REGULATION OF THE RAT OVARIAN PLASMINOGEN ACTIVATOR SYSTEM DURING FOLLICULAR DEVELOPMENT

by

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I. ABSTRACT

Successful ovarian follicular growth and ovulation require controlled and directional proteolytic activity for cell migration and follicular wall rupture. Plasminogen activator (PA) system is known to be involved in tissue remodelling during various physiologic and pathologic processes. PA system comprises two activators (tissue-type, tPA and urokinase-type, uPA), three inhibitors (PAI-1, PAI-2 and protein nexin-I) and one receptor (uPAR). *In vivo* expression of the PA system during follicular development was studied in the immature female rat. Total RNA was isolated from granulosa and theca-interstitial preparations of preantral and early antral [diethylstilbestrol- (DES) treated rats, undifferentiated granulosa cells], mid and late antral [equine chorionic gonadotropin- (eCG) treated rats; differentiated granulosa cells] and preovulatory [eCG + human chorionic gonadotropin- (hCG) treated rats; luteinized granulosa cells] follicles and mRNA abundance for tPA, uPA, PAI-1 and uPAR was determined by Northern blot analysis. In addition, the presence of these proteins in various follicular cells at different stages of follicular development was immunolocalized on ovarian sections with specific antibodies. Whereas tPA, PAI and uPAR mRNA expression and protein distribution increased in both granulosa and theca-interstitial layers during follicular maturation and reached maximum levels during the preovulatory period, the opposite was true for uPA. Urokinase PA was highly expressed at early stage of development and markedly decreased prior to the expected time of ovulation. To examine the regulation of the PA system at the early stage of follicular maturation, granulosa cells from DES- and eCG-treated immature rats were cultured with and without follicle stimulating hormone (FSH) and in the absence and presence of a differentiating [growth hormone releasing factor (GRF) and vasoactive intestinal peptide (VIP)], mitogenic [transforming growth factor alpha

and beta (TGFα and TGFβ)] or anti-differentiating [tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β)] factors. Net PA activity, specific PA type and PAI activity were determined by established fibrinolysis, fibrin overlay and reverse fibrin overlay assays, respectively. Net secreted PA (PAs) and cell associated PA (PAc) activities were higher in differentiated cells and PAs accounted for 70-80% of the total PA activity in both cell stages of follicular development. Whereas both uPA and tPA appeared to be present in cultures of granulosa cells from DES-treated rats, only tPA could be detected in cell cultures from eCG-treated animals. FSH stimulated net PA activities in concentration-dependent manner and these activities increased in undifferentiated cells and decreased in differentiated ones with the duration of culture. Both GRF and VIP stimulated PA activities in concentration-dependent fashion after 24 h of culture. Although both peptides enhanced FSH-induced net PA activities in undifferentiated granulosa cells, only GRF was effective in differentiated ones. The stimulation of PA activities by these agonists decreased with increased incubation period. TGFα, but not TGFβ, stimulated PA activities in concentration-dependent manner. A biphasic effect (stimulatory in the first 24 h and inhibitory thereafter) of TGFα on FSH-induced PA activities was observed in cultures of undifferentiated granulosa cells. Irrespective of the presence of gonadotropin, TGFα inhibited uPA activity but stimulated tPA activity in cultures of undifferentiated granulosa cells. Both TNFα and IL-1β attenuated FSH-induced net PA activities in a concentration-dependent fashion. In the presence of FSH, TNFα inhibited tPA but stimulated uPA activity. IL-1β suppressed the activity of both PA types. The suppressive effect of cytokines on FSH-induced PAs activity was associated with an increase in PAI activity in 72 h cultures of granulosa cells from the two stages of follicular maturation. The action of cytokines on PAI activity was more pronounced in the

differentiated cells, with IL-1β being more effective than TNFα. Whereas GRF and VIP stimulated basal progesterone (P) and 20α-dihydroprogesterone (20α-OH-P) secretion in both cell preparations, they only enhanced FSH-induced progestin production in undifferentiated cells. TGFα was more effective in stimulating basal progestin production in differentiated granulosa cells. Although FSH-induced total progestin (P + 20α-OH-P) secretion was not affected by TGFα, FSH-stimulated P production was suppressed by the growth factor in cultures of undifferentiated granulosa cells. Both TNFα and IL-1β attenuated FSH-induced granulosa cell progestin secretion independent of the stages of follicular maturation. Whereas GRF, but not VIP, stimulated [^{3}H]thymidine incorporation in cultures of granulosa cells from preantral and small antral follicles, this proliferative effect was significantly suppressed in the presence of gonadotropin. TGFα induced [^{3}H]thymidine incorporation in undifferentiated but not differentiated granulosa cells cultures. While FSH inhibited DNA synthesis in both cell preparations in the presence of TGFα, the antimitogenic effect of the gonadotropin was attenuated by TNFα and IL-1β. Our results suggest that 1) a coordinated expression of ovarian PA system exists during follicular development when granulosa cells undergo transformation from undifferentiated and proliferatively active cells to highly differentiated but mitogenically relatively quiescent ones, and 2) in addition to gonadotropins, several intra-ovarian factors play an important role in the regulation of the ovarian PA system during follicular development.

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III. LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic 3',5' adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DEPC	Diethylpyrocarbonate
DES	Diethylstilbestrol
20α-OH-P	20α-dihydroprogesterone
eCG	Equine chorionic gonadotropin
EGF	Epidermal growth factor
E_2	Estradiol
FSH	Follicle stimulating hormone
GH	Growth hormone
GRF	Growth hormone releasing factor
GTC	Guanidine thiocyanate
hCG	Human chorionic gonadotropin
huPA	Human urokinase plasminogen activator
3β-HSD	3β-hydroxysteroid dehydrogenase
IGF	Insulin-like growth factor
IgG	Immunoglobulin-G

IL-1	Interleukin-1
Kb	Kilobase
K_d	Dissociation constant
Kda	Kilodalton
LH	Luteinizing hormone
LDL	Low density lipoprotein
M199	Medium 199
MEM	Minimum essential medium
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered-saline
PBSG	Phosphate buffered-saline with gelatine
P	Progesterone
P-450scc	Cholesterol side chain cleavage
PA	Plasminogen activator
PAc	Cell-associated plasminogen activator
PAs	Secreted plasminogen activator
PAI-1	Plasminogen activator inhibitor-1
PAGE	Polyacrylamide gel electrophoresis
PGE	Prostaglandin E
PGF2α	Prostaglandin F2α
PN I	Protease nexin I
RNA	Ribonucleic acid

scuPA	Single-chain urokinase plasminogen activator
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
tPA	Tissue-type plasminogen activator
TBS	Tris buffered-saline
[^{3}H]	Tritium
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
tcuPA	Two-chain urokinase plasminogen activator
UV	Ultraviolet
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase receptor
VIP	Vasoactive intestinal peptide

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VII. INTRODUCTION

In the maintenance of life, nature has developed a unique form of preservation, evolution, a process of selecting the best adapted individual to complete the life cycle: birth, growth, reproduction and death. Complex systems to defend, nourish and procreate life have been established to accomplish this goal. Of these, the reproductive system assures the continuation of the species and the ovary, a part of this system, assumes the primary responsibility for the production and release of mature fertilizable oocytes. This process is repeated during each reproductive cycle and involves a series of sequential steps that includes follicular cell proliferation and differentiation, as well as oocyte maturation, resulting ultimately in the formation of a mature follicle and ovulation.

In the past, control of oocyte production has been viewed exclusively as endocrinological. More recently attention has focused on the interactions of endocrine systems with intraovarian factors in the regulation of follicular development (Adashi, 1992a). Many soluble factors are known to be produced within the follicles and to influence the proliferative and differentiated functions of granulosa cells. These include steroid hormones, neurotransmitter molecules, prostaglandins, lipoproteins, peptide hormones, growth factors and cytokines.

In addition, evidence for extensive communication between the immune, endocrine and reproductive systems has been accumulating (Adashi, 1992b). Communication between these systems occurs within the microenvironment of the ovarian follicle and is bidirectional (Adashi, 1992b). Autocrine and paracrine interactions between the immune system and the ovary are mediated by cytokines (Adashi, 1989). Studies have demonstrated that cytokines

are produced by both immunological and endocrinological cells present in the ovary including resident ovarian macrophages, T lymphocytes and mast cells (Adashi, 1989). Thus, it is the integration of multiple regulatory influences which mediates the balance between survival, proliferation and differentiation of granulosa cells and therefore maturation of the follicle and ovulation.

The ovarian stroma forms a framework of connective tissue that determines ovarian shape and holds the ovary together. This stroma is composed of collagen, elastin, fibronectin and glycoproteins (Parr, 1974). The structural changes occurring during follicular growth and ovulation require flexibility of the ovarian stroma which involves the degradation of matrix molecules. As the follicle matures the surrounding stroma must be remodelled to give room to the growing follicle. Several lines of investigation have provided strong evidence that serine protease plasminogen activator (PA) is involved in lysis and weakening of the follicular wall, therefore permitting follicular expansion during growth and rupture during the ovulatory process (Beers, 1975; Espey, 1990; Lafrance et al., 1993a). The granulosa cell layer is known to synthesize and secrete PA (Ny et al., 1985). Although follicle stimulating hormone (FSH) and intraovarian factors have been demonstrated to play an important role in the regulation of granulosa cell PA activity (Knecht , 1986; Galway et al., 1989), the interactions between the gonadotropin and components of the follicular microenvironment in the control of granulosa cell PA system during follicular development and particularly in relation to their proliferative and differentiative functions, remain unclear. The present investigation examines the action(s) and interaction(s) of gonadotropins and intraovarian factors in the regulation of the PA system during granulosa cell proliferation and differentiation.

VIII. LITERATURE REVIEW

A. Development of the ovary

The earliest sign of an embryonic ovary appears on the ventral surface of the embryonic kidney. The coelomic epithelium is thickened and clumps of cells bud off into the underlying mesenchyme, called the germinal epithelium. The primordial germ cells migrate from the yolk sac to enter the germinal epithelium and continue to divide rapidly, resulting in the development of genital ridge. The germinal epithelium continues to proliferate for a longer time . Connective tissue develops between this epithelium to form the sex cords and later define the cortex and the medulla (Baker, 1963; Witchi, 1948). These primordial germ cells, also known as oogonia, depart the mitotic cycle to initiate the nuclear changes characteristic of prophase of the first meiotic division. These changes mark the conversion of these cells to primary oocytes. Those oogonia which do not enter the meiotic division will be subjected to oogonial death (Baker and Franchi, 1967; Ohno et al., 1962; Peters, 1976). Once formed, the primary oocyte persists in the prophase of the first meiotic division until the time of ovulation. During cell growth, many oocytes undergo degeneration, both before and after birth. The primary oocytes soon become surrounded by a single layer of flattened follicle cells that are originally derived from the germinal epithelium. These structures are now called primordial follicles and are first seen in the medulla and later in the cortex. From midgestation onward, relentless and irreversible attrition progressively diminishes the germ cell endowment of the gonad. For the most part, this oocytic depletion is accomplished through follicular (rather than oogonial) atresia and continues throughout life (Franchi et al.,1962).

B. Anatomy of the ovary

The ovaries are almond-shaped nodular organs, situated in the upper part of the pelvic cavity and rest in a slight depression on the lateral wall of the pelvis. The margin of the ovary attached to the broad ligament by the mesovarium is almost straight and designated as the hilium. The blood and nerve supply of the ovary enter through the hilium. The general structure of the ovary can be studied best in cross sections, in which two portions may be distinguished, the cortex and the medulla. The cortex, or outer layer, is composed of spindle-shaped connective tissue cells and fibers, among which there are scattered follicles that are at various stages of development. The outermost portion of the cortex, which is dull and whitish, is designated as tunica albuginea; on its surface, there is a single layer of cuboidal epithelium, the germinal or surface epithelium.

The medulla, or the central portion of the ovary is composed of loose connective tissue that is continuous with that of the mesovarium. There are a large number of arteries and veins in the medulla and a small number of smooth muscle fibers that may be functional in the movements of the ovary (Warwick and Williams, 1978).

The ovarian artery is the chief source of blood to the ovary. Though both left and right ovarian arteries originate from the abdominal aorta, the left one frequently arises from the left renal artery. The ovarian artery sends branches through the mesovarium to the ovary. An additional blood supply is formed from the anastomosis with the ovarian branch of the uterine artery (Moore, 1980).The ovarian veins leave the hila of the ovaries and form a leash of vessels, called the pampiniform plexus, in the broad ligament. Whereas the right ovarian vein drains into the inferior vena cava, the left drains into the left renal vein (Moore, 1980).

The lymph vessels of the ovary first appear in the ovarian cortex and then spread to the medulla. These vessels leave the ovary at the hilium and ascend with the ovarian vein to the lumbar lymph nodes (Goldfein and Scott, 1986; Moore, 1980).

Both sympathetic and parasympathetic nerves supply the ovary. While the sympathetic innervation is derived from the ovarian plexus that accompanies the ovarian vessel, the parasympathetic innervation is derived from the vagus nerve (Lawrence and Burden, 1980; Jakobowitz and Wallach, 1987). Other neurotransmitters, such as vasoactive intestinal peptide (VIP; Larson et al., 1977) and neuropeptide Y (McDonald et al., 1987) have also been detected in the ovary.

C. Microscopic structure of the ovary

1. The oocyte-granulosa cell compartment

The primordial follicle is comprised of an oocyte surrounded by one layer of follicle (pregranulosa) cells (Mossman and Duke, 1973). When the primordial follicle enters the stage of follicle growth, the oocyte increases in volume while the granulosa cells proliferate. This results in the formation of multi-layers of granulosa cells (Bjersing, 1978; Richards, 1980). The distinguishing structures of the oocyte-cumulus complexes are as follows: the surrounding corona radiata, the zona pellucida, the plasma membrane, the perivitelline space, a small clear zone of protoplasm, a broad finely granulated zone of cytotoplasm, and the nucleus, or germinal vesicle (Franchi et al., 1962). The oocyte and the associated granulosa cells are separated from the surrounding stroma by a membrane called the "basal lamina" (Weakly, 1966). The granulosa cells are devoid of direct vascular input and are interconnected by gap junctions, resulting in electrical coupling and an expanded integrated

and functional syncytium (Albertini and Anderson, 1974; Amsterdam et al., 1976; Amsterdam et al., 1981). These cells extend cytoplasmic processes to form gap junction-like union with the plasma membrane of the oocyte. These cellular connections regulate the exchange and transport of nutrient and metabolic products between cells and are believed to control the resumption of meiosis in the oocyte. As the follicle continues to mature to form the Graafian follicle, the oocyte attaches to one pole of the follicle. At least three populations of granulosa cells are established: the mural granulosa cells (attached to the basement membrane) rich in steroidogenic enzymes (Zoller and Weisz, 1979 a & b) and LH receptors (Channing et al., 1981; Lawrence et al., 1980), the antral granulosa cells (closest to the antral cavity) and the cumulus cells (surrounding the oocyte) with low steroidogenic activity (Zoller and Weisz, 1979 a & b).

2. Theca-interstitial cells

The cells making up the theca-interstitial compartment are heterologous in nature. In the early stages of follicular development, the theca cells are indistinguishable from fibroblasts. The stromal tissue surrounding the follicle then starts to organize to form the theca layers. The theca cells closest to the basement membrane differentiate into steroid-producing cells referred to as theca interna. The outer layer, called theca externa, remains fibroblast-like and interspersed in a network of collagen and blood vessels (Erickson et al., 1985).

3. Resident ovarian white blood cells

Unlike some gonadal compartments (e.g., the testicular seminiferous tubule), the

ovary does not constitute an immunologically privileged site. Thus, resident ovarian (extravascular) macrophages, lymphocytes and granulocytes can be observed at various stages of the ovarian cycle (Adashi, 1992a, b). Macrophages constitute a major cellular component of the interstitial compartment (Hume et al., 1984). Except for macrophages, few if any other white blood cells have been observed in early phases of follicular growth. However, this situation is altered as preovulatory events (or atresia) prompt massive ovarian infiltration by several different white blood cells (Parr, 1974; Jones et al., 1980; Kirshna et al., 1988). Although the mechanisms by which these resident white blood cells influence ovarian function remain uncertain, it is presumed that they elaborate regulatory molecules which exert profound modulatory influence on the growth and functional development of the ovarian follicle (Takemura and Werb, 1984; Adashi, 1989).

4. Ovarian stroma

The ovarian stroma forms a framework of connective tissue that determines ovarian shape and holds the ovary together. This stroma is composed of interstitial collagens, elastin, proteoglycans, fibronectin and glycoproteins. Of these proteins, the fibrous protein collagen is the major structural element that provides tensile strength and anchors the developing follicles (Woessner, 1982; Bagavandoss et al., 1983; Bortolussi et al., 1989). Thus, the structural changes occurring during follicle growth, ovulation, atresia and corpus luteum formation, require flexibility and remodelling of the ovarian stroma to give room for the growing follicle.

D. Ovarian cycle

The ovarian cycle is a continuous repetitive process of follicle maturation, ovulation and corpus luteum formation and regression. An orderly sequence of events takes place that ensures that a proper number of follicles are ready for ovulation. A series of sequential actions of hormones and growth factors on the follicle lead the follicle destined to ovulate through a period of growth from primordial follicle to the preantral, antral and preovulatory stages. Ovulation occurs as a result of the rupture of the follicular wall and release of the ovum. A corpus luteum will be formed and later degenerate unless pregnancy intervenes.

1. Follicular phase

a. Primary follicles

The primordial follicle consists of an oocyte and a single layer of flattened granulosa cells (Peters et al., 1975). In the presence of an as yet unknown stimulus, a cohort of primordial follicles are recruited to enter the growth trajectory, a process whereby the follicle departs from the resting phase to begin a well-characterized pattern of growth and development. The mechanism responsible for recruiting these primordial follicles (and yet not others) remains a mystery. The spindle-shaped granulosa cells of the primordial follicle differentiate into a single layer of cuboidal cells, thereby yielding primary follicles. These cells start to secrete around the oocyte mucopolysaccharides referred to as the zona pellucida. Importantly, cytoplasmic processes of granulosa cells traverse the zona pellucida to maintain intimate gap junctional contact with the plasma membrane of the oocyte (Weakly, 1966). As specific FSH receptors appear on the plasma membrane of the granulosa cells of the primary follicles (Richards et al., 1976; Uilenbroek and Richards, 1979), they start to acquire

aromatase activity and to convert androgen produced by the theca cells (Erickson et al., 1985) into estrogen.

A morphologically distinct theca interna layer does not appear until the end of primary follicular stage. The theca externa is then formed as the follicle expands and compresses the surrounding stroma (Erickson, 1986; Freeman, 1988). The development of the thecal layer is associated with the acquisition of follicular blood supply represented by a network of capillaries adjacent to the basement membrane (O'Shea et al., 1980). The appearance of lymphatic drainage begins during the growth of the secondary follicle (O'Shea et al., 1980). As the capillaries are formed, the stromal (pre-theca) cells start to engage in concentric layering with increasing proportions proximal to the basal lamina. These cells, known as theca interna cells, enter the differentiative stage and accumulate cytoplasm and organelles for steroid hormone production as well as expresses luteinizing hormone (LH) receptors (Magoffin and Erickson, 1982; Midgley, 1973). The peripheral layer of these cells merges with the stromal cells, differentiates into smooth muscle cells and acquires sympathetic and parasympathetic innervation (Erickson et al., 1985; Bahr et al., 1974). These muscle cells are believed to play a role in ovulation (Lipner, 1973; Espey, 1978 a,b).

b. Secondary follicles

The presence of FSH receptors on the granulosa cell membrane confers receptivity to FSH and increases aromatase activity and estradiol (E_2) production in response to the action of the gonadotropin. LH binds to its receptor on theca cells and stimulates the production of androgen which diffuses through the basal lamina to the granulosa cells. The conversion of androgens to estrogens, coincides with the appearance of estrogen receptors

on the granulosa cells (Amsterdam and Rotmensch, 1987). Estrogen exerts an autocrine influence on granulosa cells to facilitate the action of FSH via induction of more FSH receptors and thus more aromatase activity, leading to greater estrogen secretion and an estrogenic microenvironment which is necessary for follicular growth (Ireland, 1987). FSH and estrogen also enhance the proliferative activity of granulosa cells, leading to the formation of multiple layers of these cells. Ultimately, with granulosa cell proliferation and differentiation, theca cell hypertrophy and differentiation, and the growth of the oocyte, the follicle increases in size and acquires the features of a secondary (preantral) follicle.

c. Antral follicles

After the formation of the preantral follicle, localized lacunae become apparent among the granulosa cells. These lacunae coalesce to form a central fluid-filled cavity, the antrum, a characteristic of the small antral (tertiary) follicle. In these follicles, the oocyte occupies an eccentric position, surrounded by two to three layers of granulosa cells. The antral fluid contains protein-bound and free steroid hormones, gonadotropins, plasma- and locally-derived-proteins, proteoglycans and electrolytes (Edwards, 1974). The plasma-derived proteins and gonadotropins reach the antrum by diffusing from the vascular space outside the basal lamina (Edwards, 1974). In contrast, locally-derived proteins, proteoglycans and steroids are secreted by granulosa cells, theca cells and/or the oocyte (Jensen and Zacharie, 1958).

d. Preovulatory follicles

During midfollicular phase, the dominant follicle(s) produces relatively more

estrogen than the other follicles in its cohort and is responsible for the increased estrogen level in the ovarian venous effluent. The high concentration of estrogen in the circulation inhibits FSH secretion by the pituitary (Fritz and Speroff, 1982). Whereas at the early stage of follicular development FSH induces its own receptor in granulosa cells, the opposite is true during the selection of the dominant follicle and when LH receptors are expressed in granulosa cells (LaPolt et al., 1992; Tilly et al., 1992a; Lee and Takahashi, 1977). The follicles not selected for dominance lose their sensitivity to FSH, exhibit a further decrease in aromatase activity and estrogen secretion as well as enhanced androgen accumulation, favoring an androgenic microenvironment which inhibits follicular growth (McNatty et al., 1980). Importantly, the development of the dominant follicle is associated with an increased ability of the theca cells to express LH receptors and bind LH compared with the non-dominant follicles (DiZerega et al., 1980; Zeleznik et al., 1981). Moreover, the vascularity of the theca layer of the dominant follicle is twice that of other follicles. This increase in vascularity leads to increased delivery of gonadotropins to the follicular cells. As such, the dominant follicle takes an active role in ensuring its preferred status.

As the concentration of LH increases in the circulation, a series of changes occurs in the ovarian microenvironment. Granulosa cells enlarge and acquire lipid inclusions (Gospodarowicz et al., 1977). The theca interna becomes vacuolated and richly vascular and the oocyte resumes meiosis (Tsafriri et al., 1982; Byskov, 1979; Dekel and Sherizly, 1985). The synergism exerted on granulosa cells by various endocrine, paracrine and autocrine factors stimulates an increased secretion of estrogen that peaks 24 h before ovulation (Pauerstein et al., 1978). Enhanced exposure to estrogen augments the appearance of LH receptors on granulosa cells and induces the surge of LH release from the pituitary gland

(March et al., 1981). LH promotes luteinization of granulosa cells in the dominant follicle and switches the production of steroids within the granulosa cells from estrogen to progesterone (Chikasawa et al., 1986). Progesterone facilitates the pituitary release of LH and enhances the formation of LH receptors in the granulosa layer (March et al., 1981; Liu and Yen, 1983). The LH surge produces a series of changes in the follicle that prepare it for ovulation: luteinization of granulosa cells, hypertrophy of theca cells, resumption of oocyte meiosis, increases in the volume of follicular fluid and permeability of basement membranes (blood vessels and follicular wall), giving the hyperemic appearance to the preovulatory follicle (Seow et al., 1988).

2. Ovulation

The tertiary or preovulatory follicle initiates its own ovulatory stimulus through increased elaboration of estradiol, which triggers the release of an LH surge from the anterior pituitary (March et al., 1981). Ovulation is a dynamic process and, depending on the mammalian species, occurs about 10-36 h from the time of LH surge to the moment of follicular rupture. This process includes a series of biochemical and morphological events within the preovulatory follicles, which leads to its ultimate rupture and discharge of the mature oocyte. The mature preovulatory follicle is embedded within the connective tissue matrix of the ovary and only a small part of it protrudes from the ovarian surface. At the follicular apex the following layers are distinguished: (i) a single layer of surface epithelium; (ii) collagenous connective tissue consisting of tunica albuginea and theca externa which contribute to the tensile strength of the follicle wall; (iii) theca interna, a well vascularized layer containing differentiated fibroblasts; (iv) granulosa cells, which are separated from the

theca interna by the basement membrane (Espey, 1967).

Several theories for the mechanism of ovulation have been put forward since the first scientific study of ovulation conducted by De Graaf in 1672. Although an increase in intrafollicular pressure has long been assumed to be the cause of rupture and extrusion of the oocyte-cumulus complex, direct measurement of intrafollicular pressure failed to demonstrate any significant increase in follicles approaching ovulation (Blandeau and Rumery, 1963; Espey and Lipner, 1963; Rondell, 1964; Bronson et al., 1979). However, levels of progesterone in follicular fluid continue to rise with a concomitant increase in follicular volume following the LH surge. This follicular expansion is accompanied by an increase in the distensibility and a decrease in the tensile strength of the follicular wall during the preovulatory period (Rondell, 1964).

The increased distensibility of the follicle wall is attributed to an increased production and action of proteolytic enzymes, such as PA and collagenase (Curry et al., 1985; Espey and Coons, 1976; Fukumoto et al., 1981; Beers et al., 1975). The demonstration of a preovulatory increase in PA (Reich et al., 1985a; Reich et al., 1986, Politis et al., 1990), the ability of plasmin to decrease the tensile strength of Graafian follicle wall (Beers, 1975) and the suppression of ovulatory rate by PA inhibitors and antibodies (Ichikawa et al., 1983; Tsafriri et al., 1989) have led to the suggestion that PA may be involved in the ovulatory process. In addition, the level of collagenase is significantly increased during the periovulatory period and is hormonally regulated (Reich et al., 1991). Moreover, the involvement of collagenases in follicular rupture was corroborated by the finding that inhibitors to these metalloproteinases suppress ovulatory rate in the rat (Reich et al., 1985b; Brannstrom et al., 1988). In view of these observations, the proteolytic enzymes are activated

in an orderly sequence. PA transforms plasminogen in the follicular fluid into plasmin which activates procollagenase. Both latter enzymes disrupt the connective tissue arrangement in the follicular wall and ovarian stroma.

In addition, there is strong evidence for an important role of prostaglandins in the ovulatory process (LeMaire et al., 1980). During this process, the endogenous levels of prostaglandins in the follicle increase markedly and this increase is inhibited by indomethacin and aspirin (prostaglandin synthesis inhibitors). Indomethacin inhibits LH-induced ovulation which is accompanied by entrapment of oocytes within the luteinized follicle (Wallach et al., 1975; Hamada et al., 1977; Maia et al., 1978; Killick and Elstein, 1987). The inhibitory action of indomethacin was reversed by exogenous prostaglandins (Brannstrom et al., 1987; Sogn et al., 1987). The precise role of prostaglandins in follicular rupture is still unresolved. These intrafollicular products may activate proteinases required in follicular rupture (Espey et al., 1981; Downs and Longo, 1982) and may play a role in the extrusion of the oocyte-cumulus cell mass by contraction of the smooth muscle found in the perifollicular stroma (Espey 1978a,b). On the other hand, leukotrienes have also been suggested to act on the perifollicular smooth muscle and regulate the extrusion of the oocyte-cumulus complex during the ovulatory process (Espey, 1990). However, the entire ovulatory process contributes to the damage and repair of the local tissue, a paradoxical state that is characteristic of an acute inflamed tissue. During the course of ovulation, principal elements suggestive of inflammatory-like reactions are observed. These include follicular hyperemia, angiogenesis, fibroblast mobilization, tissue degradation, hemorrhage, tissue remodelling and healing (Espey, 1980; Espey, 1990). These physical changes are usually mediated by many factors, of which PA, collagenase, histamine, kallikrein and prostaglandins appear to play

a central role (Lewis, 1977; Jones et al., 1980; Espey et al., 1986; Espey et al., 1989).

These studies suggest that the ovulatory surge of gonadotropins induces an inflammation-like reaction in the ovary and that the degradative events of this acute inflammatory response cause the follicle wall to degenerate, rupture and repair.

3. Corpus luteum formation and regression.

Before rupture of the follicle and the release of the ovum, marked and rapid changes occur during the luteinization of the follicular cells, including a marked reduction or cessation of cell division and an increase in cell size (Richards and Hedin, 1988). This cellular hypertrophy involves a multifold increase in the cytoplasmic volume of the cells as well as an increase in the number of intracellular organelles, such as mitochondria and smooth endoplasmic reticulum. A yellow lipid pigment, lutein, accumulates within the cell and lends its name to the process "luteinization" and the anatomical structure "corpus luteum" (Niswender and Nett, 1988). Following follicle rupture, fibroblasts from the surrounding stroma proliferate and capillaries penetrate the basal lamina. The rapid vascularization of the corpus luteum may be guided by angiogenic factors such as angiogenic growth factor and basic fibroblast growth factor which are readily detected in the follicular fluid (Gospodarowicz and Thakral, 1978; Frederick et al., 1984). This changes the microenvironment from the avascular granulosa cell layer to a well-vascularized corpus luteum. This increase in vascularisation allows direct contact of luteal cells with not only blood-borne nutrients such as low density lipoprotein (LDL) but also hormones, cytokines and growth factors that may affect the steroidogenic activity and survival of luteal cells. (Carr et al., 1981).

The corpus luteum does not contain a homogenous cell population. Besides luteal cells, endothelial cells, macrophages, pericytes and fibroblasts are also present in the corpus luteum. In addition, the luteal cells are also non-homogeneous. Two populations of luteal cells, designated small and large cells, have been identified on the basis of size, morphology and staining properties. Evidence for ultrastructural differences between the luteal cell types has recently been reviewed (Fields, 1991). The large cells contain more smooth and rough endoplasmic reticulum and mitochondria, extensive Golgi complexes and secretory granules than the small cells. The cellular characteristics are consistent with greater secretory (steroid and/or protein) activity in the large luteal cells. Although the origin of the two luteal cell populations is uncertain, it is believed that the large luteal cells are derived from granulosa cells and the small cells are of theca interna origin. Both cell types secrete androgen and estrogen and express 17α-hydroxylase and aromatase activity, enzymes solely expressed in the theca and granulosa cells, respectively. Thus, it is possible that luteinization may have caused the de novo expression of aromatase in the theca-derived luteal cells and that of 17α-hydroxylase in the granulosa-derived cells (Nelson et al., 1992; McLean et al., 1992).

Progesterone levels normally rise sharply after ovulation, reaching a peak approximately 8 days after the LH surge in humans. The increased blood supply to the corpus luteum provides increased transport of LDL-cholesterol, a substrate for progesterone production. One of the important functions of LH is the induction of LDL receptor expression on luteal cells and the regulation of LDL receptor binding, internalization and postreceptor processing for progesterone production (Golos et al., 1985; Brannian et al., 1992). In addition, the expression of P450 side-chain cleavage and 3β-hydroxysteroid dehydrogenase (enzymes responsible for progesterone production) are markedly increased

with luteinization (Ravindranath et al., 1992), thus providing the proper enzymatic machinery for higher progesterone production.

The life span and steroidogenic capacity of the corpus luteum are dependent on continued tonic LH secretion. Thus, the slow frequency of LH pulses occuring in the normal late luteal phase may contribute to the onset of luteolysis (Casper and Yen, 1979). As the corpus luteum becomes less sensitive to LH stimulation, LH receptor binding is decreased, parallelling the secretion pattern of progesterone. The mechanism by which LH receptor binding is decreased is unknown although cytokines, estradiol and prostaglandins are believed to be involved (O'Gray et al., 1972; Schoonmaker et al., 1981; Takahide, 1990).

4. Follicular atresia

The Greek word atresia (a = not; tresia = perforated) is usually used to describe the closure of a natural opening. In the context of ovarian physiology, follicular atresia denotes the enigmatic process whereby oocytes are lost from the ovary by means other than ovulation.

In the human ovary, two million oocytes are found at birth and about 400,000 follicles are present at the onset of puberty. However, only 400 follicles are normally ovulated during female reproductive life (Baker, 1963). While more than 99.9% of human follicles undergo degenerative changes, this process represents an integral part of the normal ovarian function. Thus, it is the "norm" for a follicle to degenerate rather than to ovulate. Ovulation may in fact constitute a rescue phenomenon requiring active intervention to prevent an otherwise inevitable demise. Indeed, it is unclear why nature established a generous germ cell endowment only to subject it to life-long decline.

a. Morphological and biochemical changes

Distinct morphological and biochemical characteristics of atretic follicles have been identified. These include the degeneration and detachment of the granulosa cell layer from the basement membrane (Junqueira et al., 1989), the presence of pyknotic nuclei in this cell type (Hay et al., 1976), reduction or absence of [^{3}H]thymidine incorporation into granulosa cells (Hirshfield and Midgley, 1978) and an increase in the secretion of progesterone with a concomitant decrease in estrogen production (Uilenbroek et al., 1980; Terranova, 1981). Given that atresia is the consequence of individual cellular components of the follicle dying, the mechanism underlying follicular demise is the nature of death in these cells.

b. Necrosis vs apoptosis

There are two pathways by which cells die: necrosis and apoptosis (Wyllie et al., 1980; Wyllie, 1981). These two models of cell death differ in morphology, mechanism and incidence. In general, tissue necrosis is a consequence of cell injury or other traumatization, characterized by a haphazard loss of cell structure, swelling as the result of permeability changes in the cell membrane, and ultimately cell rupture that can damage adjacent cells, leading to immune cell infiltration and inflammation. Necrosis does not occur in a developmental context, usually affects a group of contiguous cells, and involves no mRNA and protein synthesis. This "passive" cell death does not use cellular energy, and ATP levels are usually decreased in the dying cells. In contrast, apoptosis, originally known as shrinkage necrosis or programmed cell death (Kerr, 1971; Waring et al., 1991), is an active orderly event that takes place in tissues undergoing developmental changes or responding to alterations in physiological stimuli. This active "suicide" process takes place in cells with

normal ATP levels and, in some cases, requires mRNA and protein synthesis. Apoptosis affects scattered single cells and involves cell shrinkage, chromatin condensation, and formation of small apoptotic bodies, spherical bits of membrane containing nuclear fragments and organelles. Apoptotic cells are phagocytized by neighbouring cells. Because phagocytosis usually takes place before the integrity of the plasma membrane is lost, there is no leakage of cytoplasmic components and therefore, no inflammatory reaction is induced. A unique biochemical event in apoptosis, which precedes these morphological changes, is the activation of Ca^{++}/Mg^{++}-dependent endogenous endonucleases (Duke et al., 1983; Afanas'ev et al., 1986; Arends et al., 1990). This enzyme cleaves genomic DNA at internucleosomal regions resulting in fragments which, when separated by agarose gel electrophoresis, form a distinctive ladder pattern of 180-200 base pair DNA oligonucleosomal fragments. This form of DNA fragmentation is considered a biochemical hallmark of apoptosis.

c. Apoptosis of granulosa cells

On the basis of the above considerations and the observation that degenerative changes associated with atresia appear initially in the granulosa cell layer, it has been hypothesized that apoptosis is responsible for the demise of the granulosa cells and ultimately for follicular atresia (Zeleznik et al., 1989). Evidence supporting this hypothesis is provided by ultrastructural studies of granulosa cells which identify several morphological characteristics of apoptosis, including blebbing of the cytoplasm, condensation of chromatin and cellular fragmentation forming apoptotic bodies (Hay et al., 1976; O'Shea et al., 1978; Tsafriri and Braw, 1984). In addition, the presence of Ca^{++}/Mg^{++}-dependent endonuclease

activity has been detected in differentiated granulosa cells (Zeleznik et al., 1989; Boone et al., 1995). Moreover, the DNA degradation pattern of apoptosis has been observed in granulosa cells from atretic follicles but not in follicles undergoing rapid growth and development (Hughes and Gorospe, 1991) and is regulated by steroid hormones, gonadotropin releasing hormone (GnRH) and growth factors (Tilly et al., 1992b; Billig et al., 1993; Billig et al., 1994). These findings strongly suggest that apoptosis is associated with the process of follicular atresia and may provide the cellular mechanism for the induction and progression of this phenomenon.

E. Dynamics of follicular growth.

During follicular growth, less differentiated cells become committed to particular developmental pathways as they assemble into heterogeneous tissues incorporating several cell types. These tissues undergo coordinated growth and maturation to form a morphologically and functionally complex structure and the ultimate production of fully mature, preovulatory follicles. This event occurs as a result of three successive steps: recruitment (the initiation of primordial follicle growth which furnishes the ovary with a new cohort of developing follicles), basal follicular growth, and selection and maturation of the preovulatory follicles.

1. Onset of follicular growth

Some follicles begin to grow as soon as they are formed, although most spend months or years in the quiescent state. The first sign of growth is the resumption of cell proliferation by the squamous granulosa cells, which can be demonstrated by autoradiography following

[^{3}H]thymidine incorporation (Hirshfield, 1991). The rate of granulosa cell proliferation increases with increasing follicular size (Hage et al., 1978). Follicles are growing more rapidly at the time of antrum formation than at any other time during development (Hirshfield, 1991). The appearance of the antrum heralds the final phase of cell division, when cell proliferation ceases and functional differentiative features appear. Shortly after the follicle acquires a single large antral cavity, some of the granulosa cells that border the basement membrane (mural granulosa cells) withdraw from the cell cycle and no longer incorporate [^{3}H]thymidine (Hirshfield and Schmidt, 1985; Hirshfield, 1986). It has been estimated that granulosa cells which make up a rat preovulatory follicle are about ten generations of granulosa cells present in the primordial follicle from which they arose (Hirshfield, 1991). The time it takes for each follicle to grow from one size to another may alter substantially because the rate of granulosa cell proliferation is variable. Therefore the temporal feature of follicular growth takes a long time relative to the cycle length: several weeks in rodents, perhaps several month in larger animals (Chiras and Greenwald, 1977; Oakberg, 1979).

2. Clonal expansion

The mechanisms that regulate the morphogenic events during follicular growth are probably similar to those operating during cell renewal in other adult tissues (intestinal epithelium, epidermis, and blood) where differentiated, functional cells are continuously being shed and replaced by proliferation of more primitive cells. A hypothesis proposed for the fine coordination of growth and differentiation in these tissues may be applicable to ovarian follicular development (Hirshfield, 1991).

The "stem cell maturation gradient" hypothesis (Mackillop et al., 1983) states that adult renewable tissues are composed of a hierarchy of cells: at one end of the hierarchy are stem cells which are relatively undifferentiated and have limitless capacity for cell division; at the other end are "end-stage", highly differentiated, functional cells which have no capacity for proliferation. When a stem cell divides, some of its daughters embark on a course of clonal expansion leading irreversibly to terminal differentiation. Such cells are known as "transitional cells" or "committed progenitor cells". The commitment to terminal differentiation is a key event in follicular development.

Transitional cells have limited capacity for cell division. Although they still proliferate (and do so more rapidly than their stem cell parents), committed progenitor cells can continue to divide for only a finite number of subsequent generations, undergoing limited clonal expansion as a component of their program of differentiation (Mackillop et al., 1983). With each successive generation, the proliferative potential of descendant daughter cells becomes further diminished. In reciprocal fashion, as the daughter cells lose their proliferative ability, they become more and more differentiated. By the time granulosa and theca cells are recognizable as distinct morphological components of the follicles, their developmental fate appears to be irreversibly preprogrammed. Only two alternative outcomes are possible: they can differentiate along their pathways or they can die. Thus, the stem cell must receive a specific signal telling it in which direction to differentiate (Gurdon, 1987). None of these critical signals for the follicle has been identified although FSH and estrogen are believed to play key roles in this respect.

These committed progenitor cells assemble together to form the "fundamental proliferative units" (the follicles in the case of the ovary) made up of all the various cell types

which comprise the organ. It has been hypothesized that the different cell types within the proliferative unit produce inductive substances which influence the growth and differentiation of other cell types within the unit (Hirshfield, 1991). Thus, the growth kinetics of all of the tissue elements are highly coordinated. In this way, the proliferative unit starts its existence as a whole, matures as a whole and is shed as a single unit.

