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Serine protease and ovarian paracrine factors in regulation of ovulation

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1. ABSTRACT

The controlled target extracellular matrix (ECM) degradation generated by serine protease and regulated by serine protease inhibitor and ovarian paracrine/autocrine factors is an event that affects a wide variety of physiological and pathological processes in the ovary. Evidence cumulated in the past decade clearly showed that the hormone-induced coordinated expression of the tissue-type PA (tPA) produced mainly by granulosa cells and oocyte, and its inhibitor PAI-1 secreted by theca cells in the preovulatory follicles may be responsible for a controlled and directed proteolysis leading to the rupture of selected follicles in the rat, monkey and other mammals. In recent years increasing evidence further demonstrated that oocyte maturation and ovulation may also be modulated by other serine protease and inhibitor, as well as endogenously- produced ovarian paracrine/autocrine factors. Thus, it is important to identify the interrelationship between the serine protease system and the multiple factors, and to know how they regulate the ovarian physiological and pathological processes during oocyte maturation and ovulation.

2. INTRODUCTION

Ovary produces and releases mature oocytes with subsequent corpus luteum (CL) formation. These processes are repeated during each reproductive cycle and involve a series of sequential steps including cell proliferation, differentiation, oocyte maturation, ovulation, CL formation and its regression. In each of these diverse physiological processes, extracellular proteolysis must be conducted with a great specificity in order to maintain the integrity of the ovary. Accommodation of such structural changes in the ovary demands flexibility of surrounding ovarian matrix and stroma tissues. This flexibility mainly depends on degradation of matrix substances by the extracellular proteolytic system. Such specificity is achieved by a strict regulation of the biosynthesis of the molecules and complex interactions involving the catalytic enzymes, zymogens, specific surrounding ovarian matrix and stroma tissues (1). Evidence has shown that the precise multi-level regulation seems to fine-tune expression of the serine protease system and provides a controlled proteolytic activity (2-5). The serine protease, mainly the plasminogen activator (PA) system, a multi-component, contains not

only the proteolytic enzymes, but also the regulatory components including inhibitors, cofactors, cell surface receptors and binding proteins, where plasminogen is activated to the proteolytic enzyme plasmin by either of the two physiological PAs, tissue type PA (tPA) and urokinase PA (uPA), that are regulated by hormones, growth factors, and cytokines at various levels of its synthesis and secretion in ovarian cells (1,6), and modulated by specific PA inhibitors (PAI), which are released in cells in response to stimulatory signals (6-8).

During periovulatory period, LH triggers major changes in the ovarian somatic and germ cells. The oocyte completes meiotic cell cycle to become a fertilizable egg, whereas a various dramatic physiological changes in gene expression and secretion take place in the somatic cells for preparation of follicular rupture and the cumulus-oocyte complex release. It is known that the concerted changes are mainly regulated by activation of serine protease and other related intracellular signaling molecules, as well as by endogenously produced ovarian paracrine and autocrine factors (9). LH causes oocyte meiotic resumption, cumulus expansion, and follicle rupture through direct activation of granulosa cells, but also indirectly by acting on cumulus cells and oocyte. LH is a component critical for such events and the regulatory mechanisms are conserved in rodents and primates. A better understanding of these signaling networks and the related molecules activated during oocyte maturation and ovulation will provide new pharmacological opportunities for the manipulation of fertility (10).

3. REGULATION OF OOCYTE MATURATIOIN

3.1. Tissue plasmonogen activator in oocyte and its regulation

Rat or mouse oocytes express measurable amount of tPA activity (11, 12). When they underwent spontaneous meiotic maturation in culture, the oocyte tPA activity increased progressively and reached a plateau level at the time of germinal vesicle break down (GVBD). Addition of GVBD inhibitor to the culture prevented the increase in oocyte tPA activity, indicating that GVBD is required for tPA accumulation (11). Further evidence in a well-controlled *in vivo* study indicated that only tPA mRNA, but not its protein activity was detected in the rat primary oocytes of developing follicles. However, oocyte tPA activity was dramatically expressed in the follicles undergoing atresia (13). Production of inhibin subunits in the granulosa cells (GCs) of developing follicles was demonstrated, and negatively correlated with the expression of the oocyte tPA protein activity. Oocyte in a follicle does not express tPA protein activity if its surrounding GCs express normal level of inhibin subunits. Therefore, we suggest that the inhibin originated from ovarian somatic cells might control the oocyte tPA mRNA translation, and responsible for controlling oocyte GVBD. When the follicle approaches to the ovulatory stage under the action of LH pick, GC inhibin subunit expression sharply decreases, and the oocyte tPA mRNA starts to translate into its protein activity leading to oocyte GVBD and cumulus cell expansion/dispersion. On the other hand, in the developing/pathological follicles the decrease in GC inhibin subunit might induce certain morphological changes in the oocyte similar to GVBD, leading to the follicle atresia (Figure 1). Rat oocytes in the atretic follicles do contain high level of tPA activity (13).

Plasminogen activator activity was analyzed in rat and mouse cumulus-oocyte complexes. Only tPA activity was detected in the freshly prepared cumulus-oocyte complexes (14, 15). FSH, GnRH and vasoactive intestinal peptide (VIP) are capable of stimulating the complex tPA activity, but not the denuded oocyte tPA activity (11, 16, 17), suggesting that the oocyte tPA activity was regulated by the hormones via cumulus cells. *In vivo* experiments indicated that tPA activity in the oocytes was dramatically elevated immediately before ovulation, and well correlated with the morphological changes in cumulus expansion and oocyte maturation (14). Morphological analysis indicated that increase in oocyte tPA activity was correlated with the extent of cumulus cell expansion and dispersion (Figure 2). PAI-1 activity was detected only in the cumulus cells, but not in the denuted oocyte (14). To examine hormonal responsiveness, the cumulus-oocyte complexes obtained from the follicles of PMSG-treated rats were incubated with FSH and hCG for 24 h, the total tPA activity (medium plus cell lysate) in the complexes was determined. The tPA activity was markedly increased by the addition of both FSH and hCG. *In vivo* experiments with the PMSG-primed immature rats showed that after injection of hCG at various time points the cumulated cumulus-oocyte complexes were further cultured *in vitro* for 24hs with the FSH and hCG, the elevated tPA activity in the complexes was not further affected by the addition of the gonadotropins. Thus, the gonadotropin stimulation of cumulus expansion and oocyte maturation may be associated with increase in a limited amount of the cellular content and secretion of tPA activity by the cumulus-oocyte complexes. The oocyte tPA activity may be also involved in the process of detachment of cumulus oophorus from stratum granulosum prior to ovulation (11, 14). A similar temporal pattern of PA expression and matrix degradation was demonstrated in the mouse cumulus-oocyte complexes treated with the gonadotropins (15). Plasminogen activators in the bovine cumulus- oocyte complexes during *in vitro* maturation were also examined by Park *et al.* who demonstrated that tPA-mediated degradation of the matrix and the cumulus-oocyte complex dissociation is required in the complexes before ovulation (18). Szymanski *et al* analyzed the follicular fluid tPA and its inhibitor PAI-1 levels in patients undergoing COH compared to the healthy fertile women, the tPA activity in the patients with COH was lower, whereas PAI-1 was higher significantly compared to the health women. It is therefore they suggested that follicular fluid tPA and PAI-1 concentrations may be a crucial factors reflecting oocyte maturity (19).

