then declined after the LH injections. Bioactive LH levels rose to greater than surge levels within 0.5 h followed by peak levels (951 ± 205 ng/ml) within 2 h of the first recLH injection (Fig. 6D). LH levels then declined but remained above 100 ng/ml until the second LH injection caused another increase in circulating bioactive LH to peak values that were similar to those elicited by the first treatment. LH surge levels were maintained for 36-48 h. Progesterone levels increased (4.0 ± 0.3 ng/ml) within 24 h of the initial recLH injection and were similar to those in the pitLH x 2 and hCG groups. The two recLH injections increased peak progesterone values (8.1 ± 1.5 ng/ml) comparable to those in pitLH x 2 group, but still tended to be less than in the hCG group. Progesterone values returned to baseline within 7 days of the first recLH injection. A functional luteal phase of 8.8 ± 0.3 days was not significantly ($p > 0.05$) shorter than the 12-day luteal phase in the pitLH x 2 and hCG groups.

The meiotic status and quality of oocytes collected from large follicles after hCG or hLH treatment following multiple follicular development is summarized in Table 1. The number of ova collected per animal among treatment groups was similar. The proportion of atretic oocytes was low (< 8%) in all treatment groups. The percentage of immature oocytes with an intact germinal vesicle (prophase I) did not differ following the various ovulatory stimuli, although it tended to be highest (25%) in the recLH x 1 group. Additionally, the percentage of oocytes reaching MI and MII by the

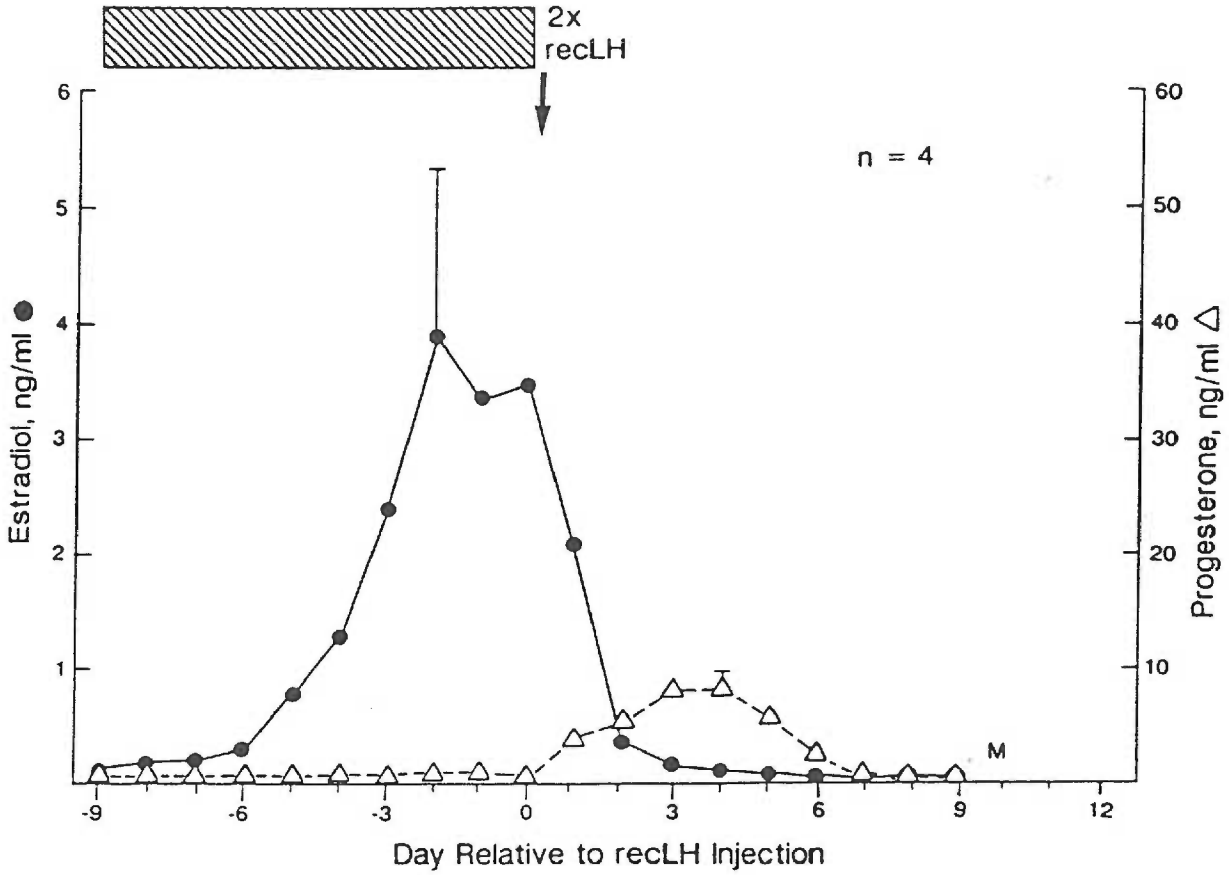


Fig. 5. Steroid patterns in monkeys receiving 2 injections (2 x) of recLH for ovulatory maturation following 9 days of human gonadotropin treatment (hatched bar). Values are normalized to the day of recLH injection (Day 0). The SEM is included for peak steroid levels, and an M designates the mean day of menses.

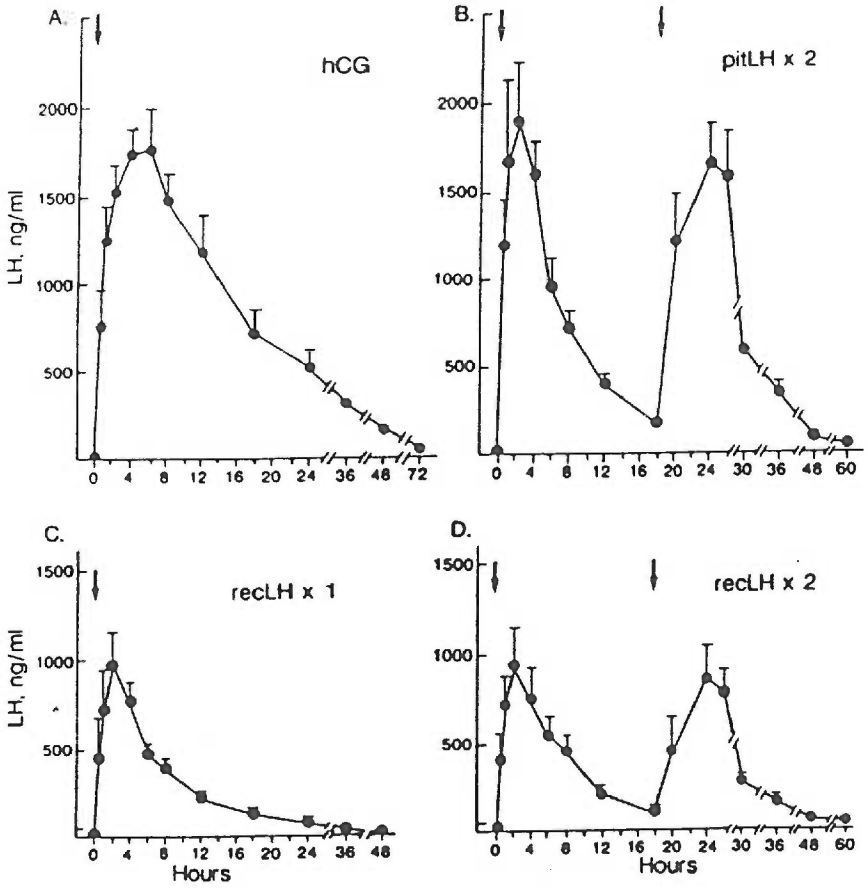


Fig. 6. LH or LH-like bioactivity in rhesus monkeys after ovarian stimulation. Multiple samples for up to 72 h were obtained from animals receiving an ovulatory dose (arrow) of hCG (A), two injections of pitLH (B), or one (C) or two (D) injections of recLH. Values are the mean ± SEM of 4-5 experiments.

Table 1. Meiotic status and quality of oocytes collected after hCG, pitLH x 2, recLH x 1, or recLH x 2, for ovulatory development following multiple follicular development in macaques.

Number of ova, stage at collection and performance	Ovulatory stimulus			
	hCG	pitLH x 2	recLH x 1	recLH x 2
No. of animals	5	4	3	4
No. of ova	189	133	114	77
Prophase I (%)	30 (16)	31 (23)	28 (25)	15 (19)
Metaphase I (%)	108 (57)	78 (59)	71 (62)	50 (65)
Metaphase II (%)	36 (19)	15 (11)	6 (5)	8 (10)
Atretic (%)	13 (7)	9 (7)	9 (8)	2 (3)
No. inseminated	143	93	84	59
% Fertilized	61	30	26	36

time of follicle aspiration among the four groups was similar (68-76%), except recLH x 1 treatment yielded a significantly ($p < 0.01$) lower proportion of MII oocytes compared to the hCG group (5 vs 19 %). Insemination of oocytes *in vitro* leading to higher fertilization percentages for the hCG group (61%) versus the groups receiving hLH (30,26,36%) was due mainly to one animal where 96% of oocytes fertilized. The percentage of fertilized eggs that cleaved was greater than 96% for all groups.

Progesterone production by granulosa cells from each of the four groups receiving different ovulatory stimuli is shown in Fig. 7. Granulosa cells from monkeys in the LH-treated groups produced comparable amounts of progesterone under basal conditions to granulosa cells from hCG monkeys. The presence of hCG *in vitro* increased progesterone production 2- to 3-fold over control levels in all treatment groups.

No nuclear staining was observed when granulosa cells were treated with the control antibody, antitimothy grass pollen (Fig. 8). Most (50-90%) granulosa cells from all monkeys receiving hCG treatment exhibited nuclear PR staining, appearing as a dark precipitate. Intense positive staining for PR was also noted in many granulosa cells from all animals receiving either recombinant or pituitary hLH for ovulatory maturation (44-85%). The only exception was in granulosa cells from one monkey receiving pitLH x 2 where positive PR staining was seen in only 12% of the cells.

Discussion

Ovarian stimulation protocols routinely use a pharmacologic bolus of hCG as the signal for ovulatory changes in follicles (Wolf *et al.*, 1989; Jones *et al.*, 1982). Due partly to its long half-life, an ovulatory hCG

Fig. 7. *In vitro* progesterone production by granulosa cells from monkeys receiving various ovulatory stimuli after ovarian stimulation. Cells were obtained from animals receiving an ovulatory dose of hCG, two injections of pitLH, or one or two injections of recLH. Cells were cultured for 24 h in the absence (control) or presence of 100 ng hCG/ml. Granulosa cells from monkeys in the LH-treated groups produced similar amounts of progesterone under control conditions to granulosa cells from hCG monkeys. In all treatment groups, 2- to 3-fold (x) increases in progesterone production over control levels occurred in the presence of hCG *in vitro*. Values are the mean ± SEM of 3-5 experiments.

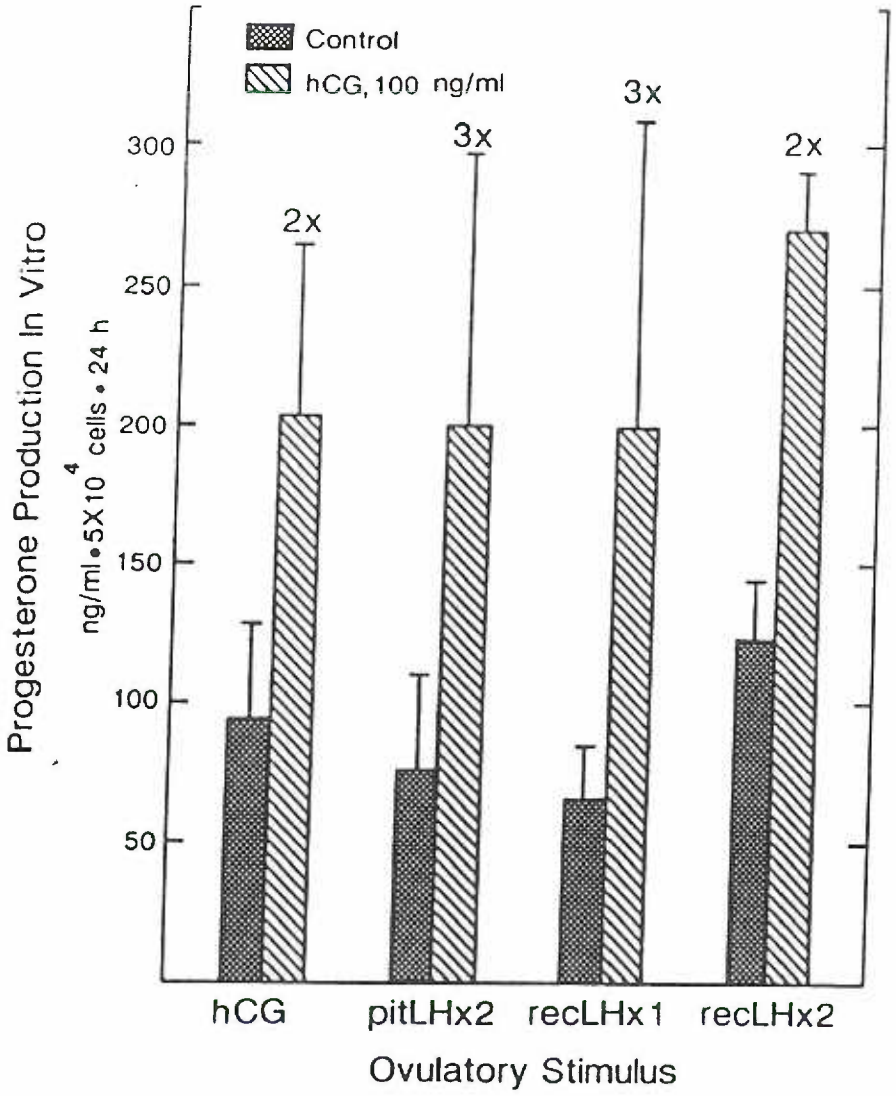
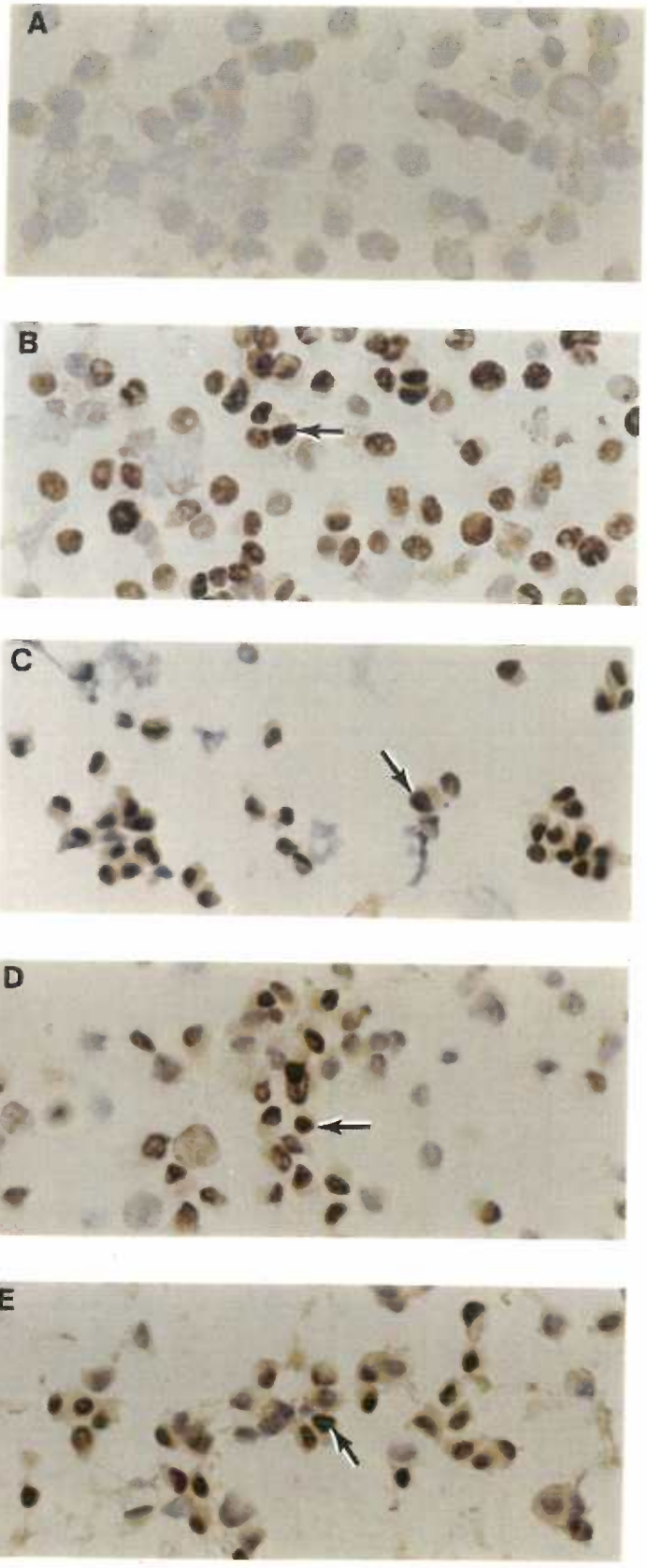