3. Induction of follicular growth.

Reactivation of quiescent primordial follicles appears to be a continuous process, beginning immediately after the first follicles are formed and continuing until the end of the reproductive period (Peters, 1969). Granulosa and theca cells proliferate without gonadotropin support. Not only does cell proliferation occur in ovaries of hypophysectomized animals given no hormone replacement (Jonassen et al., 1982), follicular growth continues normally until the preantral stage of development. In the human, folliculogenesis is not impaired in various physiologic (pregnancy and lactation), pathologic (amenorrhea and pituitary failure) or pharmacologic (oral contraceptives) situations where the circulating level of gonadotropin is low or absent (Gougeon, 1990). Moreover, ovarian histogenesis can occur *in vitro* in the absence of exogenous hormone (Hirshfield, 1991). These observations suggest that gonadotropins are not absolutely required for the initiation of follicular growth and that the presence of some intragonadal factors are more important than of extragonadal ones in initiating and controlling follicular growth and assembly.

Signals from one follicular compartment may influence cell proliferation in another compartment. For example, the growing oocyte may coordinate granulosa cell proliferation during the early stages of growth (Satoh et al., 1985). Theca cells appear to secrete factors

that influence the rate of granulosa cell proliferation. Co-culture of granulosa cells with theca cells, or culture of granulosa cells in conditioned medium from theca cell cultures increases cell numbers and [^{3}H]thymidine incorporation (Bendell et al., 1988). The microvascular network of maturing follicles is at least two times as dense as that around less mature follicles (Zeleznik and Hillier, 1984). Differences in blood flow would be expected to alter nutrient availability to proliferating cells, which in turn, would influence their generation times (Hirshfield, 1991).

While intraovarian signals appear to play a central role in initiating granulosa and theca cell proliferation, influences originating outside the ovary are also important in sustaining follicular growth. The rate of rat granulosa cell proliferation is higher in estrous than in proestrous (Hirshfield and Schmidt, 1985). These effects could be ascribed to variations in circulating steroids or gonadotropins in these different stages of the ovarian cycle. Environmental cues are also likely to have an influence on follicular growth. Diet and time of the day may affect the rate of rat granulosa cell proliferation (Hirshfield, 1991). Moreover, since the rate at which each follicle progresses through clonal expansion is dependent on many factors and since there is no feedback mechanism in place to modulate the effects of these variables, differences in the numbers of different size ovarian follicles within individual animals can be expected.

F. Terminal differentiation

Most signs of functional maturation of granulosa and theca cells first appear during the early formation of the follicular antrum. The timing of withdrawal from the cell cycle and the appearance of differentiated features are tightly coupled. Mature functional features begin

to appear in the follicle during the eighth to ninth generation, at a time when granulosa cells are proliferating most rapidly and starting to exit the cell cycle (Hirshfield, 1991).

Progression to the antral stage of follicular development is dependent upon the competence of granulosa cells to respond to FSH (Zeleznik and Hillier, 1984). FSH acts as a permissive signal of granulosa cell maturation; it is the granulosa cells, rather than the FSH that determine the nature of the cellular response (Hirshfield, 1991). All follicles in the ovary are probably exposed equally to FSH, but only follicles in the late stage of clonal expansion respond to the hormone by acquiring features of functional maturation. The nature of the response has already been predetermined (Gurdon, 1987) and the FSH stimulus is simply needed to trigger its expression.

After exposure to FSH, the follicle acquires the machinery needed for the end stage maturation (luteinization). Exposure to LH promotes the follicle into completion of the preprogrammed developmental process that was set in place during the initial stages of folliculogenesis, weeks or months earlier (Hirshfield, 1991).

1. Gonadotropin receptor expression and activation

FSH receptors are the only gonadotropic receptor expressed on granulosa cells of preantral follicles and receptors are present on granulosa cells from around the time the oocyte ceases to grow, although in a masked non-functional form (Richards and Midgley, 1976; Uilenbroek and Richards, 1979). The conversion of the receptor to functional membrane-bound protein is induced by the presence of the hormone (Ford and LaBarbera, 1988). FSH- receptor interaction causes an increase in FSH receptor number in the antral follicles (Uilenbroek and Richards, 1979). Continuous exposure of these follicles to the

gonadotropin down-regulates FSH receptors on granulosa cells of preovulatory follicles (LaPolt et al., 1992) but increases binding of [^{125}I]hCG to granulosa cells *in vivo*, indicating that FSH is capable of inducing granulosa cell hCG/LH receptor (Magoffin and Erickson, 1982). Induction of LH receptors in granulosa cells has been confirmed *in vitro* (Piquette et al., 1991) and *in vivo* (Segaloff et al., 1990). Withdrawal of FSH results in the loss of both FSH and LH receptors and death of granulosa cells (Richards, 1980; Erickson, 1986).

LH plays a key role in the differentiation of theca cells. There is evidence that the growth of small antral follicles to the preovulatory stage is dependent on subtle increases in serum LH concentrations (Bogovich et al., 1981). *In situ* hybridization studies showed the localization of LH receptor mRNAs exclusively in the theca cells of immature follicles but present in both theca and granulosa cells of mature antral follicles (Camp et al., 1991). The major change in thecal cell function in response to LH stimulation is increased LH receptor expression (Richards and Bogovich, 1982; Segaloff et al., 1990).

2. Steroid secretion

The key steroidogenic enzymes are lacking or weakly expressed in the granulosa cells of follicles at early stages of follicular development. In the presence of FSH, the granulosa cells acquire aromatase activity (for the conversion of androgen to estrogen) *in vivo* and *in vitro* (Zeleznick et al., 1974; Erickson and Hsueh, 1978). Both FSH and LH stimulate granulosa cell progesterone secretion (Hsueh et al., 1984) by inducing the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) involved in the conversion of pregnenolone to progesterone. Moreover, LH stimulates the production of aromatizable androgens and progesterone by the theca cells (Hsueh et al., 1984). The expression of enzymes involved in

estrogen and progesterone synthesis and secretion are often used as functional indices of granulosa cell differentiation.

3. Protein biosynthesis

In addition to the steroidogenic enzymes and gonadotropin receptors, many proteins are known to be synthesized in the granulosa and theca-interstitial cells during follicular maturation. Inhibin and activin synthesis in granulosa cells is regulated by gonadotropins (Bicsak et al., 1986; Ling et al., 1986; Burger and Findlay, 1989). Granulosa cells exhibit insulin-like growth factor (IGF) binding sites, the number of which is increased by FSH (Veldhuis and Furlanetto, 1985).

Components of extracellular matrix (collagen, fibronectin, laminin and proteoglycans) are secreted by follicle cells and are hormonally regulated during follicular development (Schittny and Yurchenco, 1989). Cultures of differentiated granulosa cells secrete less fibronectin than undifferentiated ones. FSH suppresses fibronectin secretion (Skinner and Dorrington, 1984; Skinner et al., 1985) but stimulates proteoglycan biosynthesis by granulosa cells (Salustri et al., 1990). In hen granulosa cells, fibronectin production increases with follicular maturation and is induced by LH (Asem et al., 1992).

The role of follicle cell-derived proteases in remodelling of extracellular matrix has been well documented. Plasminogen activators and metalloproteinases as well as their inhibitors are known to be secreted by both granulosa and theca cells and are hormonally regulated. The production of these proteases is significantly induced during cytodifferentiation (Hsueh et al., 1989; Sappino et al., 1989; Bicsak et al., 1989; Rapp et al., 1990; Mann et al., 1991).

4. Prostaglandin production

Prostaglandins act as local regulators of cell function (Curry et al., 1987). They are synthesized from membrane phospholipids via the action of phospholipases. The synthesis of prostaglandins occurs in the theca and granulosa cells and is regulated by gonadotropins (Evans et al., 1983). The enzymes involved in prostaglandin synthesis have been localized in the membranes of granulosa cells of preovulatory follicles and are induced by gonadotropins (Curry et al., 1987; Pridham et al., 1990) and transforming growth factor alpha (Li and Tsang, 1995; Li et al., 1996)

5. Morphology

Alterations in follicular cell shape and size due to gonadotropin stimulation have been well described (Amsterdam and Rotmensch, 1987; Carnegie et al., 1987; Tsang et al., 1988). Immature, flattened, epitheloid granulosa cells exposed to FSH or LH in culture adopt a spherical morphology which correlates closely with transformation of columnar-shaped cells late in folliculogenesis (Lawrence et al., 1979, Ben Zeev and Amsterdam, 1987). The response of granulosa cells to gonadotropin is also characterized by an increase in cell size, cytoplasmic volume and nuclear to cytoplasmic ratio (Amsterdam and Rotmensch, 1987).

The enhancement of steroidogenesis after the induction of aromatase and other steroidogenic enzymes by FSH and LH is reflected by significant changes in the intracellular organelles. Immature, undifferentiated granulosa cells contain mainly rough endoplasmic reticulum, which reflect the high level of protein synthesis required for cell growth and proliferation. A gradual shift towards the development of smooth endoplasmic reticulum occurs as steroidogenesis increases (Rigby et al., 1986). The tendency to tubular

mitochondrial cristae rather than lamellar cristae is accentuated during granulosa cell differentiation (Amsterdam and Rotmensch, 1987) which accounts for the increase in the steroidogenic activity.

Gap junctions develop throughout the granulosa cell compartment and are also seen between cumulus cells and oocytes. These junctions first appear during cell differentiation in the antral follicle and attain maximal size and frequency in the mature preovulatory follicle (Rotmensch et al., 1986). FSH increases the number of gap junctions both *in vivo* and *in vitro* (Amsterdam and Rotmensch, 1987).

Cytoskeletal elements (actin and microtubules) are believed to be involved in many structural alterations that coincide with differentiation-dependent metabolic activity of granulosa cells. The complex network of filaments which extends throughout the cytoplasm enables the cells to adopt a variety of shapes to carry out directed movement and provide the machinery for moving organelles from one intracellular locale to another (Ben Zeev and Amsterdam, 1987; Carnegie et al., 1987; Tsang et al., 1988). After exposure of granulosa cells to gonadotropin, lysosomes and mitochondria, which are involved in steroid biosynthesis, concentrate in the perinuclear region. This clustering facilitates the movement of substrate among organelles and thus enhances steroid synthesis (Soto et al., 1986). A significant reorganization of actin filaments occurs when cultured immature rat granulosa cells are stimulated with FSH (Tsang et al., 1988). Treatment of granulosa cells with colchicine causes depolymerization of microtubules and inhibits FSH-stimulated progestin production. FSH-induced progestin secretion is also inhibited by taxol which stabilizes microtubules (Carnegie et al., 1987).

G. Local factors affecting granulosa cell functions

In addition to steroids and gonadotropins, many soluble factors are known to be produced within follicles and to mediate the balance between granulosa cell survival, proliferation, and differentiation (Tonetta and DiZerega, 1989). Neurotransmitters, growth factors and cytokines represent a few classes of peptides secreted by ovarian cells which are known to regulate both proliferative and differentiative characteristics of granulosa cells or can modulate the influence of gonadotropin (Yamada, 1984; Adashi and Resnick, 1986). Thus, it is the integration of multiple regulatory influences which is of fundamental significance in controlling granulosa cell functions.

Extracellular matrix can also affect the expression of granulosa cell proliferative and differentiative characteristics (Carnegie et al., 1988). Granulosa cells cultured in collagen gels produce more progesterone than those cultured without collagen. Intimate intercellular associations (gap junction connections) between granulosa cells are essential for the acquisition of LH receptor (Farookhi and Desjardins 1986).

1. Growth hormone releasing factor

Growth hormone releasing factor (GRF) is a 43-amino acid hypothalamic peptide which belongs to the glucagon superfamily. This peptide regulates primarily the synthesis and secretion of growth hormone (GH) in the anterior pituitary gland (Frohman and Jansson, 1986). In the rat, GRF was isolated from hypothalamic extracts on the basis of its ability to stimulate GH secretion from cultured anterior pituitary cells (Spiess et al., 1983). The rat GRF gene is 10 kb in length and contains 5 exons that encode a 104-amino acid precursor to the 5.2 KDa rat GRF peptide (Mayo et al., 1985). The intron-exon organization of the

GRF gene is similar to those genes of the glucagon superfamily, each of which encodes large precursors that generate several biologically active and structurally related peptides by proteolytic processing (Bell, 1986).

Like other neuropeptides, GRF is also present in extrahypothalamic tissues (Thorner et al., 1986) including the gastrointestinal tract (Bruhn et al., 1985), tumors of neuroendocrine origin (Rivier et al., 1982), placenta (Meigan et al., 1988), testis (Berry and Pescovitz, 1988) and ovary (Bagnato et al., 1992), suggesting that this peptide may also have a regulatory role in peripheral tissues.

At the ovarian level, GRF-like peptide has been detected in human ovarian tissue (Moretti et al., 1990a) and in follicular fluid (Moretti et al., 1989). GRF-like substances and their mRNAs have also been identified in the rat ovary (Bagnato et al., 1992). Rat granulosa cells release immunoreactive GRF to the culture medium following 3 h of incubation (Bagnato et al., 1992). GRF and members of the glucagon superfamily compete for the same receptors on granulosa cells (Moretti et al., 1990b). GRF enhances FSH-induced rat granulosa cell steroidogenesis *in vitro* (Moretti et al., 1990b), activates folliculogenesis and stimulates the development of the dominant follicle *in vivo* (Moretti et al., 1989).

2. Vasoactive intestinal peptide

Vasoactive intestinal peptide (VIP) is a linear peptide with 28 amino acids that belongs to glucagon superfamily (Bell, 1986) and shares 32% sequence homology with GRF (Bataille, 1986; Christophe et al., 1989). VIP was originally considered an intestinal hormone involved in smooth muscle relaxation and vasodilatation (Said and Mutt, 1970). However, specific recognition sites for VIP synthesis outside the intestine, including rat liver

(Bataille et al., 1974), fat (Bataille et al., 1977), brain (Staun-Olsen et al., 1982), pancreas (Christophe et al., 1976), prostate (Prieto and Carmena, 1983) and the female genital tract of animals (Larson et al., 1977) and human (Alm et al., 1980) have been identified, suggesting that VIP may exert local regulatory influences in these tissues.

VIPergic nerve fibers are in close association with the smooth muscle of ovarian stroma (Larson et al., 1977). VIP-like immunoreactive substances and mRNAs have been reported in the ovary (Ahmed et al., 1986; Gozes and Tsafriri, 1986). VIP and GRF are believed to bind to the same receptors and to stimulate basal (Davoren and Hsueh, 1985) and FSH-induced (Moretti et al., 1990b) steroidogenesis in cultured rat granulosa cells. VIP regulates the synthesis of cholesterol side-chain cleavage enzyme (Trzeciak et al., 1986) and enhances aromatase activity in the neonatal ovary before the development of primary follicles, a stage in which the follicles are non-responsive to FSH (George and Ojeda, 1987). It also stimulates oocyte maturation in isolated preovulatory rat follicles (Tornell et al., 1988) and PA activity in cultured rat granulosa cells (Liu et al., 1987a).

3. Epidermal growth factor and transforming growth factor alpha

Epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) are structurally related but with distinct single chain polypeptides, of 53- and 50- amino acids, respectively (May and Schomberg, 1989). Although encoded by different genes, these growth factors bind to the same receptor (Todaro et al., 1980). EGF and TGFα are produced by theca cells (Skinner et al., 1987a, Rall et al, 1985) and their production is regulated by gonadotropins (Roy and Greenwald, 1990; Kudlow et al., 1987). Receptors for EGF (and therefore for TGFα) are expressed on granulosa cells (Vlodavsky et al., 1978; Jones et al.,

1982; Buck and Schomberg, 1988).

EGF and TGFα can stimulate or inhibit proliferation or differentiation of granulosa cells *in vitro*, depending upon prior hormonal stimulation and the presence of other regulatory factors in the culture medium (Leal et al., 1990). The proliferative activity of granulosa cells from many species including human (but not rat) is induced by EGF or TGFα (Gospodarowicz and Bialecki, 1979; Lafrance et al., 1993a;). Subsequent studies have confirmed species variability and revealed that the mitogenic effects of EGF on granulosa cells may be dependent upon the presence of serum or other growth factors (May and Schomberg, 1989; Bendell and Dorrington, 1990; Leal et al., 1990).

While both EGF and TGFα have been shown to inhibit estrogen and progesterone synthesis and LH receptor expression in rodent granulosa cells (Hsueh et al., 1984; Mondschein and Schomberg, 1981), they stimulate progesterone production in human granulosa-lutein cells (Richardson et al., 1989). More recently it has been reported that TGFα, like EGF, is a potent inhibitor of gonadotropin-induced granulosa cell differentiation (May and Schomberg, 1989; Schomberg et al., 1983). Both growth factors stimulate PA activity in cultures of granulosa cells (Galway et al., 1989; Lafrance et al., 1993a,b).

4. Transforming growth factor beta

Transforming growth factor beta (TGFβ) is a homodimeric polypeptide comprising two identical 112-amino acid chains (Sporn et al., 1986). TGFβ is produced by both granulosa and theca-interstitial cells and acts through a receptor system different from that of TGFα (Adashi et al., 1992a; Kim and Schomberg, 1989; Skinner et al., 1987a,b). In the ovary, TGFβ stimulates FSH-induced aromatase activity, enhances FSH-dependent

progesterone production, promotes granulosa cell proliferation, augments FSH-induced granulosa cell LH receptor expression and induces inhibin and prostaglandin biosynthesis in granulosa cell cultures (Mondschein et al., 1988; Dorrington et al., 1988; Skinner et al., 1987b; Bendell and Dorrington, 1988; Adashi and Resnick, 1986; Knecht et al., 1987; Zhang et al., 1988; Feng et al., 1986). TGFβ is likely to participate in granulosa-theca interactions, since it exerts significant actions on basal, LH-, and IGF-I-stimulated androgen production by theca cells (Magoffin et al., 1989). TGFβ is involved indirectly in the regulation of cellular responses through modulating the deposition of extracellular matrix (Roberts and Sporn, 1989). Granulosa cell hyaluronic acid production can be induced by TGFβ (Salustri et al., 1990). Thus, it is possible that TGFβ serves as an important paracrine/autocrine intrafollicular factor regulated by gonadotropin (Hernandez et al., 1987; Mulheron and Schomberg, 1990).

5. Tumor necrosis factor alpha

Cytokines secreted by macrophages, monocytes and lymphocytes can regulate ovarian cell function (Halme et al., 1985; Standaert et al., 1990). One such cytoactive substance is tumor necrosis factor alpha (TNFα) which is a 157 amino acid polypeptide with a molecular weight of 17.3 KDa. It was originally named for its oncolytic activity and its ability to induce tumor necrosis (Harrison and Campbell, 1988). Although TNFα was initially thought to be tumor selective, it has become clear that certain non-tumor cells also possess TNFα receptors and that this cytokine regulates non-cytotoxic activities in these cells (Adashi, 1989).

TNFα may be locally derived from resident ovarian macrophages (Adashi, 1989). In

addition, immunohistochemical and in situ hybridization studies demonstrate that follicle cells (oocyte, granulosa and theca) are also sources of TNFα (Roby and Terranova, 1989; Chen et al., 1993; Marcinkiewicz et al., 1994). Moreover, the production of TNFα by granulosa cells is under the control of FSH and hCG (Zolti et al., 1990) and the cytokine is known to accumulate in follicular fluid (Roby et al., 1990). Granulosa cells contain specific high-affinity, low capacity binding sites for TNFα (Veldhuis et al., 1991). TNFα is capable of attenuating the differentiation of cultured granulosa cells (Adashi et al., 1989; Darbon et al., 1989). While this cytokine inhibits FSH-stimulated aromatase activity and progesterone production in cultured granulosa cells (Emoto and Baird, 1988; Darben et al., 1989, Adashi et al., 1990), it stimulates granulosa-lutein cell proliferation (Wang et al., 1992). TNFα increases progesterone production by preovulatory follicles and prostaglandin $F_{2\alpha}$ synthesis in granulosa cells (Roby and Terranova, 1988; Zolti et al., 1990). Although TNFα has been implicated in follicular atresia, the mechanism by which it affects the process is not known (Takahide, 1990). In perfused rat ovaries, the ovulatory rate in response to LH is significantly inhibited by TNFα (Hales et al., 1994).

6. Interleukin-1 (IL-1)

Interleukin-1 (IL-1), another secretory product of ovarian macrophages, is a polypeptide of 116 amino acids. It exists in two forms (alpha and beta) and has a molecular weight of 17 KDa (Duff, 1985; Gray et al., 1986; Dinarello, 1988). Although first identified as a substance that augments proliferation of activated T-cells (Gery et al., 1972), IL-1 has subsequently been shown to modulate differentiative functions of many endocrine cells (Kennedy and Jones, 1991).

At the ovarian level, a significant amount of IL-1-like activity has been detected in follicular fluid (Khan et al., 1980; Takakura et al., 1989). IL-1s are produced by resident ovarian macrophages which are present at various stage of folliculogenesis (Adashi, 1992a, b). In addition, IL-1β and its receptor have been localized in theca-interstitial and granulosa cells (Simon et al., 1994), suggesting local synthesis and actions of this cytokine. Moreover, the intraovarian IL-1 system (ligands, receptors and receptor antagonist) is highly compartmentalized and hormonally regulated (Hurwitz et al., 1992). IL-1β inhibits the FSH induction LH receptors in granulosa cells (Gottshall et al., 1988a). Although the cytokine suppresses gonadotropin-induced follicle cell steroidogenesis (Gottshall et al., 1987; Gottshall et al., 1988b; Hurwitz et al., 1991), it has a growth promoting effect on cultured granulosa cells (Fukuoka et al., 1992). IL-1β stimulates of PGE and $PGF_{2\alpha}$ release from preovulatory follicles and enhances LH-induced ovulation in perfused ovaries (Brannstrom et al., 1993; Takehara et al., 1994).

H. Follicular steroidogenesis

Three major classes of steroid are produced by follicular cells: progestins, androgens, and estrogens. The precursor of all ovarian steroids is cholesterol (27-carbon) which is composed of a steroid nucleus (three cyclohexane rings and a cyclopentane ring), two methyl groups and an eight membered side chain. Fission of side chain at 20,22-carbon leads to the formation of a 21-carbon steroid, progesterone. Further cleavage of the side chain produces 19-carbon steroids (androgens). Removal of C-19 methyl group forms 18-carbon steroids (estrogens). The production of these steroids are dependent on the activation of key steroidogenic enzymes present in follicular cells (Fig 1).

1. Progestins

Granulosa and theca-interstitial cells are capable of progestin biosynthesis. However, central to this process is the availability of an abundant supply of cholesterol. Three possible sources of cholesterol are available to follicle cells: a) blood borne cholesterol taken up from circulating lipoproteins (Brown and Goldstein, 1976), b) preformed cholesterol stored in ovarian cells or constituent of cell membranes (Strauss et al., 1981) and c) cholesterol synthesized *de novo* from acetate in the ovarian cell (Andersen and Dietschy, 1978). Low density lipoproteins (LDL) binds to specific membrane receptors and the receptor-ligand complex enters the cell by receptor-mediated endocytosis (Goldstein et al., 1979). The endocytotic vesicles fuse to lysosomes, where LDL cholesterol esters are hydrolysed to yield free cholesterol (Brown et al., 1975). Therefore, the availability of LDL to various ovarian compartments could influence progesterone production and the relative avascularity of the granulosa cell layer during early follicular development limits progestin biosynthetic capacity.

Progesterone biosynthesis in the theca cells is under the influence of LH (Evans et al., 1981; Hsueh et al., 1984). Whereas granulosa cells of antral follicles secrete minimal progesterone in response to FSH, production of this steroid by these cells during the preovulatory period is dramatically increased by the LH surge (Hsueh et al., 1984). The main site of action of gonadotropin on progesterone biosynthesis is the conversion of cholesterol to pregnenolone. This rate-limiting reaction is catalyzed by cholesterol side chain cleavage enzyme P-450scc (Funkenstein et al., 1984) which is low or negligible in granulosa cells of small follicles, is increased in cells of the preovulatory follicles (Goldring et al., 1987) or in granulosa cells cultured in the presence of FSH (Hinshelwood et al., 1993). This enzyme is

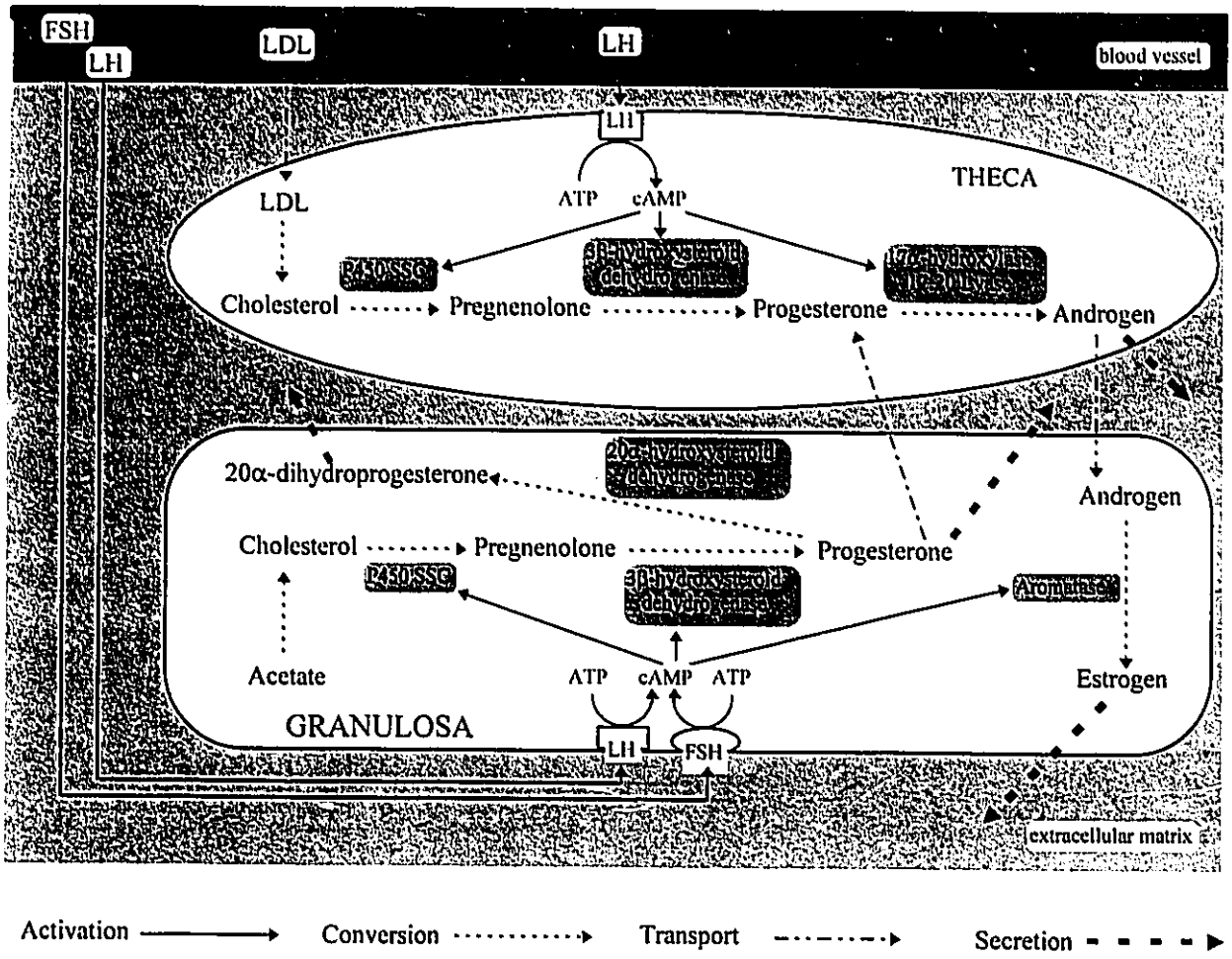

Fig 1: Schematic representation of follicular steroidogenesis and its regulation by gonadotropins.

also expressed in the theca-interstitial cells (Goldring et al., 1987). Pregnenolone is converted to progesterone by 3β-HSD, an enzyme present in both theca-interstitial and granulosa cells (Juneau et al., 1993; Teerds and Dorrington, 1993). The presence of 3β-HSD in granulosa cells of antral and preovulatory follicles but not in small follicles indicates that it is likely to be regulated by gonadotropins (Hinshelwood et al., 1993). In addition to those involved in the production of androgen in the theca cells, alternate routes of progesterone catabolism also exist. Progesterone is irreversibly converted in both granulosa and theca cells to less active metabolites, 17α-hydroxyprogesterone in human and 20α-dihydroprogesterone in rodents, by 17α-hydroxysteroid dehydrogenase and 20α-hydroxysteroid dehydrogenase, respectively (Aono et al., 1981; Hsueh et al., 1984).

2. Androgens

Direct evidence that theca/interstitial cells are the sole source of ovarian androgen comes from studies showing exclusive expression of 17α-hydroxylase/17,20-lyase (enzyme involved in the conversion of pregnenolone and progesterone to 19-carbon androgens) in these cells (Richards et al., 1987; Sasano et al., 1989). Since LH receptor is the only gonadotropic receptor present in the theca cells, it is LH rather than FSH that stimulates androgen production by these cells (Fortune and Armstrong, 1977; Tsang et al., 1979). LH increases of 17α-hydroxylase/17,20-lyase activities in the theca cells during follicular development (Fukuda et al., 1979; Bogovich and Richards, 1982).

Androstenedione is the principal aromatizable 19-carbon steroid produced by the theca interna; only a small amount of testosterone is secreted (Hamberger et al., 1978; McNatty et al., 1979; Markis and Ryan, 1980). Rat ovarian interstitial preparations are also

active in 5α-reduction of 19-carbon steroids; the major LH-stimulated product is androsterone (Magoffin and Erickson, 1982). An alternate route of androgen catabolism is the conversion of aromatizable androgens to estrogens by granulosa cells (Dorrington and Armstrong, 1979).

3. Estrogens

Whereas the steroidogenic pathways in the theca cells function primarily in the de novo production of androgens, the pathways in the granulosa cells are organized principally for the metabolism of 19-carbon steroids (i.e. androgens) to estrogen (Hsueh et al., 1984). The cooperation of the theca and granulosa cell compartments is crucial in the control of follicular estrogen biosynthesis by gonadotropins .

Treatment of hypophysectomized rats with LH enhances ovarian androgen content but concomitant administration of FSH is required to increase estradiol levels (Moon et al., 1975). Substitution of LH with aromatizable androgen (testosterone or androstenedione) leads to similar increases in estradiol production, provided that the rats had also been treated with FSH (Armstrong and Papkoff, 1976). Studies with follicular cell cultures reveal that FSH acts directly on granulosa cells to stimulate estrogen secretion in the presence of an aromatizable androgen (Dorrington et al., 1975). Co-culture of theca and granulosa cells causes a substantial increase in estrogen secretion in the presence of gonadotropins (Moor, 1977). Culture of follicle wall preparations (granulosa and theca) were far more effective in the production of estrogen than either cell type alone (Armstrong et al., 1981). These findings together with the observations that 1) theca cells, but not immature granulosa cells possess LH receptors and exclusively produce androgens and 2) granulosa cells, but not

theca cells, possess FSH receptors and respond to FSH stimulation by increasing aromatase mRNA (Fitzpatrick and Richards, 1991), protein (Hickey et al., 1988) and activity (Fitzpatrick and Richards, 1991) *in vitro* and *in vivo*, led to the development of the current "two-cell, two-gonadotropin" theory for follicular estrogen production (Dorrington and Armstrong, 1975; Armstrong and Papkoff, 1976; Hillier et al., 1994). This hypothesis requires that androgens of theca origin diffuse across the basement membrane to gain access to the aromatase enzyme in the granulosa cells. The presence of aromatizable androgens in follicular fluid are consistent with the role of these steroids to serve as substrates for aromatization by granulosa cells (Evans et al., 1981; Fortune and Hansel, 1985).

I. The plasminogen activator system

Controlled and targeted extracellular proteolytic activity is required in many biological process involving tissue remodelling (Strickland and Beers, 1976), tissue destruction (Ossowski et al., 1976; Busso et al., 1989) and cell migration (Strickland and Reich, 1976; Ossowski and Reich, 1983). The PA system is a general system for providing such extracellular proteolysis. Although this system has been viewed primarily as one responsible for dissolving blood clots, it is now clear that the same components are involved in many other biological processes that require extracellular proteolysis such as mammary gland involution (Busso et al., 1989), blastocyst implantation (Sappino et al., 1989), tumor metastasis (Ossowski and Reich, 1983) and ovulation (Beers et al., 1975; Strickland and Beers, 1976).

The two PA types catalyze the cleavage of their substrate plasminogen into a wide spectrum protease, plasmin. Whereas the "classical" plasmin substrate is fibrin, it is now

apparent that other matrix proteins can also be cleaved by this enzyme. In addition, plasmin is one of the activators of collagenase and metalloproteinase precursors, and thus plays a pivotal role in extracellular matrix remodelling (He et al. 1989)

The PA system comprises plasminogen, two types of PA (urokinase-type and tissue-type PA), various PA inhibitors (PAI-1, PAI-2 and protein nexin-I), plasmin, PA receptors and binding sites (Robbins et al., 1967; Loskutoff et al., 1988; Nielsen et al., 1988). The regulation of the PA system is complex and occurs at many levels. Since plasminogen is present in excess in plasma and body fluid (Bachmann, 1987), the regulation of this system must mainly be at the level of synthesis and activity of the rate-limiting PAs as well as the specific PAIs. The PA receptors and binding sites are important for the localization of the proteolytic activity at the site(s) of tissue remodelling which functions in the overall control of the PA system. Together this multi-level but precise regulatory mechanism appears to be important in fine-tuning the expression and function of the PA system and provides the controlled proteolytic activity that characterizes many physiologic and pathologic processes (Fig 2).

1. Components of the PA system

a. Plasminogen

Plasminogen is a glycoprotein with a molecular weight of 92 KDa and is present at a concentration of approximately 2 μM in plasma and extracellular fluids (Bachmann, 1987), including follicular fluid (Beers, 1975), ascites fluid (Le Blanc and Back, 1975) and uterine fluid (Casslen and Ohlsson, 1981). The major site of plasminogen biosynthesis is the liver (Raum et al., 1980). Due to a difference in glycosylation, two variants exist with slightly

different electrophoretic mobilities (Hayes and Castellino, 1979). The N-terminal amino acid of the native form of plasminogen is glutamine (Robbins et al., 1973) and often termed Glu-plasminogen. This plasminogen type is readily converted proteolytically to form Lys-plasminogen, with lysine as the N-terminal residue (Wallen and Wiman, 1972) and a molecular weight 8 KDa lower than the native form.

b. Plasminogen activators

Tissue-type PA (tPA) has been purified from various sources, including heart, kidney, ovary, uterus, plasma and conditioned cell culture medium (Granelli-Piperno and Reich, 1978; Rijken et al., 1981; Levin and Loskutoff, 1982). The genes encoding the human, porcine, mouse and rat tPA have been identified (Ny et al. 1984; Fisher et al., 1985; Degen et al., 1986; Heaton and Gelehrter, 1990; Rickles et al., 1988). The 29-32.7-kb human tPA gene consists of 14 exons and codes for 68 KDa protein via a 2.7-kb mRNA (Fisher et al., 1985). The structural and functional domains of tPA are coded by separate exons and include a finger domain, a growth factor-like domain, two triple-disulfide structures called kringles and a domain in the carboxy terminal region that forms the active site of the enzyme (Pennica et al, 1983). The second kringle accounts for most of the fibrin affinity of tPA (Van Zonneveld et al., 1986). Fibrin is known to strongly enhance plasminogen activation by tPA (Ranby, 1982). The first kringle in tPA has no known function.

Tissue-type PA is mostly secreted as a single-polypeptide chain (Ranby et al., 1982; Rijken et al., 1982). Conversion of the one-chain form to the two-chain form is catalysed by

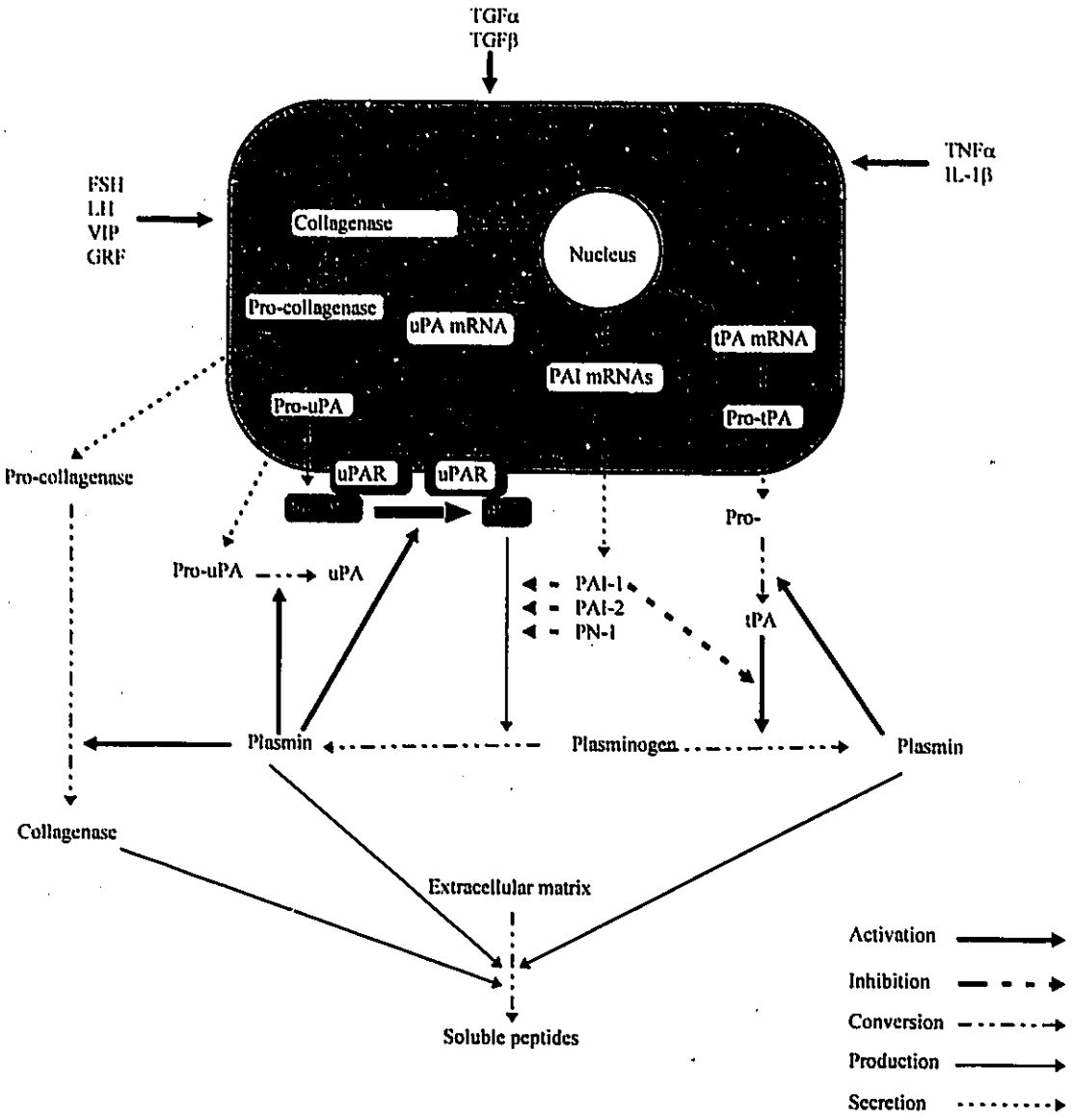

Fig 2: Schematic representation of various components of the plasminogen activator system and its regulation

plasmin, tissue kallikrein and blood coagulation factor X (Ranby et al., 1982; Ichinose et al., 1984). The one-chain tPA has little or no activity and most of the enzyme activity is attributed to the activity of the two-chain molecule (Andreasen et al., 1984, Ichinose et al., 1984), suggesting that the single-chain protein is the inactive proenzyme form of tPA.

Various cellular binding sites for tPA have been described. Some of these are clearly involved in the clearance (particularly in the liver) of the enzyme or of the tPA-PAI complexes (Rijken et al., 1990) while others are detected on endothelial cells and neurons (Verrall and Seeds, 1989; Hajjar and Hamel., 1990). Tissue-type PA bound to the endothelial cell surface is less susceptible to its specific inhibitor than the free form of the enzyme (Hajjar et al., 1987) and tPA binding sites are different from the characterized uPA receptor.