In addition to tPA and PAI-1, Hägglund *et al* observed another serine protease inhibitor, protease nexin-1 (PN-1) was present in the granulosa and cumulus cells, and high in periovulatory follicle, thereby PN-1 may provide an inhibitory activity to protect the mucified matrix of the cumulus oocyte complex from proteolytic degradation (20).

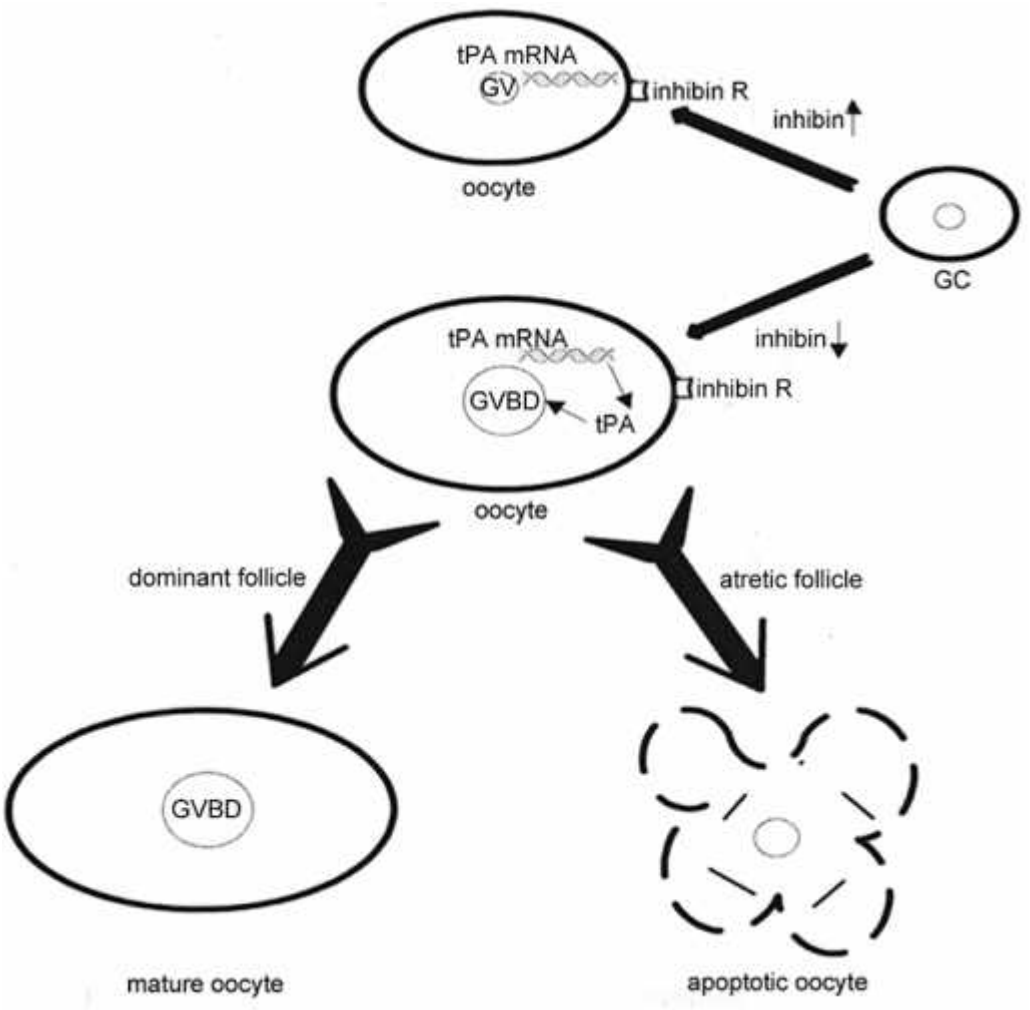


Figure 1. Effect of inhibin emanated from GC on oocyte tPA mRNA translation (Reproduced from Ref. 84). Inhibin originating from the GCs inhibits the oocyte maturation by controlling the tPA mRNA translation. Once inhibin subunit expression in GCs is decreased, the oocyte tPA mRNA starts to translate into its active protein which subsequently induces the oocyte GVBD in the dominant follicle leading to the oocyte maturation and ovulation; On the other hand, the decreases in GC inhibin subunit expression in the developing follicle, the oocyte tPA mRNA is triggered to translate tPA protein which is capable of inducing its certain morphological changes similar to GVBD in the developing follicle, subsequently leading to the oocyte and/or the follicle atresia.

Evidence also suggested the granulosa PN-1 may be involved in the process of atresia of non-ovulatory dominant follicles (21).

3.2. Role of epidermal growth factor-like growth factors in regulation of oocyte maturation

Mammalian reproduction depends on release of a mature oocyte from ovarian follicle. Maturation of the oocyte and rupture of the follicle wall constitute part of responses to the preovulatory LH surge, which is also important for the cumulus expansion and granulosa cell luteinization. The LH surge promotes ovulation via activation of multiple signaling networks in the ovarian follicle. Studies have shown the importance of LH-induced activation of the epidermal growth factor (EGF) signaling network. Hsieh *et al* found that LH-induced expression of EGF-like growth factors and EGF receptor (EGFR) transactivation are essential for regulation of a critical physiological process of ovulation (22). It was previously shown that EGFR mediates ovulatory response to LH in ovarian follicles; the LH-induced responses were only generated upon a prolonged activity of the EGFR. In addition, the continuous activity of the EGFR is essential for the chronic phosphorylation of the ERK1/2 downstream signaling molecules, which were shown to be essential for oocyte maturation and cumulus expansion. Interestingly, EGFR-sustained activity was also necessary to maintain the upregulation of Ptgs2, a gene essential for cumulus expansion. The unusual prolonged duration of ERK1/2 activity may be possibly attributed to the late induction of the ERK-specific phosphatase. It is suggested that the ovulatory process involves a nonclassical activation of this