Control
hCG, 100 ng/ml
Progesterone Production In Vitro
ng/ml • 5X10^4 cells • 24 h
300
250
200
150
100
50
2x
3x
3x
2x
hCG
pitLHx2
recLHx1
recLHx2
Ovulatory Stimulus

Fig. 8. Immunocytochemical staining for PR in granulosa cells collected from large antral follicles of monkeys after ovarian stimulation. Cells were obtained from animals receiving an ovulatory dose of hCG (A,B), two injections of pitLH (C), and one (D) or two (E) injections of recLH. Aliquots of cells were incubated with either a control AT antibody (A) or anti-hPR antibody (B-E). Cells from all monkeys exhibited intense nuclear staining (arrow) when PR antibodies were used. Initial magnification, X400.


A
B
C
D
E

stimulus has a longer duration (Zelinski-Wooten *et al.*, 1991) than the LH surge in a normal menstrual cycle (Weick *et al.*, 1973; Hoff *et al.*, 1983). In addition, there are several possible nonphysiologic effects of using hCG. These include the possibility that hCG does not entirely mimic LH action (Niswender *et al.*, 1987). Moreover when hCG is used, there is less control over ovulation timing in heterogeneous populations of growing follicles (Abbasi *et al.*, 1987). Impaired development or function of the corpus luteum may also result, perhaps due to desensitization by high levels or lack of pulsatile exposure to gonadotropins (VandeVoort *et al.*, 1988). A final consequence of exposure to a bolus of hCG is the resultant antibody production in nonhuman primate species and hence further refractoriness to exogenous human gonadotropin treatment (Wolf *et al.*, 1989). Therefore, replacement with a more physiologic stimulus such as LH is an important consideration.

The current study continues our efforts to titrate the duration and amplitude of the gonadotropin surge required for ovulatory maturation of the primate follicle and its enclosed oocyte. Recent reports from this laboratory showed that endogenous LH surges of 4-14 h elicited by GnRH or GnRH agonist following multiple follicular development were of insufficient duration to induce early ovulatory changes in rhesus macaques (Zelinski-Wooten *et al.*, 1991; Chandrasekher *et al.*, 1991). In contrast, a single injection of 2452 IU of urinary hLH, resulting in a surge of one-half (24 h) the normal duration induced reinitiation of oocyte meiosis and granulosa cell luteinization, but failed to support corpus luteum function (Zelinski-Wooten *et al.*, 1992). In this study, results following a single injection of 2500 IU recLH x 1 which elicited LH surges of up to 24 h, confirmed those from the prior LH study. Two injections of recLH or pitLH, which increased the

duration (48 h) of the LH surge to that in spontaneous cycles further promoted the development and function of corpora lutea.

Injections of either recLH or pitLH elicited surge levels of bioactive LH that were greater than or comparable to those seen in spontaneous menstrual cycles (Stouffer *et al.*, 1992). Whereas pitLH treatment elicited surge levels similar to those following hCG treatment, recLH injection resulted in a peak amplitude that was approximately half that value. In retrospect, the lower levels of serum LH achieved with the recombinant hormone preparation are consistent with evidence from *in vitro* Leydig cell assays that less bioactivity was administered. RecLH x 1 treatment produced LH surges that were similar in duration (18-24 h), but 3/4 of the amplitude to those produced by a single injection of urinary hLH (Zelinski-Wooten *et al.*, 1992). Notably, a second injection of either recLH or pitLH increased the duration of the LH surge up to 48 h. Even though the duration was similar to the 48-50 h surges in the normal menstrual cycle, the LH pattern differed in that levels increased and then declined following each LH injection.

These studies support the concept that duration of LH surge levels required for reinitiation of oocyte meiosis is > 14 h (Zelinski-Wooten *et al.*, 1991) but less than 24 h (Zelinski-Wooten *et al.*, 1992). Reinitiation of meiosis by the time of follicle aspiration was observed in most oocytes (68-76%) during 18-24 h surges of recLH x 1 and 36-48 h surges of recombinant or native hLH. Moreover, the percentage of maturing oocytes was similar to that during an ovulatory hCG stimulus of 72 h. Interestingly, the amplitude of the LH surge elicited by recLH, though lower than that elicited by pitLH, was sufficient for reinitiation of oocyte meiosis. However, LH surges of 18-24 h may not be optimal for oocyte maturation. As in our prior study with urinary LH x 1 (Zelinski-Wooten *et al.*, 1992), the percentage of oocytes

reaching metaphase II in the recLH x 1 group was less than that following hCG treatment. Two injections of recLH or pitLH resulted in a comparable proportion of oocytes reaching metaphase II as hCG treatment. Notably, fertilization rates were similar in all LH groups, as was the percentage of fertilized oocytes that cleaved. Endogenous LH surges elicited by GnRH agonist in women did not affect embryo quality (Gonen *et al.*, 1990).

Initial luteinization events in granulosa cells require LH surge exposure of 18-24 h (Zelinski-Wooten *et al.*, 1992), but a longer duration may elicit optimal changes. Recent work in several laboratories on various species (Park and Mayo, 1991; Iwai *et al.*, 1991a; Iwai *et al.*, 1991b) including primates (Hild-Petito *et al.*, 1988; Iwai *et al.*, 1990) suggests that the LH surge induces PR expression in granulosa cells of the periovulatory follicle. Repeated injections of GnRH/GnRHa that produced surge levels of endogenous LH for up to 14 h failed to induce PR expression (Chandrasekher *et al.*, 1991), whereas 18-24 h surges elicited by urinary LH induced PR in granulosa cells from some, but not all monkeys (Zelinski-Wooten *et al.*, 1992; Appendix). In this study, granulosa cells from all groups, regardless of the ovulatory stimulus administered, contained immunoreactive PR at follicle aspiration. Thus exposure to LH surge levels for 18-27 h is sufficient for PR expression in granulosa cells, but ≥ 24 h exposure may be necessary for consistent or optimal induction.

The degree of luteinization of granulosa cells, in terms of acute (24 h) progesterone production *in vitro*, was similar among treatment groups. Basal progesterone production by granulosa cells from all groups was much higher than that by granulosa cells from monkeys receiving no ovulatory stimulus following multiple follicular development (Chandrasekher *et al.*, 1991). Circulating levels of progesterone also increased in all groups within

24 h after administration of an ovulatory stimulus, but tended to be lower in the recLH x 1 group. Similarly, serum progesterone was lower in monkeys exposed to urinary LH surge levels for 18-24 h than those receiving hCG (Zelinski-Wooten *et al.*, 1992). In contrast, serum progesterone levels following pitLH x 2 and recLH x 2 were more comparable to those following hCG administration and tended to be higher than those seen in the recLH x 1 group. Thus, luteinization of granulosa cells may require LH surge level exposure of greater than 24 h.

The development and function of corpora lutea differed between groups. Whereas a continual and significant increase in serum progesterone was noted after hCG treatment, a lower increase in serum levels was seen in the recLH x 1 group. Gonadotropin surges of 24 h elicited with urinary LH (Zelinski-Wooten *et al.*, 1992) or recLH x 1 (current study) resulted in a short period of luteal function, while functional luteal phases were absent following GnRH/GnRH agonist-induced LH surges of up to 14 h (Zelinski-Wooten *et al.*, 1991). However, 48 h gonadotropin surges elicited by two injections of pitLH or recLH led to serum levels of progesterone more comparable to the hCG group. Similarly, Rotten and colleagues (1986) administered 4,000-8,200 IU of pitLH for ovulation induction in monkeys and measured circulating progesterone levels of 13.8 ± 3.8 ng/ml.

Despite differences in the amplitude of the LH surge elicited by recLH x 2 and pitLH x 2 injections, there were no differences in peak progesterone levels. In contrast, shorter LH surge durations following recLH x 1 treatment resulted in peak progesterone levels that were lower. These data suggest that corpus luteum development and function is dependent on up to 48 h LH surge durations, whereas the lower amplitudes achieved with recLH injections are sufficient. This concept is supported by recent data in

the rat where a lower long-lasting LH level is as effective in inducing ovulation as is a high level of LH (Ishikawa, 1992).

However, LH supplementation in the luteal phase may be necessary to sustain corpus luteum function in artificial cycles wherein endogenous LH levels are suppressed (VandeVoort *et al.*, 1988). Progesterone production by the macaque and human corpus luteum during the menstrual cycle requires LH (Vande Wiele *et al.*, 1970; Zeleznik and Hutchison, 1987). Daily LH supplements following a bolus injection of urinary LH in monkeys produced a luteal phase of elevated progesterone that was of normal length (Zelinski-Wooten *et al.*, 1992). Similarly, low doses of rechLH administered after ovulation induction maintained luteal function in a macaque (Simon *et al., 1988*). Direct studies on luteal tissue are needed in order to better examine luteal development as a function of LH surge duration or amplitude.

CHAPTER 5

PROGESTERONE RECEPTOR, BUT NOT ESTRADIOL RECEPTOR, MESSENGER RNA IS EXPRESSED IN LUTEINIZING GRANULOSA CELLS AND THE CORPUS LUTEUM IN RHESUS MONKEYS

Abstract

Estrogens (i.e., estradiol) and progestins (i.e., progesterone) may act as local regulators of ovarian function in various species. This study tested the hypothesis that if progesterone and estradiol act via receptor-mediated pathways in the primate ovary, then receptor mRNAs should be detectable in ovarian cells. The reverse transcription-polymerase chain reaction (RT-PCR) was employed to detect progesterone and estradiol receptor (PR and ER, respectively) mRNAs in the rhesus monkey ovary. Total RNA was isolated from macaque uterine myometrium (positive control), spleen (negative control), whole ovary, germinal (surface) epithelium-enriched cortical and medullary compartments of the ovary, granulosa cells in preovulatory follicles before and after an ovulatory stimulus, and corpora lutea from early (day 3-5), mid (day 7-8), and late (day 14-15) luteal phase of the menstrual cycle. Utilizing primers to the hormone binding region encoded by the receptor mRNAs, RT-PCR products of the expected sizes were detected for PR and ER from 1 μg of myometrial RNA, whereas no product was obtained from spleen. PR mRNA product was detected in all ovaries, germinal epithelium-enriched cortical and medullary compartments, and corpora lutea from all three stages of the luteal phase (n=3/stage). PR mRNA product was detected as a strong band in one of three preparations obtained from granulosa cells

before an ovulatory stimulus. In contrast, PR mRNA was detected in granulosa cells from all animals following an ovulatory dose of hCG. ER mRNA was detected in whole ovary and in germinal epithelium-enriched cortical compartments, with a barely visible product occasionally observed in medullary compartments of the ovary. In contrast to PR mRNA, ER mRNA was not detected in any corpora lutea throughout the luteal phase nor in granulosa cells obtained before or after an ovulatory stimulus. To confirm the specificity of the RT-PCR products, restriction enzymes cleaved the PR product from myometrium, germinal epithelium-enriched cortical compartment, and corpus luteum into predicted size fragments. Similarly, the ER product from the myometrium and the germinal epithelium-enriched compartment was cleaved into expected size fragments. Sequence analysis of the PR RT-PCR product revealed 99% homology to the cDNA for the hormone binding region of human PR. Thus, PR mRNA detection supports the hypothesis of progesterone action via classical receptor-mediated pathways in the luteinizing follicle and corpus luteum of the primate ovary. The apparent absence of ER mRNA in these ovarian compartments suggests a lesser, if any, role for estradiol.