Although urokinase-type PA (uPA) was first discovered in human urine, the presence of this PA type in other body fluids and tissues is now well recognized. Urokinase is in blood plasma, seminal plasma, the prostate gland and the ovary (Ptosed et al., 1982; Propping et al, 1982; Ny et al., 1985) and has a molecular weight of 50 KDa . Two forms of the enzyme exist: a single-chain (scuPA) inactive proenzyme and a two-chain (tcuPA) active enzyme (Eaton et al., 1984). The molecular weight of the heavy chain, also termed B-chain, is approximately 30 KDa while that of the light chain, A-chain, is 20 KDa (Sudol and Reich, 1984). The two chains are held together by one disulfide bridge (Sumi and Robbins, 1983). The scuPA is activated by a cleavage of peptide bonds, which eventually leads to the loss of Lys-158 (Verde et al., 1984). This cleavage take place in the extracellular space and can be mediated by plasmin (Keilberg et al., 1985) and kallikrein (Ichinose et al., 1986).

The gene coding for uPA has been identified in human, porcine, mouse and rat tissues (Verde et al., 1984; Nagamine et al., 1984; Belin et al., 1985; Zhang et al., 1994). The cDNA

sequence of uPA from these species shows extensive homology. The human 6.4-kb uPA gene contains 11 exons (Riccio et al., 1985) and codes for the protein via 2.5-kb mRNA (Verde et al., 1984). The functional and structural domains for uPA are: one kringle structure and the growth factor-like domain (Riccio et al., 1985). It lacks the finger domain present in tPA and the second kringle structure which gives the characteristic fibrin-binding property of tPA, and thus the affinity to fibrin. The growth factor-like domains of uPA and tPA are structurally similar to the receptor-binding regions of EGF and TGFα (Gunzler et al., 1982; Derynck et al., 1984; Komoriya et al., 1984). Whereas the function of this domain is unknown in tPA, the growth factor domain of uPA contains the receptor-binding amino acid sequence (Appella et al., 1987). Plasmin which permits its binding to its receptors further processes uPA to a 33-KDa molecule that lacks the growth factor domain (receptor-binding) and kringle domain, but is still fully active (Stump et al., 1986).

Several growth regulating peptides affect the levels of PA in cultured cells. This is of interest with respect to the suggested association of PA activity and the cell cycle (Scott et al., 1987). An increase of uPA mRNA was associated with the transition of cells through the cell cycle (Grimaldi et al., 1986). These findings suggest that a close relationship exists between the expression of uPA and the growth state of cells, possibly for changing cell-matrix interactions during cell division and movement (Pollanen et al., 1987; Lafrance et al., 1993a,b). Several observations suggest that cell surfaces represent the more relevant sites for uPA-mediated plasminogen activation (Ellis et al., 1989; Stephens et al., 1989). This hypothesis is supported by the existence of a highly specific cell-surface receptor which binds uPA with high affinity (Vassalli et al., 1985).

c. Urokinase receptor

Among the more interesting new aspects of PA regulation is the putative role of the high-affinity membrane receptor for uPA (uPAR; Vassalli et al., 1985, Stoppelli et al., 1985). The receptor has been purified and appears to be a 55-60 KDa glycosylated protein (Nielsen et al., 1988). The K_d for binding of both scuPA and tcuPA is 0.1-0.5 nM (Cubellis et al., 1986). The binding region in uPA has been localized to the cystein-rich part of the growth factor domain of uPA (Appella et al., 1987). Despite the partial amino acid homology between this sequence and EGF, the binding of uPA to the receptor is not inhibited by EGF. A synthetic peptide with the same amino acid sequence as the growth factor domain of uPA (residues 12-32) competes with uPA for binding to uPAR (Appella et al., 1987). This sequence differs from the respective sequence in tPA, which may account for the lack of tPA binding to the receptor. The growth factor domain-defective 33 KDa uPA generated by digestion with plasmin does not bind to the receptor.

The interaction of uPA with uPAR exhibits the following properties: 1) receptor binding does not require the active site for uPA, 2) scuPA binds with the same affinity as the active enzyme (Ellis et al., 1989), 3) receptor-bound scuPA can be activated on the site of the receptor (Ellis et al., 1989), 4) the region of uPA that binds to uPAR is located in the amino-terminal fragment of the A-chain (Stoppelli et al., 1985), 5) unlike many cellular receptors that are rapidly internalized on binding their respective ligands, uPA bound to uPAR remains accessible at the cell surface and the complex is neither internalized nor degraded to any significant extent (Vassalli et al., 1985; Stoppelli et al., 1985), and 6) receptor binding does not shield uPA from the action of PAIs although the enzyme-inhibitor complexes are bound with about ten times lower affinity compared with the enzyme itself

(Cubellis et al., 1989; Kirchheimer and Remold, 1989; Ellis et al., 1990). However, if receptor-bound uPA is inactivated by PAIs, the uPA/PAI complex is rapidly cleared from the cell surface and degraded within the cell (Estreicher et al., 1990; Cubellis et al., 1990).

Receptor-bound uPA stimulates limited cell growth (Kirchheimer et al., 1987). This effect was demonstrated in a carcinoma cell line expressing the receptor but that did not synthesize uPA. Incubation of cells with active tcuPA induced cell proliferation, whereas inhibition of the enzymatic activity with diisopropylfluorophosphate (which does not interfere with the receptor binding) abolished the mitogenic response. These findings suggest that the uPA-uPAR interaction stimulates cell division through an intracellular signalling mechanism. It is also possible that the receptor-bound uPA promotes local degradation of matrix structures, thus releasing growth stimulating substances bound to extracellular matrix components (Vlodavsky et al., 1987; Saksela et al., 1988).

Receptor-bound uPA may have an important role in cell invasion and metastasis, processes often correlated with increased uPA activity. Since uPA is first secreted into the extracellular space and is then bound to uPAR, cells not secreting uPA but possessing the receptor may bind enzyme produced by other cells and thereby utilize the uPA-mediated proteolytic machinery (Huarte et al., 1987). The presence and physiological role of uPA receptor(s) in the ovary are not known.

d. Plasminogen activator inhibitors

Plasminogen activator inhibitor-1 (PAI-1) is a single-chain polypeptide of approximately 54 KDa that was initially characterized as an endothelial cell PA inhibitor (Van Mourik et al., 1984). Since then it has been found in different tissues such as aorta,

adipose tissue, heart, lung, placenta and ovary (Ny et al., 1985; Jones et al., 1988 and 1989; Sawder and Loskutoff, 1991; Kessel et al., 1992). The function of PAI is, therefore, not restricted to the regulation of vascular fibrinolysis but rather is to serve as a general regulator of the PA system.

PAI-1 has been cloned (Ny et al., 1986; Wun and Kretzmer, 1987) in the human and the genomic fragment bearing the entire PAI-1 gene consists of 9 exons. Two mRNA species (3.4- and 2.4- kb) with different polyadenylation sites hybridize with PAI-1 cDNA (Ginsburg et al., 1986; Andreasen et al., 1987). Kinetic studies have shown that PAI-1 is the most efficient inhibitor known for both uPA and tPA (Loskutoff et al., 1988). The concentration of PAI-1 in plasma (about 1 nM) can rapidly inactivate physiological concentrations of both uPA and tPA (Bachmann, 1987). PAI-1 is often associated with the adhesive glycoprotein vitronectin or S protein (Declerck et al., 1988; Wiman et al., 1988). This association stabilizes the inhibitor in its active confirmation but does not interfere with the inhibition of the PAs (Seiffert et al., 1990).

PAI-2 was originally described as a uPA inactivator in the placenta (Kawano et al., 1970; Astedt et al., 1985). Recently, the gene coding PAI-2 has been isolated (Ye et al., 1987) and the amino acid sequence translated from the 2-kb mRNA identified (Webb et al., 1987). PAI-2 exists in two molecular and topological forms: a nonglycosylated intracellular 46 KDa form and a glycosylated extracellular 60 KDa species (Astedt et al., 1987; Wohlwend et al., 1987a; Wun and Reich, 1987). PAI-2 undergoes glycosylation upon secretion (Medcalf et al., 1988). It contains an internal signal peptide that appears to function suboptimally, thereby allowing the synthesis of both extracellular and intracellular forms of

the protein (Ye et al., 1989; Von Heijne et al., 1991). The secretion of PAI-2 varies between different cell types and is modulated by various growth promoting factors (Genton et al., 1987; Wohlwend et al., 1987b).

Protease nexin I (PN-I) is a less specific inhibitor than PAI-1 and PAI-2. It inhibits tcuPA effectively although with a slower rate than either PAI-1 and PAI-2 and does not bind scuPA (Eaton et al., 1984; Baker et al., 1987). PN-I is a slow inhibitor of both sctPA and tctPA, but is an extremely rapid inhibitor of thrombin, trypsin and plasmin (Scott et al., 1985).

PN-I cDNA has been cloned (McGrogan et al., 1988). Its mRNA exists in two slightly different forms (alpha and beta) which are synthesized from the same gene with alternative splicing. Both translation products bind uPA and thrombin. PN-I is a 45 KDa glycoprotein and contains 392 amino acids residues. PN-I-proteinase complexes are rapidly taken up by high-affinity receptors located on the plasma membrane (Howard and Knauer, 1987), delivered to lysosomes and degraded.

PN-I regulates thrombin-induced cell proliferation, and uPA-mediated matrix degradation, inhibits neuronal cell migration and promotes neurite outgrowth (Low et al., 1982; Bergman et al., 1986; Lindner et al., 1986; Rosenblatt et al., 1987). These actions of PN-I may be due to the modulation of the stability of the focal contacts between cells and the surrounding growth substratum.

2. Regulation of Ovarian Plasminogen Activator System.

As discussed in previous sections, the ovary is a heterogeneous organ that consists

of follicles at different stages of development and atresia. The molecular and cellular mechanisms involved in the development of ovarian follicles are coupled to the tissue remodelling processes needed for the successful growth and eventual ovulation. It seems that the proteolytic activity needed for these processes may involve regulation of PA and PAI activities (Liu et al., 1991). It has been shown that the level of tPA mRNA is low in granulosa and theca cells but high in the oocyte in early stages of follicular development (Cajander et al., 1989; Peng et al., 1993). However, preovulatory follicles demonstrate high levels of tPA activity with decreased level of PAI (Peng et al., 1993). Whereas FSH stimulates the production of both tPA and uPA in cultures of undifferentiated granulosa cells, it inhibits PAI secretion (Ny et al., 1985). LH is also an efficient stimulator of tPA in cultured rat granulosa cells (O'Connell et al., 1987). Various intraovarian factors such as EGF, TGFα, IGF and relaxin have also been shown to modulate rat granulosa cell PA activity (Too et al., 1984; Galway et al., 1989).

Since PA and PAI activity are expressed in the ovary and are hormonally regulated, the interaction between these molecules results in inactivation of PA activity. Thus the coordinated expression of the PA and PAI species will determine the net PA activity, while that of the PA receptor and binding sites determines the directionality of the proteolysis during follicular maturation and ovulation.

IX. HYPOTHESIS

The expression of the components of the PA system by ovarian cells and their regulation during follicular development are dependent on the action(s) and interaction(s) of gonadotropins and intra-ovarian factors with mitogenic, differentiative and anti-differentiative properties.

X. OBJECTIVE

The overall objective of the present investigation is to examine the action(s) and interaction(s) of gonadotropin and intraovarian factors in the regulation of plasminogen activator system during granulosa cell differentiation in the course of follicular development. The questions addressed are:

1. Which follicular cell type(s) is/are involved in the synthesis of the PA system? Is the expression of the various components of the PA system dependent on follicular maturation?
2. Does gonadotropic regulation of the granulosa cell PA system depend on the differentiative stage of the cell and thus on ovarian follicular development?
3. Do GRF and VIP regulate the granulosa cell PA system during follicular development? If so, how do they interact with FSH in this regulation?
4. Are TGFα and TGFβ involved in the regulation of the granulosa cell PA system? Are their actions dependent on follicular maturation? Do they modulate the action of gonadotropin on the PA system.
5. TNFα has an anti-differentiative effect on granulosa cells and attenuates the action of gonadotropin. Is this cytokine involved in the regulation of the granulosa cell PA system during follicular development?
6. Is IL-1β involved in the regulation of the granulosa cell PA system? Is its action dependent on follicular maturation and related to its interaction with gonadotropin?

XI. MATERIALS & METHODS

A. Reagents.

Culture media (Minimum Essential Medium and M199) and reagents (L-glutamine, non essential amino acids, penicillin, streptomycin and fungizone) were purchased from Gibco/Bethesda Research Laboratories (Burlington, Ontario, Canada). Growth hormone releasing factor (GRF), Vasoactive intestinal peptide (VIP), Diethylstilbestrol (DES), equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG), human urokinase plasminogen activator (huPA), bovine thrombin, fibrinogen, agarose (low gelling temperature), calf thymus DNA, normal rabbit and mouse IgG, morpholinopropanesulfonic acid (MOPS), ethylenediaminetetraacetic acid (EDTA) guanidine thiocyanate (GTC), diethylpyrocarbonate (DEPC), 3,3'-diaminobenzidine tetrachloride (DAB), ethidium bromide, salmon sperm DNA, Hoescht 33258 compound, sodium citrate, Denhardt solution, triton X-100 and Tween-20 were obtained from Sigma Chemical Co. (St Louis, MO, USA). Ovine FSH (NIAMDD oFSH-14) was obtained from NIDDK. Recombinant human interleukin-1β (IL-1β) and recombinant human tumor necrosis factor-alpha (TNFα) were purchased from Collaborative Research (Bedford, MA, USA). Radioiodinated fibrinogen (167-186 μCi/mg) was purchased from Chedoke-McMaster Hospital (Hamilton, Ontario, Canada). scintillation fluid (ScintiVerse), [1,2,6,7,21 ^{3}H(N)] progesterone (162 Ci/mmol) and [1,2 ^{3}H(N)] 20α-dihydroprogesterone (45.6 Ci/mmol) were obtained from Dupont/NEN Research Products (Mississauga, Ontario, Canada). Chemiluminescence detection kit, α[^{32}P]dCTP (250 μCi/μg), multiprime DNA labelling kit, anti-rabbit IgG-biotylinated, anti-mouse IgG-biotylinated and [methyl-^{3}H]thymidine (25 Ci/mmol) were obtained from

Amersham Life Science (Oakville, Ontario, Canada). Formamide was purchased from Clontech Laboratories, Inc (Palo Alto, CA, USA). Acrylamide (electrophoresis grade), N,N'-methylene-bis-acrylamide, ammonium persulfate, tetramethylethylene diamine, dithiothreitol, glycine, SDS-PAGE prestained molecular weight standards (low range), polyvinylidene difluoride membranes, Zeta-probe blotting membrane, anti-rabbit and anti-mouse IgG-horse radish peroxidase conjugate were products of Bio-Rad (Richmond, CA, USA). Hydroxyethylpiperazine ethanesulfonic acid (HEPES), sodium dodecyl sulphate, bromophenol blue and trizma base were obtained from BDH (Toronto, Ontario, Canada). Human plasminogen was purchased from Boehringer Mannhein (Montreal, Quebec, Canada). Human plasminogen activator inhibitor, rabbit anti-mouse uPA, mouse anti-human tPA, rabbit anti-rat PAI-1, rabbit anti-rat uPAR were obtained from American Diagnostica Inc. (Greenwich, CT, USA). Isopropanol and phenol:chloroform:isoamyl were purchased from Promega Corporation (Madison, WI, USA). 20α-dihydroprogesterone and progesterone antisera were gifts from Dr. D.T. Armstrong (University of Western Ontario, London, Ontario, Canada). cDNA probes for uPA and uPAR were kindly provided by Dr. S.A. Rabbani (University of McGill, Montreal, Quebec, Canada). cDNA probes for tPA and PAI-1 were gifts from Dr. X. Zhang (University of Calgary, Calgary, Alberta, Canada).

B. Methods.

1. Granulosa cell isolation and culture

A group of immature female 22-day-old Sprague Dawley rats (50-60 g, from Charles River Canada, Montreal, Quebec, Canada) was injected subcutaneously daily for 3 days with 1 mg DES per day (in 0.1 ml sesame oil). Rats were killed by cervical dislocation 72 h

following the first injection of DES. A second group of rats (23-day-old) was injected intraperitoneally with 4 IU eCG and was sacrificed 48 h later. These treatments synchronize ovarian follicular development at the preantral/small antral and medium antral stages, respectively and offer the preparations of undifferentiated and differentiated granulosa cells with high yield and efficiency (Farookhi, 1982; Sheela et al., 1983). The physiological characteristics of granulosa cell differentiation include increased ability of the cells to synthesize steroid hormones and to express LH receptors in response to gonadotropins. Whereas granulosa cells from DES-treated rats can be stimulated by FSH, but not LH, to produce progesterone, cells from eCG-treated rats respond to both FSH and LH (Zeleznik et al., 1974). Moreover, this stimulatory effect of FSH was more pronounced in granulosa cells isolated from eCG-treated rats (Carnegie and Tsang, 1984). In addition, the presence of LH receptor could only be detected in granulosa cells from eCG-treated rats (Camp et al., 1991; Piquette et al., 1991) and PA activity in these cells was also increased by LH (Karakji and Tsang, unpublished observation). Thus, these two models provide a convenient means for the study of granulosa cell functions *in vitro* during follicular development.

The animals were fed prolab RMH 4018 (AGWAY Inc., C.G., Syracuse, New York) and water ad libitum. A 14 h/10 h light/dark cycle was maintained with light cycle initiated at 06:00 h. Ovaries were collected in ice-cold medium 199 (M199) supplemented with HEPES (25 mM; pH 7.4). Granulosa cells from each group of animals were harvested by follicle puncture as previously described (Dorrington et al., 1975), washed (900 x g, 10 min) and resuspended in M199 by gentle agitation. The viability of granulosa cells determined by trypan blue exclusion test was 55-65%. To increase the percentage viability of the cell preparation, a trypsinization method described by Farookhi (1982) was used. Trypsin (50 μg)

was added to 1 ml of culture medium containing one million granulosa cells. The trypsin reaction was carried on at 37°C for 1 min and stopped with the addition of an excess amount of trypsin inhibitor. Dissociation of cell aggregates were facilitated with the addition of DNase (25 μg/ml). The viability of granulosa cells was increased to 95%.

One hundred thousand viable granulosa cells were cultured at 37°C for up to 72 h under humidified atmosphere of 95% air and 5% CO_2 in 16 mm-well tissue culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) containing in each well 0.5 ml of Minimum Essential Medium (MEM; pH 7.4) supplemented with sodium bicarbonate (26 mM), L-glutamine (0.29 mg/ml), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (0.6 μg/ml) but without phenol red. At the end of the culture period, the medium was removed, adjusted with triton X-100 (final concentration of 0.1% unless indicated otherwise) and stored in aliquots at -20°C. Cells in each well were scraped in 0.5 ml of fresh MEM with a rubber policeman and sonicated for 5 sec using a Fisher sonic dismembrator (model 300, Fisher Scientific, Fair Lawn NJ) set at a relative output of 35. One hundred μl of each sample was added to an equal volume of 0.05 M phosphate buffer (0.05 M NaH_2PO_4, 2M NaCl and 2 mM EDTA, pH 7.4) for DNA determination. The remaining samples were treated with triton X-100 (final concentration of 0.1% unless indicated otherwise) at 4°C for 1 h and stored in aliquots at -20°C.

2. Fibrinolysis assay.

Net PA activity (protease activity resulting from the presence of endogenous uPA, tPA and PAI) secreted into the medium (PAs) or associated with the cellular compartment (PAc) were determined by a fibrinolysis assay (Ailenberg et al., 1990). Briefly, 96-well

microtiter plates precoated with [^{125}I] fibrinogen (0.1 x 10^6 cpm; 2 μg/well) were incubated with thrombin (2 mM) for 1 h at 37°C for the conversion of fibrinogen to fibrin. The wells were then washed three times with 250 μl tris-HCl buffer (0.1 M, pH 8.1) for 20 min to reduce assay background activity. The assay was carried out at 37°C for 4 h in a final volume of 250 μl: 100 μl tris buffer, 100 μl of sample or different concentration of huPA as standard and 50μl of human plasminogen (0.05 μg). Plasminogen-independent fibrinolytic activity in the samples was evaluated in the absence of plasminogen. Basal and maximum release of iodinated fibrin degradable products were tested with plasminogen in the absence of sample and in the presence of trypsin (0.5% wt/vol), respectively. The radioactivity released into the assay buffer at the end of incubation period was determined. PA activity was expressed as mU uPA equivalence/μg DNA.

3. Zymography.

PA and PA inhibitor (PAI) activities were characterized by fibrin overlay assay and reverse fibrin overlay assay, respectively, as previously described (Granelli-Piperno and Reich, 1978). For the PA assay, proteins in 100 μl samples were adjusted to a final volume of 150 μl with a non-reducing loading buffer (5% sodium dodecyl sulfate (SDS), 20% glycerol and 0.001% bromophenol) and separated on 9% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 7.5 mA/gel until the bromophenol blue tracking dye migrated out of the gel (Lammeli, 1970). The electrophoretic gel, washed twice for 45 min with 2.5% (wt/vol) triton X-100 to remove SDS and subsequently with distilled water, was overlaid onto a 1% low melting point agarose indicator gel containing rat plasminogen [5 μg/ml; purified from rat plasma by Lysine-Sepharose 4B affinity chromatography (Deutsch

and Mertz, 1970)], fibrinogen (2 mg/ml) and thrombin (0.2 U/ml). PAI activity was measured as in PA assay described above but under reducing conditions (0.04% β-mercaptoethanol present in loading buffer) and with exogenous huPA (0.02 U/ml) present in the indicator gel.

4. Western blot.

uPA and tPA were detected with a previously described immunoblotting procedure, following minor modifications (Johnson and Tilly, 1990). Briefly, proteins were separated on a 9% polyacrylamide gel electrophoresis, equilibrated in transfer buffer (38.6 mM glycine, 47.8 mM tris base and 0.13 mM SDS were dissolved in 200 ml of methanol and adjusted to a final volume of 1 liter with H_2O, pH 8.1) and transferred to a polyvinylidene difluoride membrane using the Bio-Rad Trans-Blot System. Non-specific binding to the membranes was blocked with 5% BSA (w/v) in 0.01 M phosphate buffered-saline (PBS; 136 mM NaCl, 2.68 mM KCl, 11.8 mM Na_2HPO_4, 1.76 mM KH_2PO_4 ; pH 7.4) at room temperature for 2 h. Rabbit anti-mouse uPA (50 μg/ml) or mouse anti-human tPA (20 μg/ml) was diluted in PBS containing 0.1% BSA and added to the blot overnight at 4°C. The membrane was then washed twice with 0.1% Tween 20 (in PBS) and incubated for 1 h at room temperature with anti-rabbit or anti-mouse IgG-horse radish peroxidase conjugate (100 μg/ml; diluted 1:250 in PBS containing 0.1% BSA and 0.1% Tween-20), respectively. Binding of the second antibody was detected using the chemiluminescence detection kit.

5. Immunocytochemistry.

In addition to the two groups of animals prepared as described in section XI.B-1, a third group of rats were injected intraperitoneally with 4 IU eCG and followed 48 h later with 15 IU hCG. The rats were sacrificed 8 h after hCG injection. This treatment regimen generates preovulatory follicles which are expected to ovulate 12 h after hCG injeection (Liu et al., 1991). The ovaries from each group were fixed in 4% paraformaldehyde in PBS at room temperature for 12 h, dehydrated through a graded series of ethyl alcohol concentrations (70-100%) and embedded in low-temperature paraffin as previously described but with slight modifications (Teerds and Dorrington, 1993). Sections (5 μm thick) were mounted on poly-L-lysine-coated slides, deparaffinized in xylene, and rehydrated in a series of ethyl alcohol concentrations (100%-0%). After 2 min incubation in tris-buffered saline (TBS; 0.1 M tris-base, 0.15 M NaCl; pH 7.4), the endogenous peroxidase activity was blocked with 2% H_2O_2 in methanol for 30 min. The slides were subsequently washed with TBS, blocked with 5% bovine serum albumin (in TBS with 0.1% triton X-100) for 1 h to reduce the nonspecific binding and incubated overnight at room temperature with primary polyclonal antibody (rabbit anti-mouse uPA, rabbit anti-rat uPAR and rabbit anti-rat PAI-1; 10 μg/ml) or monoclonal mouse anti-rat tPA (30 μg/ml). After extensive rinsing with TBS, sections were incubated with biotylinated goat anti-rabbit IgG (secondary antibody; 1:100 in TBS with 5% BSA and 0.1% triton X-100; 1 h). The slides were then washed with TBS and incubated with avidin-D-conjugated horse radish peroxidase (1:100 in TBS containing 5% BSA and 0.1% triton X-100; 1 h). The reaction was visualised with 3,3'-diaminobenzidine tetrachloride (1 mg/ml) and 0.03% H_2O_2 and enhanced with 0.5% lithium chloride. Control sections were incubated in the absence of primary antibody with or without

normal rabbit IgG or normal mouse IgG (in the case of tPA). Six animals were used in each experimental group.

6. Total RNA extraction and Northern blot analysis

Granulosa were isolated from ovarian tissues at different stages of follicular development (described in section XI.B1). The residual ovaries which consist primarily of theca-interstitial cells were washed extensively with M199 to release undissociated granulosa cells. Both cell preparations were transferred to clean tubes, frozen in dry ice and stored at -80°C. Total cytoplasmic RNA were isolated by homogenizing and solubilizing follicular cells in GTC as previously described (Chirgwin et al., 1979) with some modifications. The homogenate was then extracted with acidified phenol and precipitated with isopropanol. The RNA pellet was dissolved in DEPC-treated water, quantified spectrophotometrically at 260 nm, aliquoted and stored at -80°C. RNA samples (10-15 μg) were size-fractionated by electrophoresis on formaldehyde-agarose gels (1.1%) containing 1 μg/ml ethidium bromide to confirm even loading of RNA samples and adequate separation of 28S and 18S ribosomal bands. RNAs seperated by electrophoresis were blotted onto nylon membrane via capillary action and cross-linked by UV light. Tissue-type PA, uPA, PAI-1 and uPAR cDNA probes were labelled using a Random Primed [α-^{32}P dCTP] DNA labelling kit. Membranes were prehybridized in 50% formamide, saline sodium citrate (SSC; 750 mM NaCl, 75 mM sodium citrate), 10X Denhardt's solution, 1% SDS, 4 mM EDTA and 100 μg/ml sheared salmon sperm DNA for 4 h at 42°C, using tightly closed pyrex tubes and a hybridization incubator (Robbins Scientific, Sunnyvale, CA, USA). Hybridization was performed overnight at 42°C with 20 X 10^6 cpm of labelled probes added to the prehybridization buffer. The membranes

were then washed twice with SSC (300 mM NaCl, 30 mM sodium citrate) in 0.1% SDS for 20 min at room temperature and twice with SSC (30 mM NaCl, 3 mM sodium citrate) in 0.1% SDS for 20 min at 55°C, sealed in a plastic bag and exposed to X-ray film at 80°C for visualization. Densitometric analysis of tPA, uPA, PAI-1 and uPAR mRNA bands was performed using a Molecular Dynamics Phosphoimager (BioRad, Mississauga, Ontario, Canada). Data were normalized against cyclophylin mRNA by using the same blot stripped of radioactivity (0.1% SDS) and reprobed with cyclophilin cDNA probe.

7. Progestin assays.

Concentrations of progesterone (P) and 20α-dihydroprogesterone (20α-OH-P) in the spent medium following 72 h of culture were determined by radioimmunoassay, as previously described (Yallow, 1985). Phosphate buffered saline-gelatine solution [PBSG: sodium phosphate dibasic (76 mM), sodium phosphate monobasic (20 mM), sodium chloride (154 mM), EDTA (1.27 mM), sodium azide (2.92 mM) and gelatin (1 g/L); pH 6.9] was used to dissolve radiolabelled steroids and corresponding antibodies. The total assay volume was 300 µl: 100 µl of spent medium or steroid standards (0, 5, 10, 20, 40, 80, 160, 320 and 640 pg) in MEM, 100 µl of radiolabelled P or 20α-OH-P (15,000 cpm) in PBSG and 100 µl of antibody in PBSG (at a concentration to achieve 20-40% binding of the tritiated ligand). The assay mixture was vortexed gently and incubated overnight at 4°C.

The tubes in the standard curve included those for the "total counts" (total counts of tritiated steroid added to each assay tube), "B_0" (binding of tritiated steroid to the antibody in the absence of unlabelled steroid) and an assay blank subtracted from all values calculated from the assay tubes. Steroid standards were measured in triplicate and samples were

analyzed in duplicate.

Following overnight incubation, the assay tubes were placed on an ice bath and 750 µl of charcoal mixture [charcoal (3.57 g/l) and dextran (0.357 g/) in PBS] were added to each tube to absorb unbound steroid, left on ice for 15 minutes and then centrifuged (550 x g for 15 minutes) to separate bound from free steroids. The supernatant containing the antibody-steroid complex was decanted into scintillation vials containing 4 ml of scintillation fluid and the radioactivity was determined in a liquid scintillation counter.

The sensitivity of both assays was 5 pg/tube. The intra- and inter-assay coefficients of variation were 6.7 and 11.2 % for P and 8.2 and 12.3% for 20α-OH-P, respectively.

8. Thymidine incorporation assay.

Incorporation of [methyl-^{3}H] thymidine into DNA, an index for DNA synthetic capacity of cultured rat granulosa cells, was determined as previously described (Miro et al., 1991). Following a 64 h culture period in the absence or presence of test agents, granulosa cells were cultured for an additional 8 h in the presence of 100,000 cpm [methyl-^{3}H] thymidine. The cells were washed with MEM twice with and once without unlabelled thymidine (100 µg/ml), fixed with trichloroacetic acid (5% wt/vol, at 4°C, 20 min) and finally washed twice with methanol. The DNA pellet was dissolved in NaOH (0.25 M), adjusted to neutral pH with HCl (0.25 M), aliquoted for DNA assay and counted to determine the level of radioactivity incorporated. DNA synthesis was expressed as cpm/µg DNA. To ascertain if the observed changes in [methyl-^{3}H] thymidine incorporation reflect DNA synthesis or DNA repair, the above studies were also carried out in the presence of hydroxyurea (0.3 mM), an inhibitor of DNA synthesis (Krakoff et al., 1968).

9. Cellular DNA content.

Aliquots of the above DNA pellet and calf thymus DNA (standard) were incubated with Hoechst 33258 dye (0.1 μg/ml, 5 min in the dark) as described previously (Cesarone et al., 1979; Labarca and Paigen, 1980). Changes in fluorescence intensity were measured in a Perkin-Elmer LS-5 luminescence spectrometer (Norwalk, CT, USA), with excitation and emission wavelengths set at 356 and 457 nm, respectively. The sensitivity of the assay was 15 ng/ml and the assay was linear up to 4 μg/ml. The intra- and inter-assay coefficients of variation were 1.6 and 4.2%, respectively.

10. Statistical analysis.

Results are presented as means ± S.E.M. of 3-5 replicate experiments, each consisting of 3 wells for each treatment group, and analyzed by one-, two- or three-way analysis of variance. Where required, data were transformed logarithmically before statistical analysis to remove heterogeneity of variance, as determined by the Barlett's test. Differences between groups were determined by the least significant difference multiple range test. Statistical difference was inferred at $P < 0.05$. Experiments using zymograms were repeated 3-4 times and photographic records of representative experiments are presented.

XII. RESULTS.

A. OPTIMIZATION OF PA ASSAY CONDITIONS AND PROCEDURE FOR CELL PREPARATION

1. Effect of Triton X-100 on PA Activity

Treatment of granulosa cells with triton X-100 induces cell membrane disruption and causes the release of cell-associated PA (Knecht, 1986). However, the influence of this detergent on PA activity per se is unclear. Granulosa cells from eCG-treated rats were cultured for 24 h in the absence or presence of FSH (400 ng/ml) and PA activity in the spent medium with and without triton X-100 (0.025-0.1%) addition was assayed. PA activity in the medium as well as exogenous human uPA standard (Fig 3) was significantly enhanced in the presence of triton. The detergent had no significant effect on the background count of the assay (in the absence of plasminogen and PA; Fig 4). The uPA standard curve and the volume-response curve for the PA-containing spent medium were both shifted to the left in the presence of triton (0.025 and 0.1%) and parallelism was preserved between them (Fig 3).

2. Effect of Trypsin Treatment of Granulosa Cells on PAs and PAc Activities.

Farookhi (1982) has demonstrated that pretreatment of granulosa cells with trypsin increased cell viability of the preparation. However, the effect of trypsin on the responsiveness of these cells, particularly in PA secretion, is not fully understood. Differentiated granulosa cells, with or without pretreatment with trypsin (as described in the method section B1) were cultured for 24 h in the presence of FSH (400 ng/ml). Pretreatment

of granulosa cells with trypsin (1 min) before culture significantly suppressed ($P < 0.05$) FSH-induced total PA activity (i.e. PAs + PAc; Fig 5). These findings indicate that the trypsin pretreatment decreases the responsiveness of granulosa cells to the gonadotropin, which could possibly be attributed to a loss in FSH receptors during trypsin digestion. In addition, although 0.025% and 0.1% triton X-100 protected PAs activity, a greater increase in FSH-stimulated PAc activity was elicited at the higher concentration in both trypsinized and non-trypsinized preparations ($P < 0.05$; Fig 5). Thus, 0.1% triton was used for the extraction of cell-associated PA from the samples in subsequent experiments and granulosa cells used in subsequent studies were prepared without trypsin treatment.

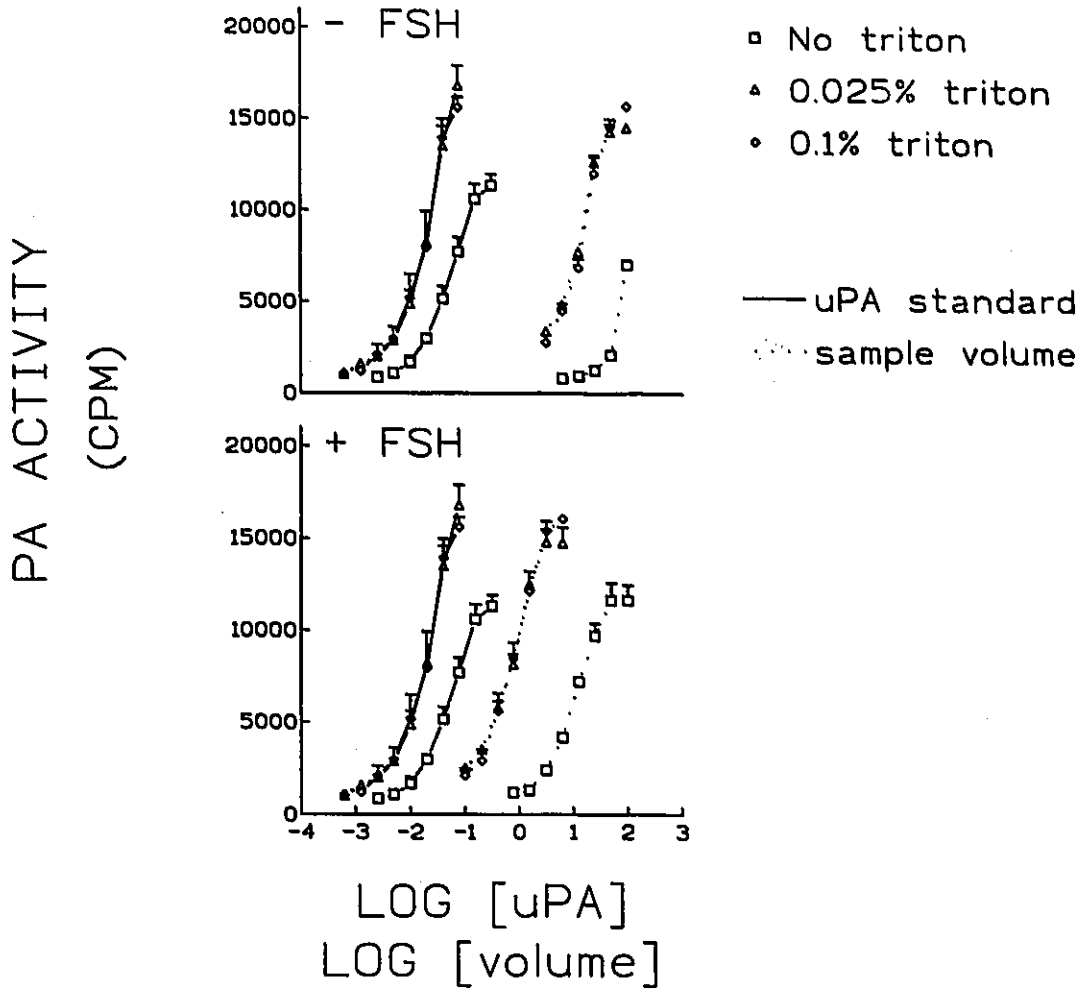

Fig 3. Effect of triton X-100 on uPA standard curve (0.78- 100 mU) and volume-response curve of PA-containing spent medium (0.78-100 µl) after 24 h cultures of granulosa cells from eCG-treated rats. PA activity is expressed in cpm (mean ± SEM of 3 experiments).

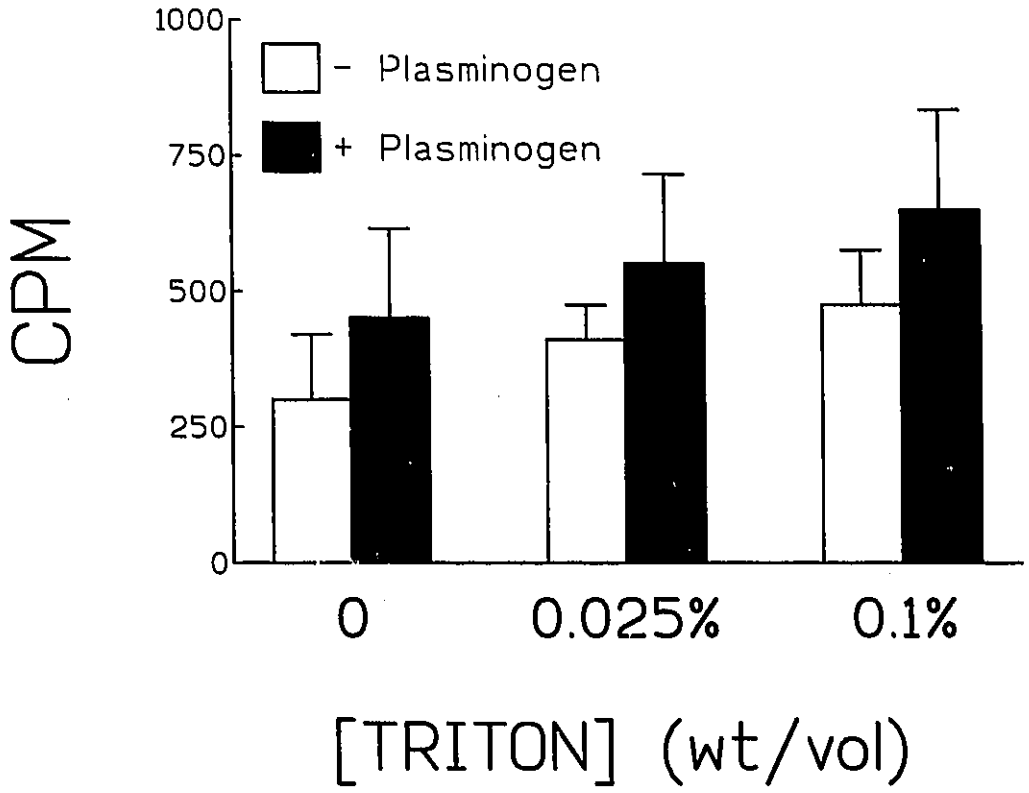

Fig 4. Influence of triton X-100 on background counts (cpm; mean ± SEM of 3 experiments) of the fibrinolysis assay in the absence and presence of plasminogen (0.05 μg).