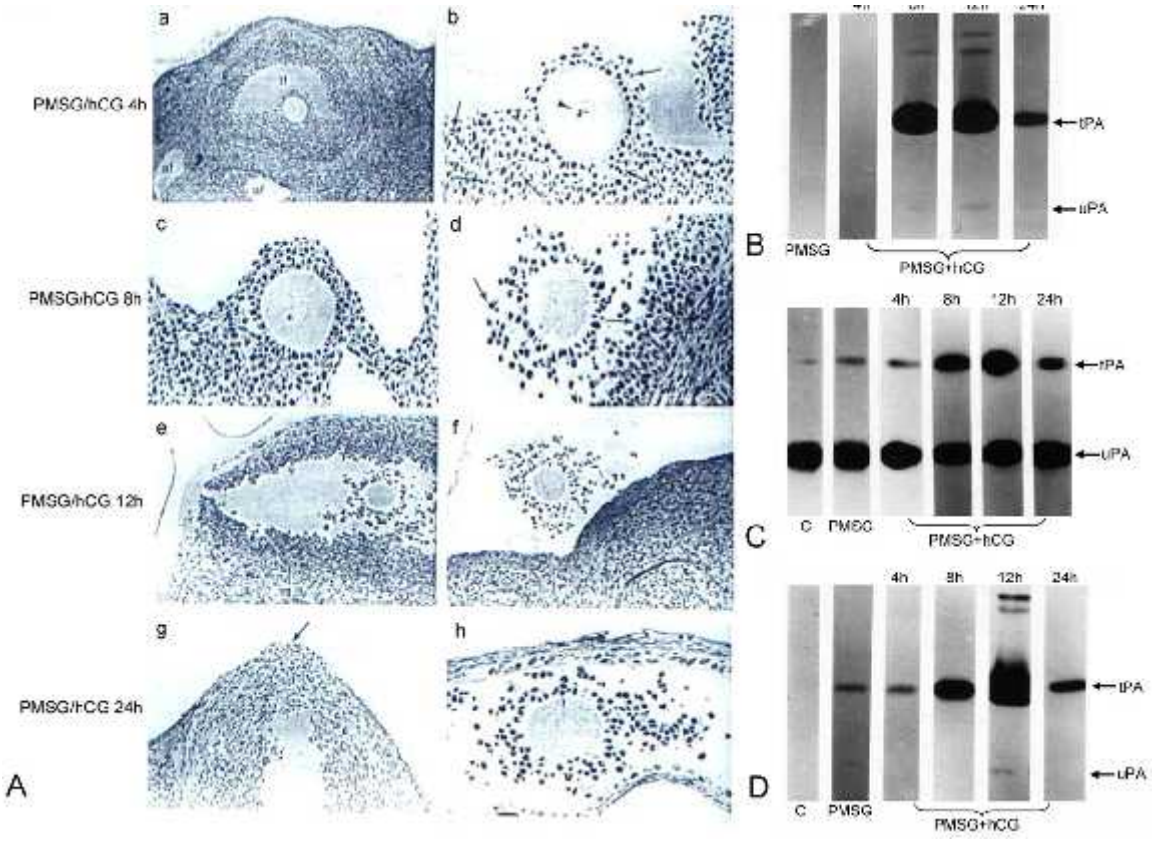


Figure 2. Injection of PMSG/hCG stimulates rat ovarian tissue plasminogen activator expression and induces ovulation (Reproduced with permission from Ref. 11, 14 and 45). A Time-dependent morphological changes of cumulus-oocyte complexes after injection of PMSG and hCG; B. Secretion of tPA activity in the cumulus-oocyte complexes in 24 hours culture after treatment with PMSG/hCG; C. tPA and uPA activities in the PMSG/hCG treated rat ovarian homogenates; D. Secretion of tPA and uPA in the granulosa cells in 24 hours culture after treatment with PMSG/hCG.

pathway (23). Su *et al* further demonstrated that oocytes promote the EGFR expression by cumulus cells. Egfr mRNA and its protein were dramatically reduced in the cumulus cells of mutant mice deficient in production of growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15). Moreover, microsurgical removal of oocytes from wild-type cumulus oocyte complexes (COCs) dramatically reduced expression of Egfr mRNA and protein, and these levels were restored by either coculture with oocytes or treatment with recombinant GDF9 or GDF9 plus recombinant BMP15(24).

Recent evidence also demonstrated that the nuclear receptor cofactor receptor-interacting protein 140 (RIP140) was essential for COC expansion and ovulation (25). Ben-Ami *et al* provided further evidence to show that the EGF family members, amphiregulin (Areg) and epiregulin (Ereg) may serve as paracrine mediators for oocyte maturation in pre-ovulatory follicles, their *in vitro* experiments showed that human GV oocytes cultured in a complex defined medium, significant more oocytes reached metaphase II stage in the media supplemented with Areg and Ereg (26). Hiradate *et al* observed that adrenomedullin (ADM) receptor and the receptor activity-modifying proteins expressed in mouse cumulus cells, but not in the oocytes. ADM in the presence of the nitric oxide donor sodium nitroprusside (SNP) significantly inhibited GVBD. Furthermore, the ADM- and SNP-dependent inhibition of GVBD was abrogated by Akt blockade, implying the possibility that ADM acting as a GVBD regulator *in vivo* (27). Zamah *et al* investigated the EGF-like growth factor-mediated mechanisms during LH stimulation in human. The amphiregulin (AREG) levels were measured in 119 follicular fluid (FF) samples from IVF/ICSI patients. FF from follicles yielding an immature germinal vesicle oocyte or from an oocyte that develops into an aberrant embryo contains lower AREG levels than that from follicles yielding a healthy oocyte, therefore they suggested that EGF-like growth factors play a role in critical peri-ovulatory events in human, and the AREG accumulation may be used as an useful marker of gonadotropin stimulation and oocyte competence (28).

3.3 Progesterone and its receptors in regulation of oocyte maturation

Progesterone (P4) plays a critical role in regulation of oocyte maturation and ovulation, which function is mediated by the two nuclear receptor transcription factors PR-A and PR-B produced from a

single gene and regulate expression of specific gene networks in the ovary. Evidence has shown that presence of both genomic and non-genomic PRs in bovine cumulus oocyte complexes (COCs), and suggested P4 with its receptors may play an important role during bovine oocyte maturation (29). Inhibition of P4 synthesis by cumulus cells or blocking of nPR and mPR alpha activity induced a decrease in bovine embryo development, indicating that P4 intracellular signaling may be mediated by its interaction with nuclear and membrane PRs, and important for oocyte developmental competence. Selective disruption of PR-A isoforms could lead to a failure of follicular rupture in response to LH stimulation, suggesting that LH triggering ovulation may be transduced by PR (30).

Yamashita *et al* further observed that progesterone was secreted from the cumulus cells and acted on the cumulus cells themselves, which required for cumulus expansion and oocyte maturation. EGF-like factors (amphiregulin, AREG; epiregulin, EREG) and TACE/ADAM17, are also expressed in cumulus cells and regulated by progesterone, they may enhance functional changes of cumulus cells and progresses meiotic maturation of oocytes during *in vitro* maturation of porcine COCs (31). Porcine COCs form an expanded cumulus ECM in response to gonadotropins during meiotic maturation. The cumulus expansion-related components TNFAIP6 and HAS2 transcripts increased significantly after the stimulation of COCs and GCs with FSH/LH. In contrast, treatment with MG132 reduced their expression, suggesting the requirement of ubiquitin- proteasome pathway-regulated protein turnover for formation of ECM during cumulus expansion and oocyte maturation (32).

3.4. Local autocrine/paracrine regulation of oocyte maturation

Recent findings indicate that mouse mural granulosa cells express natriuretic peptide precursor type C (Nppc) mRNA, whereas cumulus cells express mRNA of the NPPC receptor NPR2, oocyte-derived paracrine factors promoted cumulus cell expression of Npr2 mRNA. Therefore, the granulosa cell ligand NPPC and its receptor NPR2 in cumulus cells prevent precocious meiotic maturation, which is critical for maturation and ovulation synchrony and for normal female fertility (33).