Introduction

Two classes of steroid hormones, estrogens (i.e., estradiol) and progestins (i.e., progesterone), interact to control the development and function of female reproductive tissues such as the uterus and mammary glands (Katzenellenbogen, 1980). In addition, estradiol and progesterone may act as local modulators of ovarian function, including that of the primate corpus luteum (Gore *et al.*, 1973; Karsch *et al.*, 1973; Stouffer *et al.*, 1977;

Rothchild, 1981). The mechanisms of action of estradiol and progesterone involve binding to specific receptor proteins in target cells leading to the transcriptional activation of steroid-responsive genes (Gronemeyer, 1992). Estradiol and progesterone receptors (ER and PR, respectively) were first characterized in tissues via the specific binding of radiolabeled (^{3}H)-steroids (Jensen and Jacobsen, 1962; Milgrom *et al.*, 1970; Sherman *et al.*, 1970). Immunocytochemistry, using monoclonal antibodies to steroid receptors, also proved to be a valuable technique for the detection of ER and PR, particularly in the ovaries of several species (Iwai *et al.*, 1991a; Isola *et al.*, 1987; Korte and Isola, 1988), including the monkey (Hild-Petito *et al.*, 1988) and human (Iwai *et al.*, 1990; Press and Greene, 1988). An unexpected distribution of ER was observed in the monkey ovary, localizing this receptor to the germinal (i.e. surface) epithelium, but not to follicles or corpora lutea. In contrast, PR was detected in various monkey ovarian structures including the germinal epithelium, follicle, and corpus luteum (Hild-Petito *et al.*, 1988).

Recent studies also suggested that the regulation of steroid receptor expression in the ovary differs from that in other target tissues. In classical target tissues such as the uterus, estrogen induces PR formation through a receptor-mediated pathway (Katzenellenbogen, 1980). In certain compartments of the ovary, particularly the primate corpus luteum, PR expression may not be regulated by estrogen since ER was not detected (Hild-Petito *et al.*, 1988; Iwai *et al.*, 1990). This laboratory provided direct evidence that an ovulatory dose of hCG in stimulated cycles increases PR expression in granulosa cells during early luteinization in rhesus monkeys (Chandrasekher *et al.*, 1991), thus supporting a previously unrecognized role for the gonadotropin surge in PR induction.

Detection of PR in steroidogenic tissues by radioligand binding

techniques has proven difficult due to high endogenous levels of progesterone (Slayden *et al.*, 1992). Immunocytochemical techniques, though useful, may not detect low levels of steroid receptors and do not provide assurance of the identification of functional receptors. This study was designed to test the hypothesis that if progesterone and estradiol act via classical receptor-mediated pathways in the primate ovary, then it would be possible to detect receptor mRNAs in ovarian tissues. The reverse transcription-polymerase chain reaction (RT-PCR), utilizing primers to the hormone binding region of the receptors, was employed to detect PR and ER mRNAs in the rhesus monkey ovary, particularly in granulosa cells obtained before and after an ovulatory stimulus and in corpora lutea throughout the luteal phase of the menstrual cycle.

Results

RT-PCR Product Analysis of Monkey PR and ER

Total RNA isolated from monkey myometrium was used as a positive control in the validation of the RT-PCR reaction. Both PR and ER products of the expected size (330 and 360 bp, respectively), were detected (Fig. 1 and 2). Since immunocytochemistry suggested that the germinal (i.e., surface) epithelium of the monkey ovary contained both PR and ER protein (Hild-Petito *et al.*, 1988), RNA preparations from germinal epithelium-enriched cortical sections should contain PR and ER transcripts. RT-PCR products for both PR and ER comparable to those produced from myometrial RNA preparations were detected when RNA from germinal epithelium-enriched compartments of the ovary was analyzed (Fig. 1 and 2).

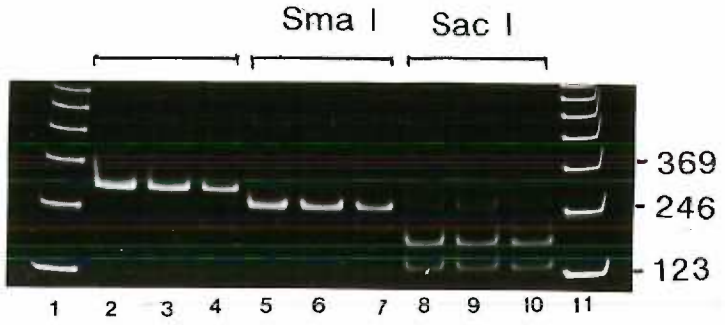


Fig. 1. Restriction enzyme analysis of RT-PCR products (PR mRNA) obtained from monkey uterine myometrium (M; Lanes 2, 5, and 8), germinal (surface) epithelium-enriched (GE) cortical compartment of the ovary (Lanes 3, 6 and 9), and the corpus luteum (CL; Lanes 4, 7, and 10) using PR primers. The products were electrophoresed in a polyacrylamide gel and stained with ethidium bromide. Lanes 1 and 11: 123 bp DNA ladder (from bottom to top: 123, 246, 369, 492, 615, 738, 861 bp). Lanes 2, 3, and 4: Undigested RT-PCR product. Lanes 5, 6, and 7: *Sma* I digest of RT-PCR product from M, GE, CL, respectively. Lanes 8, 9 and 10: *Sac* I digest of RT-PCR product from M, GE, CL, respectively. *Sma* I digestion fragments are 282 and 49 bp. Predicted sizes of fragments after *Sac* I digestion at two sites were 142, 145, and 44 bp, whereas partial digestion with cutting at one of these two sites was consistent with an additional fragment of 186 or 189 bp. The smallest digestion fragments for each enzyme were not detectable on these gels.

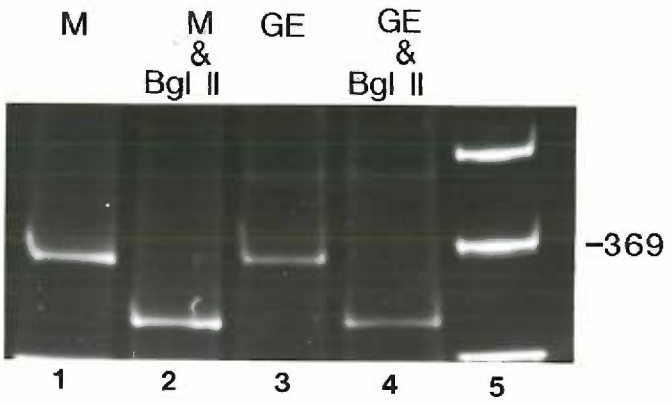


Fig. 2. Restriction enzyme analysis of RT-PCR products (ER mRNA) obtained from monkey uterine myometrium (M) and germinal (surface) epithelium-enriched (GE) cortical compartment of the ovary using ER primers. Lanes 1 and 3: Undigested RT-PCR product. Lanes 2 and 4: *Bgl* II digest of RT-PCR product. Lane 5: 123 bp DNA ladder (from bottom to top: 246, 369, and 492 bp). Predicted sizes of *Bgl* II digestion fragments were 301 and 61 bp. The small digestion fragment was not detectable. Products were electrophoresed in a polyacrylamide gel.

Product specificity was confirmed using restriction enzyme analysis. *Sac* I and *Sma* I cleaved the 330 bp PR product from myometrium, germinal epithelium-enriched sections, and corpus luteum into predicted size fragments (Fig. 1). *Sma* I digestion yielded a large fragment consistent with the formation of a 282 bp cleavage product. In contrast, predicted sizes of fragments after *Sac* I digestion at two sites are 142, 145, and 44 bp, whereas partial digestion with cutting at one of these two sites is consistent with an additional fragment of 186 or 189 bp. The smallest fragment (< 123 bp) following digestion with either restriction enzyme is not visible. The ER RT-PCR product from myometrium and germinal epithelium-enriched compartments was also specifically digested by *Bgl* II yielding a large 301 bp fragment (Fig. 2). The small digestion fragment is not visible.

To determine the similarity of the PR product of the monkey to the corresponding section of the human PR cDNA, the myometrial RT-PCR product was subcloned into pCR 1000 and sequenced. The nucleotide sequence of the partial cDNA for monkey PR (MPRRC-1) is shown in Fig. 3A. There is 99% homology between the nucleotide sequence for the monkey PR cDNA and that of the corresponding steroid-binding region of the human PR cDNA. Only two nucleotides occurring at positions 90 and 183 of MPRRC-1 differ between the two sequences. There was 100% homology (Fig. 3B) between the predicted amino acid sequence for this partial cDNA and that for the human.

PR and ER mRNA in the Monkey Ovary

PR and ER mRNA products were detected in whole monkey ovaries (Fig. 4 and 5, respectively). When the ovaries were dissected to give cortical

Fig. 3. RT-PCR product from monkey myometrium was subcloned into pCR 1000 vector and sequenced. A. The nucleotide sequence of the partial cDNA for monkey PR (MPRRC-1). There was 99% homology between the nucleotide sequence for the monkey PR cDNA and that of the corresponding steroid-binding region of the human PR cDNA. Only two nucleotides (boxed) occurring at positions 90 and 183 of MPRRC-1 differed between the two sequences. B. The predicted amino acid sequence for the partial cDNA for monkey PR. There was 100% homology between the predicted amino acid sequence for this partial cDNA and that for the human.

A

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Human    CCACAGGAGTTTGTCAAGCTTCAAGTTAGCCAAGAAGAGTTCCTCTGTATG  2638
Monkey   CCACAGGAGTTTGTCAAGCTTCAAGTTAGCCAAGAAGAGTTCCTCTGTATG  51

Human     AAAGTATTGTTACTTCTTAATACAATTCCTTTGGAAGGGCTACGAAGTCA  2688
Monkey    AAAGTATTGTTACTTCTTAATACAATTCCTTTGGAAGGACTACGAAGTCA  101

Human     AACCCAGTTTGAGGAGATGAGGTCAAGCTACATTAGAGAGCTCATCAAGG  2738
Monkey    AACCCAGTTTGAGGAGATGAGGTCAAGCTACATTAGAGAGCTCATCAAGG  151

Human     CAATTGGTTTGAGGCAAAAAGGAGTTGTGTCGAGCTCACAGCGTTTCTAT  2788
Monkey    CAATTGGTTTGAGGCAAAAAGGAGTTGTGTCTAGCTCACAGCGTTTCTAT  201

Human     CAACTTACAAAACTTCTTGATAACTTGCATGATCTTGTCAAACAACTTCA  2838
Monkey    CAACTTACAAAACTTCTTGATAACTTGCATGATCTTGTCAAACAACTTCA  251

Human     TCTGTACTGCTTGAATACATTTATCCAGTCCCGGGCACTGAGTGTTGAAT  2888
Monkey    TCTGTACTGCTTGAATACATTTATCCAGTCCCGGGCACTGAGTGTTGAAT  301

Human     TTCCAGAAATGATGTCTGAAGTTATTGCTGC  2919
Monkey    TTCCAGAAATGATGTCTGAAGTTATTGCTGC  332
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B

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Human     PQEFVKLQVSQEEFLCMKVLLLLNTIPLEGLRSQTQFEEMRSSYIRELIKAIGL
Monkey    PQEFVKLQVSQEEFLCMKVLLLLNTIPLEGLRSQTQFEEMRSSYIRELIKAIGL

Human     RQKGVVSSSQRFYQLTKLLDNLHDLVKQLHLYCLNTFIQSRALSVEFPEMMSEVIA
Monkey    RQKGVVSSSQRFYQLTKLLDNLHDLVKQLHLYCLNTFIQSRALSVEFPEMMSEVIA
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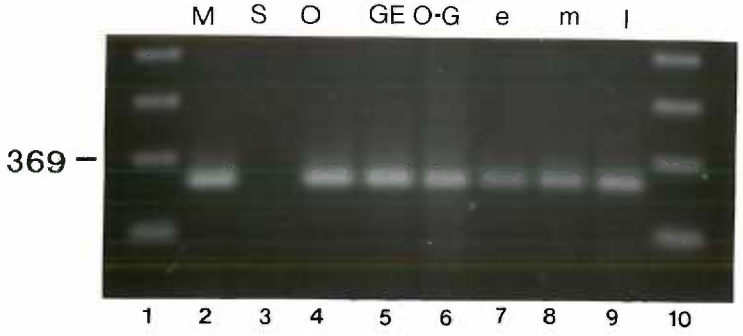


Fig. 4. Detection of PR mRNA in the monkey ovary by RT-PCR. The product was electrophoresed in a NuSieve:Seakem (3:1) agarose gel. Lanes 1 and 10: 123 bp DNA ladder (bottom to top: 246, 369, 492, 615 bp). Lane 2: myometrium (M). Lane 3: spleen (S). Lanes 4, 5, and 6: Whole ovary (O), germinal epithelium-enriched cortical (GE), and medullary compartments (O-G), respectively. Lanes 7, 8, and 9: Corpora lutea (CL) from early (e), mid (m), and late (l) luteal phase of the menstrual cycle, respectively. PR RT-PCR product of expected size (330 bp) was detected in myometrium (positive control), but not in spleen (negative control). PR mRNA was also detected in all (n=3) ovaries, in germinal epithelium and in medullary compartments, and in all corpora lutea from 3 stages (n=3/stage).