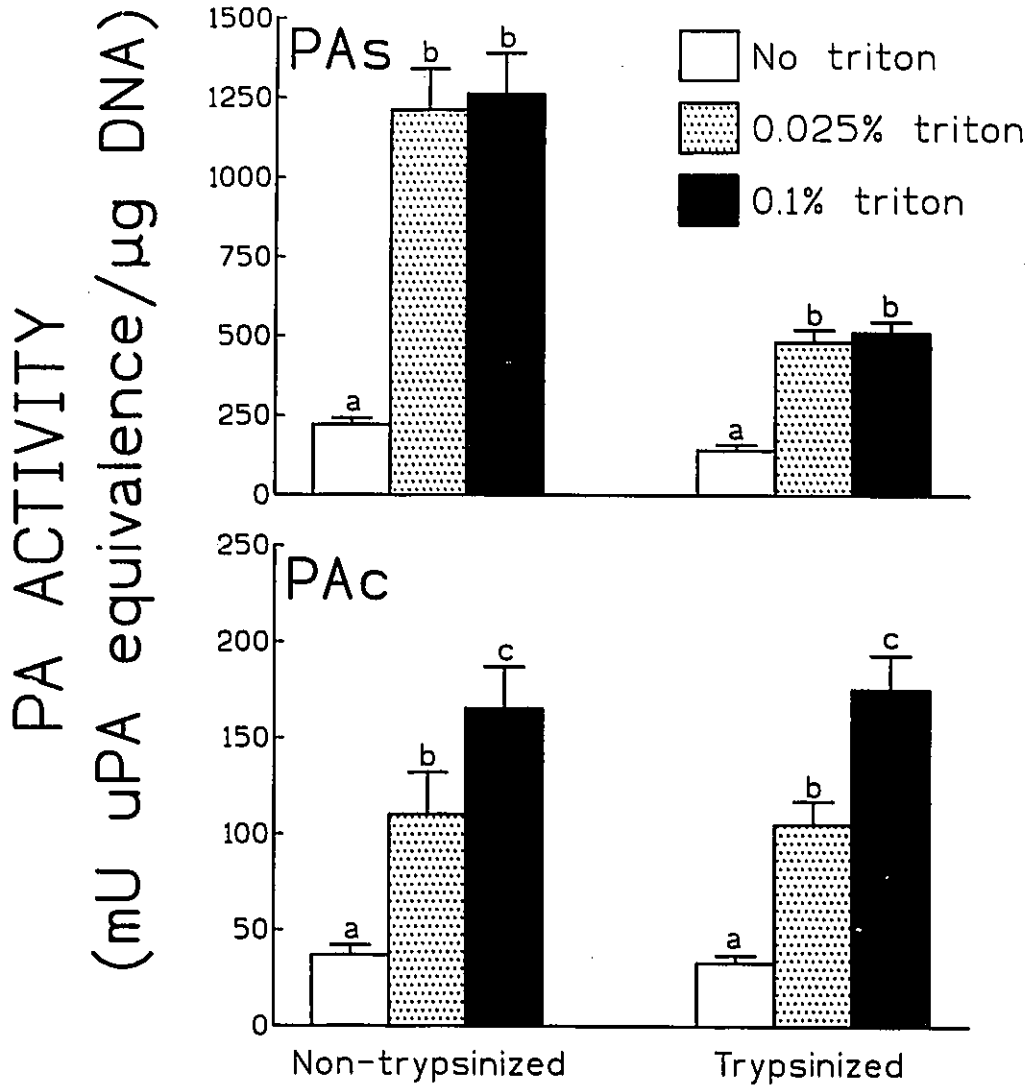

Fig 5. Effect of pretreatment of granulosa cells from eCG-treated rats with trypsin (50 µg/million cells) on FSH-induced PAs and PAc activities (mU uPA equivalence/µg DNA; mean ± SEM of 3 experiments) in a 24h culture period. Value with different alphabetical superscripts are significantly different.

B. WHICH FOLLICULAR CELL TYPE(S) IS/ARE INVOLVED IN SYNTHESIS OF THE PA SYSTEM? IS THE EXPRESSION OF THE VARIOUS COMPONENTS OF THE PA SYSTEM DEPENDENT ON FOLLICULAR MATURATION?

Total RNA were extracted from granulosa and theca-interstitial cells of ovarian follicles at preantral and small antral (DES-treated rats), medium antral (eCG-treated rats) and large antral/preovulatory (eCG and hCG-treated rats) stages of development. Changes in the level of tPA, uPA, PAI-1 and uPAR mRNA were analyzed by Northern blot. In addition, these components of the PA system were localized in ovarian sections with follicles at different stages of maturation by immunocytochemistry.

While tPA mRNA was expressed in granulosa and theca-interstitial cell preparations, the levels varied with the stages of follicular growth (Fig 6). Tissue-type PA mRNA abundance for both cell types was low in the DES and eCG groups but was markedly higher ($P < 0.05$) in the eCG + hCG group. The expression of tPA mRNA was consistently higher, at least three times, in the granulosa cells compared to the theca-interstitial cells in the eCG + hCG-treated rats (Fig 6).

Similarly, the intensity of staining for tPA protein in ovarian sections appeared to increase with follicular development (Fig 7). Whereas very little staining was observed in the granulosa cell layer of preantral follicles, the staining intensity steadily increased during the antral and preovulatory stages. Likewise, occasional tPA positive cells were detected in the theca-interstitial cell compartment at early stages of follicular maturation. However, by the preovulatory stage more intensely stained theca and interstitial cells were observed (Fig 7, E and F). The overall staining in this layer appeared more pronounced with follicular

development. Interestingly, the oocyte staining was intense but did not change with follicular maturation.

Urokinase PA mRNA abundance was determined in both granulosa and theca-interstitial cells at different stages of follicular growth (Fig 8). In contrast to tPA mRNA, levels of uPA message in both cell preparations decreased with follicular maturation and were not different at each stage of follicular development examined (Fig 8). Urokinase PA protein distribution in ovarian sections followed a similar pattern as for the uPA mRNA (Fig 8). Intense immunostaining for uPA was uniformly distributed within the granulosa and theca interna layer during early follicular development (Fig 9, C vs A and B). However, scattered cellular staining for uPA was detected in both cell compartments with follicular maturation. As observed for tPA (Fig 7), the oocytes also contained high levels of uPA throughout follicular development (Fig 9).

PAI-1 mRNA were highest in the granulosa and theca-interstitial cell preparations during the preovulatory period (eCG + hCG group; Fig 10). There was no significant difference in PAI-1 mRNA abundance between the granulosa and theca-interstitial cell preparations (Fig 10). Compared to control (Fig 11, A), low but detectable immunostaining for PAI-1 was observed in ovarian sections taken from DES- and eCG-treated rats (Fig 11, B and C). The intensity of PAI-1 staining increased with follicular development, reaching peak levels in the preovulatory period. In general, PAI staining was fairly uniformly distributed between granulosa and theca interna cells (Fig 11, D and E). Irrespective of the stages of follicular development, PAI-1 immunoreactivity was also evident in the oocyte.

Urokinase PAR transcript was detected in both granulosa and theca-interstitial cell preparations (Fig 12). While the theca-interstitial cells showed a developmental increase in

uPAR mRNA level, high levels of this message in granulosa cells was detected only during the preovulatory stage (eCG plus hCG-treated group; Fig 12). Comparable levels of the transcript were observed in granulosa and theca-interstitial cells at each stage of follicular maturation (Fig 12). The pattern of uPAR immunoreactivity was similar to the changes in uPAR mRNA levels: the most intense staining observed in the preovulatory follicles (Fig 13). This immunostaining appears to be uniformly distributed between the two cell compartments although very intensely stained cells scattered throughout the theca interna layer were evident (Fig 13, E). In contrast minimal staining was observed in the preantral as well as small and medium antral follicles (Fig 13, C and D). The oocytes were highly stained for uPAR at all stages of follicular maturation studied.

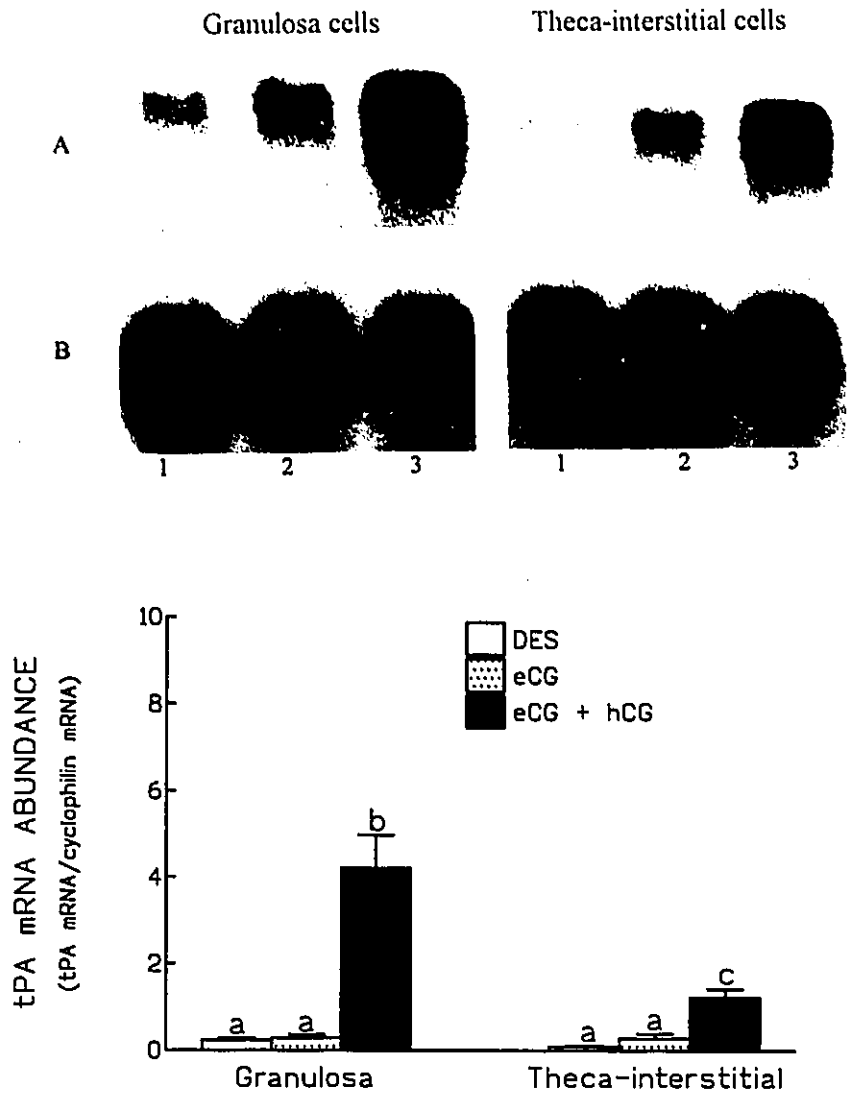

Fig 6. Tissue-type PA mRNA abundance in granulosa and theca-interstitial preparations from ovarian follicles of DES- (Lane 1), eCG- (lane 2) and eCG plus hCG- (lane 3) treated rats. Top panel shows a representative Northern blot demonstrating tPA bands (A) and cyclophilin bands (B). Lower panel shows the mean ± SEM of 4 experiments. Values with different alphabetical superscripts are significantly different $P < 0.05$.

Fig 7. Tissue-type PA immunolocalization in ovarian sections from DES- (C), eCG- (D) and eCG plus hCG- (E and F) treated rats. Controls included omission of primary antibody (mouse monoclonal anti-human tPA antibody, A) and replacement of the primary antibody with normal mouse IgG (B). O = oocyte, G = granulosa cells, T = theca interna cells, I = interstitial cells. Magnification: A-E, x100; F, x250

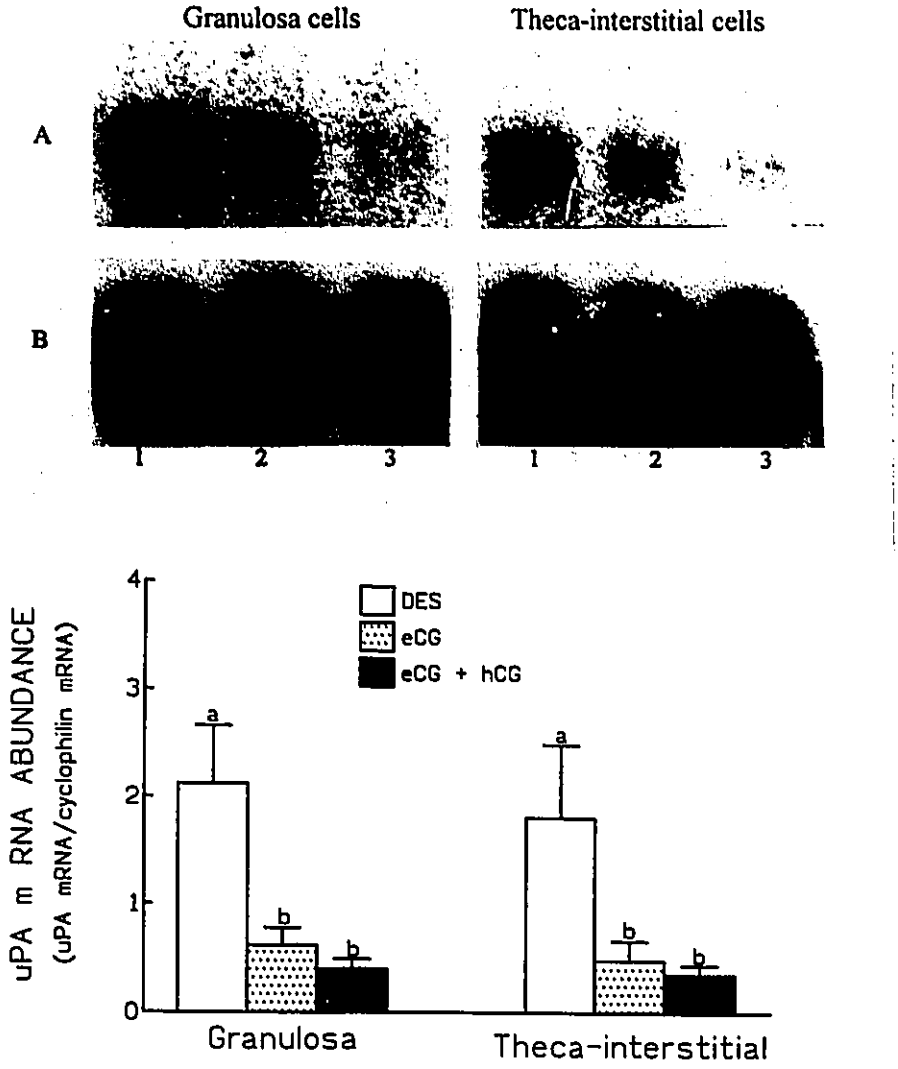

Fig 8. Urokinase PA mRNA abundance in granulosa and theca-interstitial preparations from ovarian follicles of DES- (lane 1), eCG- (lane 2) and eCG plus hCG- (lane 3) treated rats. Top panel shows a representative Northern blot demonstrating uPA bands (A) and cyclophilin bands (B). Lower panel shows the mean ± SEM of 4 experiments. Values with different alphabetical superscripts are significantly different $P < 0.05$.

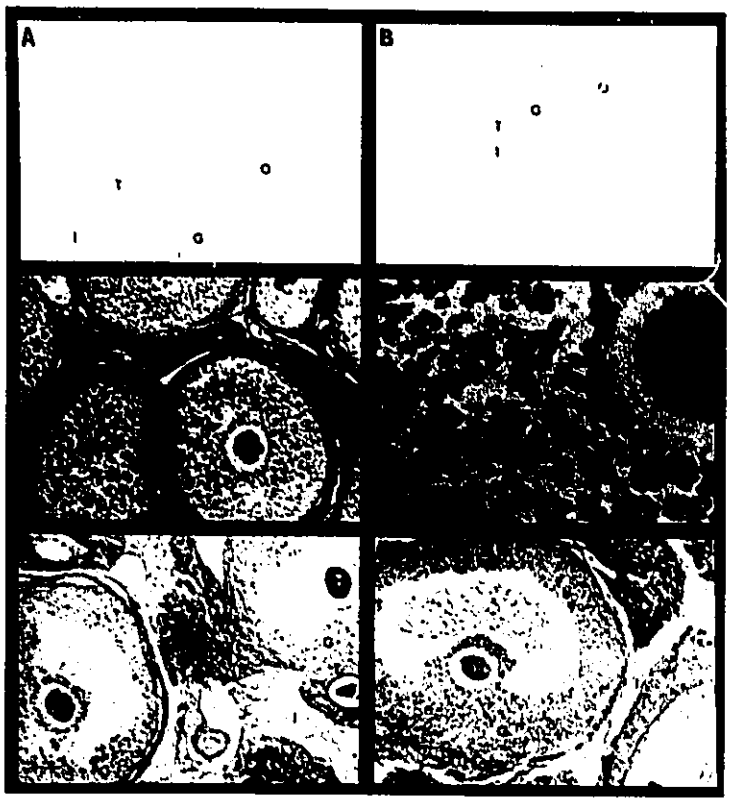

Fig 9. Urokinase PA immunolocalization in ovarian sections from DES- (C and D), eCG- (E) and eCG plus hCG- (F) treated rats. Controls included omission of primary antibody (polyclonal rabbit anti-mouse uPA antibody; A) and replacement of the primary antibody with normal rabbit IgG (B). O = oocyte, G = granulosa cells, T = theca interna cells, I = interstitial cells. Magnification: A-C and E-F, x100; D, x250.

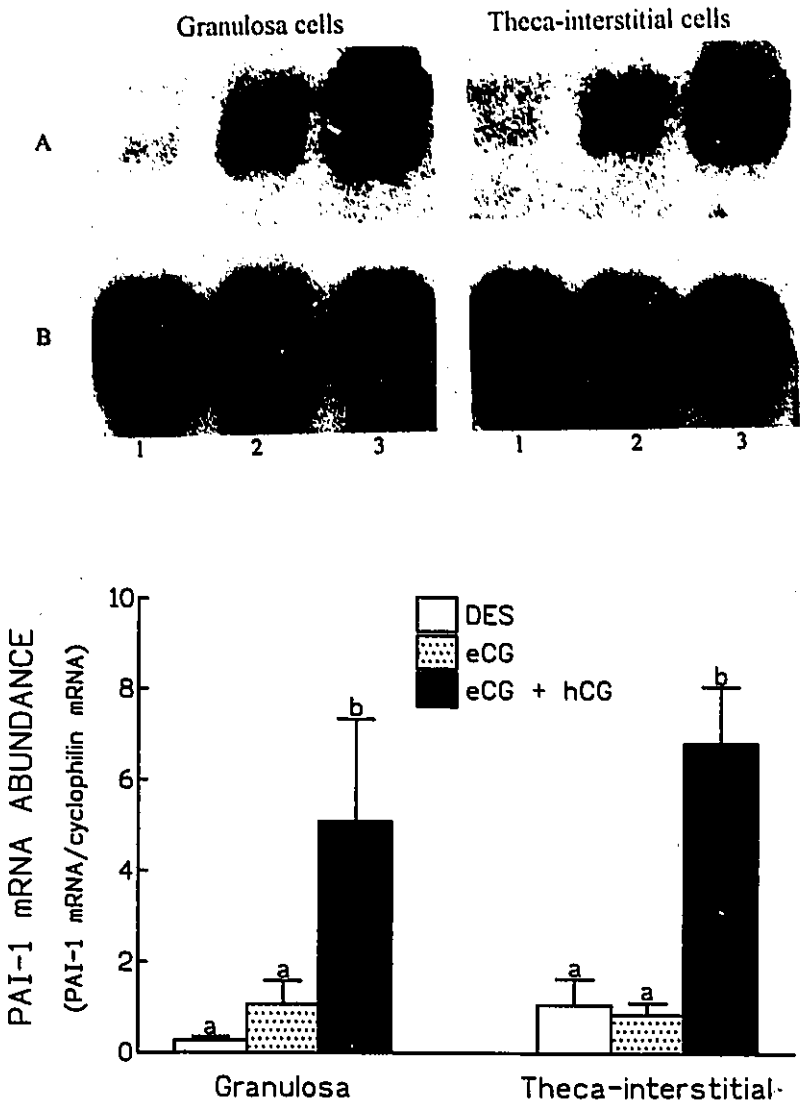

Fig 10. PAI-1 mRNA levels in granulosa and theca-interstitial preparations from ovarian follicles of DES- (lane 1), eCG- (lane 2) and eCG plus hCG- (lane 3) treated rats. Top panel shows a representative Northern blot demonstrating PAI-1 bands (A) and cyclophilin bands (B). Lower panel shows the mean ± SEM of 4 experiments. Values with different alphabetical superscripts are significantly different $P < 0.05$.

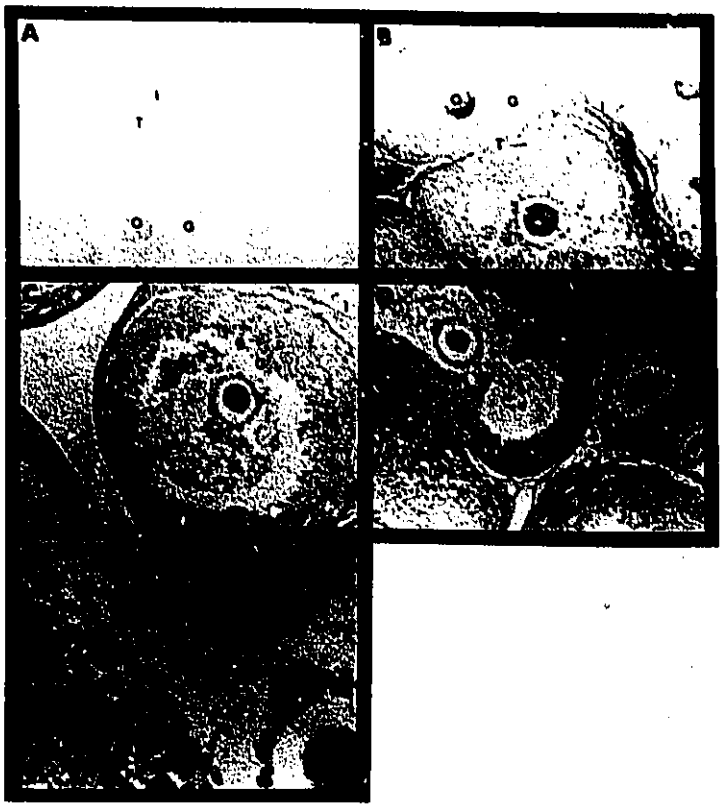

Fig 11. PAI-1 immunolocalization in ovarian sections from DES- (B), eCG- (C) and eCG plus hCG- (D and E) treated rats. Controls included replacement of the primary antibody (rabbit polyclonal anti-rat PAI-1 antibody) with normal rabbit IgG (A). O = oocyte, G = granulosa cells, T = theca interna cells, I = interstitial cells. Magnification: A-D, x100; E, x250.

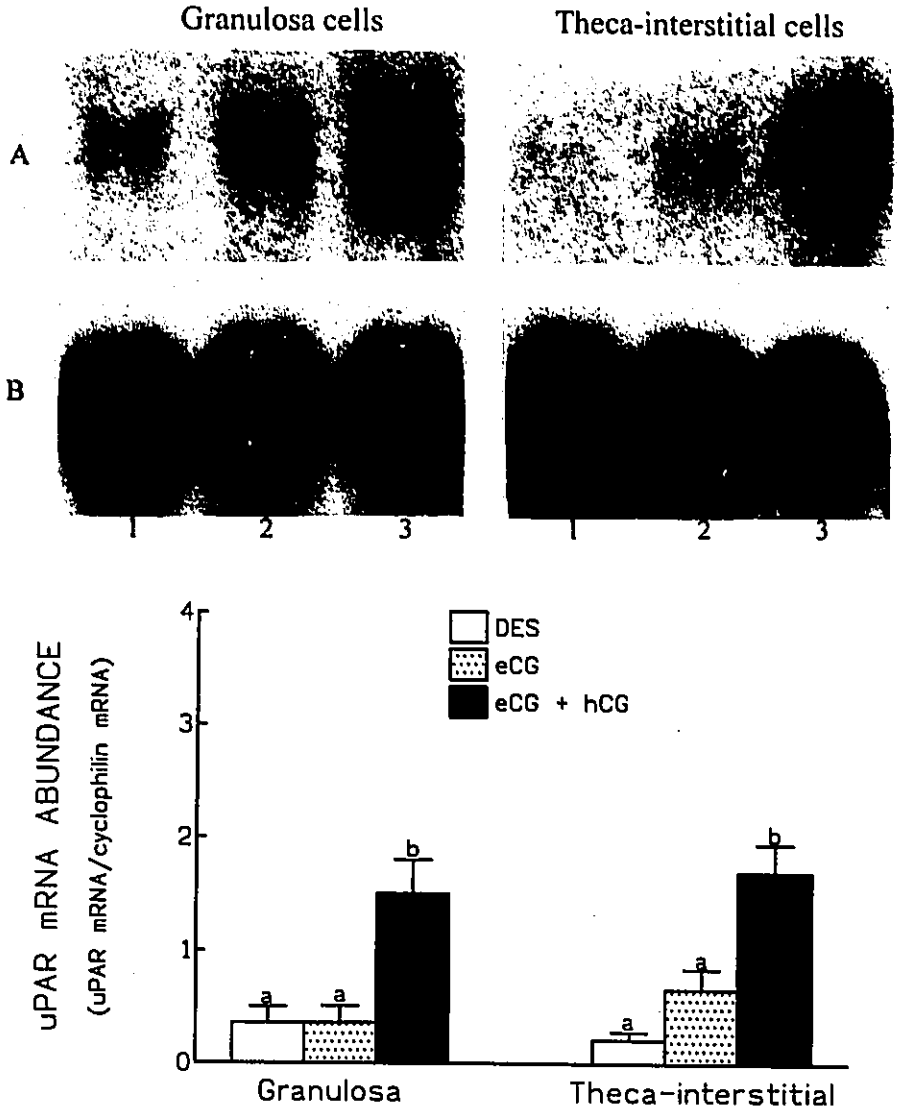

Fig 12. Urokinase PAR mRNA abundance in granulosa and theca-interstitial preparations from ovarian follicles of DES- (lane 1), eCG- (lane 2) and eCG plus hCG- (lane 3) treated rats. Top panel shows a representative Northern blot demonstrating uPAR bands (A) and cyclophilin bands (B). Lower panel shows the mean ± SEM of 4 experiments. Values with different alphabetical superscripts are significantly different $P < 0.05$.

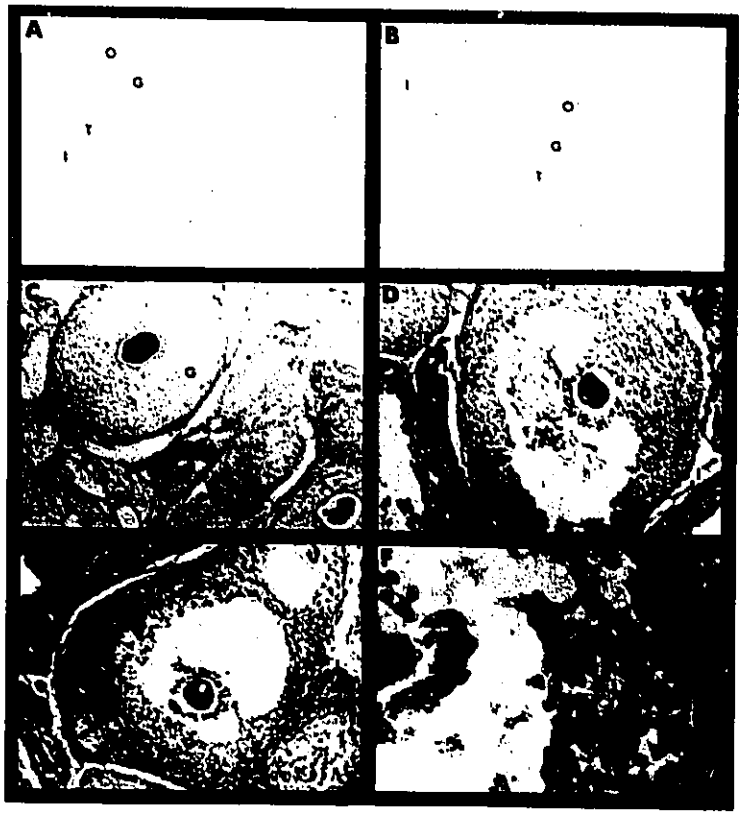

Fig 13. Urokinase PAR immunolocalization in ovarian sections from DES- (C), eCG- (D) and eCG plus hCG- (E and F) treated rats. Controls included omission of primary antibody (rabbit polyclonal anti-rat uPAR antibody; A) and replacement of the primary antibody with normal rabbit IgG (B). O = oocyte, G = granulosa cells, T = theca interna cells, I = interstitial cells. Magnification: A-E, x100; F, x250.

C. DOES GONADOTROPIC REGULATION OF THE GRANULOSA CELL PA SYSTEM DEPEND ON THE DIFFERENTIATIVE STAGE OF THE CELL AND THUS ON OVARIAN FOLLICULAR DEVELOPMENT?

1. Secreted Versus Cell-associated PA Activity

Unkeless and coworkers (1974) demonstrated that the synthesis and secretion of PA are coupled. In the present studies secreted and cell-associated PA activities in granulosa cells cultured in the presence and absence of FSH were determined to assess the relative levels of enzyme activity during gonadotropin stimulation. Irrespective of the stage of follicular development, basal and FSH-stimulated PAs activities accounted for 70-80% of the total PA activity present in granulosa cell cultures (Fig 14). FSH-induced PAs and PAc activities were markedly higher (~ 4-fold; $P < 0.05$) in 24 h cultures of granulosa cells from eCG-treated rats than those from DES-treated animals (Fig 14).

2. Concentration-dependent Influence of FSH on PA Activity in Undifferentiated and Differentiated Granulosa Cells.

Cultures of granulosa cells from DES- and eCG-treated for 24 h in the presence of various concentrations of FSH (0-800 ng/ml) showed a significant and dose-dependent enhancement ($P < 0.05$) of PAs activity by the gonadotropin (Fig 15). As the maximal stimulatory influence of FSH was observed at 400 ng/ml (Fig 15), unless indicated otherwise, this concentration was used in subsequent experiments.

3. Time Course Study of the Action of FSH on PAs Activity during Follicular Development.

To determine the time course of the effect of FSH on PA activities, granulosa cells from DES- and eCG-treated rats were cultured for 24, 48 and 72 h in the absence and presence of the gonadotropin (400 ng/ml). Whereas the maximal stimulatory effect of FSH on PAs activity was detected in differentiated granulosa cells (eCG-treated rats) after 24 h of culture (Fig 16), these activities were lost during the subsequent 48 h (Fig 16). However, FSH-induced PAs activity continue to rise during the 72 h culture period for the undifferentiated cells (DES-treated rats; Fig 16).

4. Definition of the PA Type(s) Secreted during Follicular Development and their Regulation by FSH

Zymographic analysis of the PAs activity in granulosa cell cultures showed two lysis bands (55 and 30 KDa) in undifferentiated granulosa cells (DES-treated rats) while only the band of higher molecular mass was detected in the differentiated ones (eCG-treated rats; plate 1). Western blot analysis of the high and low molecular mass PA species in 72 h cultures of granulosa cells from DES-treated rats identified the 55 and 30 KDa proteases as tPA and uPA, respectively (plate 2).

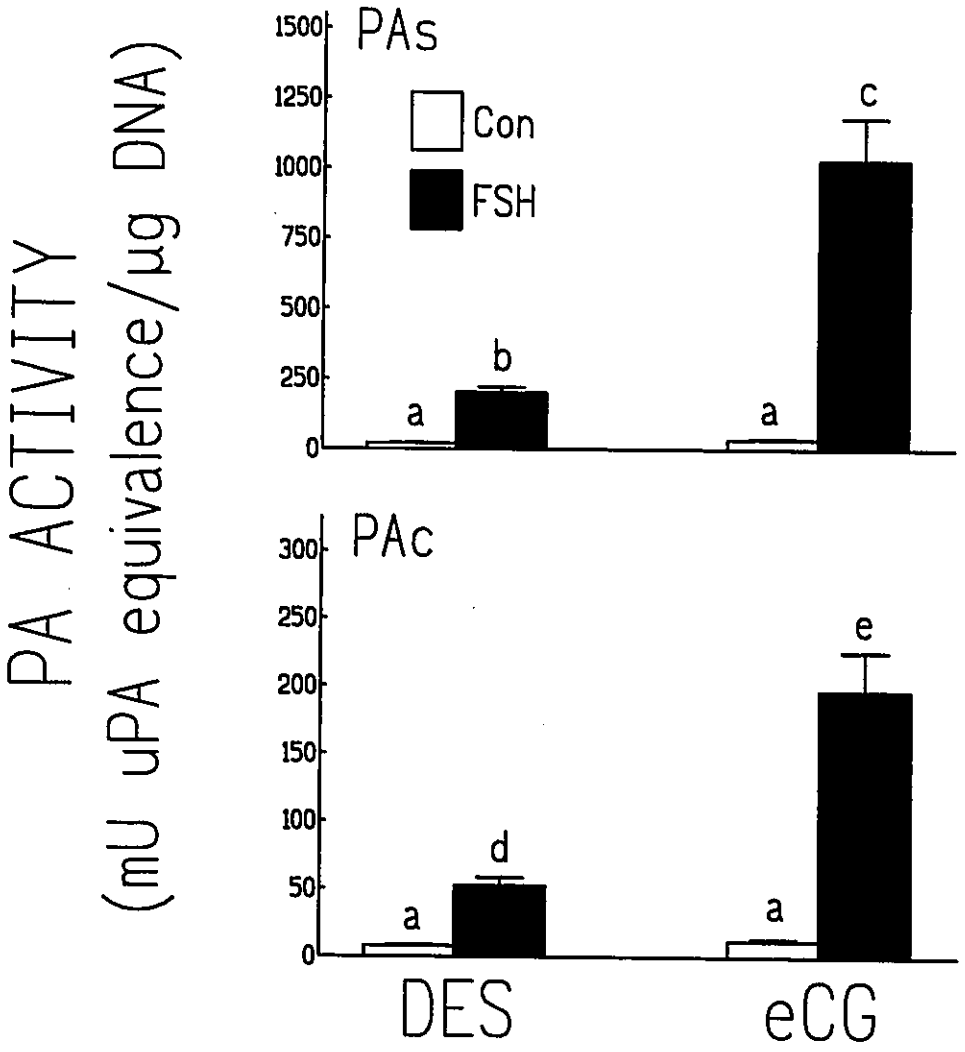

Fig 14: Effect of FSH (400 ng/ml) on PAs and PAc activities (mU uPA equivalence/µg DNA; mean ± SEM of 5 experiments) in 24 h cultures of granulosa cells from DES- and eCG-treated rats. Values with different alphabetical superscripts are significantly different ($P < 0.05$).

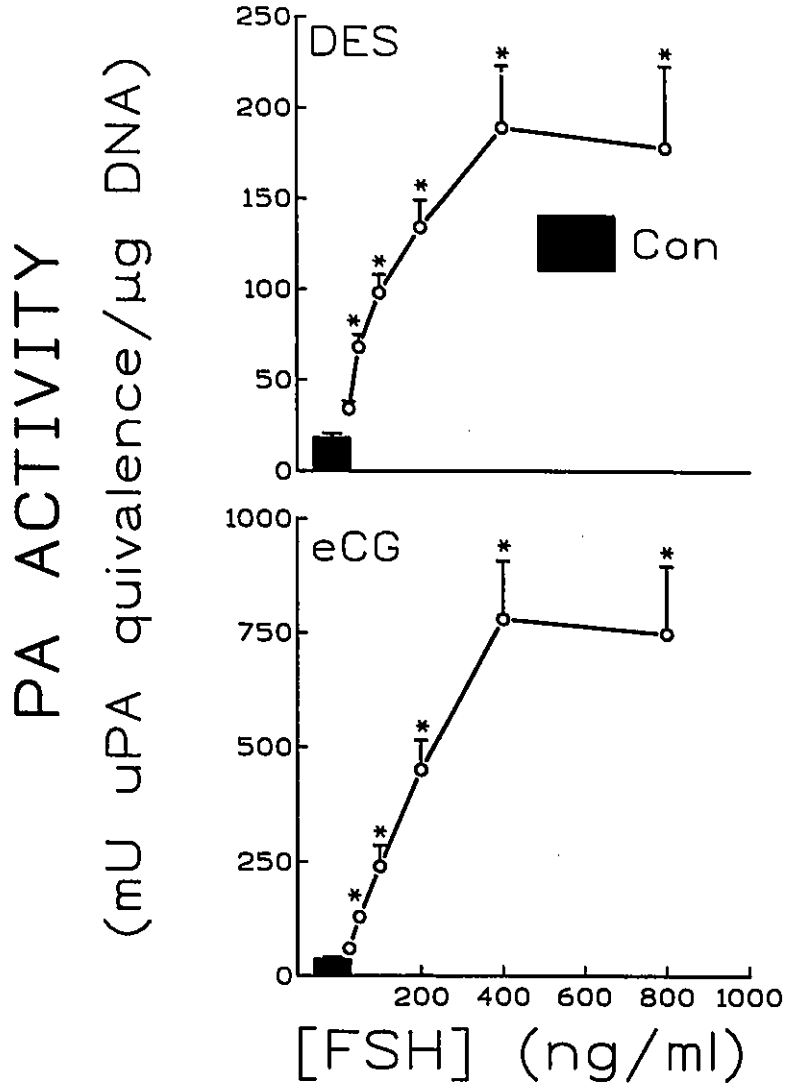

Fig 15. Concentration-dependent stimulation of PAs activity (mU uPA equivalence/µg DNA; mean ± SEM of 3 experiments) by FSH in 24 h cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ compared to control (black histogram).

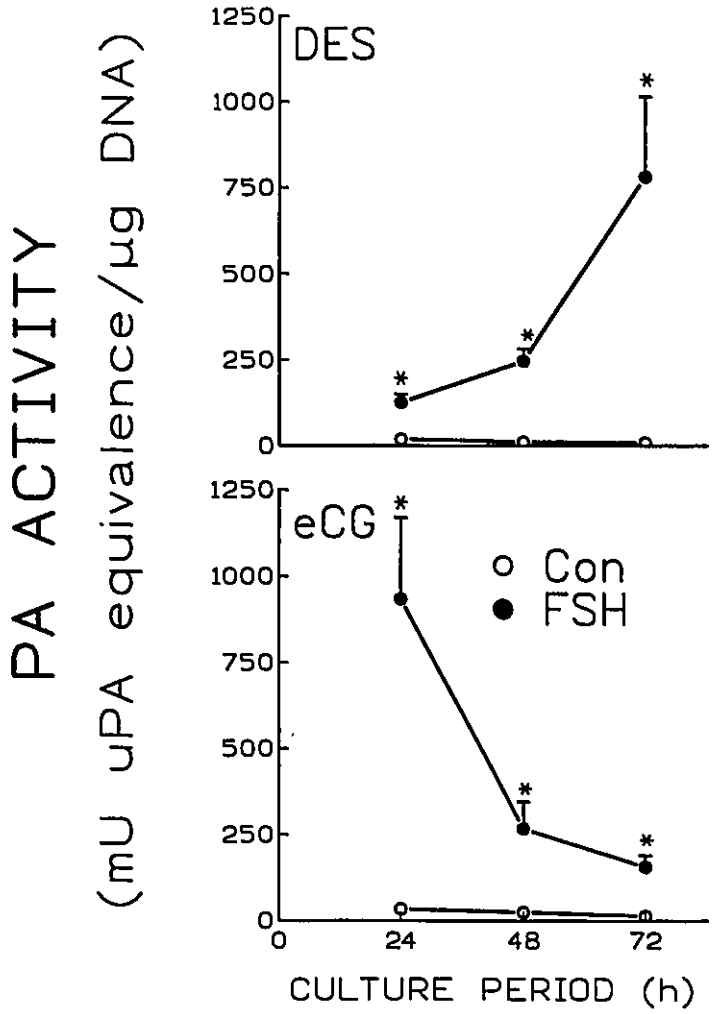

Fig 16. Time course study of the effect of FSH (400 ng/ml) on PAs activity (mU uPA equivalence/µg DNA; mean ± SEM of 3 experiments) in cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ compared to control.

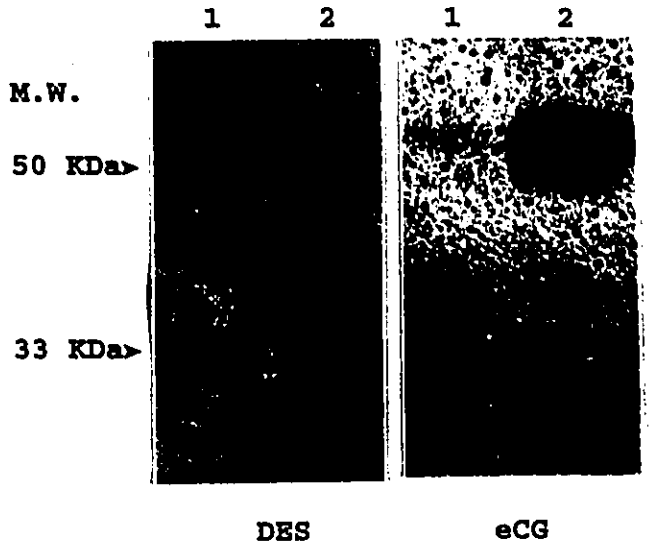

Plate 1. Effect of FSH (400 ng/ml; lane 2) on PAs activity in 24-h cultures of granulosa cells from DES- and eCG-treated rats, as determined by a fibrin overlay method. Lane 1 represents basal PAs activity (control). A representative zymogram from 3 experiments is shown.