Components of the PI3 kinase pathway, the serine/threonine kinase Akt (PKB) and an Akt substrate FKHRL1 are expressed in mammalian oocytes which are regulated by stem cell factor (SCF). Actions of granulosa cell derived SCF on primordial to primary follicle transition and subsequent follicle development may involve activation of Akt and inhibition of FKHRL1 activities in oocytes (34). The oocyte Akt might enhance follicle development, while the oocyte FKHRL1 could inhibit follicle development. The cascade from granulosa cell SCF to oocyte Kit-PI3 kinase-Akt-FKHRL1 may be possible to play an important role to regulate growth rate of mammalian oocytes, whereas the oocyte factors could regulate activation and early follicular development.

It is known that LH surge activates the signaling molecules RAS and ERK1/2 and triggers oocyte maturation and ovulation in mammals. Fan *et al* disrupted Erk1/2 in mouse granulosa cells *in vivo* and showed that these kinase are necessary for LH-induced oocyte resumption of meiosis, oocyte maturation and ovulation. Selected disruption of the Cebpb gene in granulosa cells demonstrates that C/EBPbeta (CCAAT/Enhancer-binding protein-beta) is a critical downstream mediator of ERK1/2 activation. Thus, ERK1/2 and C/EBPbeta constitute an *in vivo* LH-regulated signaling pathway that controls oocyte resumption of meiosis, maturation and ovulation-related events (35-37)

A well-known ovarian pathological disorder, polycystic ovary syndrome (PCOS) is a common metabolic dysfunction and heterogeneous endocrine disorder in women of reproductive age. Although patients with PCOS are typically characterized by increased numbers of oocytes retrieved during IVF, they are often of poor quality, leading to lower fertilization, cleavage and implantation rates, and a higher miscarriage rate. Qiao *et al* reviewed progress for searching the database MEDLINE (1950 to 2010) related to oocyte maturation and embryo developmental competence. The search results showed that alteration of many factors may directly or indirectly impair competence of maturating oocytes through endocrine and local paracrine/autocrine actions, resulting in a lower pregnancy rate in patients with PCOS. The extra- and/or intra-ovarian factors identified included gonadotrophins, hyperandrogenemia and hyperinsulinemia, members of epidermal, fibroblast, insulin-like and neurotrophin families of growth factors, as well as cytokines, any abnormality of these factors may negatively affect granulosa cell-oocyte interaction, oocyte maturation (Figure 3), contributing to unsuccessful outcomes for patients with PCOS (38). Evidence has shown that increased PAI-1 activity is an early marker of PCOS with cardiovascular risk. PCOS essentially is a disorder of hyperinsulinemic insulin resistance, and may be heralded by precocious pubarche (PP). Increased plasma PAI-1 levels in early pubertal PP girls may indicate those girls with greater risk of developing hyperinsulinemic-hyperandrogenism features of PCOS (39). Hypofibrinolytic PAI-1 activity showed an independent association with first-trimester miscarriage in the observed 430 women with PCOS (40).

4. PLASMINOGEN ACTIVATOR SYSTEM IN REGULATION OF FOLLICLE RUPTURE

4.1. Ovulation requires ovarian matrix degradation

Follicle rupture involves a series of tissue-specific and time-coordinated physiological, biochemical and morphological changes in ovary. In order for egg to escape from follicle at time of ovulation, an opening of follicle wall and detachment of cumulus oophorus from stratum granulosum are required. Factors leading to occurrence of this very complex process have fascinated biologists for many years. Nearly a century ago, Schochet suggested that proteolysis might be responsible for degradation of follicle wall during ovulation (41). Supportive evidence for this concept was not obtained until 1975, when a hypothesis for rat ovulation was proposed. Beer *et al* demonstrated that PA-generated plasmin is responsible for disruption of follicle wall (42-44). Further

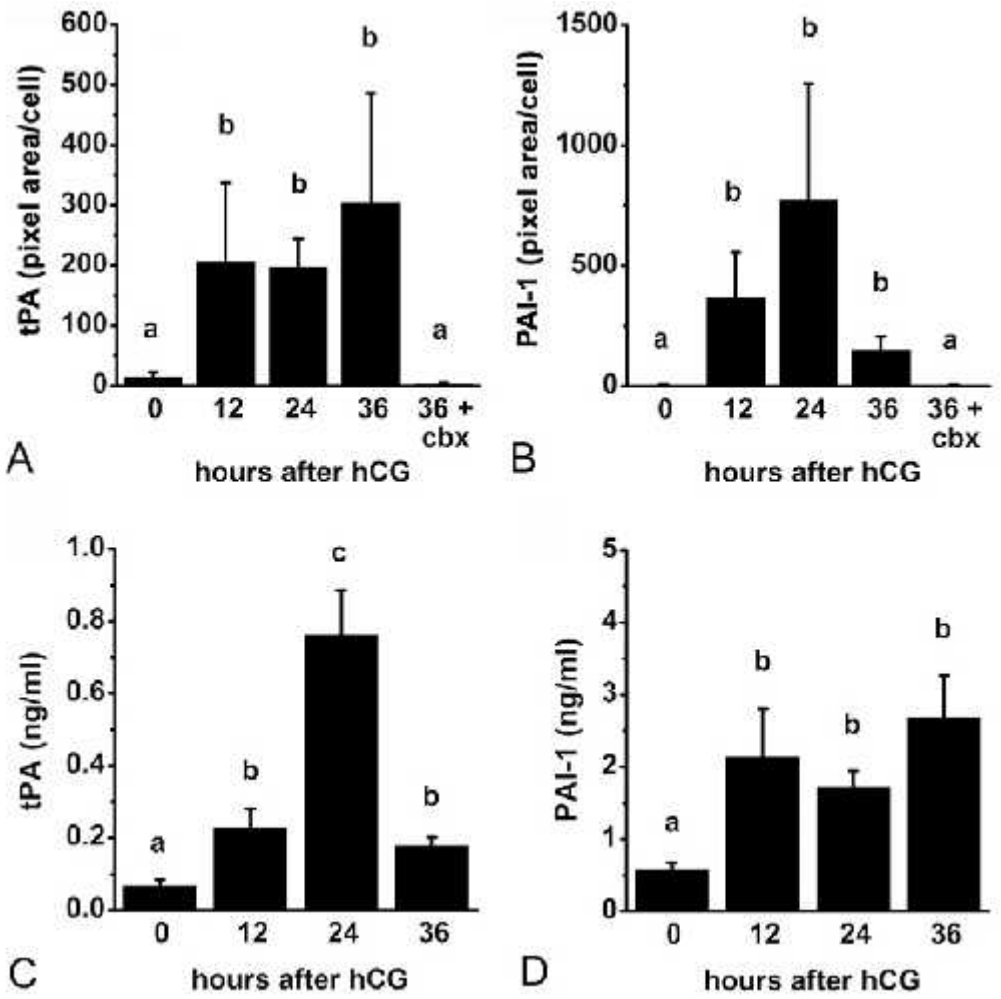


Figure 3. tPA and PAI-1 protein levels in monkey granulosa cells treated with hCG or hCG plus prostaglandin E2 inhibitor (Reproduced with permission from Ref. 38). Granulosa cells were obtained from the monkeys treated with hCG for 0, 12, 24, and 36 hours or with hCG plus prostaglandin E2 inhibitor, celecoxib(cbx) for 36 h. Experiments were assessed for tPA (A) and PAI-1 (B) proteins using MetaMorph analysis of *green* fluorescence (*pixel area*) and normalized to cell number (n = 3 animals per time point). C and D, tPA and PAI-1 proteins were measured by ELISA.

studies showed that rat granulosa cells produce two types of PA, tPA and uPA (11,14), only tPA but not uPA is a secretary protein in the ovary (45, 46), while theca-interstitial cells (TIs) secrete tPA inhibitor PAI-1(3,4). Coordinated expression of tPA, (but not uPA) and PAI-1 in rat and monkey ovaries was stimulated by gonadotropins, and responsible for follicle rupture (4, 45, 47).