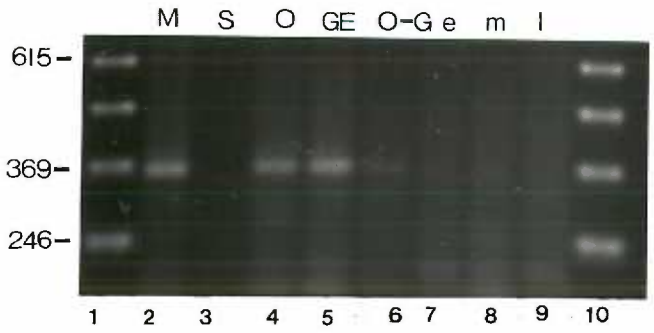


Fig. 5. Detection of ER mRNA in the monkey ovary by RT-PCR. The product was electrophoresed in a NuSieve:Seakem (3:1) agarose gel. Lanes 1 and 10: 123 bp DNA ladder (bottom to top: 246, 369, 492, 615 bp). Lane 2: myometrium (M). Lane 3: spleen (S). Lanes 4, 5, and 6: Whole ovary (O), germinal epithelium-enriched cortical (GE), and medullary compartments (O-G), respectively. Lanes 7, 8, and 9: Corpora lutea (CL) from early (e), mid (m) and late (l) luteal phase of the menstrual cycle, respectively. ER RT-PCR product of expected size (360 bp) was detected in myometrium (positive control), but not in spleen (negative control). ER mRNA was also detected in ovaries and in germinal epithelium. Only a barely detectable product was obtained from medullary compartments. ER mRNA was not detectable in any corpora lutea from 3 stages (0/3 for each stage).

and medullary compartments, PR mRNA was detected as a strong band in both compartments (n=3 for both compartments). ER mRNA was detected in two of three preparations of cortical compartments. In contrast, the ER product was only detected as a very light band in one preparation of medullary RNA. The PR and ER mRNA products were tissue specific since similar-sized products were detected in myometrium, but not in spleen.

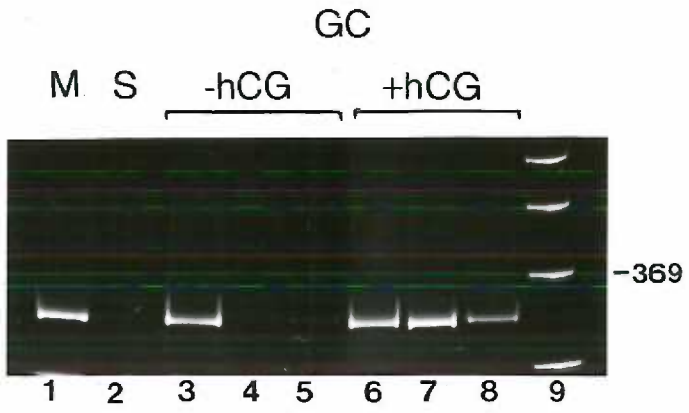
To determine if PR and ER mRNA were expressed in the corpus luteum throughout its life span in the menstrual cycle, total RNA was isolated from luteal tissue obtained at early, mid, and late stages of the luteal phase (n=3/stage). PR mRNA product was detected in all corpora lutea (Fig. 4) from early through late luteal phase (n=3 for each of 3 stages). In contrast, ER mRNA product was not detected in any corpora lutea at any stage of the menstrual cycle (Fig. 5).

Since PR mRNA was expressed in the corpus luteum during the menstrual cycle, we determined if PR mRNA product was expressed in granulosa cells of follicles before and after an ovulatory stimulus. PR mRNA product was detectable as a strong band in granulosa cells from only one of three animals not receiving an ovulatory stimulus (Fig. 6A, lane 3). No PR mRNA product was observed in granulosa cells from a second animal (lane 4), whereas a very light band was detected in the remaining preparation (lane 5). In contrast, an amplification product of the appropriate size was detected in granulosa cells from all monkeys (n=5) after administering an ovulatory bolus of hCG. An actin RT-PCR product confirmed that the granulosa cell RNA was intact (Fig. 6B). RNA obtained from granulosa cells before or after an ovulatory dose of hCG was also probed for ER mRNA product (Fig. 6B). ER mRNA was not detected in granulosa cells regardless of exposure to hCG (n=3).

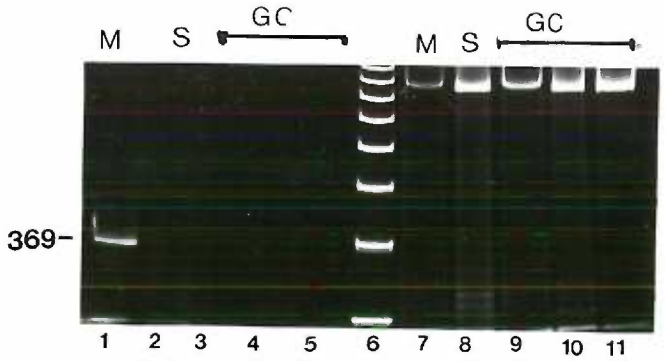
Fig. 6A. Detection of PR mRNA in monkey granulosa cells by RT-PCR. Lane 1: Myometrium (M). Lane 2: Spleen (S). Lanes 3, 4, 5: Granulosa cells obtained before an ovulatory stimulus (-hCG). Lanes 6, 7, 8: Granulosa cells obtained after an ovulatory stimulus (+hCG). Lane 9: 123 bp DNA ladder. PR mRNA product of the expected size was detected in myometrium, but not in spleen. PR mRNA was detected as a strong band in granulosa cells from 1 of 3 -hCG animals (Lane 3). PR mRNA product was not observed in granulosa cells from a second animal (Lane 4), whereas a very light band (Lane 5) was detectable in the remaining preparation. In contrast, PR mRNA product was detected in granulosa cells from all + hCG monkeys.

Fig. 6B. Detection of ER and actin mRNA in monkey granulosa cells by RT-PCR. Lanes 1 and 7: Myometrium (M). Lanes 2 and 8: Spleen (S). Lanes 3, 4, 5: Granulosa cells before/after an ovulatory stimulus. Lane 6: 123 bp DNA ladder. Lanes 9, 10, 11: Granulosa cells before an ovulatory stimulus. ER mRNA was not detected in granulosa cells regardless of exposure to hCG. An actin RT-PCR product confirmed that RNA obtained from granulosa cells before an ovulatory stimulus was intact. The products detected in this figure were electrophoresed in a polyacrylamide gel.

A



B



Discussion

This is the first study to examine the expression of PR and ER messenger RNA in the primate ovary. ER mRNA was detected in germinal epithelium-enriched cortical compartments of the macaque ovary, but not in granulosa cells of pre- and periovulatory follicles nor in corpora lutea at any stage of the luteal phase. In contrast, PR mRNA was detected in cortical sections of the ovary, in granulosa cells of periovulatory follicles, and in corpora lutea from early, mid, and late luteal phase of the menstrual cycle. Taken together, the data are consistent with receptor-mediated actions of progesterone, but not estradiol, within luteinizing granulosa cells of the periovulatory follicle and the corpus luteum in primates.

Several findings support the conclusion that the RT-PCR products reflect specific detection of PR and ER mRNA in monkey tissues using primers to the steroid binding domain of each receptor. First, tissue specificity was observed in that PR and ER mRNA RT-PCR products were detected in the uterus and the ovary, but not in the spleen. Secondly, the RT-PCR reaction and the restriction enzyme analysis resulted in products of predicted sizes that were equivalent for the uterus and the ovary. Finally, sequence analysis revealed that the nucleotide sequence of the monkey PR cDNA is 99% homologous to the corresponding steroid-binding region encoded by the human PR cDNA.

ER mRNA was detected in the germinal epithelium, but not in granulosa cells of the follicle or in corpora lutea. Similarly, ER was localized using immunocytochemistry to the germinal epithelium (Hild-Petito *et al.*, 1988), but not to granulosa cells or corpora lutea of the monkey ovary. In contrast to the immunocytochemical data on ER protein, low levels of ER

mRNA were detected in medullary compartments of the ovary. Oocytes (Wu *et al.*, 1992) or other cells could be the source of the ER RT-PCR product in medullary preparations of the ovary and could reflect low levels of ER not detectable by immunocytochemistry. The absence of ER in granulosa cells of the monkey follicle is supported by the absence of estradiol enhancement of FSH action on aromatase activity and progesterone synthesis in cultured marmoset monkey granulosa cells (Hillier *et al.*, 1987). Koering's (1987) study showing that diethylstilbestrol (DES) treatment of juvenile rhesus monkeys did not affect the number or size of growing preantral or antral follicles is also consistent with the absence of ER in monkey follicles. ER expression in human granulosa cells is controversial. ER mRNA was not detected by RT-PCR in granulosa cells obtained from patients in an IVF program (Wu *et al.*, 1992), but was present in cumulus oocyte complexes, suggesting oocytes as the source of ER mRNA. However, Iwai and colleagues (1990), detected immunohistochemical staining for ER protein in granulosa cells of human antral, but not preantral, follicles, and not in cells of the dominant follicle at the time of the LH surge. In contrast to granulosa cells, there is no controversy regarding ER absence in the primate corpus luteum (Iwai *et al.*, 1990; Hild-Petito *et al.* 1988). The absence of receptor-mediated actions of estrogen in the monkey corpus luteum are supported by several studies (Hutchison *et al.*, 1987; Ellinwood and Resko, 1983), including those from our laboratory (Hild-Petito *et al.*, 1988). However, nonreceptor-mediated effects of estrogen such as the direct inhibition of 3ß-hydroxysteroid dehydrogenase activity in corpora lutea (Depp *et al.*, 1973) or the modulation of protaglandin formation (Degan *et al.*, 1987) have been reported. Therefore, except for in the germinal epithelium, receptor-mediated actions for estrogen in the primate ovary, remain unsubstantiated.

In contrast to ER, PR mRNA was detected consistently in germinal epithelium-enriched cortical and medullary sections of the ovary, in granulosa cells following the ovulatory gonadotropin surge, and in corpora lutea. Similarly, PR was detected using immunocytochemistry in several compartments of the primate ovary including the germinal epithelium and stroma, in granulosa cells after an ovulatory stimulus, and in corpora lutea from early, mid and late luteal phases of the menstrual cycle (Hild-Petito *et al.*, 1988; Chandrasekher *et al.*, 1991). Interestingly, the number of PR-positive cells declined in corpora lutea by the late luteal phase, whereas PR mRNA continued to be detected. However, RT-PCR under the conditions utilized in the present study, is not quantitative. Further support for progesterone action comes from a recent study showing ligand binding of luteal PR following treatment of monkeys with trilostane, a 3ß-HSD inhibitor used to lower endogenous levels of progesterone (Slayden *et al.*, 1992). The binding was of a high affinity comparable to that in classical target tissues such as the uterus. Taken together, these data provide strong evidence for PR expression in the primate corpus luteum and are consistent with local PR effects beyond development and continuing throughout the luteal phase.

Support for progesterone action in the periovulatory follicle and the corpus luteum comes from a limited number of studies. Progestins reportedly enhance the activity of steroidogenic enzymes in theca and granulosa cells (Ruiz de Galarreta *et al.*, 1985; Tonetta, 1987). DiMattina and colleagues (1986) observed that RU486, an antiprogestin, inhibits progesterone production by human granulosa cells *in vitro*. A role for progesterone around ovulation has also been suggested. For example, Loutradis and colleagues (1991) reported that ovulation in PMSG-treated mice was completely blocked when the progesterone antagonist RU486 was administered with hCG.

Progesterone may also influence the development and function of the corpus luteum. Rothchild (1981) first hypothesized that progesterone stimulates its own production and release from corpora lutea. In the monkey ovary, PR was localized in luteal cells containing 3ßHSD activity (Hild-Petito *et al.*, 1988). Progestins also stimulate the expression of LH receptors in early bovine luteal cells during culture (Jones *et al.*, 1992). Thus, evidence is accumulating for a role for progesterone in regulating, via receptor-mediated mechanisms, the function of the corpus luteum.

The detection of PR mRNA in luteinizing granulosa cells of periovulatory follicles, but not typically as a strong band in granulosa cells of preovulatory follicles is consistent with recent immunocytochemical evidence from our laboratory showing that the ovulatory gonadotropin surge [LH in spontaneous cycles (Hild-Petito *et al.*, 1988) and hCG in stimulated cycles (Chandrasekher *et al.*, 1991)] stimulated PR expression in granulosa cells during early luteinization in rhesus monkeys. A quantitative analysis of PR mRNA levels in granulosa cells from preovulatory and periovulatory follicles is required to confirm that gonadotropin action may be to increase the levels of PR message. There is a growing body of evidence for the involvement of LH in PR expression in granulosa cells. In rats, PR mRNA was expressed in granulosa cells of large follicles following the LH surge (Park and Mayo, 1991). When pentobarbital was administered to block the preovulatory LH surge, PR mRNA expression was not detected. LH/hCG induced PR mRNA in porcine granulosa cells (Iwai *et al.*, 1991b) and PR in rabbit granulosa cells (Iwai *et al.*, 1991a). Gonadotropin induction of PR in primate granulosa cells did not seem to involve an estrogen-mediated mechanism, since ER was was not detected in granulosa cells at any time (Hild-Petito *et al.*, 1988). Similarly, there may not be a direct role for estrogen in rat ovarian PR gene

expression. PMSG-primed immature rats with high levels of estrogen did not express PR mRNA (Park and Mayo, 1991). Estrogen-independent expression of PR was also reported in chicken intestinal mesothelium and smooth muscle (Salomaa *et al.*, 1989). Determining whether PR expression is a direct action of LH/hCG or the result of other gonadotropin-triggered events in the ovulatory follicle remains an important aim.

In summary, the detection of PR mRNA together with earlier data on the presence of PR protein, supports the hypothesis of progesterone action via classical receptor-mediated pathways in the primate ovary. In contrast, ER mRNA was not detected in granulosa cells nor in corpora lutea confirming earlier immunocytochemical data and suggesting a lesser, if any, role for estrogen in local regulation of the primate corpus luteum.

Materials and Methods

Animal Models and Tissue Collection

The general care and housing of rhesus monkeys at the Oregon Regional Primate Research Center (ORPRC) has been described previously (Molskness *et al.*, 1987). Adult females were checked daily for menses (onset of menses = cycle day 1). Daily blood samples were obtained from unanesthetized monkeys by saphenous venipuncture from day 1 or day 8 of the menstrual cycle when tissues were collected during the follicular or luteal phase, respectively. Serum was stored at -20 C until measurement of progesterone and estradiol by RIA (Hess *et al.*, 1981; Resko *et al.*, 1975) in the ORPRC Hormone Assay Core Laboratory.