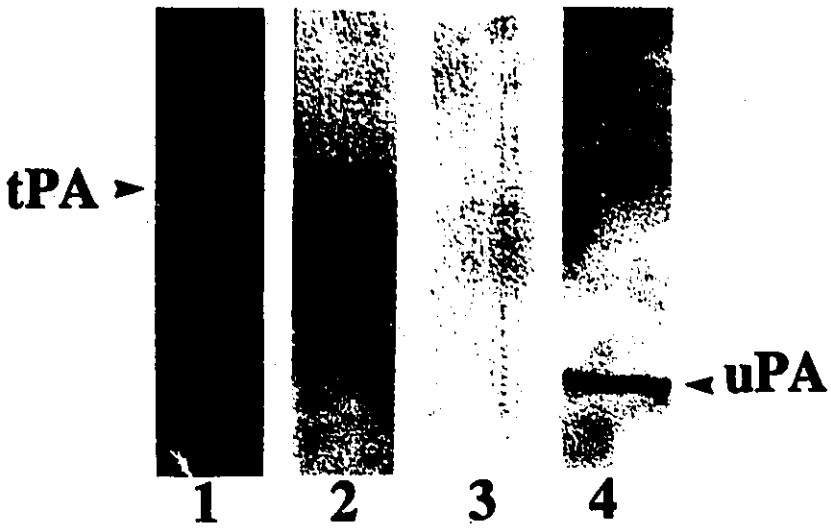

Plate 2. Western blot analysis of FSH (400 ng/ml)-induced PAs activity after 72-h cultures of granulosa cells from DES-treated rats. Assays were carried out in the presence and absence of mouse anti-human tPA (lane 1 and 2, respectively) or of rabbit anti-mouse uPA (lane 4 and 3, respectively). A representative blot of 3 experiments is shown.

5. Influence of FSH on Progestin Secretion during Follicular Development.

Progestin production (an index of cytodifferentiation) by granulosa cells from DES- and eCG-treated rats cultured in the absence and presence of FSH for 24 and 72 h was determined. With the exception of P secreted in cultures of granulosa cells from eCG-treated rats, FSH-induced P and 20α-OH-P production increased significantly ($P < 0.05$) with the incubation period in both cell preparations. Irrespective of the duration of the culture period and the cytodifferentiative state of granulosa cells, the FSH-stimulated 20α-OH-P secretion was more pronounced than P production. Although FSH-induced total progestin (P + 20α-OH-P) secretion was markedly higher ($P < 0.05$) in cultures of granulosa cells from eCG-treated than DES-treated rats, no significant difference in P production was observed in both cell preparations following 72 h of culture (Fig 17).

6. Effect of FSH on DNA Synthesis during Follicular Development.

Cultures of granulosa cells from DES-treated and eCG-treated rats in the absence and presence of FSH for 72 h showed no significant difference in [^{3}H]thymidine incorporation at the two stages of follicular maturation (Fig 18). Independent of the differentiative state of the granulosa cells, the gonadotropin significantly inhibited ($P < 0.05$) DNA synthesis, as indicated by [^{3}H]thymidine incorporation.

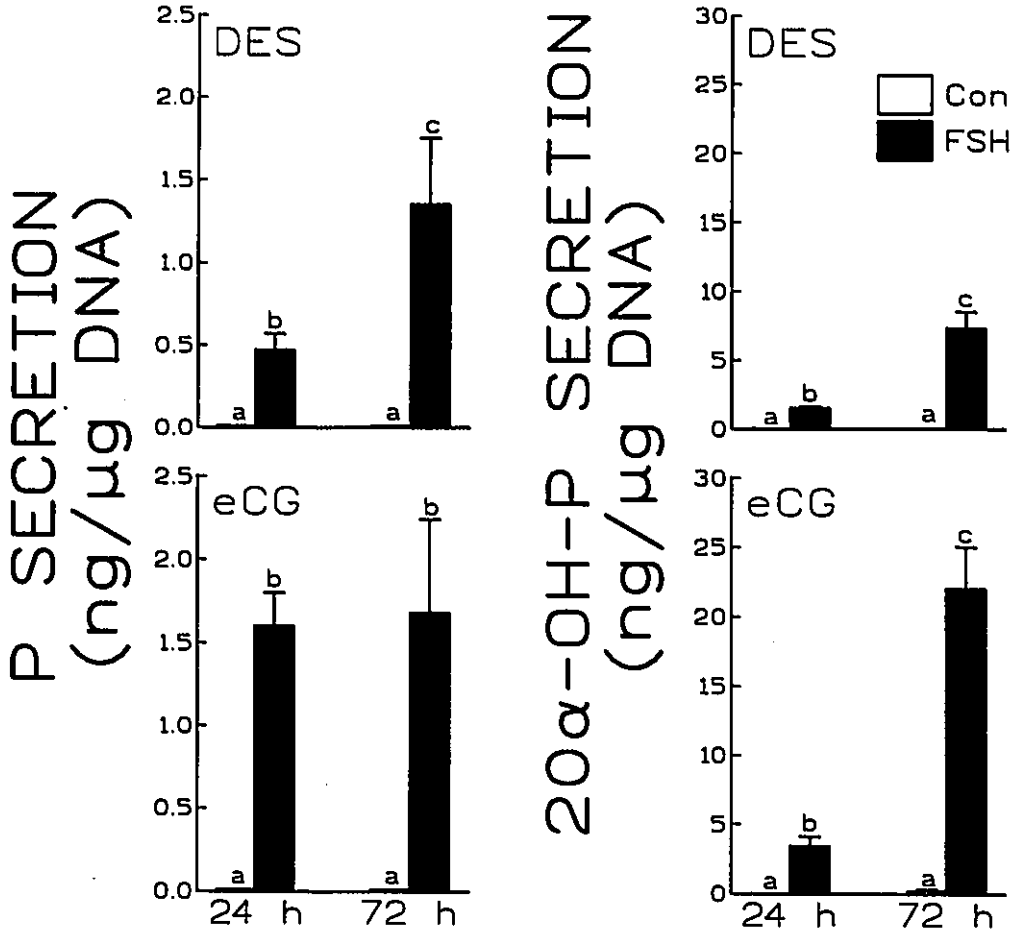

Fig 17. Influence of FSH (400 ng/ml) on progesterone (P) and 20α-dihydroprogesterone (20α-OH-P) secretion (ng/µg DNA; mean ± SEM of 3 experiments) in 24 h and 72 h cultures of granulosa cells from DES- and eCG-treated rats. Values with different alphabetical superscripts within each panel are significantly different ($P < 0.05$).

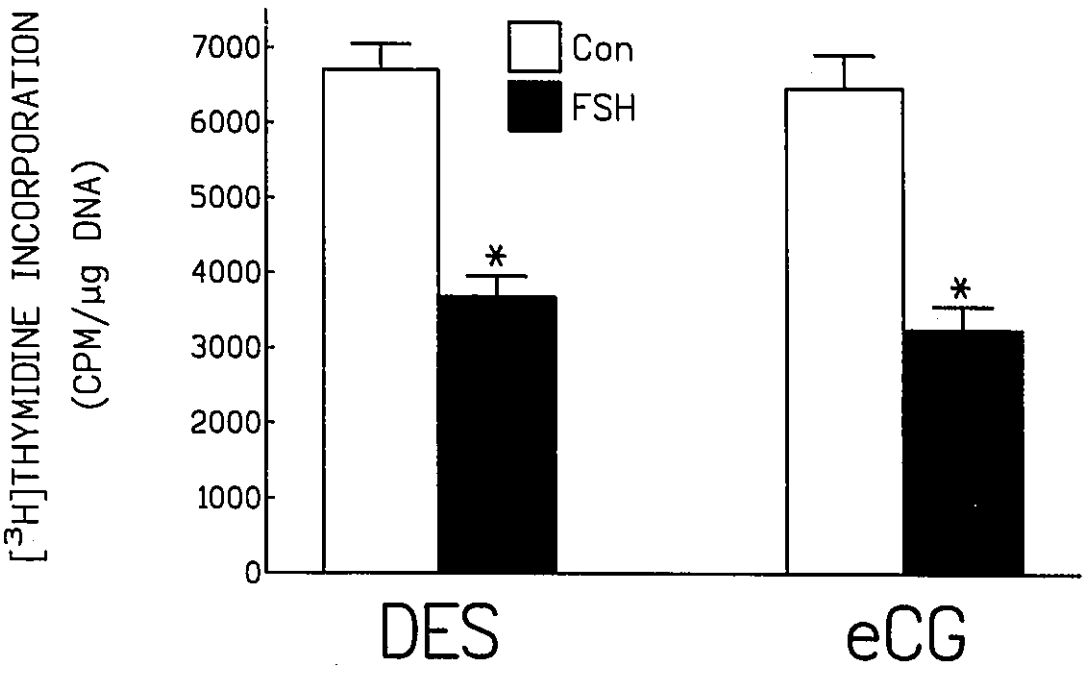

Fig 18. Influence of FSH (400 ng/ml) on [^{3}H]thymidine incorporation (cpm/µg DNA; mean ± SEM of 3 experiments) in 72 h cultures of granulosa cells from DES- and eCG-treated rats.

* $P < 0.05$ compared to control.

D. DO GRF AND VIP REGULATE THE GRANULOSA CELL PA SYSTEM DURING FOLLICULAR DEVELOPMENT? IF SO, HOW DO THEY INTERACT WITH FSH IN THIS REGULATION?

During follicular development the granulosa cells change their functional characteristics from proliferatively active to a differentiative state, the nature and extent of this alteration are dependent on gonadotropin and follicular microenvironment (Hirshfeild, 1991; Adashi, 1992b). GRF and VIP were shown to mimic an FSH effect by stimulating folliculogenesis *in vivo* and granulosa cell steroidogenesis and differentiation *in vitro* (Tornell et al., 1988; Moretti et al., 1989; Spicer and Enright, 1991). However, how these intra-ovarian peptides regulate the PA system in the context of granulosa cell proliferation and differentiation remains to be determined.

1. Influence of GRF and VIP on basal and FSH-induced Progestin Production during Follicular Growth.

GRF and VIP (10^{-6} M) significantly enhanced ($P < 0.05$) basal P and 20α-OH-P secretion in 24 h cultures of granulosa cells from DES- and eCG-treated rats (Fig 19). The effects of these peptides were more pronounced in cultures of granulosa cells from the latter experimental animals. However, both GRF and VIP significantly stimulated ($P < 0.05$) FSH-induced progestin secretion in granulosa cell cultures from only DES-treated rats (Fig 19).

2. Influence of GRF and VIP on Granulosa Cell DNA Synthesis during Follicular Development in the absence and presence of FSH.

GRF (10 μM), but not VIP, stimulated [^{3}H]thymidine uptake following 72 h of culture of granulosa cells from both stages of follicular development (Fig 20). The stimulation was due to increased DNA synthesis and not to DNA repair since [^{3}H]thymidine incorporation was markedly suppressed by the presence of hydroxyurea (0.3 mM; an inhibitor of DNA synthesis; 16614 ± 3049 versus 6124 ± 879 cpm/μg DNA). FSH significantly inhibited ($P < 0.05$) GRF-induced DNA synthesis in cultured granulosa cells from the two stages of follicular maturation (Fig 20).

3. Dose-dependent Effect of GRF and VIP on basal PAs and PAc Activities during Follicular Development

Studies on undifferentiated and differentiated granulosa cells (DES- and eCG-treated rats, respectively) cultured for 24 h in the absence and presence of GRF or VIP (10^{-9}-10^{-5} M) revealed that the peptides stimulated PAs and PAc activities at both stages of follicular development in a concentration-dependent manner (Fig 21). Maximal stimulation of PAs and PAc activities however was not observed even at a high concentration (10 μM) of these agonists irrespective of the stage of follicular maturation. Although VIP was more effective in stimulating PAs and PAc activities at a low concentration with both cell preparations, GRF elicited a greater response at a high concentration (10 μM; Fig 21). GRF- and VIP-stimulated PAs activity was more pronounced in cultures of differentiated granulosa cells than undifferentiated ones ($P < 0.05$).

4. Time Course Study of the Effect of GRF and VIP on basal PAs and PAc Activities during Follicular Maturation.

With the exception of PAc activity of granulosa cells from eCG-treated rats, PA activities in cells cultured for up to 72 h in the absence of GRF or VIP (10 μM) decreased significantly ($P < 0.05$) with the duration of culture (Fig 22). GRF- and VIP-stimulated PAs and PAc activities at both stages of follicular maturation also decreased with the increased duration of culture ($P < 0.05$; Fig 22).

5. Influence of GRF and VIP on FSH-induced PAs and PAc Activities during Follicular Development.

The effect of GRF and VIP (10^{-6} and 10^{-5} M) on FSH-stimulated PAs and PAc activities in 24-h granulosa cell cultures from DES- and eCG-treated rats is shown in figure 23. Although the gonadotropin-induced PAs activity in cultures of granulosa cells from DES-treated rats was significantly enhanced ($P < 0.05$) by both GRF and VIP, the former agonist appeared to be more effective at 10 μM (Fig 23). In cultures of granulosa cells from eCG-treated rats, only GRF at high concentration (10^{-5} M) could significantly enhanced ($P < 0.05$) gonadotropin-induced PAs and PAc activities (Fig 23). Zymographic analysis indicates that whereas both GRF and VIP enhanced basal and FSH-stimulated tPA activity during 24 h of culture irrespective of the stage of cytodifferentiation, they exhibited little or no effect on uPA activity at the early stage of follicular maturation (plate 3).

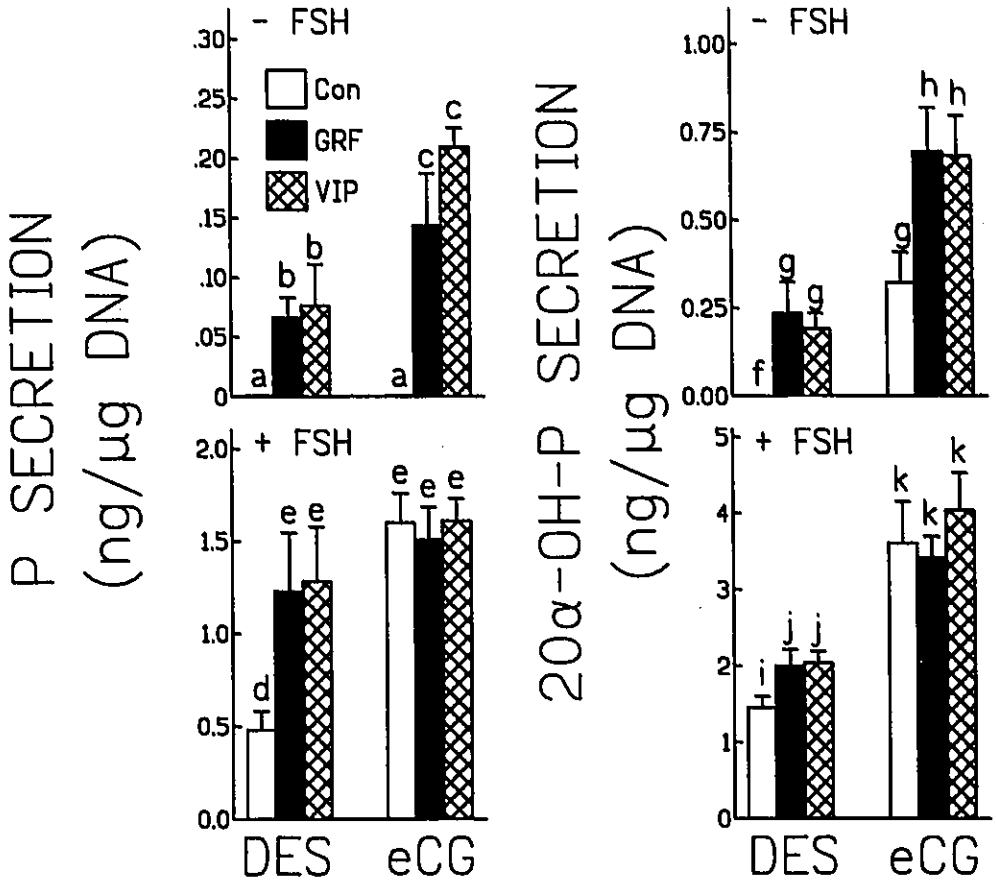

Fig 19. Effect of GRF (1 µM) and VIP (1 µM) on basal and FSH (400 ng/ml)-induced progesterone (P) and 20α-dihydroprogesterone (20α-OH-P) secretion (ng/µg DNA; mean ± SEM of 4 experiments) in 24 h cultures of granulosa cells from DES- and eCG-treated rats. Values with different alphabetical superscripts within each panel are significantly different ($P < 0.05$).

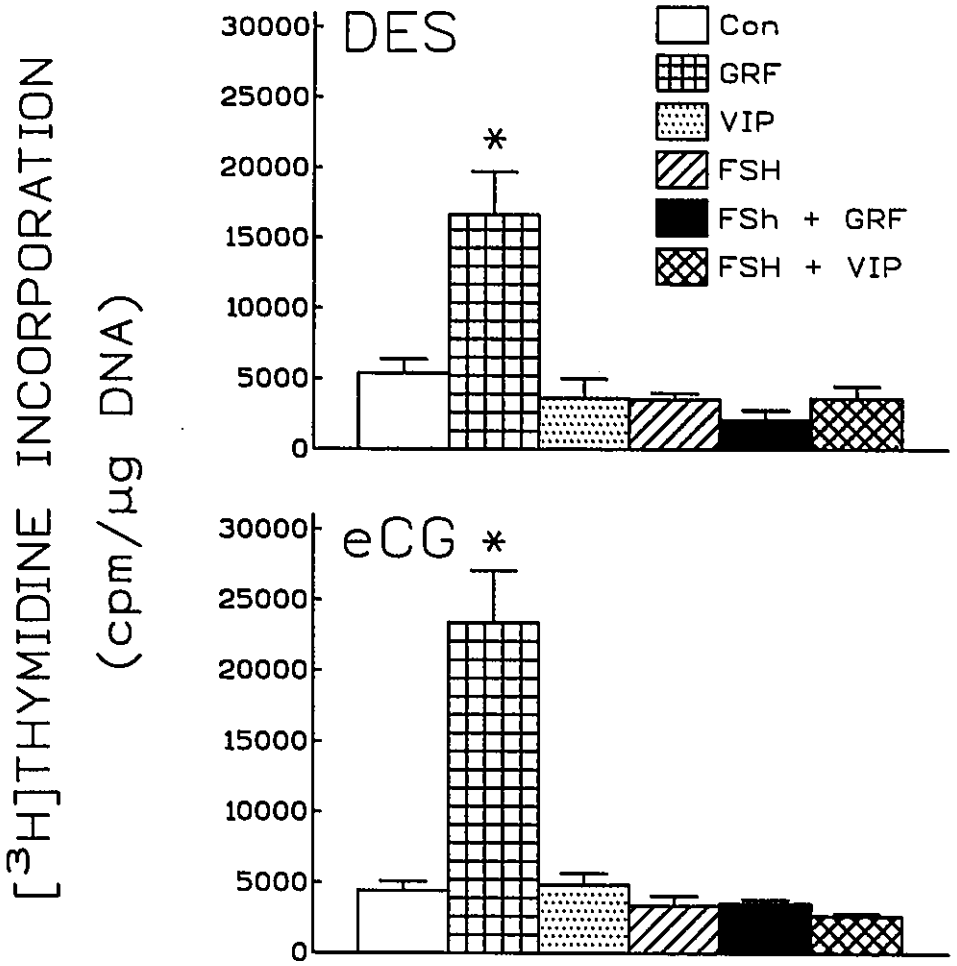

Fig 20. Influence of GRF (10 µM) and VIP (10 µM), alone or in combination with FSH (400 ng/ml), on [^{3}H]thymidine incorporation (cpm/µg DNA; mean ± SEM of 3 experiments) in 72 h cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ compared to control.

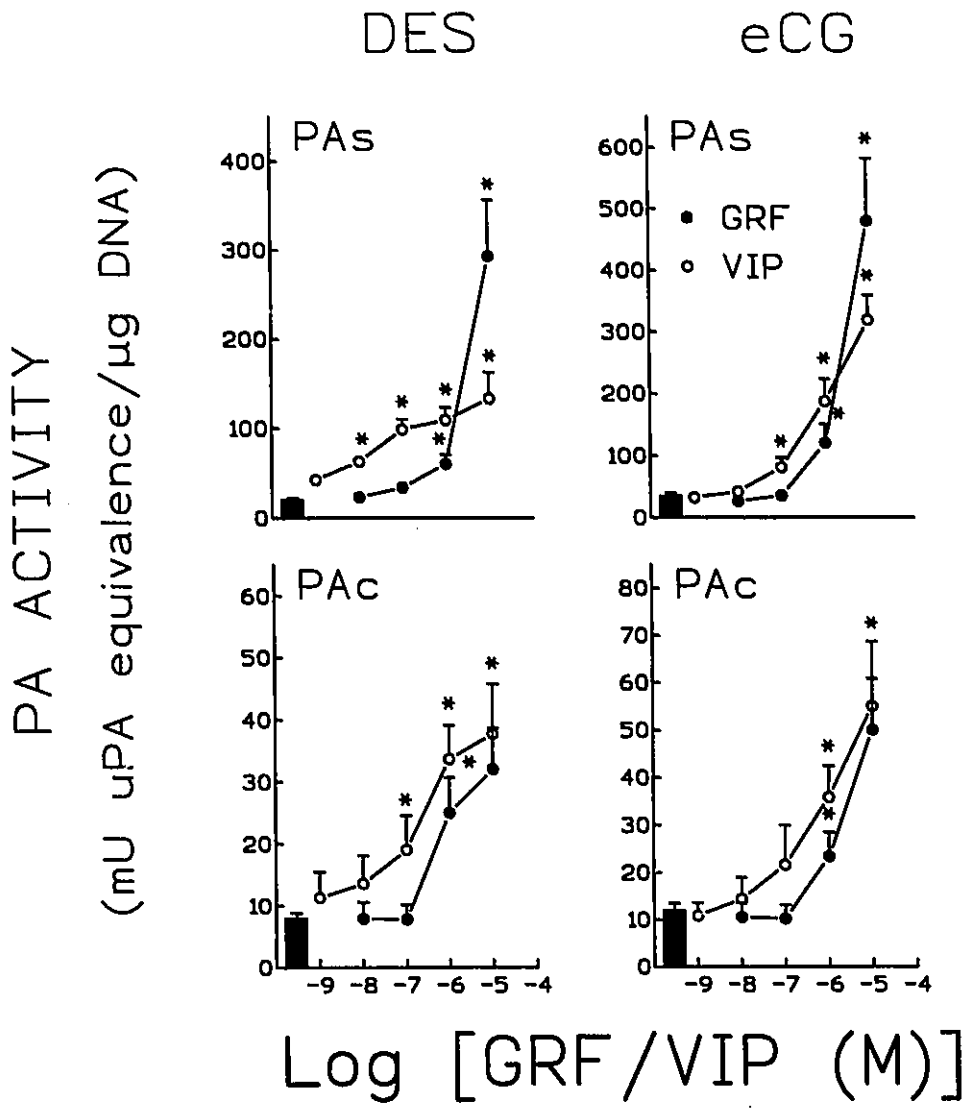

Fig 21. Concentration-dependent stimulation of PAs and PAc activities (mU uPA equivalence/µg DNA; mean ± SEM of five experiments) by GRF (10^{-8} -10^{-5} M) and VIP (10^{-9}-10^{-5} M) in 24 h cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ compared to control (black histogram).

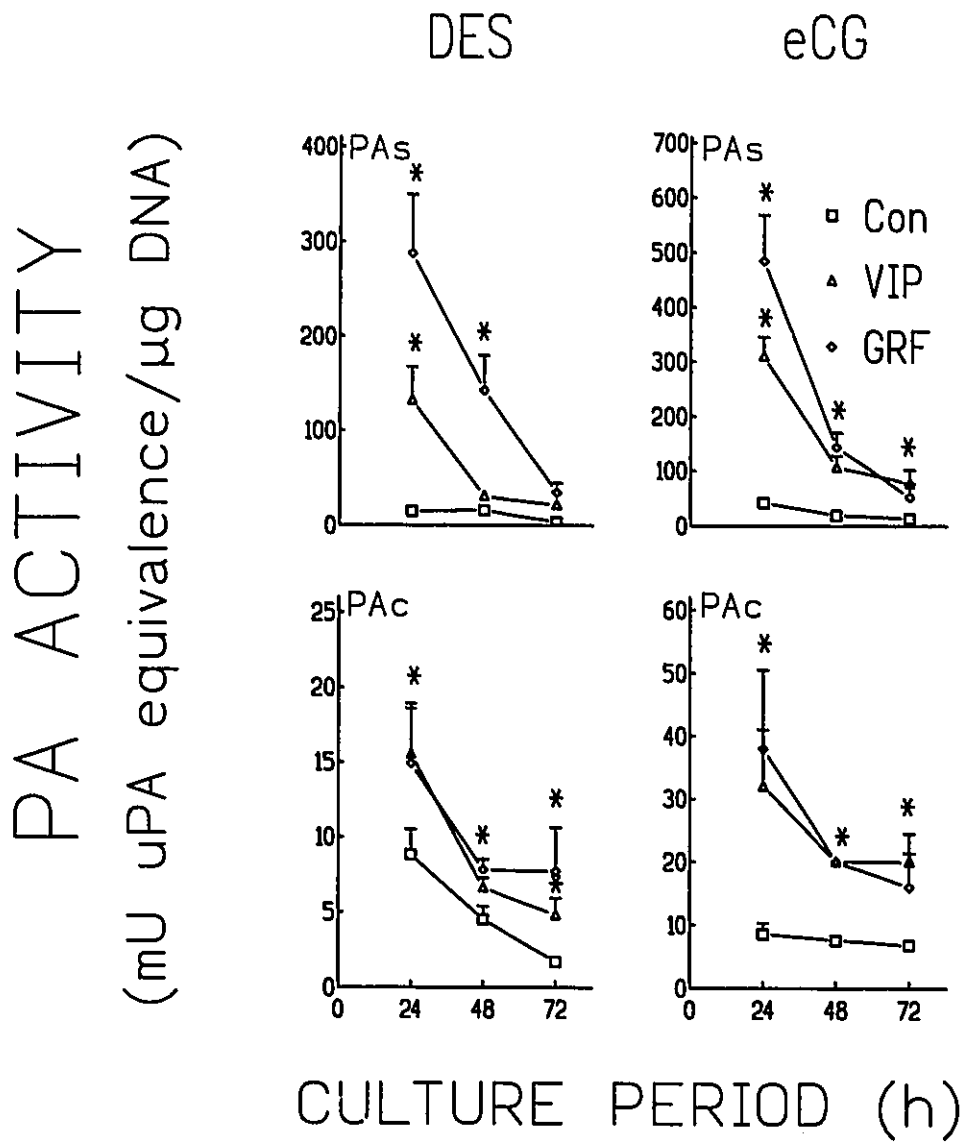

Fig 22. Time course study of the effect of GRF (10 µM) and VIP (10 µM) on PAs and PAc activities (mU uPA equivalence/µg DNA; mean ± SEM of 3 experiments) in cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ GRF or VIP compared to control.

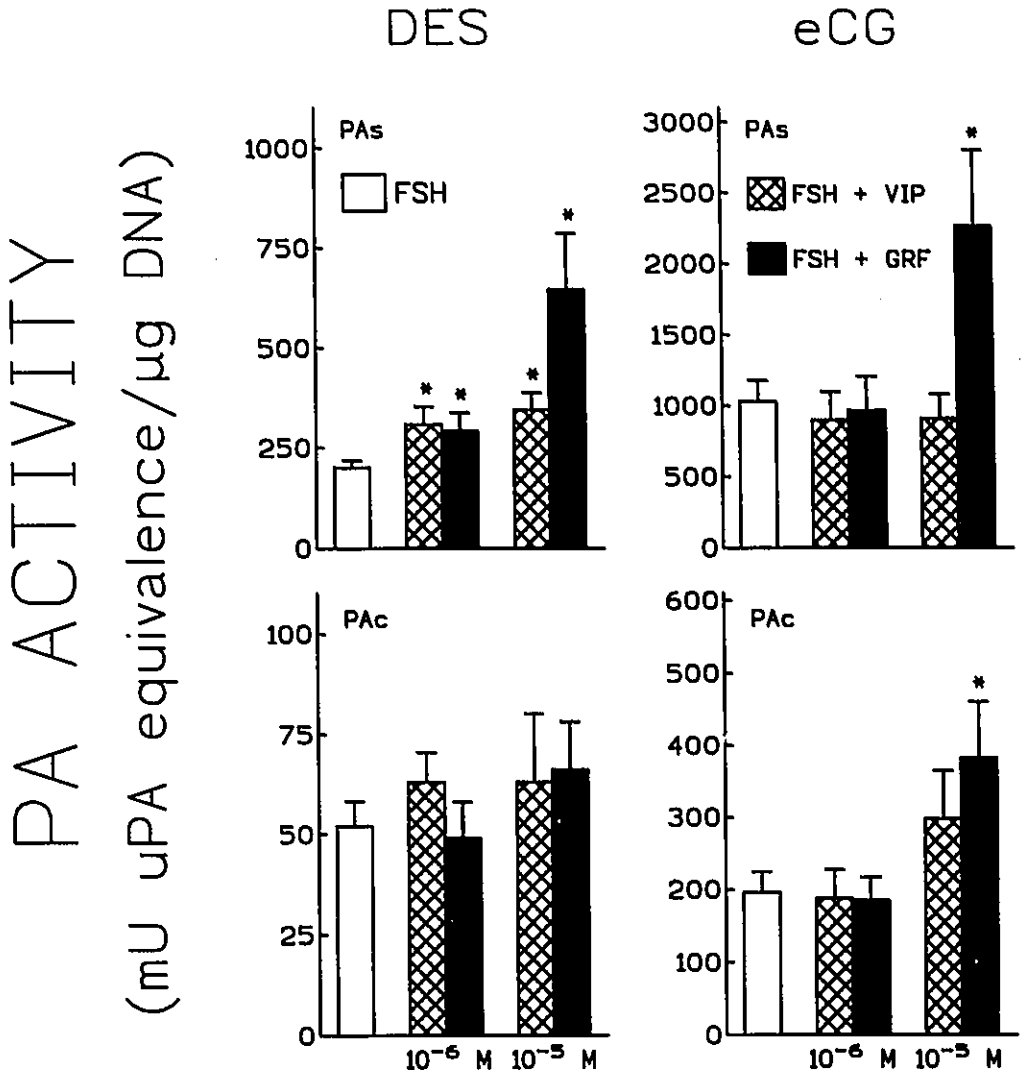

Fig 23. Influence of GRF (1 and 10 µM) and VIP (1 and 10 µM) on FSH (400 ng/ml)-stimulated PAs and PAc activities (mU uPA equivalence/µg DNA; mean ± SEM of 4 experiments) in 24 h cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ compared to FSH alone.

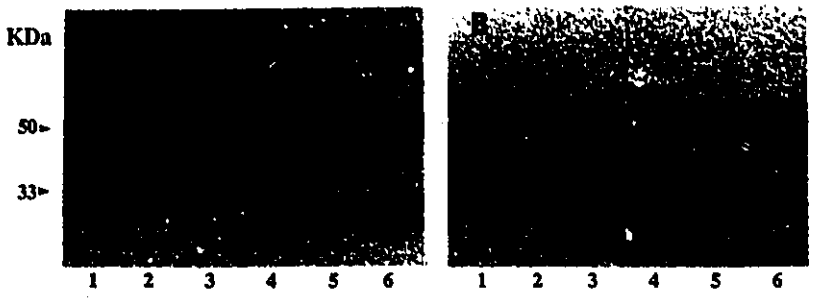

Plate 3. Effect of VIP (1 μM; lane 2), GRF (1 μM; lane 3), FSH (400 ng/ml; lane 4), GRF plus FSH (lane 5) and VIP plus FSH (lane 6) on PAs activity in 24-h cultures of granulosa cells from DES- and eCG-treated rats (A and B, respectively), as determined by the fibrin overlay method. Lane 1 represents basal PAs activity (control). A representative zymogram from five experiments is shown.

E. ARE TGFα AND TGFβ INVOLVED IN THE REGULATION OF THE GRANULOSA CELL PA SYSTEM? ARE THEIR ACTIONS DEPENDENT ON FOLLICULAR MATURATION? DO THEY MODULATE THE ACTION OF GONADOTROPIN ON THE PA SYSTEM?

Several studies have indicated that growth factors play a significant role in the regulation of ovarian cell proliferation and differentiation and in modulating gonadotropic action (Adashi, 1992b). TGFα and TGFβ are produced by follicular cells and have antagonistic effect on granulosa cell function (Adashi, 1992b). TGFα inhibits FSH-induced rat granulosa cell differentiation, promotes the proliferative capability of hen, but not rat, granulosa cells (Lafrance et al., 1993b; Bendell and Dorrington, 1990) and stimulates PA activity by undifferentiated granulosa cells both in the rat and the hen (Galway et al., 1989; Lafrance et al., 1993a,b). TGFβ enhances FSH-induced rat granulosa cell steroidogenesis and stimulates DNA synthesis in these cells (Bendell and Dorrington, 1990). However, the actions and interactions of these growth factors in the absence and presence of gonadotropin in the regulation of the proteases involved in follicular wall remodelling accompanying granulosa cell proliferation and differentiation is unclear.

1. Influence of TGFα in the Absence and Presence of FSH on DNA Synthesis during Follicular Development.

Granulosa cells from DES- and eCG-treated rats were cultured for 72 h in the absence and presence of FSH (400 ng/ml) with or without TGFα (10 ng/ml). DNA synthesis was assessed by the ability of the cells to incorporate [^{3}H]thymidine. As shown in figure 24,

TGFα markedly stimulated ($P < 0.05$) DNA synthesis in granulosa cells from DES-treated rats but not from eCG-treated rats. The mitogenic response of the cells to the growth factor was completely suppressed ($P < 0.05$) by FSH (Fig 24). Although the gonadotropin had no significant influence on basal [^{3}H]thymidine incorporation in granulosa cells from eCG-treated rats, it markedly inhibited (> 50%; $P < 0.05$) this response in the presence of the growth factor. The TGFα-induced [^{3}H]thymidine incorporation into DNA of undifferentiated granulosa cells cultured in the absence and presence of hydroxyurea (0.3 mM; a DNA synthesis inhibitor) were 13451 ± 1793 and 5348 ± 632 cpm/μg DNA ($n = 3$), respectively, indicating that the stimulatory effect of the growth factor was on DNA synthesis rather than DNA repair.

2. Effect of TGFα on basal and FSH-induced P and 20α-OH-P Secretion during Follicular Maturation.

To examine the action of TGFα on progestin production, granulosa cells from DES- and eCG- treated rats were cultured for 72 h in the absence and presence of FSH and with or without TGFα (10 ng/ml). Independent of the stage of follicular development, TGFα significantly stimulated ($P < 0.05$) basal P and 20α-OH-P secretion. The increase in progestin secretion was significantly more pronounced ($P < 0.05$) in the more differentiated cells (eCG group; Table 1). Likewise, FSH-stimulated total progestin (P + 20α-OH-P) secretion was significantly higher ($P < 0.05$) in cultures of differentiated granulosa cells than undifferentiated ones (Fig 25C). TGFα had no effect on FSH-induced total progestin secretion irrespective of the stages of follicular development although it significantly inhibited ($P < 0.05$) FSH-induced P secretion by undifferentiated granulosa cells (Fig 25A),

thus markedly increasing the progestin secretion ratio (20α-OH-P/P; Fig 25D).

3. Dose-response Effect of TGFα and TGFβ on basal PAs and PAc Activities.

The influence of TGFα and TGFβ on PA activities in 24 h-cultures of granulosa cells from eCG-treated rats is shown in figure 26. TGFα but not TGFβ significantly stimulated ($P < 0.05$) PAs and PAc activities in a concentration-dependent manner. Maximal stimulatory response was observed at 10 ng/ml (Fig 26).

4. Influence of TGFα and TGFβ on basal and FSH-induced PAs and PAc Activities during Follicular Development.

PAs and PAc activities in the absence and presence of FSH were determined in 24 h cultures of granulosa cells from DES- and eCG-treated rats with or without TGFα (10 ng/ml) or TGFβ (25 ng/ml). TGFα (10 ng/ml) significantly enhanced ($P < 0.05$) basal and FSH-induced PAs and PAc activities at both stages of follicular development (Fig 27). The magnitude of TGFα stimulation of total basal PA activity (PAs + PAc) was 5.9 and 2.2 fold in differentiated and undifferentiated granulosa cells, respectively. TGFβ had no significant influence on basal, FSH- and TGFα-stimulated PAs and PAc activities irrespective of the stage of follicular development (Fig 27; Table 2).

5. Time Course Study of the Effect of TGFα on basal and FSH-induced PAs and PAc Activities during Follicular Development.

To determine the time course of the influence of TGFα on granulosa cell PA activities during follicular development, cells from DES- and eCG-treated rats were cultured

for up to 72 h in the absence and presence of FSH and with or without TGFα (10 ng/ml). Whereas TGFα-stimulated PAs activity remained unchanged in granulosa cell cultures for the DES group, PAc activity decreased significantly ($P < 0.05$) with increased duration of incubation (Fig 28). In cultures of granulosa cells from eCG-treated rats, however, TGFα-stimulated PAs and PAc activities decreased significantly ($P < 0.05$) with increased culture duration (Fig 28). Although TGFα enhanced FSH-induced PAs and PAc activities during the first 24 h irrespective of the stages of follicular development, the gonadotropin effect on the undifferentiated granulosa cells was markedly suppressed by the presence of the growth factor in 48 and 72 h of culture (Fig 29).

6. Characterization of basal and FSH-induced PAs Activity in the Absence and Presence of TGFα during Follicular Development.

While TGFα alone stimulated tPA activity in differentiated granulosa cell throughout the culture period, it caused a time-dependent increase in both tPA and uPA activities in the undifferentiated cells (plate 4). The stimulatory effect of the growth factor on tPA and uPA activities in granulosa cells from the early stage of follicular maturation was observed after 48 h of culture (plate 4). Although FSH stimulated tPA activity in cultures of granulosa cells from DES-treated rats throughout the culture period and with maximal stimulatory influence observed following 72 h of culture, little enhancement of uPA was detected. However, the stimulatory effect of gonadotropin on tPA activity in cells from eCG-treated rats decreased with increased duration of culture (plate 4). In cells from DES-treated rats cultured for 72 h, TGFα inhibited both FSH-induced tPA and uPA activity (plate 4). This inhibitory effect of TGFα on PA activity was not associated with detectable changes in PAI activity irrespective

of gonadotropin treatment, the duration of culture, and the stage of follicular maturation.