4.2 Plasminogen activator inhibitor type 1 in ovary

Rat oocyte does not secrete PAI-1, GCs synthesize negligible amount of PAI-1 activity, the majority of PAI-1 activity in ovary is produced by TCs (3, 4, 47). PAI-1 activity is stimulated by gonadotropins in a time-dependent manner. Incubation of isolated follicles obtained from PMSG/hCG treated immature rats indicated that no measurable tPA, but uPA activity, and huge amount of PAI-1 activity were detected in the conditioned media at the all time points after the gonadotropin treatment (3), indicating that GC-secreted tPA activity may be completely neutralized by the presence of PAI-1 secreted by the TCs (follicular wall). The occurrence of a high molecular weight lysis zones on the indicator gel suggests the formation of complexes between the plasminogen activator and the inhibitor (Figure 4A&B).

To analyze the interactions between tPA produced by GCs and PAI-1 secreted by TCs in rat, two types of the purified cells obtained from the ovaries at various time points after the PMSG/hCG treatment were incubated alone or in combination. The interactions between the plasminogen activator and the inhibitor in the conditioned media of the two cell combined cultures completely inhibited the tPA activity produced mainly by GCs before 8 h after hCG injection. However, after 12 h of hCG injection, despite the presence of high level of PAI-1 activity in the conditioned media produced by both TC and

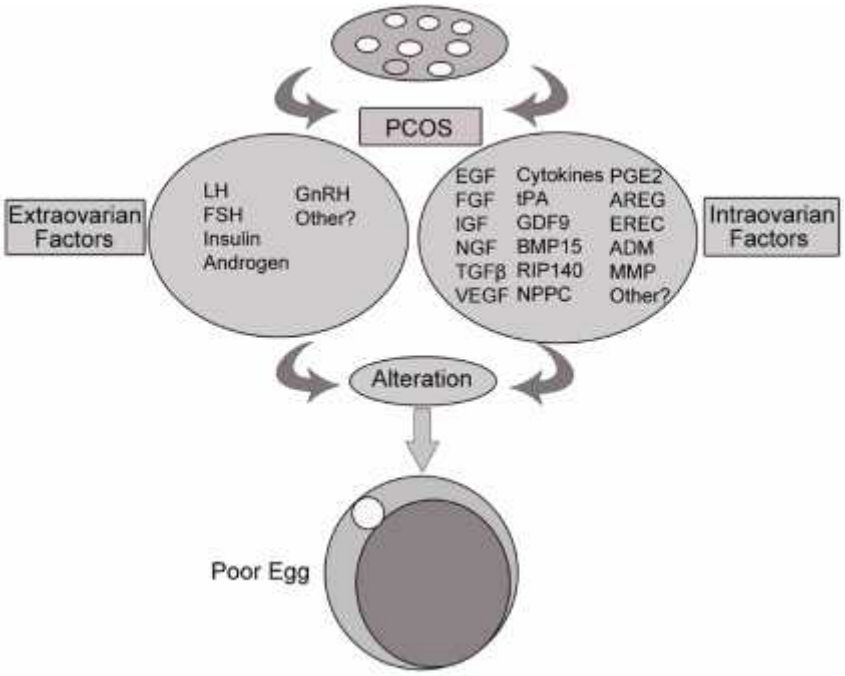


Figure 4. Interaction and regulation of tPA and PAI-1 in rat follicles (Reproduced with permission from Ref. 3, 4 and 45). Immature rats were treated with PMSG/hCG at the indicated time points, the ovaries were removed, individual follicles, granulosa (GCs) and theca-interstitial cells (TIs) were prepared, and cultured in the medium for 24 hours as shown in the figures. A. tPA activity in the conditioned media of the cultured follicles; B. PAI-1 activity in the conditioned media of the cultured follicles; C. tPA and PAI-1 protein activities in the GCs-TCs or in their combination 24 hours culture at various time points after injection of PMSG/hCG; D A diagram showing the coordinated expression of tPA and PAI-1 in the follicle induces a directed and localized window of proteolytic flow which is responsible for the follicle rupture.

GC, the total tPA activity in the culture reached a maximum level, that may be important leading to the follicle rupture (Figure 4C). Evidence also showed that the changes and regulation of PAI-1 expression in TCs of the PMSG/hCG treated monkeys (47) exactly follow the same profile as in the rat (4). Taken together, it is suggested that the interaction and regulation of the PA activator and inhibitor in mammal follicles may play an important role in maintaining normal ovarian function and mechanism of ovulation (Figure 4C).

4.3. Coordinated expression of tPA and PAI-1 in ovary induces ovulation in rat and rhesus monkey

To study whether coordinated regulation of tPA and PAI-1 takes place *in vivo* in response to physiological signals, the changes of the mRNA levels and the protein activities of both tPA and PAI-1 in the ovarian cells were examined in rat during PMSG/hCG-induced ovulation (4, 47). The mRNA levels and protein activities of both tPA and PAI-1 in the ovary were coordinately regulated by the gonadotropins in a time-dependent and cell-specific manner, such that the maximum level of tPA mRNA and activity in GCs was obtained just prior to ovulation. Both before and after ovulation, PAI-1 mRNA and activity synthesized predominantly by T1s reached the maximum levels ensuring the inhibition of proteolytic activity in the extracellular ovarian compartment. The tissue-specific and time-coordinated expression of tPA and PA-1 genes in the ovary allows a narrow window of periovulatory increase in tPA activity, which may be important for the regulation of the ovulatory process (Figure 4D). In the rat, ovulation is preceded by a transient and cell-specific expression of tPA and PAI-1, which causes a proteolytic activity localized to the surface of the ovary just prior to ovulation. Intrabursal injection of α_2-antiplasmin or antibodies against tPA to neutralize the follicular plasmin or tPA activity significantly blocked the gonadotropin-induced ovulation in rat (48), indicating a pivotal role of tPA in rodent ovulation. Using a PMSG/hCG-induced, synchronized rhesus monkey ovulation model we have demonstrated that the GC-derived tPA was substantially elevated in the preovulatory follicles, and reached its maximum level just prior to ovulation. The TC-derived PAI-1 was also stimulated by the PMSG and hCG treatments. However, the maximum level of PAI-1 appeared 12 h earlier than that of the tPA. When ovulation approached, accompanying the highest tPA level in the preovulatory follicles, the follicular PAI-1 level declined dramatically to its minimum value (47). GCs contained considerable level of uPA activity, which was not regulated by the gonadotropins before ovulation. These data suggest that in the monkey ovary as the same as in the rat, the coordinated expression tPA and PAI-1 regulated by gonadotropins is also responsible for the follicle rupture.