Tissues were collected at surgery as described previously (Hild-Petito *et al.*, 1988) from anesthetized rhesus monkeys (*Macaca mulatta*) with the

exception of one ovary which was obtained from a cynomolgus monkey (*Macaca fascicularis*). Spleen and two ovaries were obtained from monkeys undergoing autopsy for other research protocols or for diseases not affecting reproductive function. Uterus samples were obtained from ovariectomized animals undergoing treatment with estradiol implants for 14 days (Low *et al.*, 1989). Ovaries lacking a dominant structure (i.e., large follicle or corpus luteum) were either left intact (n=3) or dissected to obtain germinal (i.e., surface) epithelium-enriched cortical and medullary compartments of the ovary. Corpora lutea (n=3/stage) were obtained by lutectomy during early (day 3-5 post- LH surge), mid (day 7-8), and late (day 14-15) luteal phase of the menstrual cycle. Specimens were placed in Hank's Balanced Salt Solution at 4 C and transported to the laboratory.

An ovarian stimulation model developed for collection of numerous mature oocytes for in vitro fertilization (Zelinski-Wooten *et al.*, 1991) was used to obtain granulosa cells from pre- and periovulatory follicles. Beginning at menses, rhesus monkeys were given 60 IU human (h) FSH (Metrodin, Serono Laboratories, Inc., Randolph, MA; days 1-6) followed by 60 IU hFSH plus 60 IU hLH (Pergonal, Serono, days 7-9) to stimulate the growth of multiple follicles. Animals (n=3-5/group) received either no ovulatory stimulus or 1000 IU hCG (Profasi, Serono), im, on day 10. Granulosa cells were obtained by follicle aspiration at laparotomy 27 h after the hCG injection or on day 10 in animals receiving no ovulatory stimulus. After oocyte removal, granulosa cells were enriched from follicular aspirates on a 40% Percoll (Sigma Chemical Co., St. Louis, MO) gradient (Chandrasekher *et al.*, 1991). Tissues and granulosa cells were frozen on dry ice and stored at -70 C until RNA extraction.

RNA Extraction

Total RNA was isolated from tissues and cells using the cesium chloride ultracentrifugation method of Chirgwin and colleagues (1979). Tissues and cells were homogenized in 4 M guanidine thiocyanate. Tissue homogenates were centrifuged at 10,000 x g_{max} for 10 minutes to pellet cell debris. The supernatant was decanted and cesium chloride added (final concentration 2.38 M). Samples were loaded onto a 2 ml (1.5 ml for cells) cushion of 5.7 M cesium chloride and spun for 18-24 h at 15 C and 36,000 rpm (20 C and 41,000 rpm for cells). The supernatant was discarded and the RNA pellet resuspended in 5 mM EDTA (pH 8). Suspensions were extracted first with phenol:chloroform (1:1), followed by two extractions with chloroform:isoamyl alcohol (24:1). RNA was precipitated with 2.5 volumes of 100% ethanol and 0.1 volume of 3M sodium acetate (pH 5.2) overnight at -20 C. The RNA was pelleted by centrifugation at 15,000 x g for 30 minutes. The pellet was resuspended in DEPC-treated water and the concentration determined by absorbance at 260 nm.

RT-PCR Analysis

The oligonucleotide primer sequences used in the RT-PCR assay to detect PR and ER mRNA consisted of 20 or 21 nucleotides and were selected from the region encoding the steroid binding domain based on the reported sequences of the cloned human, rabbit, rat, and mouse cDNAs (Savouret *et al.*, 1989; Park and Mayo, 1991; White *et al.*, 1987; Koike *et al.*, 1987; Green *et al.*, 1986). The primers fulfilled the general criteria of random base distribution, approximately 50% GC content, and non-complementarity of primer sequences. In addition, primers were chosen to bracket 1) several restriction sites and 2) where possible, an area including an intron. The PR

primers bracketed 330 bp with the sense and antisense primers corresponding to nucleotides 2415 - 2434 and 2725 - 2745, respectively, of the human PR cDNA (Savouret *et al.*, 1989). ER primers bracketed a 360 bp sequence with the sense primer corresponding to nucleotides 1207 - 1227 and the antisense primer to nucleotides 1549 - 1569 of the human ER cDNA (Green *et al.*, 1986). Primers to human ß-Actin mRNA (Clontech Laboratories, Inc., Palo Alto CA) resulted in the amplification of a RT-PCR product of 1126 bp.

RT-PCR was performed according to the protocol outlined in the GeneAmp RNA PCR Kit (Perkin Elmer Cetus, Norwalk, CT) with some modifications. One microgram of total RNA dissolved in 1mM EDTA, 10 mM NaCl, 10 mM Tris-HCl (pH 8) was incubated with 1 µM of the antisense primer at 70 C for 10 minutes followed by chilling on ice. A mix of the following components was added to each sample for a total reaction volume of 20 µl: 5X RT Buffer (250 mM Tris-HCl at pH 8.3, 375 mM KCl, 15 mM $MgCL_2$; Gibco BRL, Gaithersburg, MD), 4 µl; autoclaved water, 1 µl; 10 mM each dNTP (dATP, dCTP, dGTP, dTTP; Pharmacia LKB Biotechnology, Inc., Piscataway, NJ), 2 µl each; 40 U RNasin Ribonuclease Inhibitor (Promega Corporation, Madison, WI), 1 µl; 200 U M-MLV Reverse Transcriptase (Gibco BRL), 1 µl; and 0.1 M DTT (Gibco BRL), 2 µl. The reaction mix was overlaid with 100 µl of mineral oil (Sigma Chemical Company, St. Louis, MO) and incubated in a Programmable Thermal Controller (MJ Research, Inc.) as follows: 42 C for 60 min, 99 C for 5 min, and 5 C for up to 24 h. The RT reaction sample was then either used immediately for PCR or frozen at -20 C.

The PCR protocol entailed the addition of the following mix to each RT reaction sample: Taq DNA Polymerase 10X Buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.8 at 25 C), 15 mM $MgCl_2$, 1% Triton X-100; Promega), 8 µl;

2.5 U Taq DNA Polymerase (Promega), 0.5 µl; 0.2 µM sense primer, 1 µl; DEPC-treated water to give a total PCR reaction mix of 100 µl. The DNA Thermal Cycler was programmed as follows: 2 min at 95 C, 1 min at 95 C and 1 min at 60 C for 45 cycles (to denature template complexes, and to anneal-extend complexes, respectively), 7 min at 60 C to maximize strand completion, and up to 24 h at 4 C. The PCR samples were stored at -20 C until further analysis.

PCR without RT was performed with designated RNA preparations to confirm the absence of DNA contamination in samples. In addition, several control reactions were run in parallel during each RT-PCR analysis of PR and ER mRNA in ovarian samples. These included RNA preparations from receptor-positive (uterus) or -negative (spleen) control tissues as well as a tube without RNA to check for reagent contamination. When appropriate, samples were checked for intact RNA by RT-PCR analysis for actin.

Gel Electrophoresis

A 15 µl aliquot of the PCR sample containing 0.1 volume of a loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) was size fractionated by electrophoresis on a NuSieve:SeaKem (3:1) agarose (FMC Bioproducts, Rockland, ME) gel. Samples were first stacked for 10 min at 100 V and then electrophoresed overnight at 20-25 V. Some samples were also fractionated by electrophoresis in either a 10% or 17-27% polyacrylamide gel. An aliquot of a 123 bp DNA ladder (Bethesda Research Laboratories Life Technologies, Inc.) was included as a size marker. Gels were stained in ethidium bromide and the bands visualized using a DNA transilluminator (Fotodyne Incorporated, New Berlin, WI). A photograph of the gel was obtained with positive/negative instant sheet film (Polaroid)

using a MP-4 camera system.

Restriction Enzyme and Sequence Analysis of PCR Products

RT-PCR samples were incubated for 3 h with a restriction enzyme as outlined by the manufacturer. Cloning and sequencing of the PR RT-PCR product was performed as follows. RT-PCR products were gel purified and ligated into pCR 1000 vector cut with *Hph* I (Invitrogen, San Diego, CA). DNA sequencing was done on double stranded template using dideoxy-chain termination method (Sanger *et al.*, 1977) with flanking primers on the vector and sequenase (US Biochemicals, Cleveland, OH). Database analysis and sequence alignment was done with DNASTAR software (Madison, WI).

CHAPTER 6

EXPRESSION AND MAINTENANCE OF PROGESTERONE RECEPTORS BY PRIMATE GRANULOSA CELLS *IN VITRO*: EFFECTS OF GONADOTROPINS AND PROSTAGLANDIN E_2

Abstract

Earlier studies in this laboratory (Hild-Petito *et al.*, 1988) suggested that progesterone receptors (PR) first appeared in granulosa cells of the dominant follicle in monkeys during the periovulatory interval in spontaneous menstrual cycles. Support for this hypothesis was obtained when we observed PR staining in luteinized granulosa cells (LGC) following an ovulatory dose of hCG in stimulated cycles (Chandrasekher *et al.*, 1991; Chapter 3). This study was designed to determine whether PR expression in granulosa cells could result from a direct action of the gonadotropin surge or be mediated by other gonadotropin-triggered events in the periovulatory follicle, such as enhanced prostaglandin (PG) synthesis and action. Animals were treated for up to 9 days with human gonadotropins to promote the development of multiple preovulatory follicles. Granulosa cells were aspirated from large follicles before (nonluteinized granulosa cells, NGC) or 27 h after (LGC) an ovulatory injection of hCG. NGC were cultured with or without hCG, FSH, PGE_2, and db-cAMP, while LGC were cultured in the presence or absence of hCG for 4 days. Granulosa cells were processed on days 0, 1, and 4 for PR detection by indirect immunocytochemistry using a monoclonal antibody to human PR. Progesterone (P) production by NGC was low under control conditions and was stimulated ($p < 0.05$) by exposure to

hCG, FSH, PGE_2, and db-cAMP. NGC were devoid of specific nuclear staining for PR on day 0. NGC from 3 of 4 animals did not exhibit any PR staining following 1 or 4 days of culture under control conditions. Treatment *in vitro* with hCG, FSH, PGE_2, or db-cAMP for 1 day did not result in staining for PR in these granulosa cells. However, cell maintenance was improved by the presence of these agonists, as reflected by a greater cell number compared to controls. After 4 days of culture in the presence of hCG, FSH, PGE_2 and db-cAMP, some, but not all, cells stained positively for PR. PGE_2 treatment consistently yielded the greatest response. NGC from 1 of 4 animals exhibited PR staining under control conditions on day 1; however, PR staining was more intense in the presence of hCG. P production by LGC was significantly greater than NGC under control conditions. P production was stimulated ($p < 0.05$) over control levels by hCG throughout four days in culture. Positive staining for PR was observed in 64 ± 5 % of LGC on day 0. By day 1, PR-positive cells declined in both control and hCG-treated cultures, but the decline was less in the presence of hCG. Similar results were observed on day 4 of culture. Although the culture conditions may not have been optimal, this study provides the first evidence that *in vitro* exposure of NGC to surge levels of gonadotropins can directly stimulate PR expression. In addition, the presence of LH/hCG may assist in the maintenance of PR in LGC. Also, gonadotropin action in PR induction may be mediated by autocrine factors such as prostaglandins.

Introduction

Investigators have proposed that the ovary of several species, including rodents (Schreiber and Hsueh, 1979) and primates (Hild-Petito *et*

al., 1988; Iwai *et al.*, 1990), contains progesterone receptors (PR). Evidence amassed from several experimental approaches indicates that PR is present in the follicle and corpus luteum of the rhesus monkey. First, immunocytochemical techniques detected specific nuclear staining for PR in the theca- but not the granulosa- layer of the growing follicle, in the luteinizing granulosa layer of the periovulatory follicle, and in luteal tissue throughout the menstrual cycle (Hild-Petito *et al.*, 1988). Second, high-affinity binding sites for ^{3}H-progestin were observed in nuclear and cytosolic preparations of luteal tissue following acute treatment of rhesus monkeys with trilostane, a 3ß- hydroxysteroid dehydrogenase inhibitor, to lower endogenous progesterone levels (Slayden *et al.*, 1992). Third, PR mRNA was detected in luteinizing granulosa cells of periovulatory follicles and in the corpus luteum using the reverse transcription-polymerase chain reaction (RT-PCR) assay (Chapter 5).

It was also suggested that the regulation of PR in the ovary differs from mechanisms in other reproductive organs, such as the uterus or mammary gland. In the uterus, estradiol induces PR formation through a receptor-mediated pathway (Katzenellenbogen, 1980). This appears unlikely in the luteinizing granulosa cells and luteal cells of the macaque ovary since estrogen receptor (ER) and its mRNA were nondetectable by immunocytochemistry (Hild-Petito *et al.*, 1988) or RT-PCR (Chapter 5), respectively. A novel role for the midcycle LH surge in inducing PR in luteinizing granulosa cells was proposed by Hild-Petito and colleagues (1988) based on immunocytochemical evidence that nuclear staining for PR appeared in the granulosum of the dominant follicle in monkeys during the periovulatory interval. Support for this hypothesis was obtained when PR staining was observed in luteinized granulosa cells following an ovulatory

dose of hCG in stimulated cycles. In contrast, PR staining was not detected in nonluteinized granulosa cells isolated from monkeys in the absence of an ovulatory stimulus (Chandrasekher *et al.*, 1991; Chapter 3).

PR expression in luteinizing granulosa cells could result from a direct action of the gonadotropin surge or be mediated by other gonadotropin-triggered events in the periovulatory follicle, such as enhanced prostaglandin (PG) synthesis and action. To determine whether gonadotropins act directly on macaque granulosa cells to induce PR, nonluteinized granulosa cells were cultured in the presence of LH/hCG. To examine the specificity of action, cells were also cultured in the presence of FSH or PGE_2. To test whether PR expression was mediated by cAMP-activated pathways, cells were exposed to dibutyryl cAMP. Finally, luteinized granulosa cells were cultured in the presence and absence of hCG to examine whether gonadotropins played a role in PR maintenance.