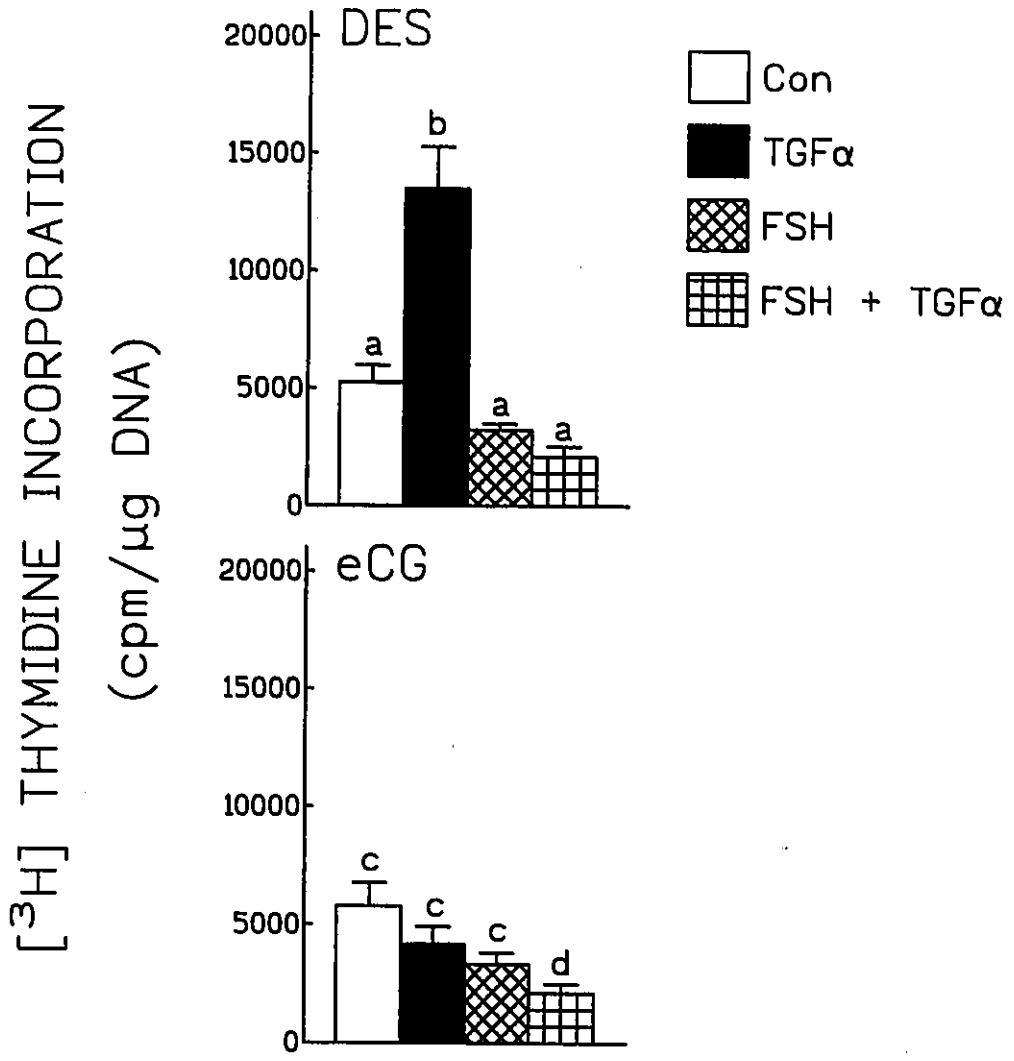

Fig 24. Effect of TGFα (10 ng/ml) and FSH (400 ng/ml), alone or in combination, on [^{3}H]thymidine incorporation (cpm/µg DNA; mean ± SEM of 5 experiments) in 72 h cultures of granulosa cells from DES- and eCG-treated rats. Values with different alphabetical superscripts are significantly different ($P < 0.05$).

Table 1. Basal and TGFα-induced progestin secretion in 72-h cultures of granulosa cells from DES- and eCG-treated rats.

	Progesterone[+]		20α-dihydroprogesterone[+]	
	DES	eCG	DES	eCG
control	ND	ND	ND	206 ± 105
TGFα	6.2 ± 1.1*	247 ± 102*	34 ± 16*	4628 ± 995*

+ ng/μg DNA; mean ± SEM of 3 experiments; ND = not detectable; * $P < 0.05$ compared to control.

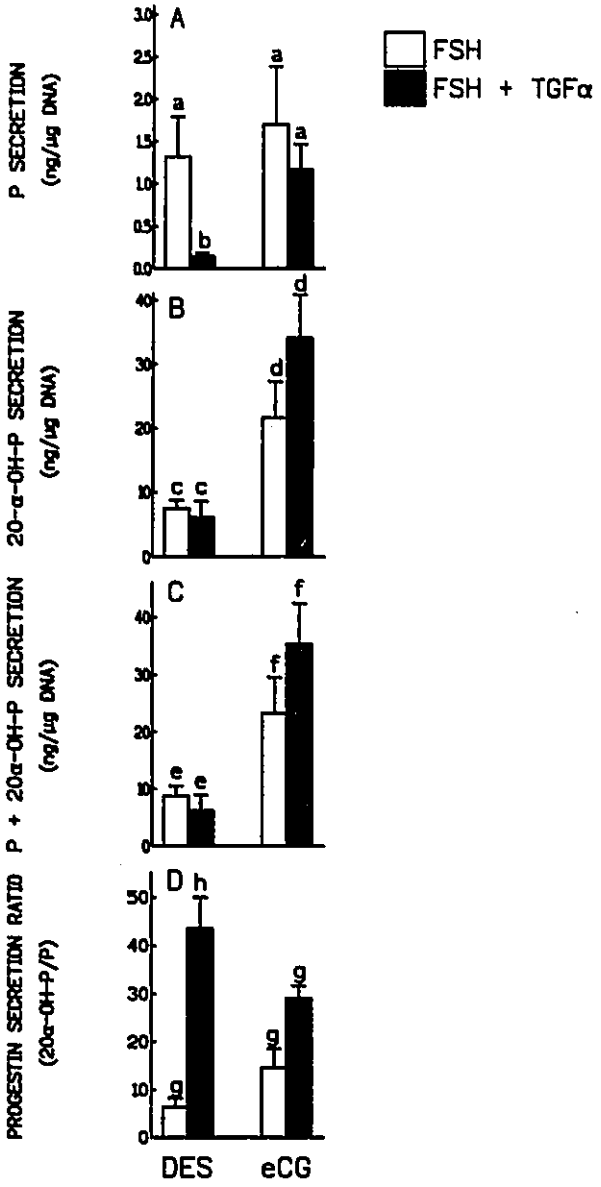

Fig 25. Effect of TGFα (10 ng/ml) on FSH (400 ng/ml)-stimulated P(A), 20α-OH-P (B), total progestin (P + 20α-OH-P; C) secretion (ng/μg DNA; mean ± SEM of 3 experiments) and the secretion ratio of 20α-OH-P to P (20α-OH-P/P; D) in 72 h cultures of granulosa cells from DES- and eCG-treated rats. Values with different alphabetical superscripts within each panel are significantly different ($P < 0.05$).

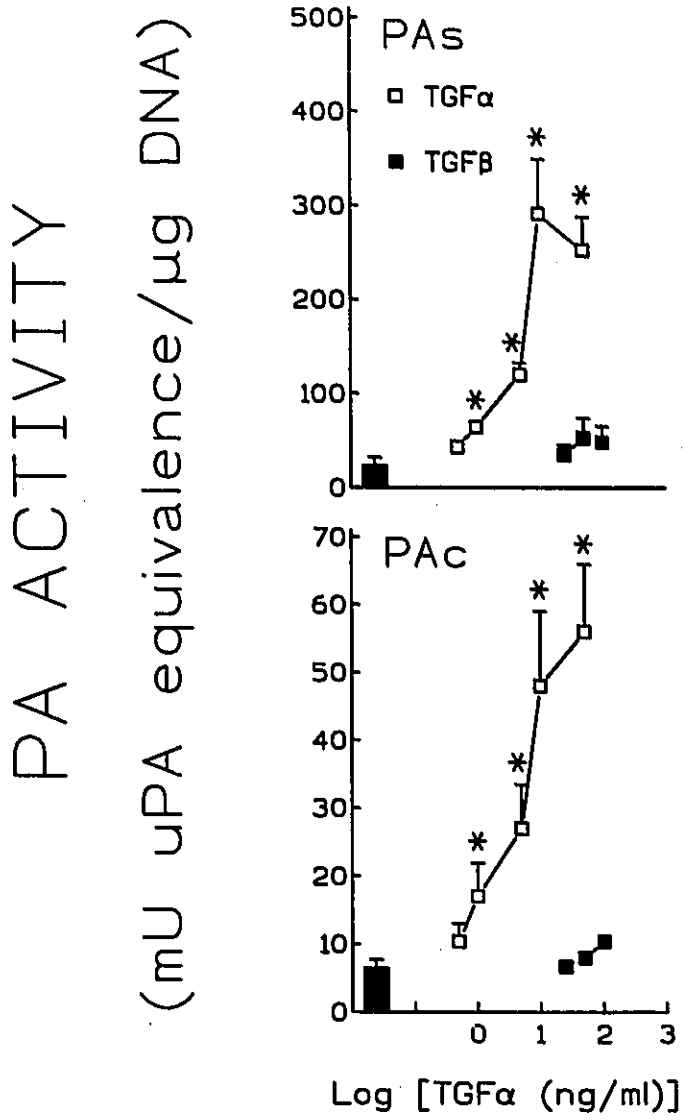

Fig 26. Dose-response studies of the effects of TGFα (0.5-50 ng/ml) and TGFβ (25-100 ng/ml) on PAs and PAc activities (mU uPA equivalence/µg DNA; mean ± SEM of 4 experiments) in 24 h cultures of granulosa cells from eCG-treated rats. * $P < 0.05$ compared to control (black histogram).

Table 2. Effect of TGFα, TGFβ and TGFα + TGFβ on basal PA activity in cultures of granulosa cells from DES- and eCG-treated immature rats.

	PAs		PAc	
	DES	eCG	DES	eCG
control	18 ± 3.1^a	42 ± 5.5^a	8.7 ± 0.9^a	12 ± 0.8^a
TGFα	40 ± 7.7^b	267 ± 50^b	19 ± 5.8^b	48 ± 11^b
TGFβ	16 ± 5.2^a	41 ± 9.6^a	6.5 ± 0.6^a	13 ± 2.2^a
TGFα + TGFβ	55 ± 11^b	227 ± 25^b	23 ± 4.3^b	38 ± 5.1^b

Influence of TGFα (10 ng/ml), TGFβ (25 ng/ml) and TGFα plus TGFβ on basal PAs and PAc activities (mU uPA equivalence/μg DNA; mean ± SEM of 4 experiments) in 24 h cultures of granulosa cells from DES- and eCG-treated rats. Values with different alphabet superscripts in each column are significantly different ($P < 0.05$).

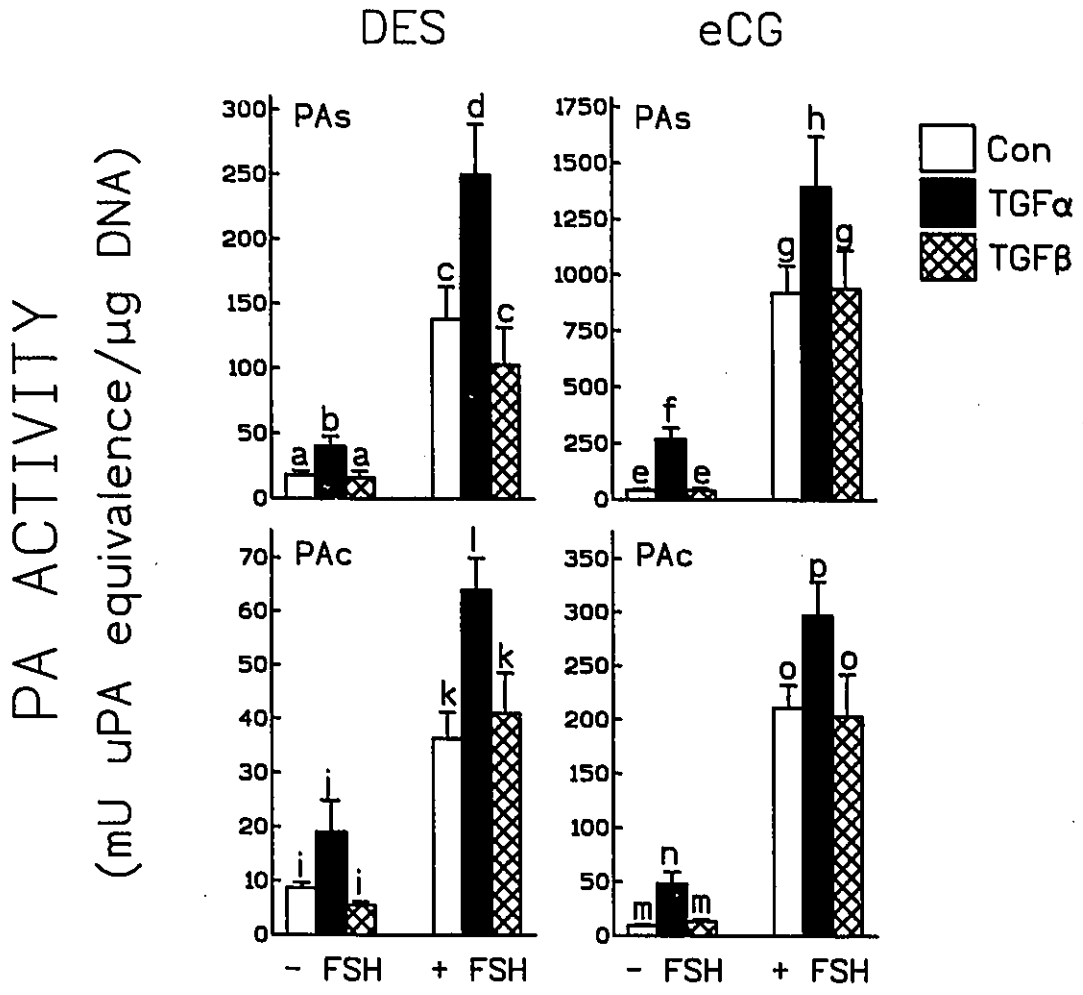

Fig 27. Effects of TGFα (10 ng/ml) and TGFβ (25 ng/ml), alone or in combination with FSH (400 ng/ml), on PAs and PAc activities (mU uPA equivalence/µg DNA; mean ± SEM of five experiments) in 24 h cultures of granulosa cells from DES- and eCG-treated rats. Values with different superscripts within each panel are significantly different ($P < 0.05$).

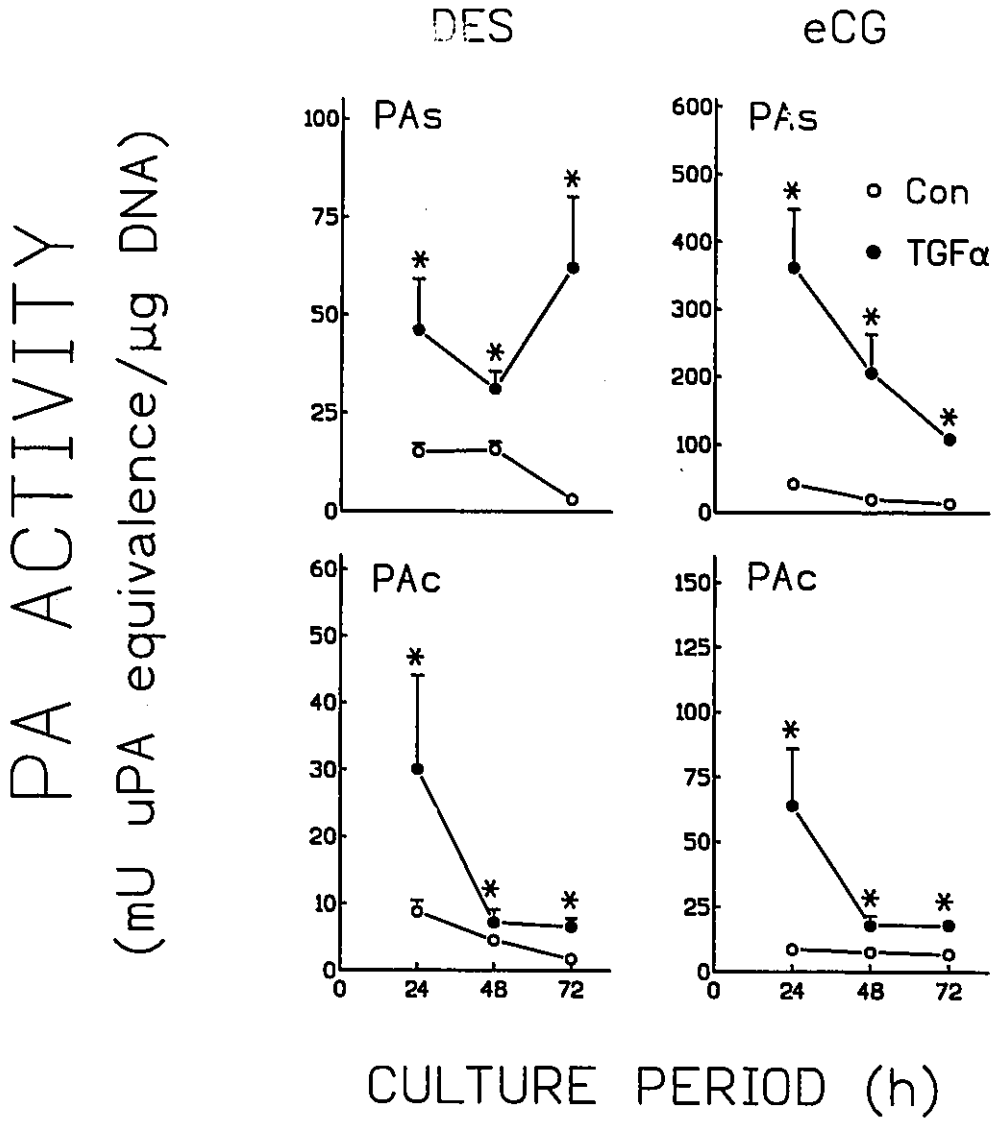

Fig 28. Effects of TGFα (10 ng/ml) on basal PAs and PAc activities (mU uPA equivalence/μg DNA; mean ± SEM of 3 experiments) in 24, 48, 72 h cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ compared to control.

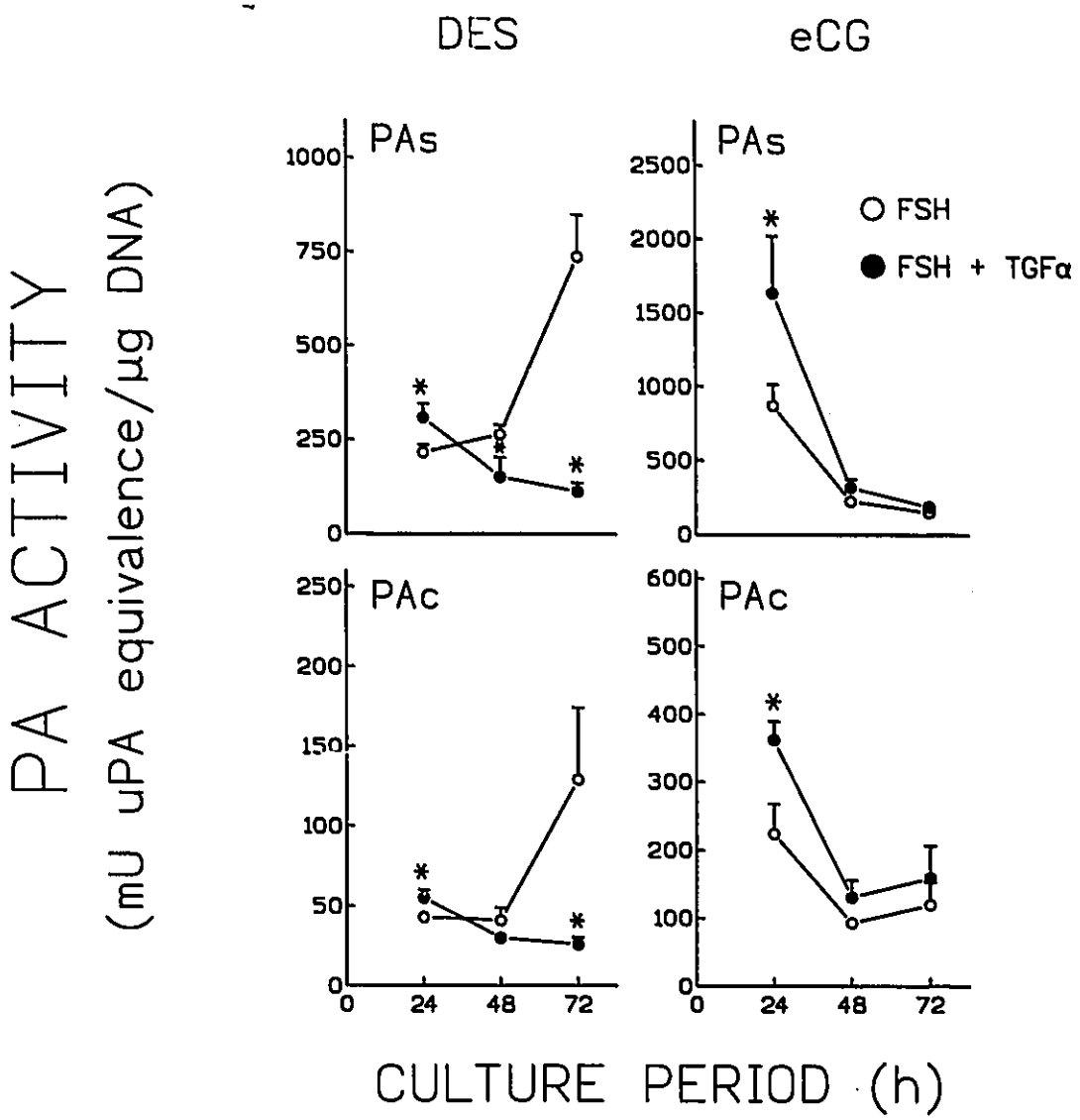

Fig 29. Influence of TGFα (10 ng/ml) on FSH (400 ng/ml)-induced PAs and PAc activities (mU uPA equivalence/µg DNA; mean ± SEM of 3 experiments) in 24, 48, and 72 h cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ compared to FSH.

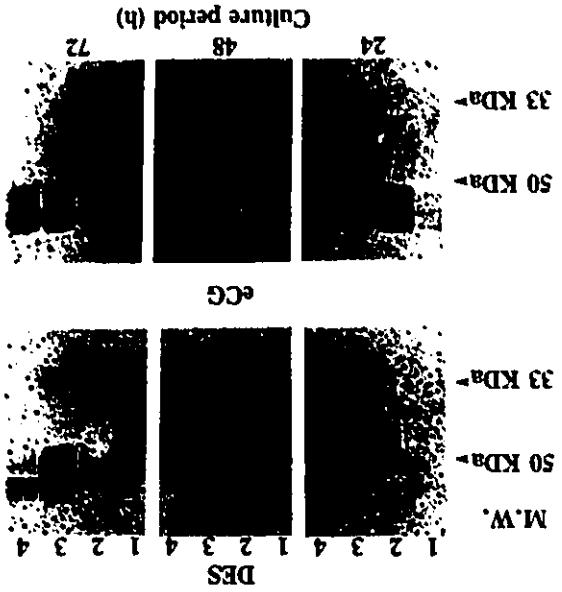

Plate 4. Influence of TGFα (10 ng/ml; lane 2), FSH (400 ng/ml; lane 3), and TGFα plus FSH (lane 4) on PAs activity in 24, 48, and 72 h cultures of granulosa cells from DES- and eCG-treated rats, as determined by a fibrin overlay method. Lane 1 represents basal PAs activity (control). A representative zymogram from five experiments is shown.

F. TNFα HAS AN ANTI-DIFFERENTIATIVE EFFECT ON GRANULOSA CELLS AND ATTENUATES THE ACTION OF GONADOTROPIN. IS THIS CYTOKINE INVOLVED IN THE REGULATION OF THE GRANULOSA CELL PA SYSTEM DURING FOLLICULAR DEVELOPMENT?

Although the interaction between granulosa, theca and germ cells play an important role in the regulation of follicular development, another cell type of the immune system, the macrophages, which permanently present within ovarian tissue and secrete several substances such as TNFα and IL-1β were shown to modulate ovarian function (Adashi et al., 1992a). TNFα, also produced by follicular cells, suppress granulosa cell steroidogenesis induced by FSH (Veldhuis et al., 1991; Tekpetey et al., 1993). Moreover, TNFα inhibits ovulation in the perfused rat ovary (Hales et al., 1994). Nevertheless, the role of TNFα in the regulation of the PA system during follicular development has not as yet been determined.

1. Effect of TNFα on FSH-induced Progestin Production during Follicular Development.

To evaluate the anti-differentiative effect of the cytokine on granulosa cells during follicular development, cells from DES- and eCG-treated rats were cultured for 72 h in the presence of FSH and with or without TNFα (10 ng/ml), and progestin secretion was examined. Independent of the cytodifferentiative state of the granulosa cells, TNFα significantly inhibited ($P < 0.05$) FSH-induced progestin secretion (Fig 30).

2. Influence of TNFα on DNA Synthesis during Follicular Development.

The effect of TNFα (10 ng/ml) on DNA synthesis in granulosa cells from DES- and

eCG-treated rats was determined following 72 h of culture in the absence and presence of FSH (400 ng/ml) and with or without the cytokine. Although the cytokine was not effective in modulating basal DNA synthesis, it significantly reversed the inhibitory effect of the gonadotropin on [^{3}H]thymidine incorporation at both stages of follicular development (Fig 31).

3. Dose-response Study of the Effect of TNFα on FSH-induced PAs and PAc Activities during Follicular Growth.

Granulosa cells from DES- and eCG-treated rats were cultured for 72 h in the absence and presence of FSH (400 ng/ml) and with or without TNFα (0.5-50 ng/ml). With the exception of PAc in the eCG group, FSH-stimulated PA activities were significantly suppressed by the cytokine in a concentration-dependent manner (Fig 32). Maximal inhibition was detected at a TNFα concentration of approximately 10 ng/ml, irrespective of the stage of follicular development (Fig 32).

4. Time course Study of the Effect of TNFα on Basal and FSH-induced PAs and PAc Activities during Follicular Development.

The time course of TNFα (10 ng/ml) action was investigated in cultures of granulosa cells from DES- and eCG-treated rats over a culture period of 72 h in the absence and presence of FSH (400 ng/ml). As shown in figure 33, the loss in basal PA activities during the 72 h culture period was significantly enhanced ($P < 0.05$) by the presence of TNFα at both stages of follicular development. Although the inhibition of PAs and PAc activities in the DES group was observed as early as the first 24 h with TNFα, the effect of cytokine only

became apparent during the second 24 h of culture granulosa cells from eCG-treated animals (Fig 33). While TNFα significantly inhibited ($P < 0.05$) FSH-induced PAs and PAc activities in cultures of granulosa cells from DES-treated rats after 48 h, it suppressed PAs activity at the later follicular stage following 72 h (Fig 34).

5. Characterization of FSH-induced PAs Activity in the Absence and Presence of TNFα during Follicular Development.

Whereas TNFα inhibited FSH-induced tPA activity at both stages of follicular maturation, it enhanced gonadotropin-stimulated uPA activity in undifferentiated cells during 72 h of culture (Plate 5). The decrease in granulosa cell PA activity was accompanied by a marked increase in PAI activity which was more pronounced in cultures of granulosa cell from eCG-treated rats (plate 6). No increase in PAI activity was detectable in 24 and 48 h cultures of granulosa cell irrespective of the stage of follicular maturation or the presence of the cytokine (data not shown).

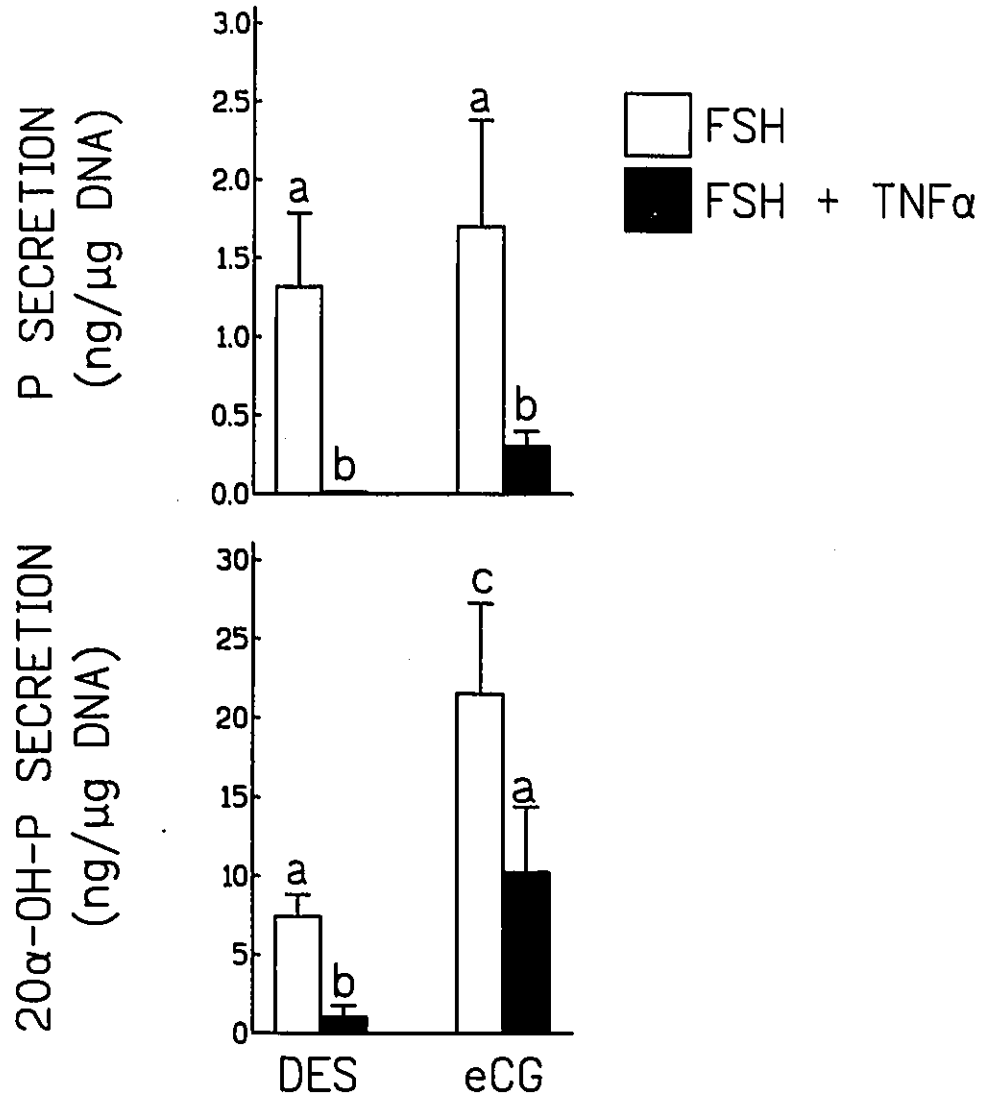

Fig 30. Effect of TNFα (10 ng/ml) on P and 20α-OH-P secretion (ng/µg DNA; mean ± SEM of 4 experiments) in presence of FSH (400 ng/ml) in 72 h cultures of granulosa cells from DES- and eCG-treated rats. Values with different alphabetical superscripts in each panel are significantly different ($P < 0.05$).

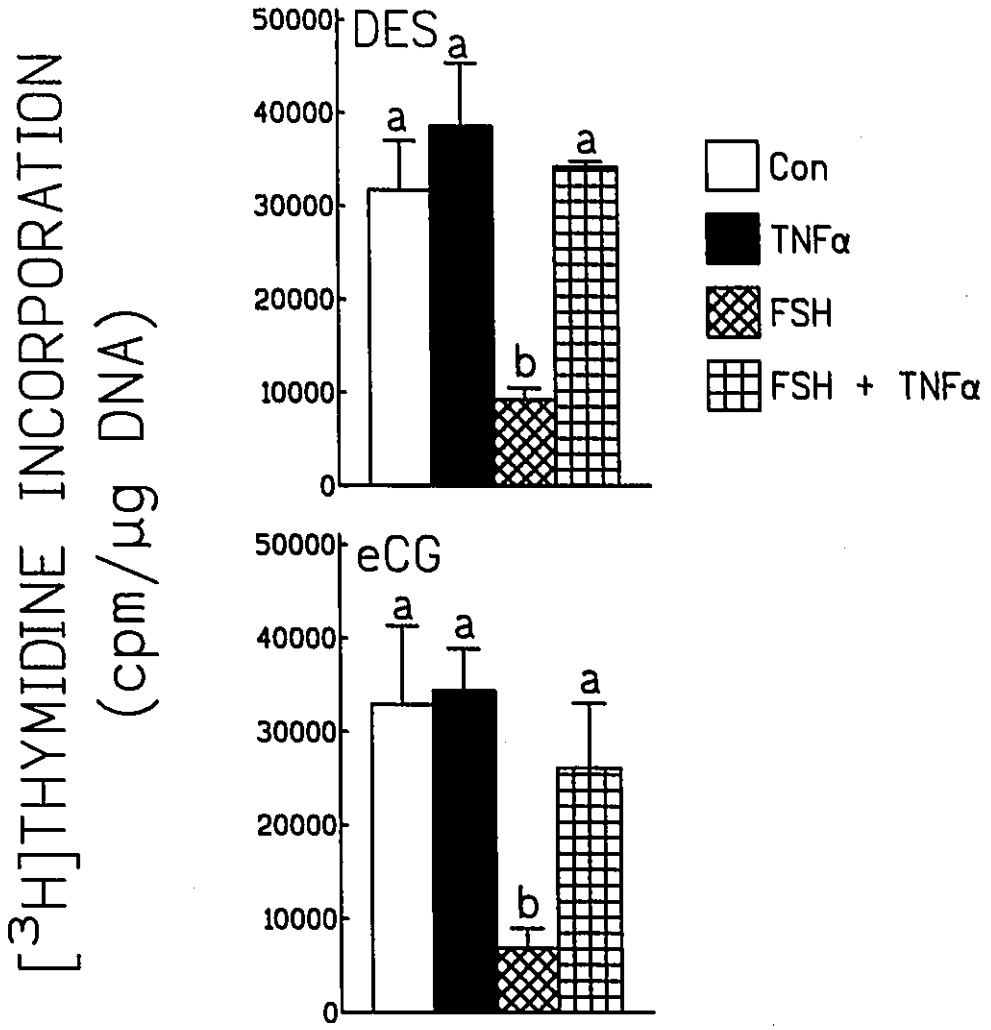

Fig 31. Influence of TNFα (10 ng/ml) alone or in combination with FSH (400 ng/ml) on [^{3}H]thymidine incorporation (cpm/µg DNA; mean ± SEM of 4 experiments) in 72 h cultures of granulosa cells from DES- and eCG-treated rats. Values with different alphabetical superscripts in each panel are significantly different ($P < 0.05$).

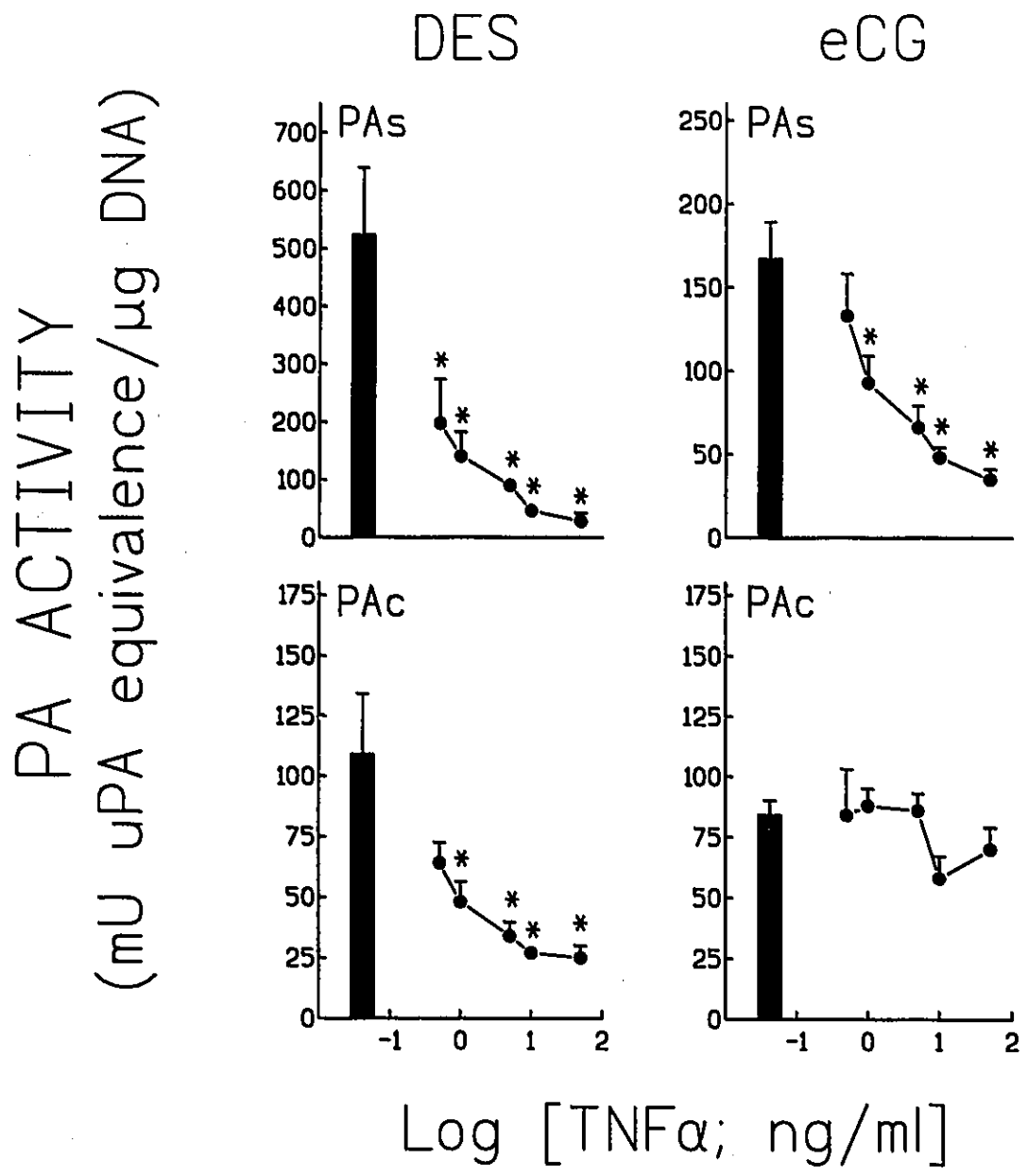

Fig 32. Concentration-dependent inhibition of FSH (400 ng/ml)-induced PAs and PAc activities (mU uPA equivalence/µg DNA; mean ± SEM of 4 experiments) by TNFα in 72 h cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ compared to FSH alone (black histogram).

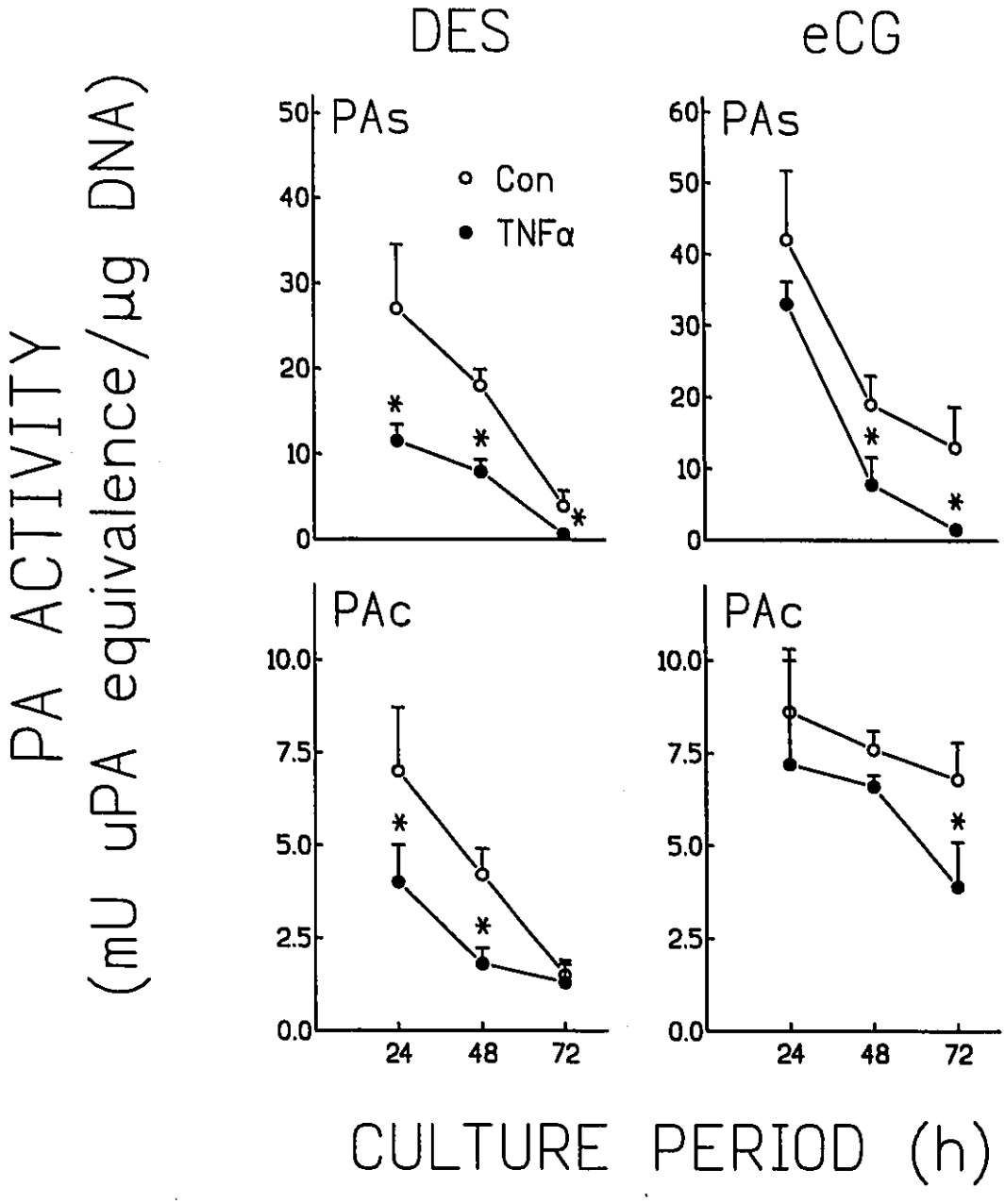

Fig 33. Time course study of the effect of TNFα (10 ng/ml) on basal PAs and PAc activities (mU uPA equivalence/µg DNA; mean ± SEM of 3 experiments) in cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ compared to control.