In recent years Markosyan and Harris *et al* have demonstrated that prostaglandin E2 (PGE2) mediates many effects of LH surge within the periovulatory follicle, and

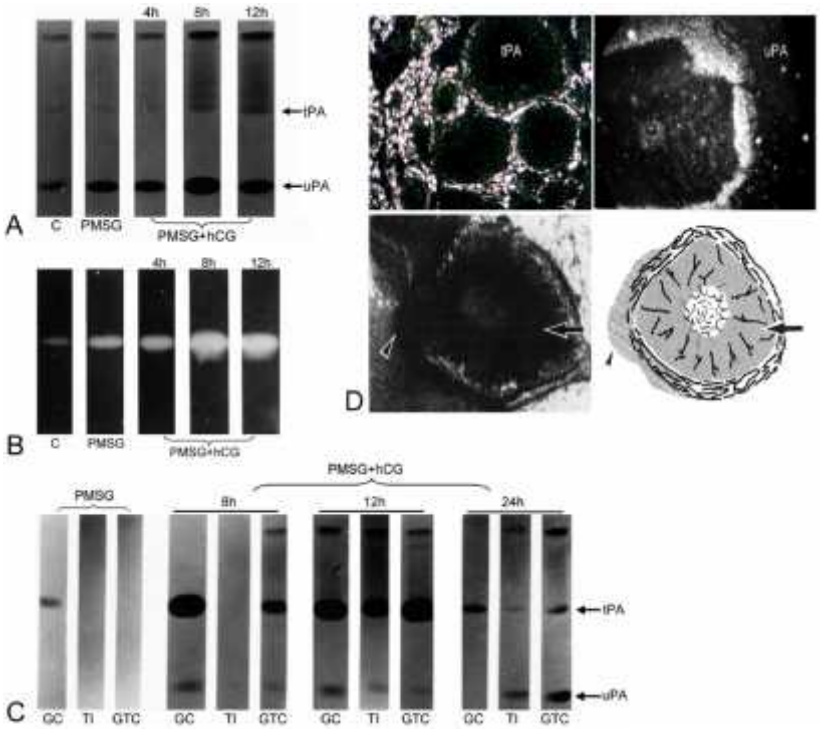


Figure 5. Intra-and extra-ovarian factors associated with the PCOS pathology that negatively affects oocyte maturation (Reproduced with permission from Ref. 50). The listed intra- and extra-ovarian factors confirmed in recent years refer to abbreviations in the text.

observed PGE2 also increased tPA and PAI-1 protein levels in GCs in rat and monkey. Treatment of hCG with the PG synthesis inhibitor celecoxib, tPA and PAI-1 protein activity were considerably decreased, as shown in Figure 5, suggesting that the elevated PGE2 late in the periovulatory interval acts to stimulate proteolysis and follicle rupture. (50). Differential expression of 4 PGE2 (EP) receptors in monkey ovarian GCs have been observed, they may contribute to a specialized function of each of the cell subpopulation. The cumulus cells likely respond to PGE2 via EP2 and EP3, whereas PGE2 controls the rupture of a specific region of the follicle via EP1. Differential EP receptor expression may permit each GC subpopulation to generate a unique response to PGE2 during process of ovulation (51).

4.4. GnRH and FSH stimulate oocyte and GC tPA activity and induce ovulation

To further examine whether hormones or compounds which stimulate tPA expression in oocyte and GCs could induce ovulation, GnRH, FSH and vasoactive intestinal peptide (VIP) were tested. GnRH and its agonist (GnRHa) are known to stimulate tPA expression in cultured rat GCs and cumulus oocyte complexes (16) and to induce ovulation in the hypophysectomized rats by acting directly on the ovary (52). To clarify the specific role of tPA in ovulation induced by GnRHa, we have examined the effect of an ovulatory dose of GnRHa on the ovarian tPA and PAI-1 expression in hypophysectomized rats (53, 54). GnRHa stimulated the induction of tPA (but not uPA) activity in GCs in a time-dependent manner as hCG does (55). GnRH and hCG seem to elicit the similar responses in the ovaries.

Both *in vitro* and *in vivo* studies have indicated that FSH is capable of inducing rat ovarian tPA expression (14). A LH-free recombinant FSH (rcFSH) has been tested for the induction of ovulation in the hypophysectomized immature and adult rats (56). The data showed that rcFSH induced ovulation with associated increases in ovarian tPA, but not uPA expression.

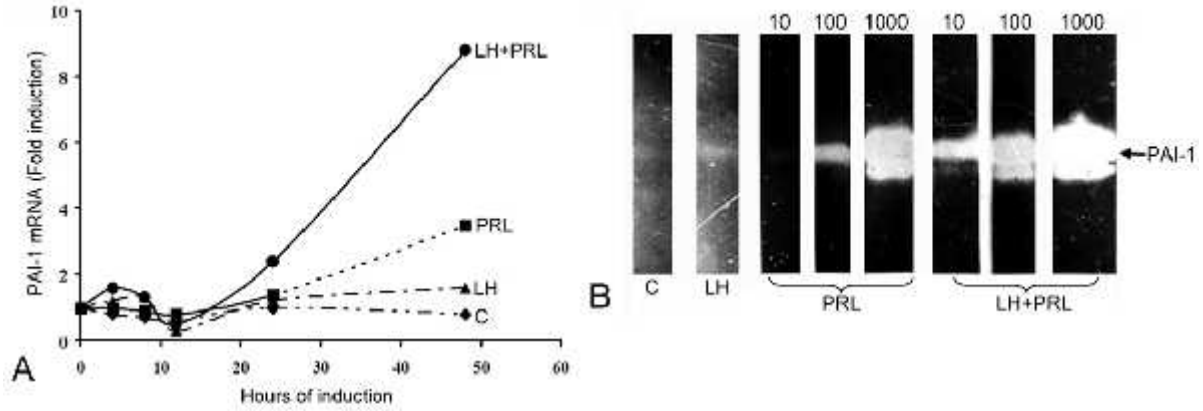
4.5. Vasoactive interstitial peptide, forskolin, cAMP, PMA stimulate GC tPA activity and induce ovulation

Vasoactive interstitial peptide (VIP), originally considered to be a gut hormone, has recently been found to increase tPA activity in cultured rat GCs and cumulus-oocyte complexes (17, 57). The ovulatory effect of VIP was also studied using *in vitro* perfused ovaries from immature rats primed with PMSG. VIP-induced ovulation could be observed in all perfused ovaries (58). Furthermore, forskolin and cAMP (an activator of protein kinase A), and PMA (an activator of protein kinase C) that are all strong inducers of ovarian tPA activity *in vitro* (14), have been demonstrated to be able to induce ovulation in perfused rabbit and rat ovaries (59,60).