Materials and Methods

Animals and follicular development

General husbandry and housing of rhesus monkeys at the Oregon Regional Primate Research Center (ORPRC) were described previously (Molskness *et al.*, 1987). Adult, regularly cycling females were checked daily for menses (onset of menses = cycle day 1). From day 1 of the menstrual cycle until the next onset of menses, daily blood samples were obtained at 0800 h from unanesthetized monkeys by saphenous venipuncture. Serum was stored at -20 C until measurement of progesterone and estradiol by radioimmunoassay (Hess *et al.*, 1981; Resko *et al.*, 1975) in the ORPRC Hormone Assay Core Laboratory.

The follicular stimulation protocol was described previously (Zelinski-Wooten *et al.*, 1991). Beginning at menses, rhesus monkeys were given one of two regimens of human gonadotropins to stimulate the growth of multiple follicles: 60 IU human follicle stimulating hormone (hFSH; Metrodin, Serono Laboratories, Inc., Norwell, MA) for six days, followed by 60 IU hFSH + 60 IU hLH (Pergonal, Serono) for 3 days or 60 IU hFSH plus 60 IU hLH (days 1-7 or 9). Half of the gonadotropin dose was injected im twice daily at 0800 and 1600 h. To induce ovulatory maturation, one group of animals (n = 4) received hCG (1000 USP units im, Profasi, Serono), while the other group (n=4) received no ovulatory stimulus.

Granulosa cell isolation

Granulosa cells were obtained by follicle aspiration (Zelinski-Wooten *et al.*, 1991) 27 h after the hCG injection (luteinized granulosa cells), and on day 8 or 10 in animals receiving no ovulatory stimulus (nonluteinized granulosa cells). After removal of oocytes, follicular aspirates were pooled and the cell suspension was centrifuged on a 40% Percoll (Sigma Chemical Co., St. Louis, MO), 60% Hanks' Balanced Salt Solution containing 0.1% BSA to collect granulosa cells with reduced blood cell contamination (Chandrasekher *et al.*, 1991; Chapter 3). Granulosa cells were counted using a hemacytometer and viability was assessed by trypan blue exclusion.

Cell Cultures

Granulosa cells (80,000 cells/well) were cultured on glass chamber slides coated with Cell-Tak (Collaborative Research, Inc., Bedford, MA). Nonluteinized granulosa cells were cultured in Ham's F-10 containing 10% anovulatory monkey serum with or without the addition of hCG (100

ng/ml,CR123), hFSH (100 ng/ml; 2-NIAMDD), prostaglandin E_2 (PGE_2; 14 μM; Cayman Chemical, Ann Arbor, MI), or dibutyryl-cAMP (db-cAMP; 5mM; Sigma). The dosages that were used maximally stimulate progesterone production by monkey ovarian cells (Stouffer *et al.*, 1978; Zelinski-Wooten and Stouffer, 1990). Luteinized granulosa cells were cultured in Ham's F-10 containing 10% anovulatory monkey serum with or without hCG (100 ng/ml). Cell cultures (n = 2-4 replicates/monkey for each *in vitro* treatment) were incubated at 37 C in humidified 95% air/5% CO_2. Media were collected on days 1-4 of culture and frozen for measurement of progesterone content by radioimmunoassay (Hess *et al.*, 1981) at a later date.

Immunocytochemistry for PR

Granulosa cells were frozen in liquid propane on days 0, 1 and 4 of culture and stored at -20 C until analysis of PR by indirect immunocytochemistry as described previously (Chandrasekher *et al.*, 1991; Chapter 3). Cells were incubated with either a monoclonal antibody against human PR (JZB39; 2.5 μg/ml) or a nonspecific antibody (AT, antibody to antigen B of timothy grass pollen; 10 μg/ml). The antibody complex was visualized by incubation with avidin-DH and biotinylated horseradish peroxidase-H complex (ABC kit from Vector Laboratories, Burlingame, CA) followed by treatment with diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide. The percentage of nuclei staining for PR was determined for each *in vitro* treatment/monkey in nonblinded observations by a random count, if possible, of 500 cells.

Statistical Analyses

Media levels of P were transformed prior to statistical analyses when

heterogeneity of variance was present. Differences in P content in media were analyzed by ANOVA, followed by Newman-Keuls' or *t* test for comparison of means. Differences at $p<0.05$ were considered significant.

Results

Nonluteinized Granulosa Cells

P Production

Under control conditions, P production by nonluteinized granulosa cells was low on day 1 (12.1 ± 4.1 ng/ml) and declined during 4 days (0.6 ± 0.2 ng/ml) in culture ($p=0.06$; Fig. 1). The presence of agonists (hCG, FSH, PGE_2, and db-cAMP) stimulated ($p<0.05$) P production over control levels throughout culture. P production stimulated by hCG and FSH did not change over 4 days in culture. In contrast, the stimulatory effect of PGE_2 tended to increase over time, with P production increased more than 2000-fold by day 4 of culture. Treatment with db-cAMP tended to be most effective in stimulating P production on day 2, with stimulation comparable to that with PGE_2 thereafter.

Induction of PR

Nonluteinized granulosa cells from all 4 animals were devoid of PR staining on day 0 (Fig. 2, A and B), as shown previously (Chandrasekher *et al.*, 1991; Chapter 3). There was no specific nuclear staining for PR on day 1 or 4 (Fig. 2,C) of culture under control conditions in cells from 3 animals. Moreover, treatment *in vitro* with hCG, FSH, PGE_2, or db-cAMP for 1 day did not result in staining for PR in granulosa cells from these animals. Maintenance of cells under control conditions was not ideal, resulting in decreasing cell numbers from day 1 to 4 of culture. In contrast, maintenance

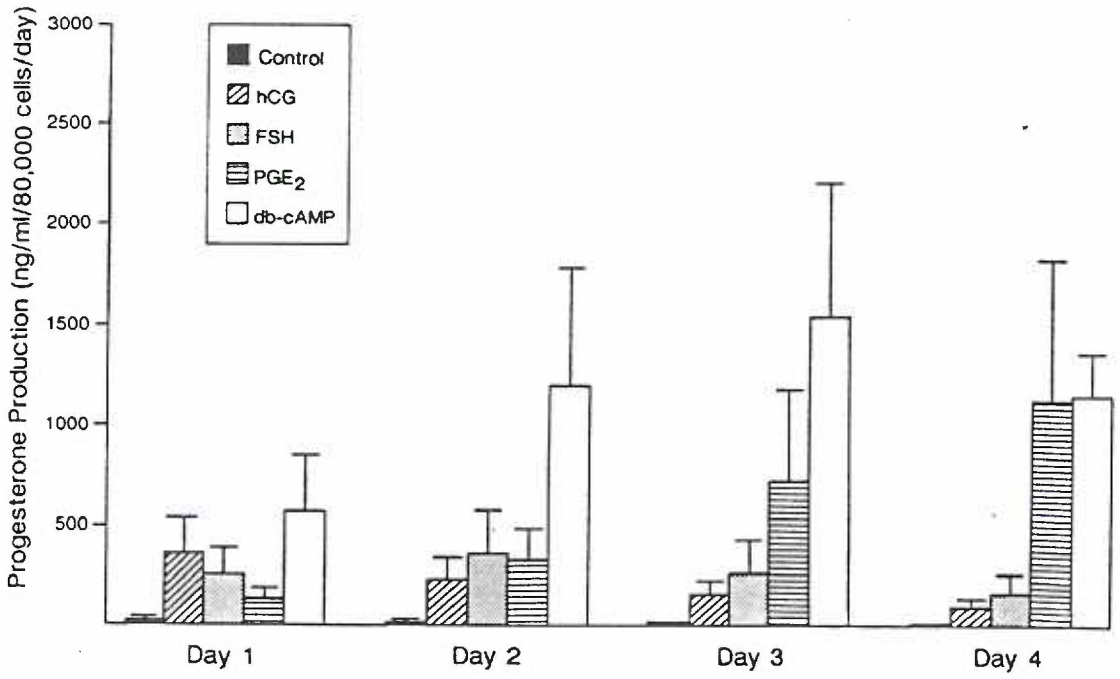


Fig. 1: P production by nonluteinized granulosa cells cultured for 4 days with or without hCG (100 ng/ml), hFSH (100 ng/ml), PGE_2 (14 μM), or db-cAMP (5 mM). The presence of agonists stimulated ($p < 0.05$) P production over control levels throughout culture. Values are the mean ± SEM of 3 experiments.

was improved by the other treatments, reflected by a greater cell number compared to controls. Fig. 2 summarizes the immunocytochemical staining for PR in granulosa cells from one monkey cultured for 4 days in the presence of hCG, FSH, PGE_2 and db-cAMP. Whereas cells were devoid of specific nuclear staining for PR under control conditions, some, but not all, cells stained positively for PR in the presence of hCG (Fig. 2D; 30% of cells), FSH (Fig. 2E; 23%), PGE_2 (Fig. 2F; 37%), and db-cAMP (Fig.2G; 10%). The results were variable from animal to animal, but PGE_2 treatment consistently yielded the greatest response (range: 8-37% PR-positive cells) and db-cAMP treatment provided the least response (0-10%).

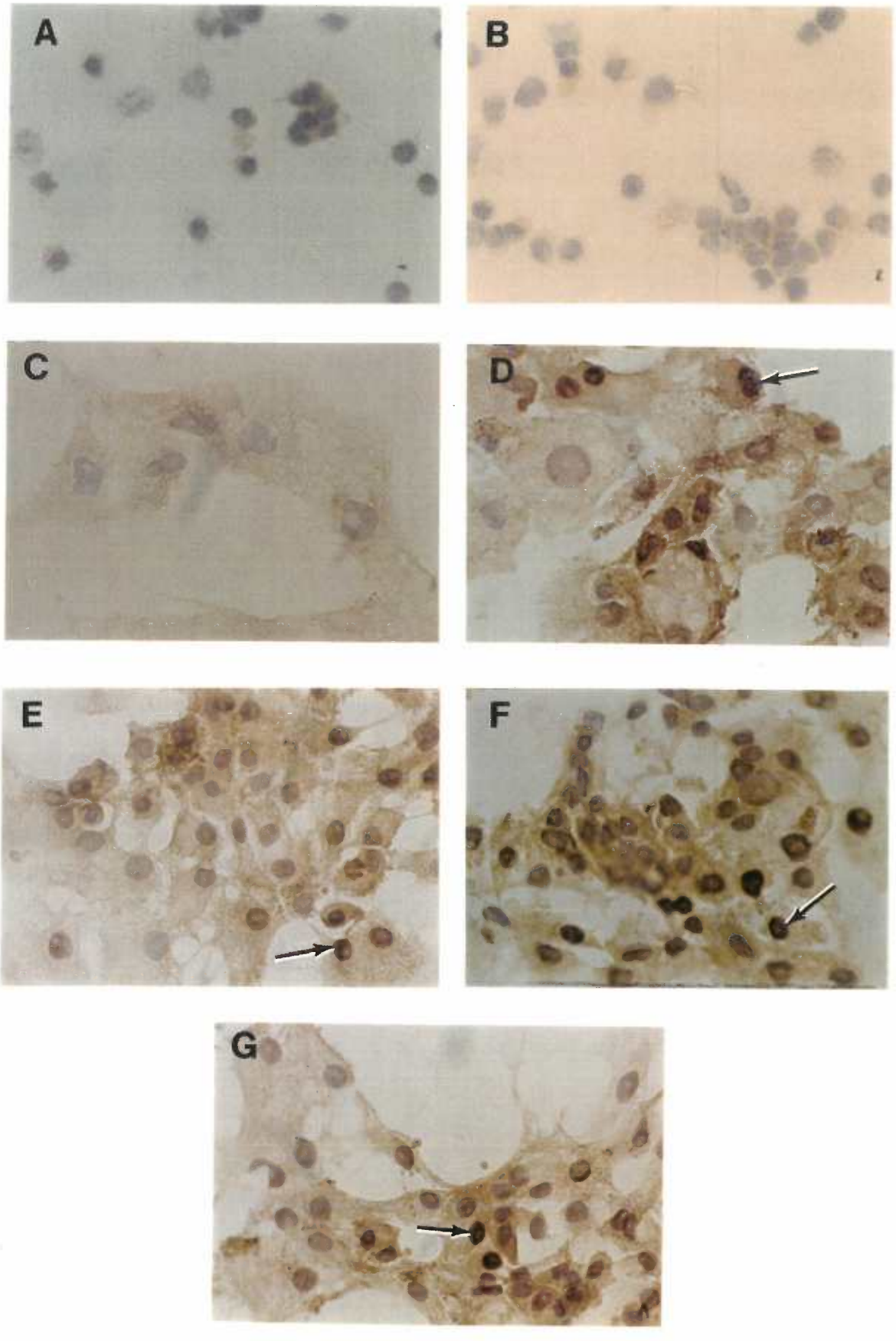
Granulosa cells from 1 of 4 monkeys exhibited nuclear PR staining (39% of cells) under control conditions on day 1 (Fig. 3A). However, the staining had a stringy appearance which was unlike that observed in luteinized granulosa cells on day 0. Exposure to hCG resulted in increased PR staining that was more intense than that under control conditions (Fig. 3B). Cells from this animal continued to express PR on day 4.

Luteinized Granulosa Cells

P Production

Granulosa cells from monkeys receiving hCG as an ovulatory stimulus produced significantly greater amounts of P under control conditions than did cells from the group receiving no ovulatory stimulus (323.6 ± 123.6 versus 12.1 ± 4.1 ng/ml, mean ± SEM, respectively). Moreover, P production by luteinized granulosa cells was maintained at a similar level during 4 days of culture (Fig. 4). Throughout culture, hCG stimulated P production over control levels ($p<0.05$).

Fig. 2: Immunocytochemical staining for PR in nonluteinized granulosa cells from rhesus monkeys following ovarian stimulation. Cells were stained on the day of follicle aspiration (A, B) or following 4 days of culture in the absence (C) or presence of hCG (D), FSH (E), PGE_2 (F), and db-cAMP (G). Aliquots of cells were incubated with anti-PR antibody (B-G) or a nonspecific AT antibody (A). Nuclear PR staining (arrow) was seen in the presence of hCG, FSH, PGE_2, and db-cAMP. Initial magnification, X400.