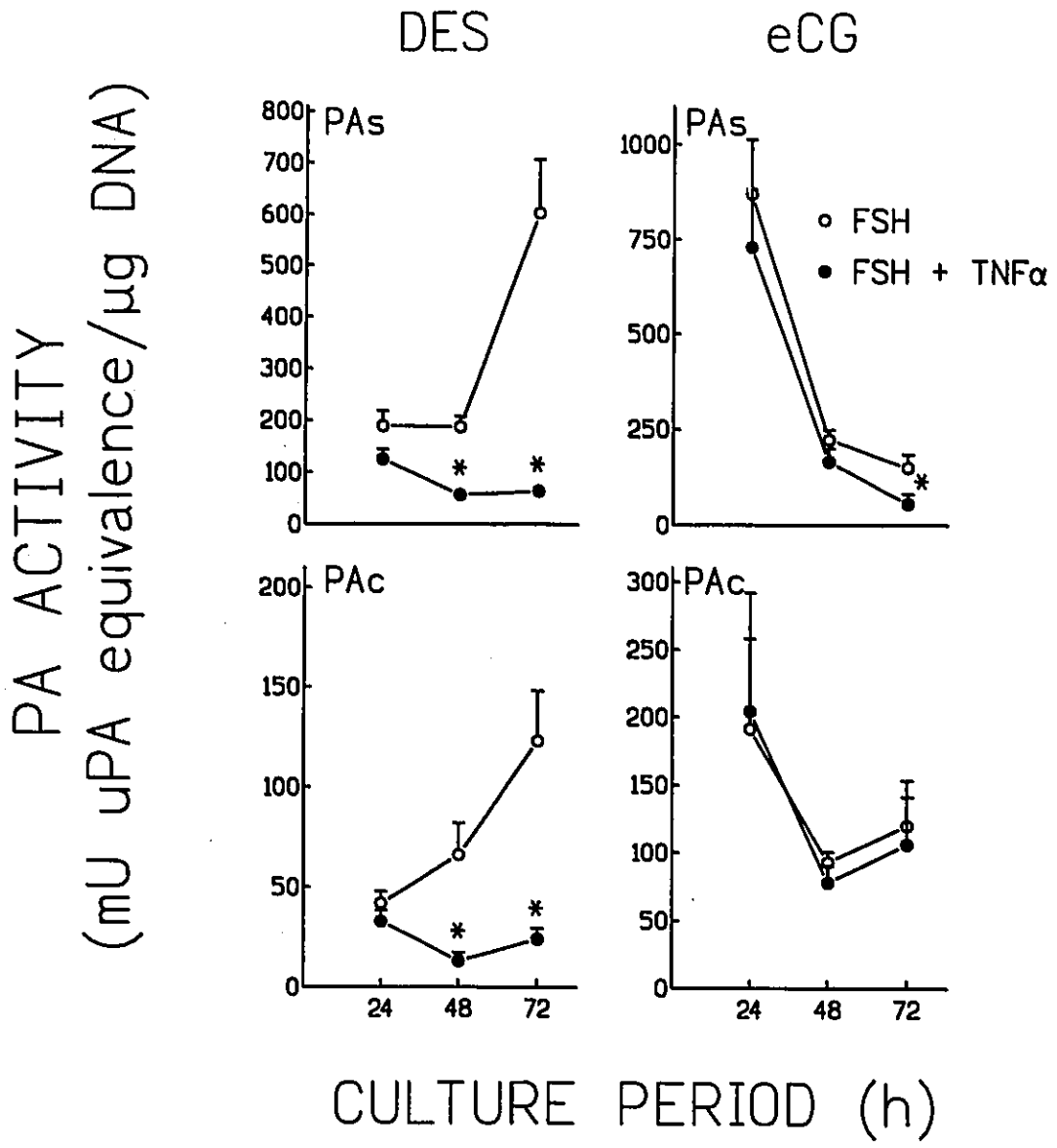

Fig 34. Time course study of the suppression of PAs and PAc activities (mU PA equivalence/µg DNA; mean ± SEM of 3 experiments) by TNFα (10 ng/ml) in the presence of FSH (400 ng/ml) in cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ compared to FSH.

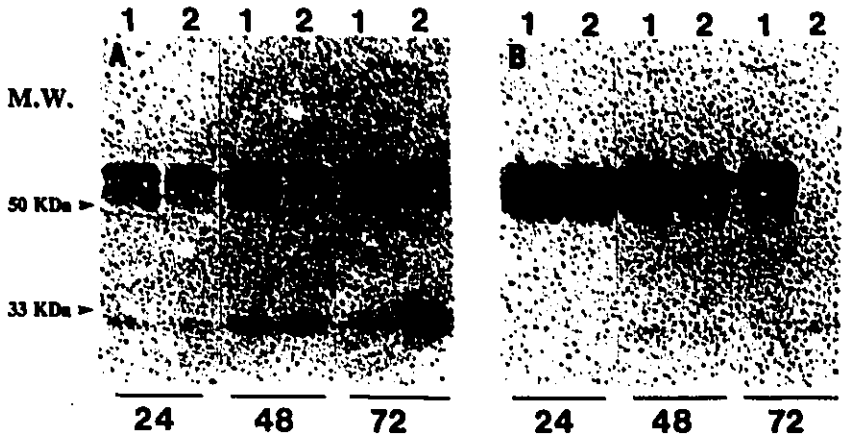

Plate 5. Effect of TNFα (10 ng/ml) on FSH (400 ng/ml)-induced PAs activity in 24, 48, 72 h cultures of granulosa cells from (A) DES-treated and (B) eCG-treated rats, as determined by a fibrin overlay method. A representative zymogram from 3 experiments. Lane 1, FSH; lane 2, FSH plus TNFα.

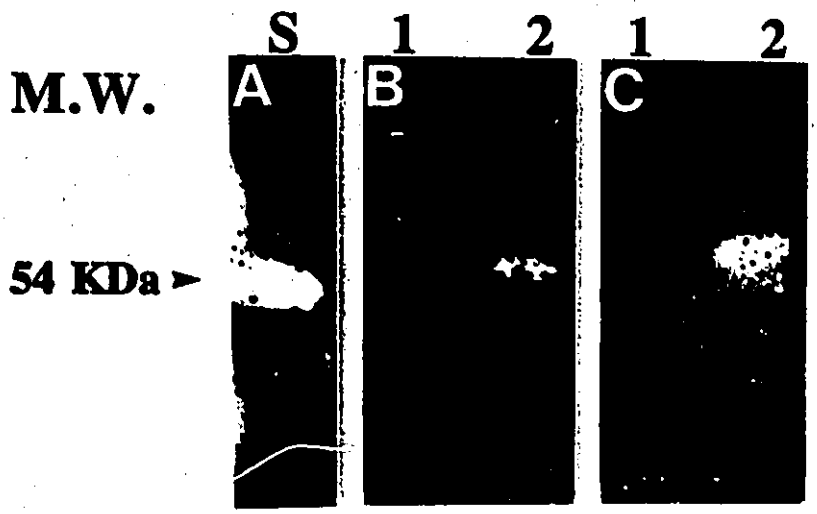

Plate 6. PAI activity in granulosa cells from (B) DES-treated and (C) eCG-treated rats cultured for 72 h in presence of FSH (400 ng/ml; lane 1) and FSH + TNFα (10 ng/ml; lane 2), as determined by a reverse fibrin overlay method. (A) activity of human PAI-1 standard (0.1 units). A representative zymogram of 3 experiments.

G. IS IL-1β INVOLVED IN THE REGULATION OF THE GRANULOSA CELL PA SYSTEM? IS ITS ACTION DEPENDENT ON FOLLICULAR MATURATION AND RELATED TO ITS INTERACTION WITH GONADOTROPIN?

Although IL-1β attenuates the gonadotropic action on granulosa cell differentiation (Gottshall et al., 1988a,b), it stimulates the production of gelatinase (enzyme involved in tissue remodelling) by cultured granulosa cells (Hurwitz et al., 1993) and enhances LH-induced ovulation in perfused ovary (Takehera et al., 1994). However, how this cytokine regulate the proteases, e.g. PA system believed to be involved in extracellular matrix degradation during follicular maturation remains unclear.

1. Influence of IL-1β on FSH-induced Progestin Production during Follicular Development

To examine the influence of IL-1β on progestin secretion, granulosa cells from DES- and eCG-treated rats were cultured in the presence of FSH with or without IL-1β (10 ng/ml). Independent of the cytodifferentiative state of granulosa cells, the cytokine significantly suppressed ($P < 0.05$) FSH-induced progestin secretion (Fig 35).

2. Effect of IL-β on DNA Synthesis in the Absence and Presence of FSH.

The effect of IL-1β on DNA synthesis in granulosa cells from DES- and eCG-treated rats cultured for 72 h in the absence and presence of FSH (400 ng/ml) and with or without IL-1β (10 ng/ml) is shown in figure 36. While the cytokine is ineffective alone, it

significantly attenuated the suppressive effect of the gonadotropin on [^{3}H]thymidine incorporation at both stages of follicular development (Fig 36).

3. Dose-response Study of the Effect of IL-1β on FSH-induced PAs and PAc Activities during Follicular Development.

To determine the effect of IL-1β on PA activities, granulosa cells from DES- and eCG-treated rats were cultured for 72 h in the presence of FSH (400 ng/ml) and different concentrations of IL-1β (0-50 ng/ml). Independent of the cytodifferentiative state of the granulosa cells, the cytokine significantly attenuated ($P < 0.05$) gonadotropin-induced PAs and PAc activities in a concentration-dependent manner (Fig 37) and with maximal inhibition observed at a concentration of 10 ng/ml.

4. Time Course Study of the Influence of IL-1β on Basal and FSH-induced PAs and PAc Activities during Follicular Development.

The changes in PAs and PAc activities were investigated on granulosa cells from DES- and eCG-treated rats maintained in culture for different durations in the absence or presence of FSH (400 ng/ml) and with or without IL-1β (10 ng/ml). While the cytokine significantly stimulated ($P < 0.05$) PAs and PAc activities in granulosa cells of DES-treated rats in a 72 h culture period, it markedly inhibited ($P < 0.05$) these activities by 48 h in cells from the eCG group (Fig 38). IL-1β significantly suppressed ($P < 0.05$) FSH-induced PAs and PAc activities by the second 24 h of culture irrespective of follicular maturation (Fig 39).

5. Characterization of FSH-induced PAs Activity in the Absence and Presence of IL-1β.

As shown in the zymogram in plate 7, IL-1β suppressed FSH-induced tPA activity in cultures of granulosa cells from both DES- and eCG-treated rats , with the effect being more pronounced with increased duration of culture. Urokinase-type PA activity in the 48 and 72 h cultures of cells from the former group was likewise inhibited by the cytokine. The suppression of gonadotropin-induced PAs activity by IL-1β was accompanied by an increase in PAI activity which was maximum in cultures of granulosa cells from eCG-treated rats at 72 h (Plate 8). In addition, three species of PAI with molecular mass of 54, 40, and 30 KDa were evident in these cultures. However, no PAI activity was detectable in 24 and 48 h of cultures irrespective of the stage of follicular development or the presence of cytokine (data not shown).

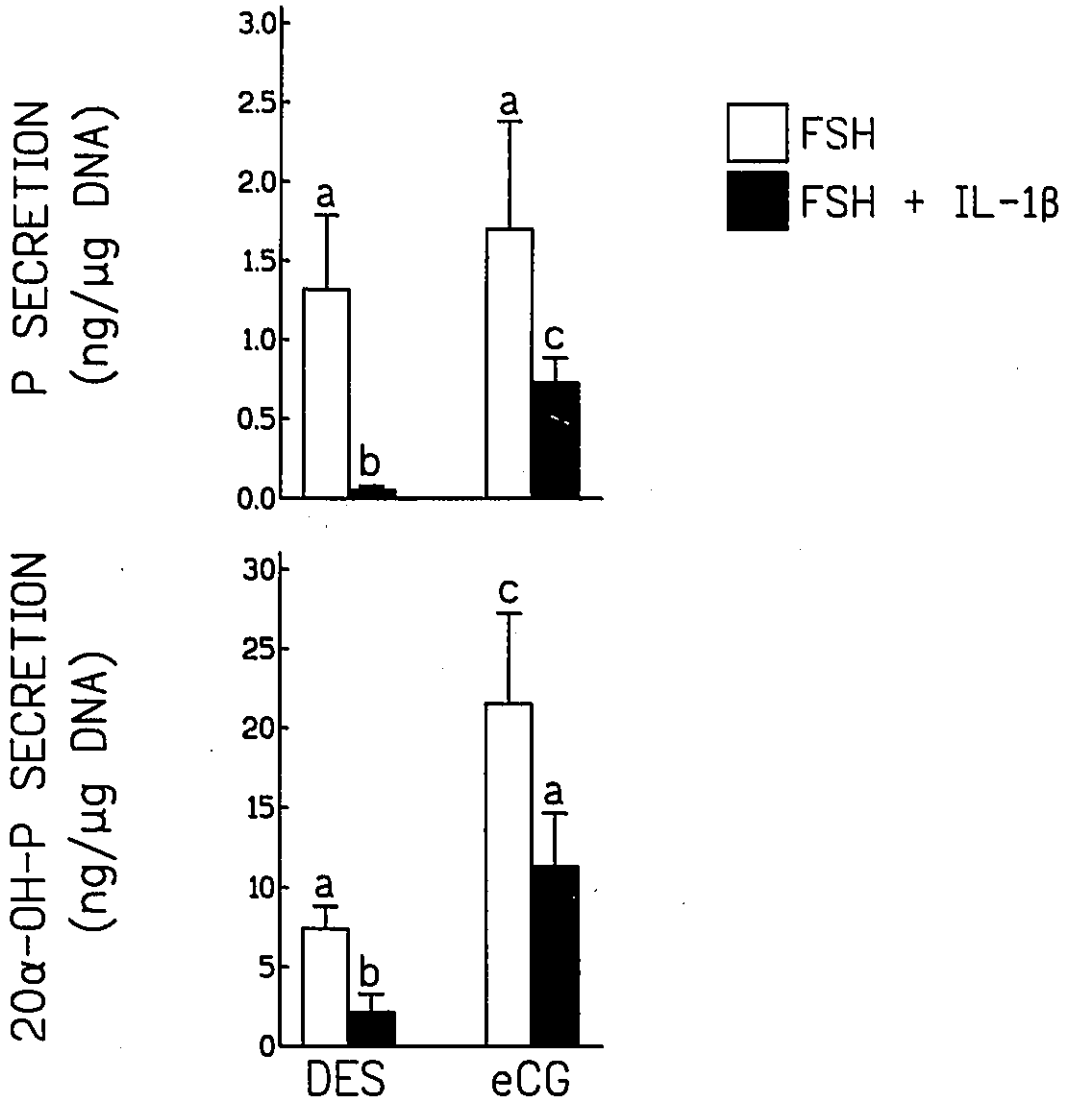

Fig 35. Effect of IL-1β (10 ng/ml) on FSH (400 ng/ml)-stimulated P and 20α-OH-P secretion (ng/µg DNA; mean ± SEM of 4 experiments) in 72 h cultures of granulosa cells from DES- and eCG-treated rats. Values with different alphabetical superscripts within each panel are significantly different ($P < 0.05$).

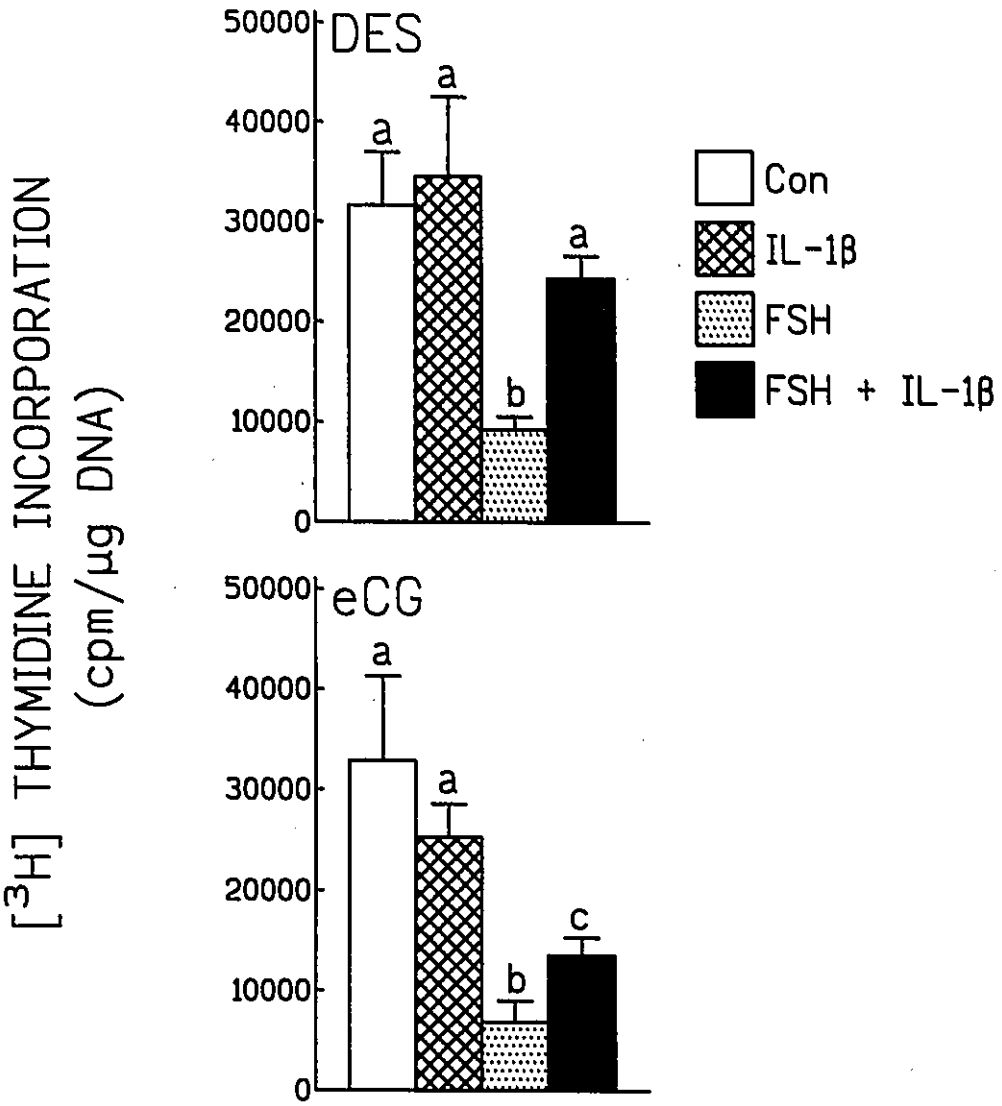

Fig 36. Influence of IL-1β (10 ng/ml) and FSH (400 ng/ml) on [^{3}H]thymidine incorporation (cpm/µg DNA; mean ± SEM of 4 experiments) in 72 h cultures of granulosa cells from DES- and eCG-treated rats. Values with different alphabetical superscripts within each panel are significantly different ($P < 0.05$).

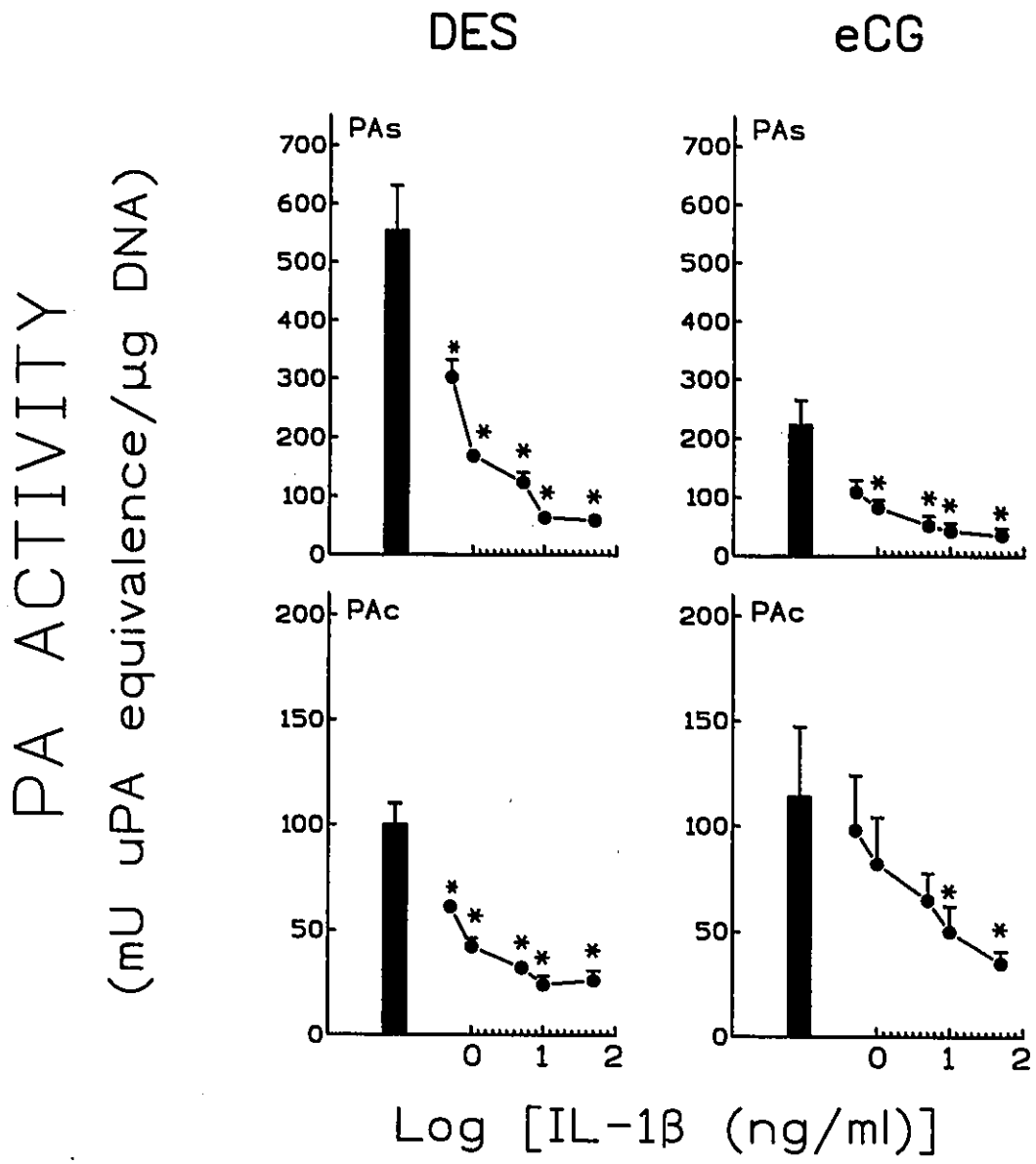

Fig 37. Concentration-dependent inhibition of FSH (400 ng/ml)-induced PAs and PAc activities (mU uPA equivalence/µg DNA; mean ± SEM of 4 experiments) by IL-1β (0.5-50 ng/ml) in 72 h cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ compared to FSH alone (black histogram).

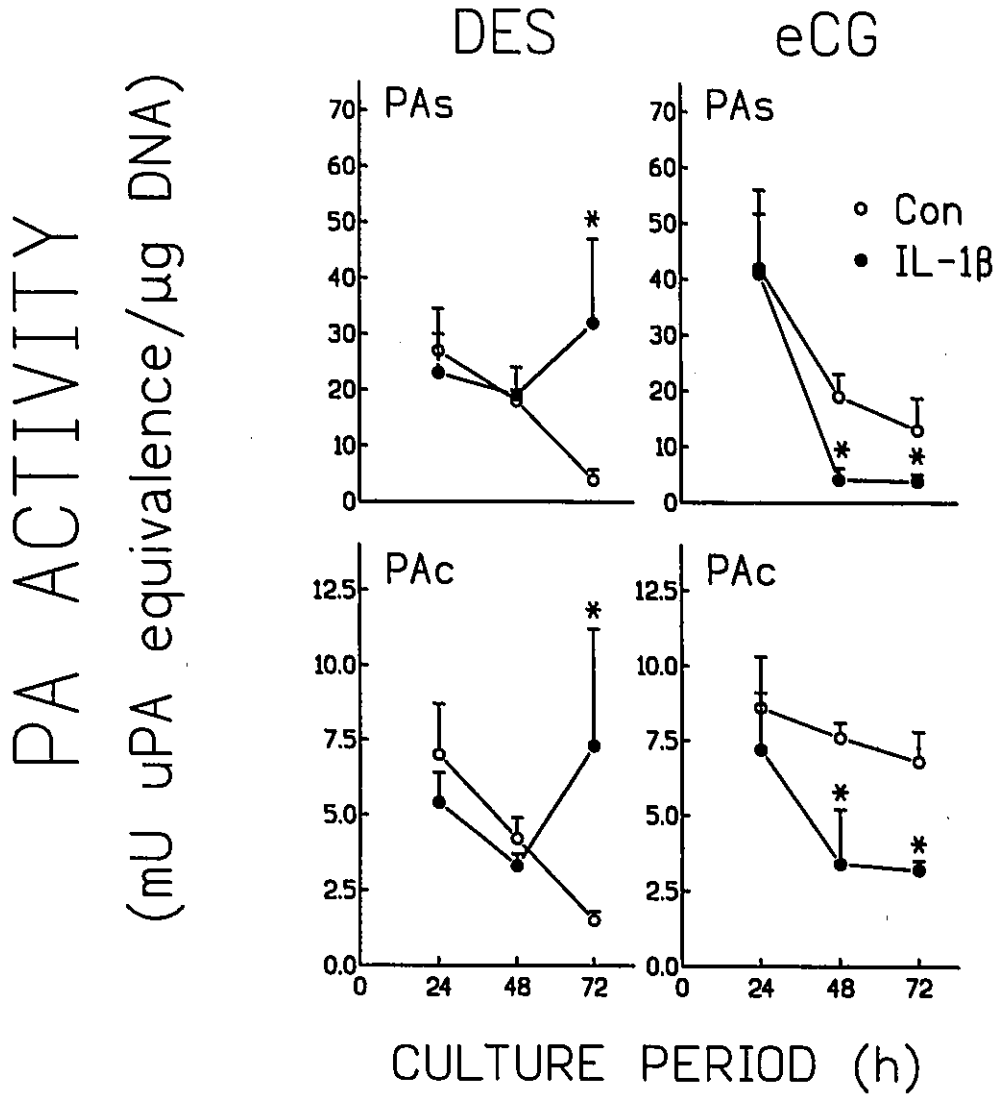

Fig 38. Time course study of the effect of IL-1β (10 ng/ml) on basal PAs and PAc activities (mU uPA equivalence /µg DNA; mean ± SEM of 3 experiments) in 24, 48 and 72 h cultures of granulosa cells from DES- and eCG-treated rats. * $P < 0.05$ compared to control.

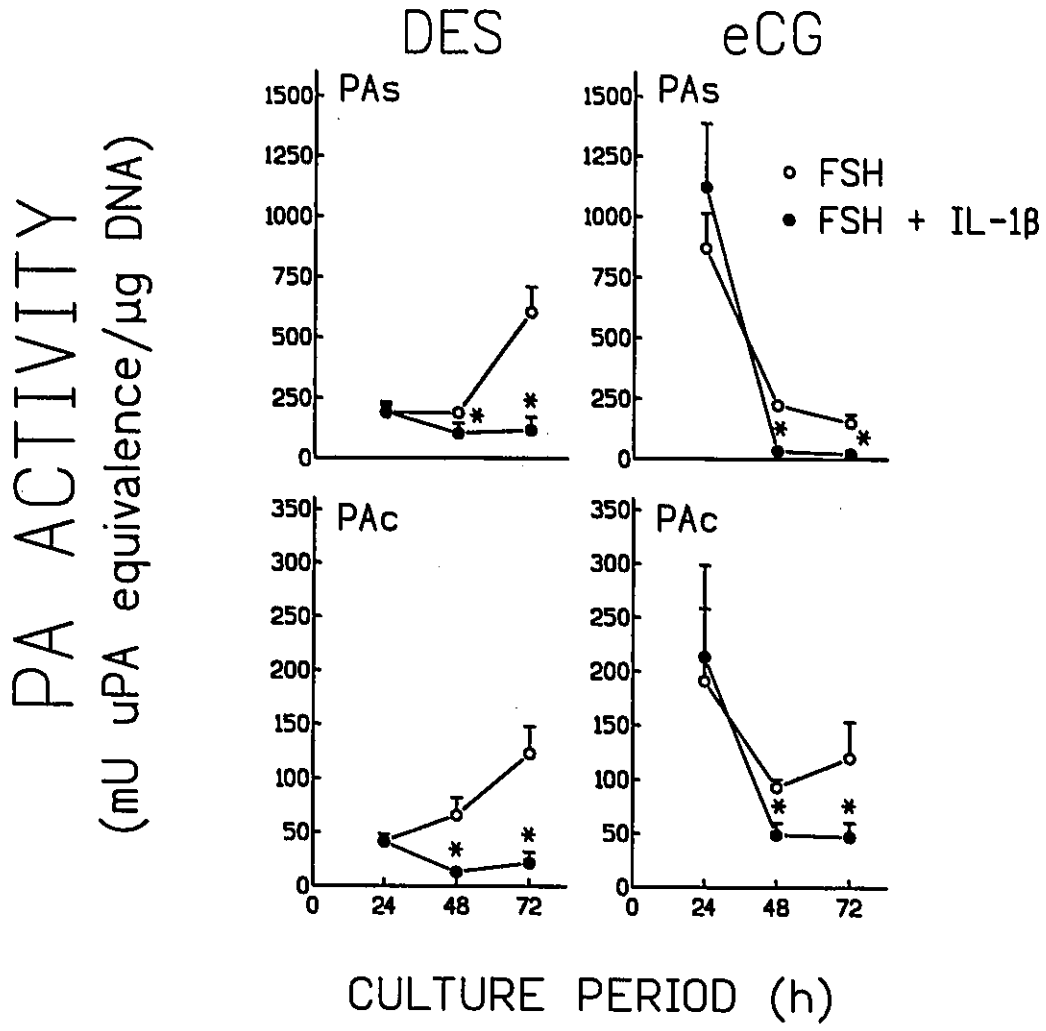

Fig 39. Time course study of the influence of IL-1β (10 ng/ml) on PAs and PAc activities (mU uPA equivalence/μg DNA; mean ± SEM of 3 experiments) in granulosa cells from DES- and eCG-treated rats cultured for 24, 48, and 72 h in the presence of FSH (400 ng/ml). * $P < 0.05$ compared to FSH only group.

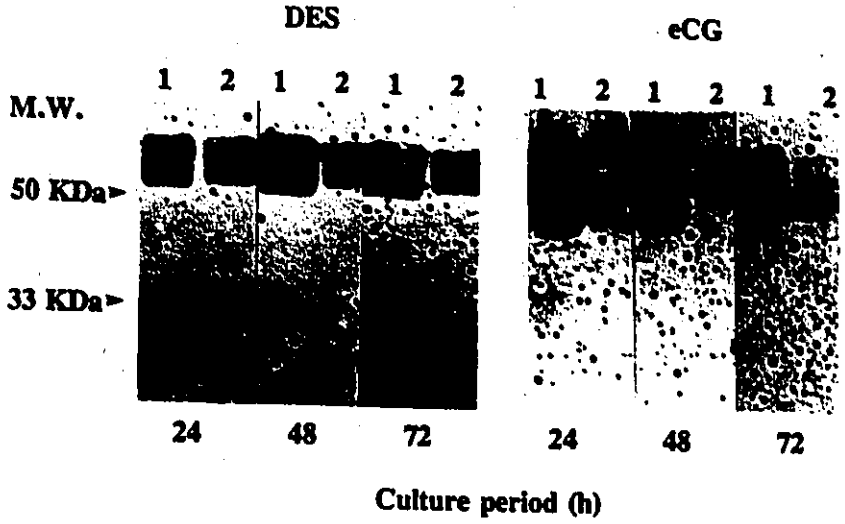

Plate 7. PAs activity in 24, 48 and 72 h cultures of granulosa cells from DES- and eCG-treated rats in the presence of FSH (400 ng/ml; lane 1) and FSH + IL-1β (10 ng/ml; lane 2), as determined by a fibrin overlay method. A representative zymogram of 3 experiments is shown.

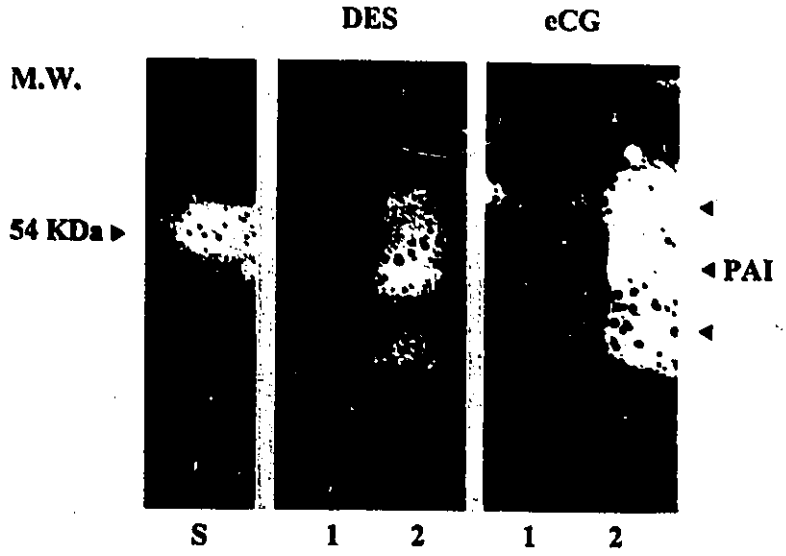

Plate 8. Effect of FSH (400 ng/ml; lane 1) and FSH + IL-1β (10 ng/ml; lane 2) on PAI activity in 72-h cultures of granulosa cells from DES- and eCG-treated rats, as determined by a reverse fibrin overlay method. "S" represents human PAI-1 standard (0.1 unit). A representative zymogram from 3 experiments is shown.

XIII. DISCUSSION

In mammal, the PA system is believed to play an important role in a variety of physiologic functions associated with proteolysis, including blood clot dissolution, embryo implantation, cell invasion and migration and ovulation (Friezner-Degen et al., 1986). This fibrinolytic system comprises an inactive proenzyme, plasminogen, which is activated to the proteolytic enzyme plasmin by two different plasminogen activators, tPA and uPA (Blasi et al., 1987). Although both PA types share the characteristic ability to activate plasminogen, they have different protein structures, tissue-specific expression and biological activities and play distinct roles in different biological processes (Vassali et al., 1985). They are products of two distinct genes with different genomic organization and chromosome localization.

Whereas tPA has specific affinity for fibrin and produces clot-restricted plasminogen activation, uPA lacks affinity for fibrin and requires conversion from inactive single-chain precursors to catalytically active two-chain derivatives (Blasi et al., 1987). Urokinase PA binds to a specific cell-surface receptor and is believed to be primarily involved in cell-mediated proteolysis (Vassalli et al., 1985). Its localization on the migrating front of cells has led to the suggestion that it may be involved in cell invasion through extracellular matrices (Pollanen et al., 1987; Lafrance et al., 1993a). Directional proteolysis could play a role in physiological processes such as follicular growth, ovulation, angiogenesis and corpus luteum formation and regression (Carmeliet et al., 1994).

Whereas inactivation of either tPA or uPA genes in mice had no significant influence on reproductive performance, deletion of both PA genes from these animals resulted in

marked reduction of fertility (Carmeliet et al., 1994). These findings suggest that the PA system may play an important role during the reproductive period and that the two types of PA may complement each other in their regulatory function. However, their precise role during ovarian follicular development remains to be established. Thus, it is important to study the regulation of these proteases during follicular maturation and ovulation and to investigate the potential roles of endocrine, paracrine and autocrine factors in the control of the PA system.

A. Expression of the Components of the Ovarian PA system during Follicular Development

Many biological processes involving tissue remodelling, tissue destruction and cell migration are equipped with an inducible enzyme system to tightly control extracellular matrix degradation and overcome intercellular barriers. Plasminogen activation is one such system which may be involved in the regulation of follicular wall remodelling during follicular development and ovulation (Liu et al., 1987b; Pellicer et al., 1988). The present study examines the compartmental expression of the PA system during follicular maturation using an established *in vivo* rat model (Liu et al., 1987b). Our data are consistent with previous findings that tPA mRNA and protein expression by the granulosa and theca-interstitial cells increased markedly during the preovulatory period and that tPA synthesized by granulosa cells accounted for 70-80% of the total tPA produced during this period (Cajander et al., 1989; Peng et al., 1993). The observation of these developmental changes in tPA message is supported by the current demonstration of high levels of tPA immunoreactivity in granulosa and theca-interstitial cells in ovarian sections from eCG plus

hCG-treated rats (compared to the DES- and eCG- treated rats) and an earlier observation that tPA activity significantly increased in ovarian homogenates, granulosa cells and theca-interstitial cells following hCG injection (Liu et al., 1987b). The high level of tPA in the preovulatory period and the induction of tPA activity by gonadotropin in cultured granulosa cells suggest that this protease may be involved in the cascade of biochemical events leading to follicular wall rupture and expulsion of the oocyte.

Moreover, changes in PAI-1 mRNA abundance and protein content during follicular maturation followed a close temporal pattern of changes to those of tPA. Whereas the expression of PAI-1 mRNA and protein were low in both granulosa and theca-interstitial cells in early stages of follicular development, they were markedly increased during the preovulatory period. Since the interaction between tPA and PAI-1 results in the formation of tPA-PAI-1 complexes and in the neutralization of the protease activity, synchrony in their synthesis may be important in keeping the functional proteolytic activity low and preventing premature rupture of the ovulatory follicle until the expected time of ovulation when PAI-1 expression and activity are down-regulated (Liu et al., 1991).