4.6. Compounds which decrease tPA and/or increase PAI-1 expression inhibit ovulation

To examine whether the inhibitory effect of

indomethacin on ovulation is associated with a decrease in



C

Dose-dependent inhibition of gonadotropin-induced ovulation by prolactin in PMSG-primed immature rats

µg PRL	# animals	ovarian wt mean±SE	# ovulating rats	# ova/ovary	% inhibition
0	13	39.2±1.7	13	13.0±1.3	0
50	8	39.2±2.6	6	6.3±1.0	47.4
100	10	40.6±2.9	7	4.4±0.8	66.9
200	17	34.5±2.3	8	3.2±0.8	76.0

Figure 6. Prolactin stimulates LH-induced PAI-1mRNA (A) and protein (B) expression and inhibits hCG-induced ovulation (C) in rats (Reproduced with permission from Ref. 64&65).

ovarian tPA expression in ovary, the action of indomethacin on secretion of both tPA and uPA in GCs has been carefully investigated following hCG or GnRH administration in the PMSG-primed hypophysectomized immature rats (61). The results showed that (1) ovulation induced by both hormones was effectively blocked by the concomitant administration of indomethacin (1 mg/rat); (2) indomethacin remarkably suppressed tPA (but not uPA) secretion in GCs obtained at 12 h after hCG or GnRHa injection; (3) this compound also dose-dependently inhibited the GnRHa- and FSH-stimulated tPA secretion in GCs *in vitro.* Indomethacin inhibition of ovulation and tPA secretion was further confirmed by Tanaka *et al.* (62).

Prolactin (PRL) is a pituitary hormone, mainly involved in stimulating milk production. *In vitro* studies have demonstrated that PRL was capable of inhibiting LH- and FSH-induced tPA mRNA and protein activity in rat GCs and of stimulating ovarian PAI-1 expression (63, 64). Further experiments showed that injection of PRL inhibited hCG-induced rat ovulation in a dose-dependent manner. PRL involvement of regulation of ovarian PAI-1 gene expression and inhibiting hCG-induced ovulation is outlined in Figure 6.

Above serous evidences clearly demonstrated that hormones or compounds which stimulate ovarian tPA activity could induce ovulation, while the hormones or compounds that enhance ovarian PAI-1 expression could inhibit ovulation. Because GCs and oocyte synthesize the most of follicular tPA activity, whereas the follicular walls (TCs) contribute the most of PAI-1 activity, PAI-1 may therefore serve as a specific barrier to localize the tPA activity within the follicles. As ovulation approaches, the level of PAI-1 in TCs dramatically decreases, while tPA activity in the follicle rises to its maximum level. The coordinated expression of tPA and PAI-1 in the follicle may therefore lead to a short pulse of proteolytic activity and induces the follicle rupture (65-67).

4.7. Serine proteases in regulation of ovulation in various other species

In addition to rodent and primate we have also examined ovarian serine protease activities in other species, such as rabbit, cat, giant panda and amphioxus (chordata). Ovaries of these species contained mainly tPA activities, which were regulated by gonadotropins (68). Cao *et al* measured expression/activity of the PA system in bovine follicles at different stages of development. Abundance of mRNA encoding PN-1, tPA, uPA and PAI-1 were initially up-regulated by hCG in the preovulatory follicular wall homogenates. PN-1, PAI-1 and tPA mRNA expression then decreased near the expected time of ovulation, whereas uPA mRNA levels remained high. PN-1 protein levels in FF were significantly higher in non-atretic than in atretic follicles, and plasmin activity was correspondingly higher in the atretic follicles. These results indicate that PN-1 may be involved in the process of atresia in non-ovulatory dominant follicles and prevention of precocious proteolysis in periovulatory

follicles (21). Using a chicken model, Tilly and Johnson (69) and Jackson *et al*. (70) have found that dramatic increase in PA activity, specifically in stigma region, is correlated with ovulatory process (71). Evidence has also suggested interaction of the ovarian PA and its inhibitor PAI-1 in pig is important in ovulatory process (72). Dow *at al* observed both PAI-1 and PAI-2 mRNAs were up-regulated in preovulatory bovine follicles induced by GnRH in a cell-specific manner and suggested that regulation of PAI-1 and PAI-2 may help to control plasminogen activator activity associated with ovulation (73). To examine whether proteases play a role in ovulation of non-mammalian vertebrate species, various serine protease inhibitors were given to the fish (teleost medaka), their ovulation rates were also dramatically reduced. This finding was an indication that a plasmin-like protease, similar if not identical to plasmin, plays a role in follicle rupture during ovulation in the fish. Data also indicate that this serine protease participates in the follicle rupture for only a few hours prior to the activation of MMP-mediated hydrolysis at ovulation, suggesting that two different proteolytic enzyme systems, the serine protease and MMP, may be involved in the fish follicle rupture (74). Crespo *et al* further demonstrated TNF alpha could have an important role in the follicle rupture and oocyte expulsion during ovulation in teleost fish (75).

Surprisingly, however, evidence showed that single-deficient mice lacking tPA, uPA, or PAI-1 gene function were found to have normal reproduction, although mice with a combined deficiency of tPA and uPA were significantly less fertile by reducing 26%. The loss of an individual PA seems to be functionally complemented by the remaining PA, but this compensation does not appear to involve any compensatory up-regulation, implying that a functionally redundant mechanism for plasmin formation operates during gonadotropin-induced ovulation, and the PAs together with other proteases may generate the proteolytic activity required for follicular wall degradation（76）. To further study the mechanism of the reduced ovulation efficiency in PA-deficient mice, Ny *et al.* comparatively examined plasmin activity and regulation in the ovaries of wild-type mice and mice with deficient PA gene function during PMSG- and hCG-induced ovulation. In mice lacking either tPA or PAI-1, the plasmin activity in the ovary prior to ovulation was similar to that of the wild-type mice, indicating the amount of plasmin generated by PAs prior to ovulation in wild-type mice greatly exceeds the amount required for efficient ovulation (20, 77).

5. MATRIX METALLOPROTEINASE IN REGULATION OF OVULATION

Many studies have suggested that the PA system and the MMP system, either separately or in combination, may provide the proteolytic activity required for rupture of follicular wall at the time of ovulation. Several studies have shown that plasmin in plasminogen (plg)-deficient mice is not required for normal ovulation. To investigate the role of MMPs and the possibility of a functional overlap or synergy between the MMP and the PA system during ovulation, ovulation efficiency in wild-type and plg-deficient mice treated with the broad-spectrum MMP inhibitor galardin were detected. Both wild-type mice and heterozygous plg-deficient (plg+/-) mice treated with galardin prior to ovulation; there was a mild (18-20%) reduction in ovulation efficiency. Surprisingly, galardin treatment of plg-deficient (plg-/-) mice only caused an additional 14% reduction in ovulation efficiency as compared to vehicle-treated plg-/- mice. The data therefore suggest that although MMPs may play a role in degradation of the follicular wall, they may not be obligatory for ovulation. In contrast to previous studies on tissue remodeling during wound healing and placental development, there is no obvious functional overlap or synergy between the PA and MMP systems (78). Peluffo *et al* further examined if metalloproteinase involvement in primate ovulation. Vehicle or matrix metalloproteinase inhibitor (GM6001) was injected into the preovulatory follicle at the time of hCG administration. Histological analysis revealed that vehicle- injected follicles ruptured, whereas GM6001-injected follicles did not. These findings demonstrate metalloproteinases are critical for follicle rupture in the primates (79). Furthermore, expression of the stromelysins (MMP3, MMP10, MMP11) was analyzed in the periovulatory human and rat ovaries, the data showed that there are divergent patterns of stromelysin expression associated with ovulation, and only MMP 10 induction may be associated with ovulation (80).