A
B
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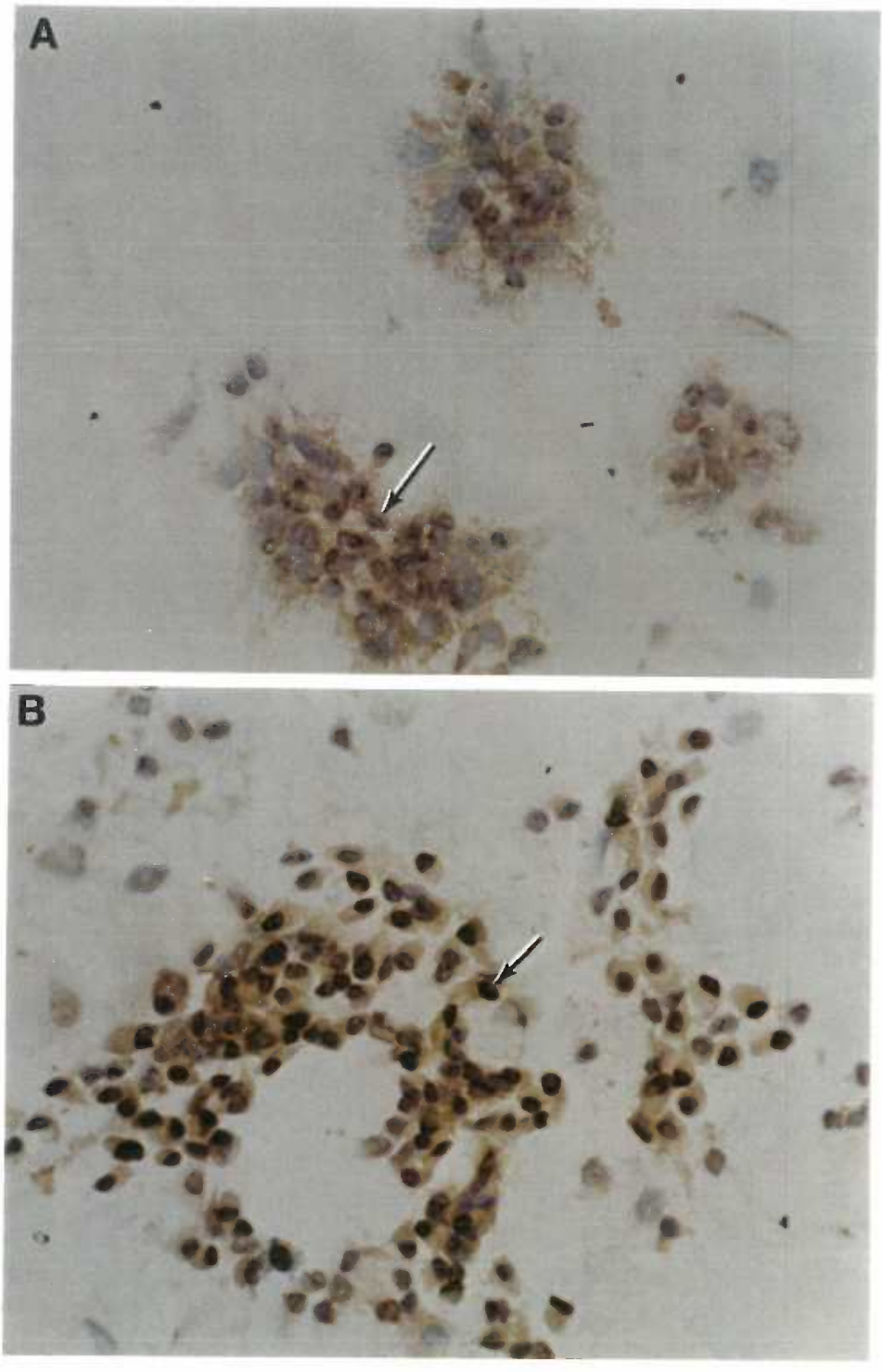
G

Fig. 3: Immunocytochemical staining for PR in nonluteinized granulosa cells from one monkey cultured in the absence (A) and presence (B) of hCG for 1 day. Aliquots of cells were incubated with anti-PR antibody. Nuclear PR staining (arrow) was observed in some cells under control conditions, whereas increased PR staining was detected in the presence of hCG.


A
B

Maintenance of PR

As reported previously by our laboratory (Chandrasekher *et al.*, 1991; Chapter 3) the majority (64 ± 5%) of granulosa cells collected from monkeys following an ovulatory dose of hCG stained intensely positive for PR (Fig. 5, B). By day 1 of culture under control conditions, the percentage of cells staining positively for PR decreased dramatically (9% ± 3; n = 4). The percent of PR-positive cells also decreased in cultures treated with hCG *in vitro*, but the decline was less than that seen in control cultures (25% ± 7 positive). Cells from two animals stained more intensely in the presence of hCG than under control conditions (Fig. 5, C and D). Similar results were observed on day 4 of culture.

Discussion

This study provides the first evidence that *in vitro* exposure of nonluteinized granulosa cells from rhesus monkeys to surge levels (100 ng/ml) of gonadotropins will stimulate PR expression. Earlier studies in this laboratory (Hild-Petito *et al.*, 1988) indicated that PR first appeared in granulosa cells of the dominant follicle in monkeys during the periovulatory interval in spontaneous menstrual cycles. In artificial cycles, PR staining was not detected in granulosa cells isolated from monkeys receiving no ovulatory stimulus following ovarian stimulation. In contrast, granulosa cells from animals receiving hCG as an ovulatory stimulus showed intense PR staining within 27 hours (Chandrasekher *et al.*, 1991). The current studies show that LH has a direct action on macaque granulosa cells to promote PR expression. *In vivo* expression of PR mRNA in granulosa cells of ovulatory follicles following the LH surge has been reported in the rat

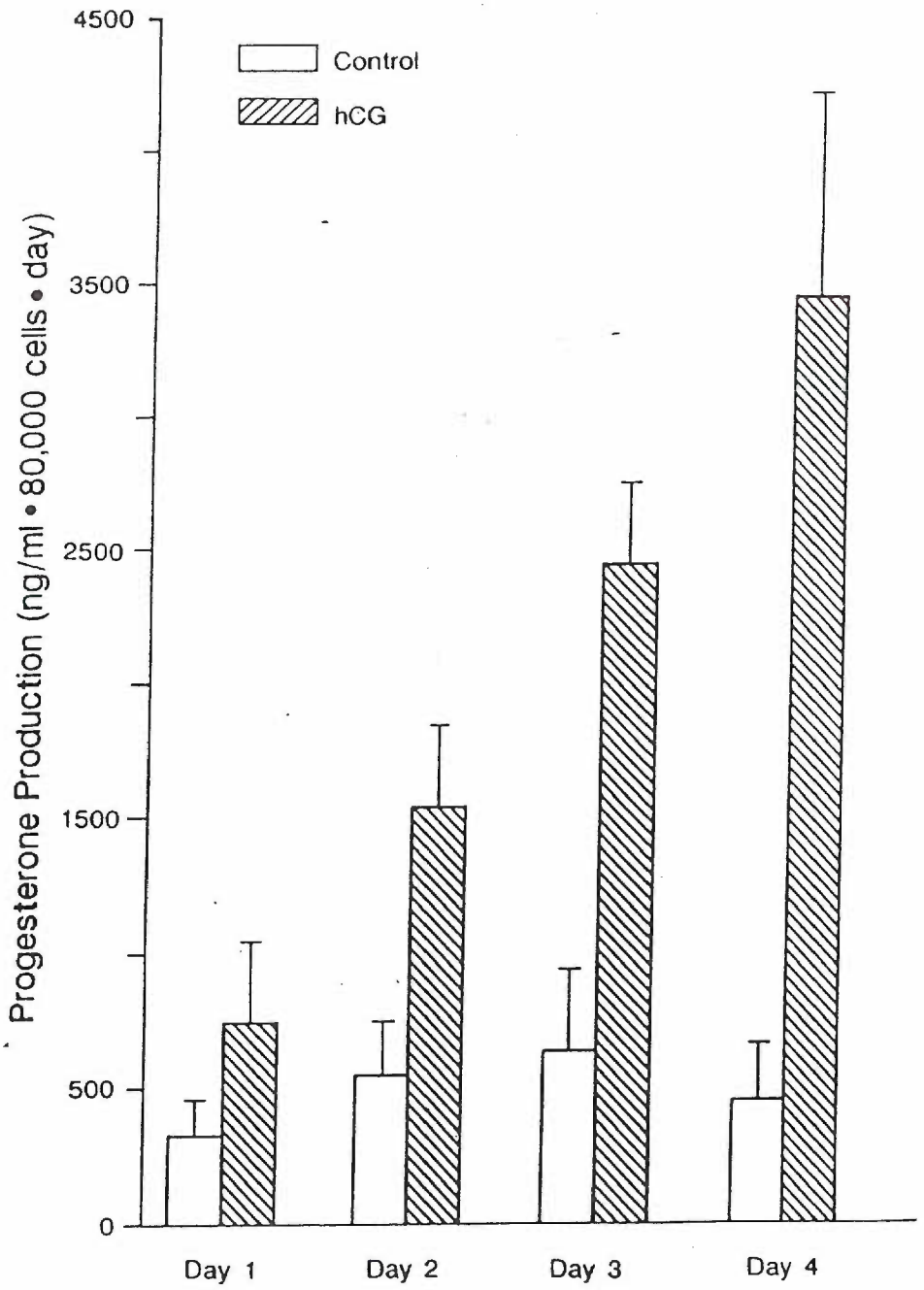
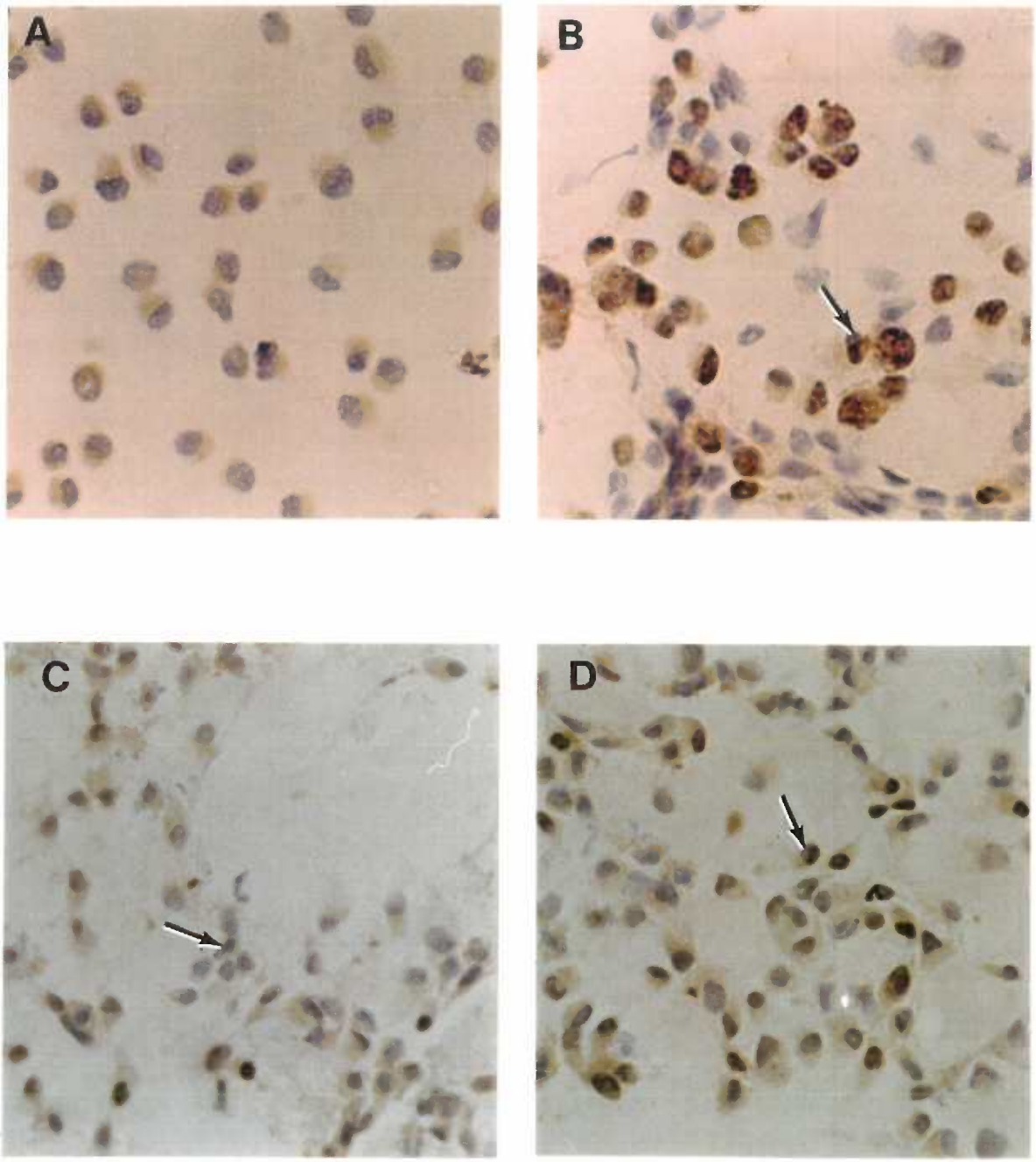


Fig. 4: P production by luteinized granulosa cells cultured for 4 days with or without hCG (100 ng/ml). P production was maintained at a similar level under control conditions during 4 days of culture. Throughout culture, hCG stimulated P production over control levels ($p < 0.05$). Values are the mean ± SEM of 4 experiments.

Fig. 5: Immunocytochemical staining for PR in luteinized granulosa cells on the day of follicle aspiration (A, B). Cells from 1 monkey are also shown following 1 day of culture in the absence (C) and presence (D) of hCG. Aliquots of cells were incubated with AT (A) or anti-PR antibody (B-D). Nuclear PR staining was observed in the majority of cells on the day of follicle aspiration (B). The percentage of cells staining positively for PR decreased following 1 day of culture, but the decline was less in cells exposed to hCG (D) than in cells cultured under control conditions (C).