Cell-cell and cell-matrix interfaces represent physical barriers to cell migration. The attachment and detachment of proliferating and migrating cells to and from neighboring cells or extracellular matrix must occur via sequential breakdown and reformation of protein bonds which serve as anchorage sites. Urokinase PA and its receptor are believed to play an essential role in extracellular tissue remodelling during cell proliferation and migration (Blasi et al., 1987). Folliculogenesis is a physiological process that occurs as a consequence of a controlled proliferative activity of granulosa and theca cells. Our data indicate that proliferatively active granulosa and theca cells (DES-treated rats) showed maximal uPA

mRNA abundance and protein content which markedly decreased with follicular maturation, reaching lowest levels during the preovulatory stage. In contrast, uPAR is expressed in granulosa and theca-interstitial cells in a developmentally regulated manner. Whereas uPAR mRNA and protein are detectable at early stages of follicular growth, maximal levels of uPAR expression were observed at the preovulatory period. Although the physiological significance of this apparent dichotomy is presently unknown, it is possible that the presence of high levels of uPAR serves to ensure appropriate levels of uPA activity during follicular maturation and ovulation. Urokinase PA is synthesized and secreted as an inactive enzyme, pro-uPA, and the conversion of pro-uPA to uPA may involve plasmin-mediated proteolysis (Blasi et al., 1987). Whereas the binding of the pro-uPA to uPAR increases the rate of its conversion at least twenty-fold as compared to that of the free proenzyme (Stephens et al., 1989; Picone et al., 1989; Estreicher et al., 1989), this mechanism appeared insignificant at the preovulatory period since pro-uPA expression was low, as evident by the low uPA mRNA abundance and protein content. Moreover, receptor-bound active uPA is not internalized nor degraded until it binds PAI and form uPAR-uPA-PAI complexes (Blasi et al., 1987). After internalization, the uPA-PAI component is degraded and uPARs are recycled and distributed to new positions on the cell surface (Blasi et al., 1987). Thus, the coincidental rise in PAI-1 and uPAR mRNAs and protein contents at the preovulatory period could facilitate the action uPAR in the clearance of uPA and PAI-1 from the system, thus permitting a more active role of tPA for the ovulatory process. In contrast, the low level of uPAR and PAI-1 could be insufficient for uPAR-uPA-PAI-1 internalization to significantly affect the high level of uPA produced at early stages of follicular development, an observation consistent with an important role of urokinase in extracellular matrix

remodelling believed to be associated with cell proliferation and migration during folliculogenesis.

It is particularly important to consider that the mRNA abundance for the various components of the PA system in the theca-interstitial preparations may, in part, be attributed to granulosa cell contamination. However, the presence of high immunoreactive cells for tPA, uPA, PAI-1 and uPAR within theca-interstitial compartment and the detection of tPA and PAI-1 mRNAs in the theca-interstitial layer by *in situ* hybridization (Peng et al., 1993) suggest local synthesis of these molecules. Whether these substances are produced by theca cells or other cell types present in this compartment, e.g. endothelial cells, macrophages and fibroblasts, remains to be determined.

It is of interest that the oocyte also exhibited heavy immunostaining for tPA, uPA, PAI-1 and uPAR at all stages of follicular development. These observations raise the intriguing possibility that the oocyte plays a significant role in the control of extracellular matrix remodelling, particularly in the cumulus region where the cells are proliferatively most active. This notion is consistent with the concept that the oocyte secretes various factor having important regulatory roles in the function of its support cells (Vanderhyden et al., 1992; Vanderhyden et al., 1993; Vanderhyden, 1993). The cross-talk between the oocyte and the granulosa and theca-interstitial cells in the regulation of extracellular matrix remodelling is an important area of future investigation.

In summary, the precise nature and multiplicity of level of control of the PA system is important for extracellular matrix remodelling during ovarian follicular development and ovulation. Whereas there is ample evidence for the role and regulation of follicular wall remodelling during the periovulatory period, little is known concerning the participation and

relative importance of the PA types, as well as their regulation by gonadotropins and intra-ovarian factors during early follicular maturation. These latter aspects were the focus of my subsequent investigations, the results of which are discussed hereafter.

B. The role of FSH in the regulation of PA activity during follicular development

Ovarian follicular development and ovulation involve proliferation and differentiation of the granulosa and theca cells, the nature and extent of which are dependent on the complex actions and interactions of gonadotropins, growth factors and cytokines (Gottschall et al., 1987; Adashi, 1992a, b). The expansion of the follicle during follicular growth and the rupture of the follicular wall during ovulation require considerable extracellular matrix remodelling around and within the follicle. This process is believed to result from the synthesis and action of various proteases such as PA (Beers, 1975; Beers et al., 1975). FSH is a potent stimulator of PA activity in undifferentiated rat granulosa cells *in vitro* (Too et al., 1984; Knecht, 1986, Canapari and Strickland, 1986) and this protease activity increases with follicular maturation in response to gonadotropins *in vivo* (Liu et al., 1987a; Liu et al., 1987b; Hsueh et al., 1988). The present study confirms and extends these observations and demonstrates that the *in vitro* stimulatory effect of FSH (progestin secretion and PA activity) was more pronounced in differentiated cells of the antral follicles (eCG-treated rats) than proliferatively active ones from preantral follicles (DES-treated rats). Although the physiologic basis for this difference is unknown, our findings are consistent with the observation that differentiated rat granulosa cells exhibit higher FSH receptor expression than those from immature follicles (LaPolt et al., 1992). In this context, Tilly et al. (1992a) have demonstrated that FSH stimulates the expression of its receptor mRNA in 72 h cultures

of granulosa cells from immature rat follicles which, as demonstrated in the present investigation, is associated with an increase in PA activity. In contrast, the studies by Lee and Takahashi (1977) and Schwall and Erickson (1983) have shown that FSH induced down-regulation of FSH binding sites and internalization of its own receptor during 72 h culture of differentiated granulosa cells. Results from the present investigation clearly demonstrated that whereas granulosa cells from immature preantral follicles undergo cytodifferentiation in response to FSH *in vitro* and secrete higher PA activity and progestin, exposure of already differentiated cells to the gonadotropin not only failed to maintain their differentiated function (FSH receptor induction as demonstrated by Lee and Takahashi, 1977) but exhibited a significant loss of PA activity.

Both types of PA (uPA and tPA) have been identified in the rat ovary and are believed to have different physiological functions and hormonal regulation (Ny et al., 1985). In addition, PAI, an important regulatory element of the PA system, is known to be produced and hormonally controlled in cultured granulosa cells (Ny et al., 1985). Although both uPA and tPA have the same biological activity, they differ in their tissue distribution and physiological functions (Blasi et al., 1987; Freizner-Degen et al., 1986). The increase in tPA activity during the preovulatory period (Reich et al., 1985a; Liu et al., 1987 a,b; Hsueh et al., 1988) and the suppression of ovulation by exogenous tPA antibody (Tsafriri et al., 1989) suggest a key role of this PA type in the initiation of the ovulatory cascade and follicular wall rupture during ovulation. However, it has been hypothesized that uPA is associated with tissue remodelling accompanying cell proliferation and migration during various physiological and pathological conditions (Ossowski et al., 1976; Sappino et al., 1989). The increase of uPA activity in hen granulosa cells *in vitro* at early stages of follicular

development and its suppression by the ovulatory hormone (luteinizing hormone) in the preovulatory follicle (Tilly and Johnson, 1987; Lafrance et al., 1993a,b) suggested that at least in the hen ovary, this PA type is important in follicular wall remodelling accompanying cell proliferation and migration during folliculogenesis and not ovulation. In the present study, we have observed that tPA is present in cultures of granulosa cells from both preantral and antral follicles while uPA is detectable only in granulosa cells from the less mature developing follicles. The stimulator effect of FSH on tPA activity with little or no effect on uPA activity suggests that this gonadotropin plays an important role in the regulation of the differentiative function of granulosa cells during follicular development.

In addition, our data show that secreted PA activity accounts for much of the total PA activity measured in granulosa cell cultures irrespective of the stage of follicular maturation or the presence of different agonists, suggesting that the synthesis and secretion of PA are tightly coupled as previously shown in other cell types (Unkeless et al., 1974). This conclusion is however at variance with the earlier study of Knecht (1986) which suggested that the enzyme is synthesized by granulosa cells and remains closely associated with them throughout the culture period. The reasons for the differences in our findings are unclear but may possibly be related to the pretreatment of our spent medium samples with triton X-100 (not performed in Knecht's study) or to different methods of measuring PA activity.

Moreover, FSH has been shown to stimulate granulosa cell proliferation *in vivo* (Hirshfield, 1991) but not *in vitro* (Bendell and Dorrington, 1990), suggesting that the action of the gonadotropin *in vivo* may be mediated by the secretion and the action of a paracrine factor(s). Our present findings have indicated that FSH suppresses DNA synthesis in granulosa cell cultures at both stages of follicular development and are consistent with the

notion that granulosa cells leave the cell cycle before the initiation of cell differentiation (Hirshfield, 1991).

C. Effect of GRF and VIP on granulosa cell PA activity during follicular development

Whereas the synthesis and secretion of GRF and VIP by ovarian cells has been well established (Moretti et al., 1990a; Gozes and Tsafriri, 1986), little is known about the role of these peptides in the regulation of ovarian folliculogenesis. Earlier studies have indicated that both peptides regulate granulosa cell differentiation (Moretti et al., 1990b; Trzeciak et al., 1986; Davoren and Hsueh, 1985; Johnson and Tilly, 1988) and that VIP stimulates PA activity in cultures of granulosa cells from preantral follicles (Liu et al., 1987a). The present investigation extends these observations and demonstrates that GRF and VIP induce PA activity and progestin secretion (P and 20α-OH-P) in granulosa cell cultures and that the stimulatory effect of the peptides was more pronounced in cells from antral than preantral follicles. Whether the difference in the granulosa cell responses between the two developmental stages is due to higher receptor levels in differentiated cells remains to be determined. However, our present results and the earlier report that VIP increases aromatase activity in the fetal rat ovary before acquiring the responsiveness to FSH (Tornell et al., 1988) suggest that these peptides may be important for the initiation of follicular development.

In infertile women resistant to gonadotropin therapy, the administration of GRF together with FSH stimulate folliculogenesis and ovulation (Moretti et al., 1989). Long term infusion of cattle with GRF analog increases ovarian follicular size and progesterone content (Spicer and Enright, 1991). The actions of GRF and its analogue on the human and bovine

ovary could be mediated by growth hormone (GH), an agonist known to potentiate the gonadotropin-induced steroidogenic capabilities of granulosa cells (Jia et al., 1986) or via direct interaction of GRF with its specific receptor on granulosa cells as suggested by the present investigation and earlier studies (Moretti et al., 1990b). It has been demonstrated that GRF and VIP potentiate FSH-induced rat granulosa cell steroidogenesis *in vitro* (Moretti et al., 1990b). The present study shows that both peptides enhance FSH-induced PA activity and progestin secretion in cultures of undifferentiated granulosa cells. Thus, in light of their direct stimulatory influence on basal granulosa cell PA activity, DNA synthesis and progestin secretion, GRF and VIP appear capable of both mimicking and enhancing the actions of FSH in the ovarian follicle. This is not surprising in light of the observation that granulosa cells from immature, estrogen-treated rats are heterogenous and consist of a large subpopulation which responds readily to FSH with the induction of LH receptors and steroidogenic enzymes and a subpopulation exhibiting lower sedimentation rate and greater sensitivity to VIP (Kasson et al., 1985).

In contrast to an earlier report that GRF and VIP bind to common receptors to stimulate steroid secretion in undifferentiated granulosa cells (Moretti et al., 1990b), the present investigation demonstrates that only GRF can potentiate the gonadotropic stimulation of differentiated granulosa cell PA activity and can induce DNA synthesis in cells from antral and pre-antral follicles, indicating a possible involvement of distinct receptors for these peptides in these regulations. Whether the difference in the responses of granulosa cells to these peptides is due to changes in the affinities of the two classes of GRF/VIP receptors (Moretti et al., 1990b) during cytodifferentiation, the presence of different GRF/VIP receptors as demonstrated in other cell types (Pandol et al., 1984; Christophe et al., 1988),

the ability of FSH to induce the expression of GRF receptor with different sensitivity than the GRF/VIP receptors (Bagnato et al., 1991) or the action of GRF via two different cytoplasmic signal pathways (i.e. cAMP and Ca^{2+}) is not readily apparent and awaits further investigation.

The proliferation of granulosa cells is in response to intraovarian factors stimulating cell cycle progression (Skinner and Coffey, 1988; Yoshimura and Tamura, 1988; Spicer et al., 1992). The present study demonstrates for the first time that GRF stimulates DNA synthesis in cultures of rat granulosa cells irrespective of the stage of follicular maturation and that this mitogenic response is suppressed by FSH. The physiological significance of this inhibitory effect of FSH is not readily apparent. It is possible that the differentiative function of the gonadotropin is dominant over the mitogenic influence of GRF and, as such, prevents the progression of granulosa cells from G1/G0 to S phase of the cell cycle and results in the "switching off" of the GRF-stimulated proliferative response during the transition of these cells into a differentiated state, as evident by a suppression of GRF-stimulated granulosa cell DNA synthesis and a concomitant increase in progestin (P and 20α-OH-P) and PA secretion.

Tissue-type PA activity was stimulated by GRF, VIP and FSH irrespective of the stage of granulosa cell differentiation. These findings are consistent with the concept that these ovarian peptides are important regulators of follicular wall remodelling associated with growth and rupture.

In summary, whereas VIP potentiates FSH-induced granulosa cell differentiation (PA and progestin secretion) during early follicular development, GRF is effective throughout follicular maturation. In addition, GRF is a potent mitogen, the activity of which is modulated by FSH. Taken together, these results cast doubt on an earlier notion that GRF

and VIP bind to common receptors to regulate granulosa cell function, and strongly support an important facilitatory role of the peptides in the gonadotropic control of the transition of granulosa cells from a proliferative to differentiative state and in the weakening of follicular wall in the preovulatory period.

D. Regulation of granulosa cell PA activity by TGFα during follicular development

Various intraovarian factors are known to modulate granulosa cell PA activity in the rat and domestic hen (Too et al., 1984; Liu et al., 1987a; Tilly and Johnson, 1990; Lafrance et al., 1993 a,b). The synthesis and secretion of TGFα by the theca-interstitial cells of mammalian ovaries (Kudlow et al., 1987; Skinner and Coffey, 1988) and the expression of its common EGF/TGFα receptor on granulosa cells (Chabot et al., 1986; Feng et al., 1987), suggest that a paracrine mechanism may exist whereby TGFα from the theca cells regulates the protease activity in granulosa cells during follicular development. Galway et al. (1989) have demonstrated that TGFα increases tPA mRNA level and activity in cultures of undifferentiated rat granulosa cells. Moreover, our present studies further demonstrate that TGFα stimulates basal granulosa cell PA activity at both stages of follicular maturation with the growth factor being more effective in cells at an advanced stage of differentiation. In this regard, Feng et al. (1987) have shown that there is a greater abundance of TGFα receptor in granulosa cells from eCG- than DES-treated rats. Moreover, our data demonstrate that TGFα interacts with FSH in the regulation of the granulosa cell PA system during cell differentiation and that these interactions are complex. TGFα enhances FSH-stimulated granulosa cell PA activity during 24 h of culture irrespective of the stage of follicular development. Whereas the physiologic mechanism(s) of this potentiation is not readily

apparent, this may be due to cross-talk between distinct cytoplasmic signalling pathways operated by TGFα and FSH [i.e. tyrosine kinase (Tilly et al., 1992b); and protein kinase-A, (Knecht and Catt, 1981), respectively], induction of TGFα receptors by FSH (Feng et al., 1987; Fujinaga et al., 1992) and/or the presence of different subpopulations of rat granulosa cells with different agonist sensitivity (Kasson et al., 1985). However, in 72 h of culture during which granulosa cells from immature follicles undergo further cytodifferentiation in the presence of gonadotropin (increased progestin secretion and PA activity), TGFα suppressed FSH-stimulated PA activity and P secretion while exhibiting no effect on differentiated cells under similar conditions. Moreover, the inhibitory effect of TGFα on FSH-stimulated PA activity could not be due to an influence on the activity of PAI, since our results show no detectable presence of this inhibitor irrespective of the stage of follicular maturation, the presence of the growth factor and the duration of culture. Alternatively, it is possible that during the cytodifferentiative process the acquisition and increase in FSH receptors which are essential for the amplification of gonadotropin action (Camp et al., 1991) are attenuated by the presence of TGFα during the early stages of follicular development, as observed by Tilly et al. (1992a) and Dunkel et al. (1994). The present data suggest that the growth factor may be an important modulator of the gonadotropic action during the transition of granulosa cells from a proliferatively active to a differentiated state during follicular development and are consistent with earlier observation that the growth factor attenuates the differentiative responses (increased aromatase activity, progestin secretion and LH receptor expression) of granulosa cells from estrogen-treated rats cultured for 72 h in the presence of FSH (Adashi and Resnick, 1986; Adashi et al., 1987; Bendell and Dorrington, 1990).

TGFβ is secreted by the theca-interstitial and granulosa cells and appears to have an

antagonistic action on the regulation of granulosa cell functions by TGFα (Skinner et al., 1987a,b; Kim and Schomberg, 1989; Bendell and Dorrington, 1990; Adashi et al., 1989a). The present study demonstrates for the first time that, irrespective of the stage of follicular maturation, TGFβ has no significant influence on basal, TGFα- and FSH-induced PA activity . In contrast, Lafrance et al. (1993a,b) observed that TGFβ stimulates basal and TGFα-induced PA activity in hen granulosa cells from immature (F5-6), but not in mature (F1) follicles *in vitro*. Whether the differences in the response to TGFβ in these studies could be attributed to species differences remains to be determined. A difference in the presence of functional TGFβ receptor in these granulosa cell preparations could not be a contributing factor since TGFβ is known to enhance FSH-induced aromatase activity in cultures of granulosa cells from DES-treated rats (Bendell and Dorrington, 1990).

The stimulatory effect of TGFα on tPA activity at both stages of follicular development is coincident with increased granulosa cell differentiation (progestin production). In addition, the inhibitory influence of this growth factor on uPA activity in the presence of FSH at earlier stages of follicular maturation is coupled with a suppression of proliferative activity of granulosa cells (DNA synthesis). Collectively these findings is consistent with the notion that in the rat, tPA is associated with granulosa cell differentiation and follicular rupture, whereas uPA is involved in follicular wall remodelling accompanying granulosa cell proliferation and migration during folliculogenesis.

In summary, we have shown that TGFα stimulates basal granulosa cell PA activity during follicular development, potentiates FSH-induced PA activity at early stage of follicular maturation but attenuates this activity during the gonadotropin-induced granulosa cell differentiation. Moreover, the mitogenic response of granulosa cells to the growth factor

is suppressed by FSH at early stages of follicular growth and TGFα acts synergistically with the gonadotropin to inhibit DNA synthesis as the cells undergo differentiation. These findings support the hypothesis that the cytodifferentiative function of FSH is modulated by intrafollicular regulatory factors (e.g. TGFα) that control the transition of granulosa cells from a proliferative to a differentiated state at defined stages of follicular development and may determine the fate of the ovarian follicles (i.e. atresia vs growth).

E. The role of TNFα in the regulation of PA activity during granulosa cell differentiation and proliferation

There is an increasing body of evidence that the intricate interactions between granulosa, theca and germ cells play an important role in the regulation of ovarian follicular development and ovulation (Hirshfield, 1991). This formidable correlation now appears to be complicated by another cell type of the immune system, the macrophages. Since resident ovarian (i.e. extravascular) macrophages and their secretory products (e.g. TNFα and IL-1β) are present at distinct stages of the ovarian cycle (Hume et al., 1984) and TNFα is capable of modulating the endocrine function of granulosa and theca cells *in vitro* (Roby and Terranova, 1990; Veldhuis et al., 1991; Zachow et al., 1992; Yan et al., 1993), the concept that the immune system may also constitute a key regulatory component of ovarian folliculogenesis has recently been forwarded (Adashi, 1992a, b). However, precisely how the cytokine regulates this process at the cellular level is unclear. In the present study we have demonstrated for the first time an important regulatory role for TNFα in granulosa cell proliferation and differentiation during follicular development. While TNFα alone did not appear to have a significant effect on rat granulosa cell DNA synthesis, it markedly

attenuated the suppressive effect of gonadotropin on cell proliferation. These findings are consistent with an earlier report that TNFα stimulates *in vitro* proliferation of highly differentiated human granulosa cells previously primed with gonadotropins *in vivo* (Yan et al., 1993).

The influence of TNFα on granulosa cell differentiation has been well documented (Emoto and Baird, 1988; Adashi et al., 1990; Veldhuis et al., 1991; Tekpetey et al., 1993). TNFα inhibits FSH-induced cAMP production in rat and porcine granulosa cells *in vitro* (Emoto and Baird, 1988; Adashi et al., 1989b; Veldhuis et al., 1991). In addition, the cytokine has also been shown to suppress the secretion of estradiol and progestin by porcine, rat and human granulosa cells stimulated by gonadotropin (Emoto and Baird, 1988; Adashi et al., 1989b; Adashi et al., 1990; Veldhuis et al., 1991, Tekpetey et al., 1993). Results from the present studies extend these observations and demonstrate that the cytokine suppresses not only FSH-induced progestin secretion but also tPA activity in cultures of granulosa cells irrespective of the stage of follicular maturation. It is of interest to note that the duration of culture required for TNFα to exert its influence on basal and FSH-stimulated PA activity was considerably shorter in the undifferentiated than differentiated granulosa cells. Moreover, the effect of the cytokine on granulosa cell progestin secretion was more pronounced at the early stage of follicular development. Whether these differences in TNFα response was due to a decrease in binding affinity and capacity of its receptor during granulosa cell differentiation remains to be determined. Thus, considering the influence of TNFα on granulosa cell DNA synthesis, progestin secretion and tPA activity, it appears that TNFα is an important modulator of the gonadotropin-induced transition of granulosa cells from a proliferative to a differentiative state. Although the cellular mechanism of this regulation is yet to be

determined, it is possible that the mitogenic influence of the cytokine is dominant over the differentiated function of FSH and, as such, promotes the progression of the granulosa cell from G_1-G_0 to S phase of the cell cycle and results in the "switching off" of FSH-induced suppression of cell proliferation.

The present study demonstrates that uPA activity is lost with the progression of granulosa cells towards a more differentiated state and that TNFα increases this enzyme activity at early stage of follicular development, concomitant to the increase in the DNA synthetic response of granulosa cells. Furthermore, our results show that TNFα attenuates FSH-induced tPA activity irrespective of the stage of follicular development, an effect associated with a suppression of progestin secretion. Thus, these results further support the hypothesis that whereas tPA is produced during cell differentiation and ovulation, uPA is involved in granulosa cell proliferation and migration. The suppressive effect of TNFα on net PA and more specifically on tPA activity can be attributed at least in part to an increase in PAI activity in both cell preparations. This apparent difference between the results in net PA activity (fibrinolysis assay) and uPA activity (fibrin overlay assay) might be due to the higher activity rate of tPA (compared to uPA) determined in the presence of fibrin in the former assay (Knecht, 1986).

Although the precise mode of action of TNFα in the inhibition of gonadotropin-induced granulosa cell differentiation (PA and progestin secretion) is not well established, Adashi et al. (1989b) have demonstrated that the cytokine exerts its effects at a level distal to cAMP generation and not by decreasing FSH receptor binding capacity or affinity. On the other hand, TNFα can increase progesterone production in preovulatory follicles without affecting cAMP production (Sancho-Tello and Terranova, 1991), thus raising the possibility

that TNFα may act via protein kinase C or other signalling systems independent of the adenylate cyclase-protein kinase-A pathway as suggested by Sancho-Tello and Terranova (1991). Moreover, studies on hen granulosa cells from our laboratory (Soboloff et al., 1995) have shown that the action of TNFα involves intracellular Ca^{++} mobilization and have suggested, as observed in other cell types (Mathias et al., 1991; Schutze et al., 1992) and more recently in rat granulosa cells (Santana et al., 1995), that sphingolipid breakdown products (ceramide and sphingosine) play an important role in this regulation. Whether this intracellular signalling pathway is activated in the regulation of rat granulosa cell proliferation and PA system by TNFα awaits further investigation.

In summary, our findings demonstrate that TNFα inhibits basal and FSH-induced tPA activities, attenuates FSH-stimulated progestin secretion and reversed the suppressive effect of gonadotropin on DNA synthesis, thus supporting the hypothesis that the antigonadotropic influence of TNFα on granulosa cell differentiation (PA activity and progestin secretion) is inversely coupled with its ability to promote granulosa cell proliferation (DNA synthesis). In addition, TNFα may control tissue remodelling to prevent premature follicular rupture during granulosa cell proliferation and differentiation and suppresses the potential weakening of the follicular wall until the time of ovulation. Thus, in addition to TGFα, TNFα may play a key role in determining the fate of the ovarian follicle (growth, ovulation or atresia).

F. Effect of IL-1β on PA activity during granulosa cell differentiation and proliferation

The existence of a complete IL-1 system in the mammalian ovary (Hurwitz et al., 1991; Simon et al., 1994) and the ability of IL-1 to modulate the endocrine function of

granulosa and theca cells *in vitro* (Gottshall et al., 1987; Gottschall et al., 1988a; Gottschall et al., 1988b, Fukuoka et al., 1988; Hurwitz et al., 1991) have led to the belief that interleukin-1s play a regulatory role in follicular maturation, ovulation and corpus luteum formation. In the present investigation, we have obtained novel evidence for the importance of IL-1β in the regulation of granulosa cell proliferation and differentiation during follicular maturation. Our results are consistent with an earlier demonstration (Gottschall et al., 1987; Gottschall et al., 1988a,b; Fukuoka et al., 1988) of the inhibitory effect of the cytokine on gonadotropin-stimulated granulosa cell differentiation (P secretion and LH receptor expression) *in vitro* and further demonstrate that the cytokine not only inhibits FSH-induced progestin secretion but also PA activity irrespective of the stage of follicular development. Moreover, IL-1β antagonizes the inhibitory influence of FSH on granulosa cell DNA synthesis in both cell preparations. Interestingly, TNFα has more pronounced biological actions than IL-1β in the regulation of granulosa cell proliferation and differentiation in the presence of FSH. These findings are consistent with the contention that the ability of cytokines to inhibit granulosa cells differentiation is inversely associated with their capacity to induce proliferation (Fukuoka et al., 1989). Thus, it appears that IL-1β promotes the mitogenic capability of the granulosa cells and their progression from G0-G1 to S phase of the cell cycle and, as such, may also modulate the differentiative influence of gonadotropins.

Although the mechanism of action of IL-1β on granulosa cells is not known, it has been shown that the cytokine exerts it actions via: a) mobilization of intracellular calcium as in the case of T-lymphocytes (Luckasen et al., 1974); b) the synthesis and secretion of prostaglandins such as during muscle proteolysis and fever induction (Bernheim et al., 1980; Baracos et al., 1983) and c) sphingolipids and sphingolipid breakdown products (ceramide

and sphingosine) in transformed cells (Mathias et al., 1993). Whether one or more of these biochemical signals are involved in eliciting IL-1β action on rat granulosa cell differentiation and DNA synthesis awaits further investigation. Moreover, it is known that the stimulation of granulosa cell differentiation (steroid production and PA activity) by FSH is mediated via cAMP (Knecht and Catt, 1983; Adashi and Resnick, 1984; Ranta et al., 1984) and the accumulation of this second messenger is critical in the first 24 h (Gottschall et al., 1988c). Gottshall et al (1988c) have demonstrated a discordance in the effect of IL-1 on rat granulosa cell differentiation stimulated by FSH and cAMP. They showed that IL-1 suppresses FSH-induced cAMP accumulation after 48 or 72 h of culture followed by an attenuation of P secretion and LH receptor expression. In the presence of 8-bromo cAMP the cytokine enhanced P secretion but inhibited LH receptor expression. Thus, irrespective of the intracellular mechanism involved in action of IL-1β on granulosa cells, it appears that its cross-talk with the adenylate cyclase-protein kinase A pathway is significant in the regulation of granulosa cell differentiation by the cytokine.

Our present demonstration that IL-1β stimulates basal PA activity in cultures of undifferentiated granulosa cells is consistent with previous report on the stimulatory influence of the cytokine on a 92-kilodalton gelatinase (a metalloproteinase also involved in tissue remodelling) in cultures of granulosa cells from immature untreated rats (Hurwitz et al., 1993). In addition, IL-1β inhibits basal PA activity in more differentiated cells. Although the physiologic significance of this dual and opposite effect of IL-1β during follicular development is not readily apparent, it may be possible that different intracellular signalling pathways are involved in its regulation of the PA system and different remodelling functions (c.f. granulosa cell proliferation and migration vs follicular rupture) exist at various

cytodifferentiative states. Moreover, IL-1β also inhibited FSH-induced tPA activity in undifferentiated and differentiated granulosa cells and this inhibition is accompanied by a suppression of progestin secretion, a response consistent with the anti-differentiative action of the cytokine (Gottshall et al., 1987; Fukuoka et al., 1988).

Since PAI binds and neutralizes PA activities, net PA activity and ultimately follicular wall remodelling during development and ovulation are dependent on the relative abundance and interaction of these molecules. PAI activity and mRNA levels have been detected in human preovulatory follicular fluid and granulosa cells, respectively (Jones et al., 1989) and its expression by rat granulosa and theca cells is hormonally regulated (Liu et al. 1991; Ny et al., 1985). Although IL-1β enhances PAI production in endothelial cells (Nachman et al., 1986), the role of the cytokine in the regulation of ovarian PAI synthesis is not known. We have demonstrated that IL-1β and TNFα stimulate granulosa cell PAI activity *in vitro* in the presence of FSH during follicular development, with the cytokines being more effective at late stages of maturation and with IL-1β more potent than TNFα. Thus, the loss of net PA activity in these cells observed in the presence of the cytokines can be attributed at least in part to increased PAI production.

In summary, the present data demonstrate that IL-1β inhibits FSH-induced granulosa cell differentiation (PA activity and progestin secretion) and attenuates the suppressive influence of gonadotropin on granulosa cell DNA synthesis. These findings suggest that the cytokine may modulate the transition of these cells from a proliferative to a differentiated state and may play an important role in the control of tissue remodelling during ovarian follicular growth.

G. General conclusion

In conclusion, the maturation of the ovarian follicles involve several sequential stages: initiation , growth, selection and ovulation. Once the primordial follicles are recruited to grow, they are irreversibly committed to their destiny, i.e. ovulation or atresia. As each follicle progresses through the developmental process, the granulosa cells proliferate rapidly but their limited proliferative potential diminishes with follicular maturation. Upon receipt of an appropriate stimulus the cells change their proliferative course towards differentiation or death. It is becoming increasingly apparent that the regulation of normal ovarian follicular development and function is the consequence of the regulation of cell division, differentiation and cell death. Accommodation of these changes in the ovary demands flexibility of the surrounding ovarian stroma which depends mainly on the degradation of extracellular matrix. This matrix consist of adhesion proteins (e.g. fibronectin, laminin and entactin) for cell attachment and structural proteins (e.g. collagen, elastin and glycoproteins) which hold the ovary together. The PA system has been shown to play a central role in regulating extracellular proteolysis. In the ovary, the two known forms of PA are produced by follicular cells. These activators catalyze the conversion of plasminogen to plasmin, which has wide enzymatic specificity. Plasmin is capable of decreasing the tensile strength of follicular wall either directly by degrading components of the extracellular matrix (e.g. as laminin and fibronectin) or indirectly by causing tissue disruption through the activation of other proteases (e.g. collagenases). Although both uPA and tPA has the same biochemical function, our results and previous data (Vassalli et al., 1991) suggest that these enzymes may have different physiologic function. The production of uPA by the proliferatively active granulosa and theca cells at early stages of follicular growth and by other mitogenically

active and invasive cell types, e.g. trophoblast and tumor cells, is in agreement with the concept that this protease may contribute to the remodelling of follicular wall which characterizes actively growing and migrating adherent cells. On the other hand, the activity of tPA is enhanced in the presence of fibrin, suggesting that this protease may catalyze fibrinolysis. In this view, the marked increase in tPA secretion by granulosa and theca cells during the ovulatory period may cause the rupture of the follicle and prevent premature clot formation in the ovarian stigma, therefore, giving time for the expulsion of the oocyte-cumulus complex. However, PA activity must be controlled in time and space to prevent unnecessary damage to the follicular wall. This control is complex and occurs at many levels: a) the synthesis and secretion of the activators, b) the production of PAIs which neutralize PA activity and c) the expression of uPAR which enhances the activation of pro-uPA and localizes uPA action. Our data indicates that these components of the PA system are developmentally regulated and their production by follicular cells, specifically the granulosa cell, are modulated by gonadotropin and intra-ovarian factors (Fig 40).

Although gonadotropins are known to promote granulosa cell differentiation and thus follicular maturation, several intra-ovarian regulatory influences are now known to either permit or prevent granulosa cells from completing the maturation process. Results from the present thesis have identified some of these factors and classified them as mitogenic (TGFα), differentiating (GRF and VIP) and anti-differentiating (TNFα and IL-1β) molecules which act via a paracrine and/or autocrine manner to regulate granulosa cell function (Fig 40). TGFα modulates granulosa cell function in a stage-dependent manner. Whereas, the growth factor stimulates granulosa cell proliferation at early stages of follicular growth, it acts synergistically with FSH in promoting the differentiation of these cells, a process associated

with an increase in tPA activity. On the other hand, GRF and VIP mimic and enhance FSH action on granulosa cell differentiation. In addition, both cytokines, TNFα and IL-1β, antagonizes the action of FSH independent of follicular stages (Fig 40). These factors not only control the fine tuning of the transition of granulosa cells from a proliferative to differentiative state but also provide these cells with the appropriate biochemical machinery for extracellular tissue remodelling to accommodate clonal expansion at early stages of follicular development and follicular wall rupture during ovulation. Thus, the actions and interactions of gonadotropin and intra-ovarian factors play an important role in the regulation of granulosa cell functions and in determining the fate of these cells (i.e. proliferation, differentiation or death) and ultimately that of the follicle (ovulation versus atresia).

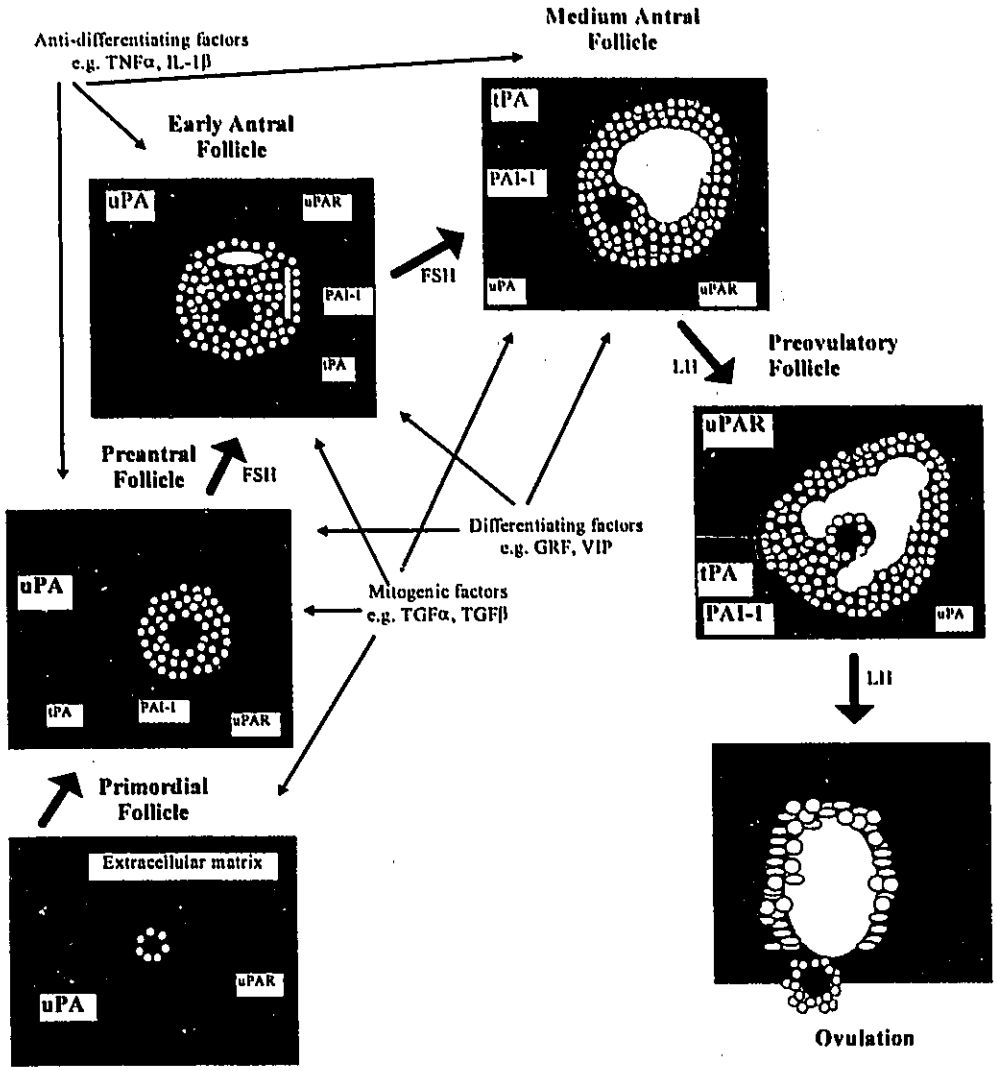

Fig 40: Schematic representation demonstrating the actions and interactions of mitogenic, differentiating and anti-differentiating factors in the regulation of ovarian follicular development

XIV. FUTURE STUDIES

Although the regulation of plasminogen activator system during follicular development by gonadotropin and local ovarian factors has been extensively studied, these investigations can be extended to examine the interactions between these intra-ovarian factors in the control of PA system accompanying granulosa cell proliferation and differentiation, particularly the potential role of intra-ovarian factors in the regulation of uPAR expression by granulosa cells during follicular development. It is also important to determine the synthesis and secretion of these intra-ovarian regulators during follicular maturation and their mechanisms of action in the regulation of granulosa cell functions. In addition, it will be of interest to analyse if the change in the expression of uPAR is associated with increased proliferative activity of the granulosa cells and the possible action of uPA as a mitogen.

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Feb 1987-Apr 1987	Research elective, Department of Physiology, American University of Beirut.
Nov 1990-Apr 1991	Visiting scholar. Loeb Medical Research Institute. Ottawa Civic Hospital
May 1991- present	Ph.D.graduate research, Department of physiology, University of Ottawa.

Publications:

1. Nassar CF, **Karakji EG**, Habbal ZM, Nasser MG (1986) Dopamine receptors in normal and diabetic liver plasma membrane. Gen Pharmacology 17:367

2. Nassar CF, Abdallah LE, **Karakji EG** (1991) Effect of chlorpromazine on proline absorption across rat jejunum. Pharmacol Res 24:53.

3. Nassar CF, Abdallah LE, Nuwayri-Salti N, **Karakji EG** (1991) Colchicine inhibition of duodenal absorption of calcium. Gen Pharmacol 22:755.

4. **Karakji EG**, Tsang BK (1995) Follicular stage-dependent regulation of rat granulosa cell plasminogen activator system by transforming growth factor-alpha *in vitro*. Biol Reprod. 52:411.

5. **Karakji EG**, Tsang BK (1995) Growth hormone releasing factor and vasoactive intestinal peptide stimulate rat granulosa cell plasminogen activator activity in vitro during follicular development. Mol Cell Endocrinol 107:105

6. **Karakji EG**, Tsang BK (1995) Tumor necrosis factor-alpha inhibits rat granulosa cell plasminogen activator activity in vitro during follicular development. Biol Reprod 52:745

7. **Karakji EG**, Tsang BK (1995) Regulation of rat granulosa cell plasminogen activator system: influence of interleukin-1β and follicular development. Biol Reprod 53:1302.

Presentations at National and International Conferences:

1. **Karakji EG**, Croze F, Tsang BK (1992) Effect of growth hormone releasing factor (GRF) and vasoactive intestinal peptide (VIP) *in vitro* on basal and FSH-stimulated rat granulosa cell plasminogen activator (PA) activity during follicular development. Annual Meeting of the Canadian Fertility and Andrology Society, Kananaski, Alberta. Canada. Nov 25-28

2. **Karakji EG**, Tsang BK (1993) Transforming growth factor (TGF) alpha stimulates rat granulosa cell plasminogen (PA) activity during follicular development. 26th Annual Meeting of the Society for the Study of Reproduction. Colorado State University, Fort Collins, Colorado, USA. Aug 1-4

3. **Karakji EG**, Tsang BK (1993) Tumor necrosis factor-alpha (TNFα) inhibits rat granulosa cell plasminogen activator (PA) activity during follicular development and ovulation. 49th Annual Meeting of the American Fertility Society/39th Annual

Meeting of the Canadian Fertility and Andrology Society, Montreal, Quebec, Canada. Oct 9-14

4. **Karakji EG**, Tsang BK (1994) Interleukin-1β modulates the FSH regulation of rat granulosa cell proliferative and differentiative activity *in vitro* during follicular development. Xth Ovarian Workshop, Ann Arbor, Michigan, USA. Jul 21-23

5. Li M, **Karakji EG**, Xing R, Rabbani SA, Carnegie JA, Wade M, Tsang BK (1996) Urokinase Receptor (uPAR) expression in the ovary during follicular development. 29th Annual Meeting of the Society for the Study of Reproduction, London, Ontario, Canada. July 27-30