A
B
C
D

(Park and Mayo, 1991). Recent *in vitro* data also support a role for LH in the induction of PR mRNA in porcine granulosa cells (Iwai *et al.*, 1991b). However, it is not clear whether this transcription activity is translated into the expression of PR in these species.

A possible role for surge levels of FSH in PR induction in granulosa cells cannot be excluded by the present study. It is known that the preovulatory FSH surge is important for follicular development and maturation in some species (Erickson, 1986). In contrast, the role for FSH in ovulation has been thought to be minimal. However, a recent study showed that recombinant FSH can induce ovulatory responses in rats (Galway *et al.*, 1990). Taken together, these observations do not rule out a role for either gonadotropin (LH or FSH) in the induction of PR of the periovulatory follicle.

Nevertheless, the time course of PR expression and its correlation with P production *in vitro* differs from that observed *in vivo*. PR expression was detected in granulosa cells *in vivo* within 27 h following administration of an ovulatory dose of hCG to monkeys undergoing ovarian stimulation protocols; at this time point P levels were also increasing. In contrast, gonadotropin treatment *in vitro* induced PR expression in granulosa cells within four days at a time when P secretion was declining. As discussed later, the culture conditions in the present study may not be optimal for granulosa cell survivability and could account for the differences observed. However, it is possible that other factors that are absent or diluted in the culture system may be required for the optimal induction of PR. In uterine cells, the expression of PR is modulated by multiple factors including P and growth factors (Aronica and Katzenellenbogen, 1991). This study provides evidence of a novel role for another local factor, i.e., PGs, in the induction of PR expression in primate granulosa cells. PGs are proposed mediators of LH

action in ovulation in rodents (Le Maire *et al.*, 1987). Human granulosa cells produce PGs (Feldman *et al.*, 1986), supporting a role for cyclooxygenase products in the periovulatory interval. However, the integration of LH/hCG and PGE_2 action in PR expression awaits further study.

This study extends the concept of LH regulation to include the maintenance of PR expression. PR levels in luteinizing granulosa cells declined *in vitro*, but the decline was partially prevented by LH/hCG. This suggests that the expression of PR is not constitutive, but is regulated at least in part by LH. The decrease in granulosa cell PR expression occurred while P production continued to increase over 4 days in culture. One action of P in the uterus is to downregulate the expression of PR. Greenberg and colleagues (1990) did not see any effects of RU486, an antiprogestin, on PR levels in human luteinizing granulosa cells during a week of culture. Similar studies in porcine (Iwai *et al.*, 1991b) and rabbit (Iwai *et al.*, 1991a) granulosa cells suggested no effect of P on PR mRNA and protein expression, respectively. However, it is not clear if the RU486 used in these studies was effective against high levels of P generated in the culture media. The effect of P on PR expression in monkey granulosa cells remains to be tested.

Since gonadotropins and PGE_2 activate adenylate cyclase and stimulate cAMP-dependent pathways in the ovary, including macaque luteal tissue, their promotion of PR expression may be cAMP-mediated. However, the effect of db-cAMP on PR expression in this study was less than that by hCG or FSH, whereas the effect of PGE_2 was the greatest. Similarly, db-cAMP induced lower levels of PR mRNA in porcine granulosa cells than did LH (Iwai *et al.*, 1991b). Thus cAMP may partially mediate the actions of gonadotropins and PGs. However, the involvement of other signal transduction pathways in PR expression cannot be ruled out. Recent

observations suggest that some of the periovulatory actions of LH/hCG in rat granulosa cells are mediated by cAMP-independent mechanisms, e.g., activation of tyrosine kinases (Morris and Richards, 1992). The mechanisms whereby gonadotropins and PGs influence PR expression await clarification.

Several factors regarding the cell culture system need to be considered when evaluating the results obtained in this study. First, a decrease in cell numbers was observed over time in culture. The decline in cell numbers was not prevented even though glass slides coated with Cell-Tak, which purportedly enhances cell attachment, were used. Second, the granulosa cells were cultured in the presence of serum which may contain factors that inhibit (Greco and Gorski, 1989) or stimulate (Katzenellenbogen and Norman, 1989) PR concentrations. Third, the variability in granulosa cell response may be due to follicle heterogeneity or the presence of cell subpopulations. The stimulation protocols used to obtain granulosa cells for these studies result in the development of multiple follicles of varying maturity. This is consistent with the observation that the majority of luteinized granulosa cells, but not all, stain positively for PR on day 0. However, not all luteal cells in the macaque corpus luteum stained positively for PR (Hild-Petito *et al.*, 1988). Finally, the disruption of cellular interactions that may occur *in vivo*, such as that between theca and granulosa cells, needs to be considered. Estrogen-dependent intercellular interactions have been proposed in the female monkey genital tract where ER is present only in stromal cells, while the adjacent epithelium shows estrogen-dependent effects (Brenner *et al.*, 1990). Thus, the cell culture system, though valuable for preliminary observations was probably not optimal for evaluating PR expression.

In summary, although the culture conditions may not have been

optimal for examining steroid receptor induction, this study provides the first evidence that *in vitro* exposure of nonluteinized granulosa cells to surge levels of gonadotropins can stimulate PR expression. However, the effect of varying gonadotropin levels on PR stimulation *in vitro* remains to be tested. Evidence is also provided for PGE_2 action in PR expression in nonluteinized granulosa cells. Also, the presence of LH/hCG may assist in the maintenance of PR in luteinized granulosa cells during culture. Taken together, our findings show that gonadotropins can act directly on macaque granulosa cells to stimulate PR expression. However, the integration of LH/hCG and PGE_2 action in PR expression awaits further study.

CHAPTER 7

DISCUSSION AND CONCLUSIONS

The research presented provides direct evidence that an ovulatory dose of hCG in stimulated cycles induces progestin receptor expression in granulosa cells during early luteinization in rhesus monkeys. Earlier studies in this laboratory (Hild-Petito *et al.*, 1988) suggested that progestin receptors first appeared in granulosa cells of the dominant follicle in monkeys during the periovulatory interval in spontaneous menstrual cycles. The current study showed that progestin receptor staining was not detected in granulosa cells isolated from monkeys receiving no ovulatory stimulus after ovarian stimulation. In contrast, many granulosa cells from animals receiving hCG as an ovulatory stimulus showed intense progestin receptor staining within 27 hours of the hCG injection and prior to ovulation. Specific nuclear staining for progestin receptors was also detected immunocytochemically in human granulosa cells obtained from *in vitro* fertilization protocols (Greenberg *et al.*, 1990). Similarly, *in vivo* expression of progestin receptor in human granulosa cells of ovulatory follicles following the LH surge was reported recently (Iwai *et al.*, 1990). Taken together, these observations are consistent with a previously unrecognized role for the gonadotropin surge to stimulate progestin receptors in primate luteinizing granulosa cells during natural and artificial menstrual cycles.

This is the first attempt to titrate LH surge requirements for progestin receptor expression and luteinization of granulosa cells in primate follicles. GnRH-1 treatment after follicular stimulation elicited endogenous LH surge levels (>100 ng/ml) that were maintained for 4 hours (Zelinski-Wooten *et al.*,

1991). GnRH-3 and GnRHa treatment extended the duration of LH surge levels to 8 and 14 hours respectively (Zelinski-Wooten *et al.*, 1991), while 24 and 48 hour durations were elicited using recombinant or pituitary LH injections. The data indicate that surges of up to 14 hours were typically unable to induce progestin receptor expression, with cells from these groups responding similarly to the group receiving no ovulatory stimulus. Similarly, *in vitro* progesterone production by granulosa cells from monkeys exposed to LH surges of 4-14 hours was not comparable to that by cells exposed to LH surge levels for > 18 -24 hours. Thus LH surge durations comparable to those in rodents and domestic animals were not sufficient for granulosa cell progesterone receptor expression or luteinization. LH surges of up to 24 hours induced reinitiation of oocyte meiosis and granulosa cell luteinization, but failed to support corpus luteum function. Increasing the duration of the LH surge to that in spontaneous cycles (48 hours) further promoted the development and function of corpora lutea. Thus exposure to LH surge levels required for ovulatory events differs in that 18-24 hours is sufficient for progestin receptor expression in granulosa cells, whereas optimal luteinization and full corpus luteum development of these cells may require exposure of greater than 24 hours.

This is the first study to examine the expression of both progestin and estrogen receptor messenger RNA in the primate ovary. Estrogen receptor mRNA was detected in germinal epithelium-enriched cortical compartments of the macaque ovary, but not in granulosa cells of pre- and periovulatory follicles nor in corpora lutea at any stage of the luteal phase. In contrast, progestin receptor mRNA was detected in cortical sections of the ovary, in granulosa cells of periovulatory follicles, and in corpora lutea from early, mid, and late luteal phase of the menstrual cycle. This is consistent with

immunocytochemical data on the distribution of estrogen and progestin receptors in the monkey ovary (Hild-Petito *et al.*, 1988). Thus there is strong evidence for the presence of progestin, but not estrogen, receptors in the follicle and corpus luteum of the primate ovary. Recent studies in the rat and the pig provide evidence for the involvement of LH in progestin receptor mRNA expression in granulosa cells (Park and Mayo, 1991; Iwai *et al.*, 1991). It remains to be determined whether LH action is to stabilize PR levels by preventing degradation of the receptor or its mRNA, or alternatively, to play a role in transcription. Taken together, the data are consistent with receptor-mediated actions for progesterone, but not estradiol, within luteinizing granulosa cells of the periovulatory follicle and the corpus luteum in primates.

Finally, initial experiments indicate that LH can act directly on granulosa cells in *vitro* to promote progestin receptor expression. A possible role for FSH in the induction of progestin receptors is also supported by this study. Although the culture conditions were not optimal for examining steroid receptor induction, the study provides the first evidence that *in vitro* exposure of nonluteinized primate granulosa cells to surge levels of gonadotropins can stimulate progestin receptor expression. Evidence is also provided for PGE_2 action in progestin receptor expression in nonluteinized granulosa cells. However, the integration of LH/hCG and PGE_2 action in PR expression awaits further study. The effect of db-cAMP on progestin receptor expression was less than that by hCG or FSH, and PGE_2 thus suggesting that cAMP may only partially mediate the effects of these substances. In addition, LH/hCG may assist in the maintenance of progestin receptors in luteinized granulosa cells during culture.

In summary, the current study provides direct evidence for the

hypothesis that the gonadotropin surge induces progestin receptor expression in granulosa cells of primates. Furthermore, it supports the concept that LH has a direct action in inducing progestin receptors in granulosa cells and that this action may be mediated by prostaglandins. New information is provided for the presence or absence of progestin and estrogen receptor mRNAs in compartments of the primate ovary. Finally, important data concerning the duration of the LH surge required for primate granulosa cell progestin receptor expression and luteinization is provided.

APPENDIX

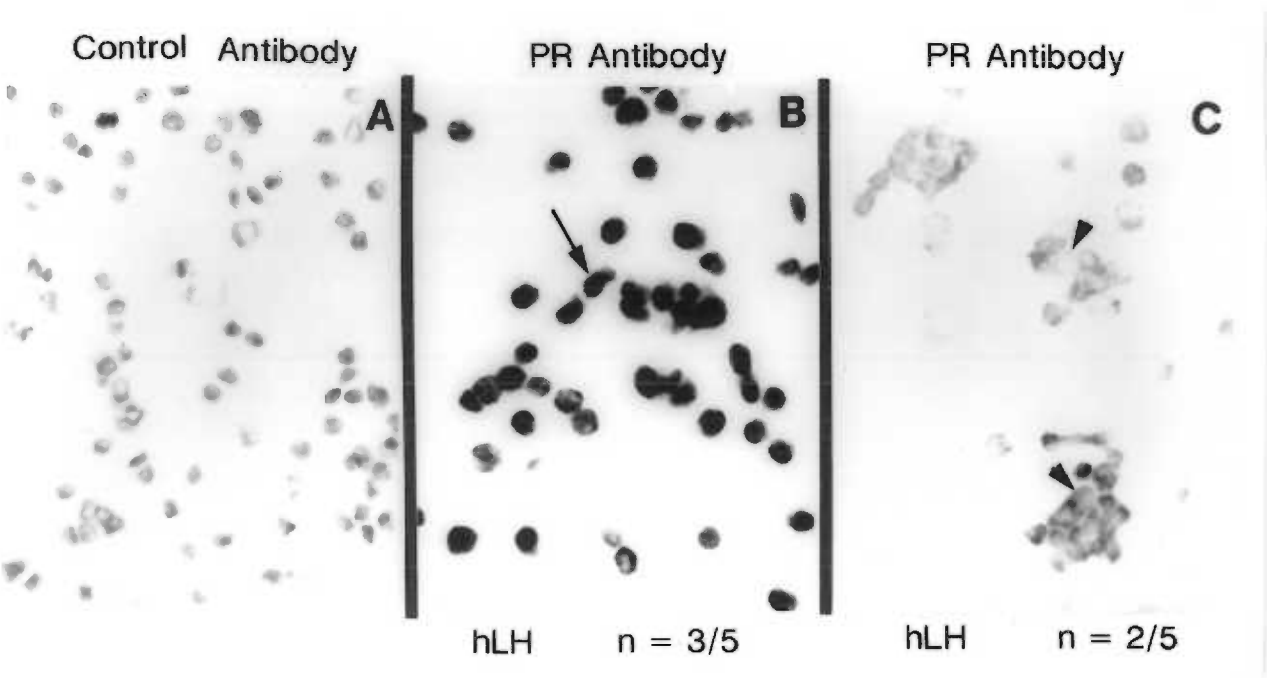


Fig. 1. Immunocytochemical staining for PR in luteinizing granulosa cells collected from 5 monkeys 27 h after urinary hLH injection for ovulatory maturation after multiple follicular development. Cells were incubated with a control antibody (A) or antihuman PR (B,C). Intense nuclear staining (B; DAB precipitate appears black, arrow) was evident in three animals receiving hLH. Cells from the other two hLH treated animals (C) did not exhibit nuclear staining (arrowheads). Cytoplasmic staining was nonspecific, because it was present when cells were incubated with either antibody. Initial magnification, X400.

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