6. SIGNALING INVOLVED IN REGULATION OF OVULATION

The molecular and structural changes in the follicle triggered by LH at ovulation require complex interactions between somatic cells and oocyte. It is well established that the signals originating from oocyte play an essential role in orchestrating growth and development of follicle. Conversely, the exact contribution of local signals in the two compartments to ovulation is less clear. LH causes oocyte meiotic resumption, cumulus expansion, and follicle rupture through direct activation of granulosa cells, but also indirectly by acting on cumulus cells and oocyte. Recent studies have identified some of components of signaling network activated at this critical transition (81). LH initially activates cAMP signaling but then the stimulus branches into a number of contiguous and interacting pathways. Activation of the EGF network is one of these pathways, and accumulating evidence strongly indicates that this is a component critical for oocyte maturation, cumulus expansion, and follicle rupture. Progesterone plays a critical role in regulation of ovulation. Selective disruption of PR-A isoforms leads to a failure of ovulation. The mural granulosa cells where inducing expression of paracrine signals that interact with cumulus cells to control cumulus matrix function and expansion to facilitate follicular rupture. Harris *et al* demonstrated that prostaglandin E2 (PGE2) mediates many effects of LH surge within the periovulatory follicle. Differential expression of four PGE2 receptors may contribute to the specialized functions of each of the monkey granulosa cell subpopulation. Cumulus cell EP2 and EP3 protein levels increased between 0 and 36 h after hCG, mural granulosa cells expressed low levels of EP1 protein at 0 h and higher

levels 24-36 h after hCG. However, EP1 protein levels were higher in the granulosa cells away from the follicle

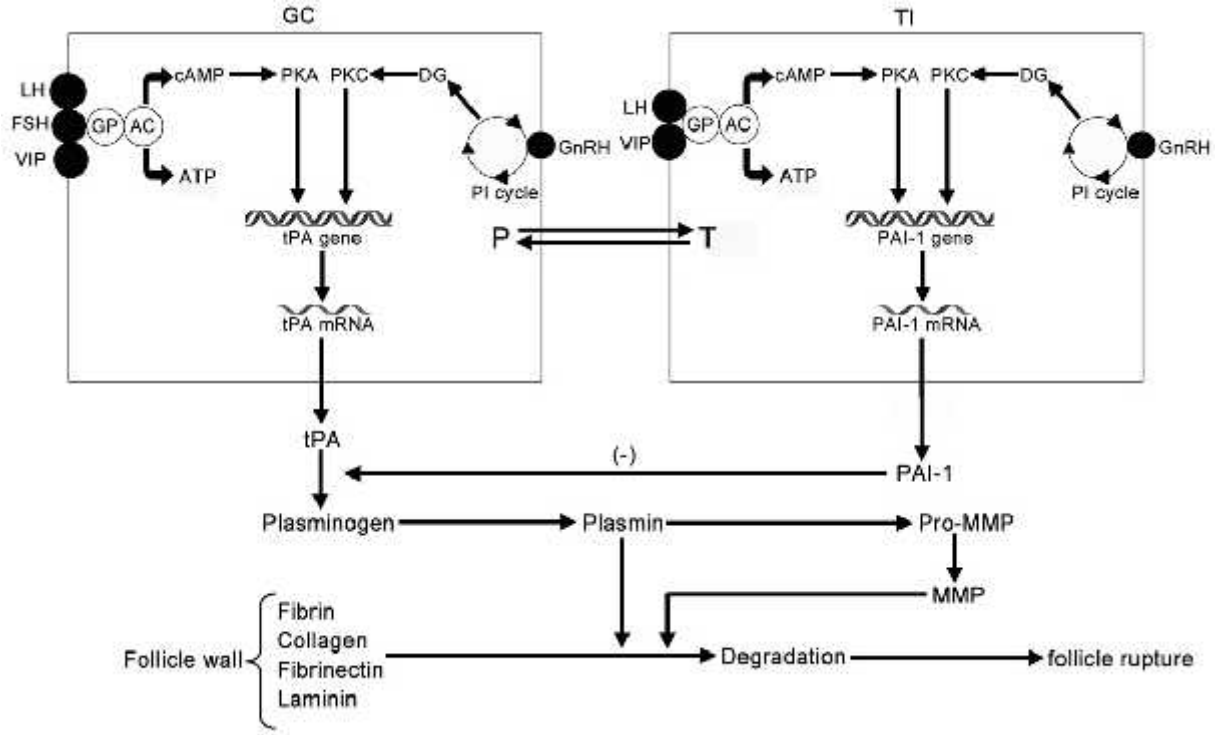


Figure 7. Schematic representation of involvement of ovarian tPA produced mainly by GC and PAI-1 secreted by TC in the process of ovulation. P, Progesterone; T, testosterone; GP, G-protein; AC, adenylate cyclase; DG, 1, 2-diacylglycerol; PI, phospholipid; PKA, protein kinase A; PKC, protein kinase C. For the explanation of the other abbreviations, see the text.

apex compared with apex cells 36 h after hCG. Higher level of PAI-1 protein was also measured in the non-apex cells induced by EP1. The authors suggest that cumulus cells likely respond to PGE2 via EP2 and EP3, whereas PGE2 controls rupture of a specific region of the follicle via EP1. Therefore, differential expression of EP receptors may permit each granulosa cell subpopulation to generate a unique response to PGE2 during the process of ovulation (50, 51).

Evidence available from the published literatures showed that serine proteases and inhibitors, mainly the tissue type plasminogen activator and the inhibitor PAI-1 may play the most important role in oocyte maturation and ovulation. A schematic representation of involvement of ovarian tPA produced mainly by GC and PAI-1 secreted by TC in the process of ovulation is summarized in Figure 7. Other proteases and inhibitors, as well as ovarian autocrine/paracrine factors may be also involved in the processes of oocyte maturation and ovulation.

7. PERSPECTIVE

In spite of importance of tPA in ovulation both in rat and monkey, species differences exist in expression of the PA system in ovaries (5, 68). Mouse ovary mainly secretes uPA activity, while rat ovary secretes only tPA activity. Furthermore, studies with tPA and uPA double knock-out mice only have a 26% decreased ovulation rate, indicating that plasmin in mouse may be not necessary required for efficient follicular rupture. Other proteases in the mouse ovary may be also involved in the ovulatory process in the absence of plasmin. Studies have indicated that other families of proteases including the MMP system may also involve in ovarian function. The complicated natures of various protease families have increased the tediousness of such studies. In recent years increasing evidence suggested that oocyte maturation and ovulation may also be modulated by other serine protease and inhibitor, as well as endogenously-produced ovarian paracrine/autocrine factors. Thus, it is important to identify the interrelationship between the serine protease system and the multiple factors, and to know how they regulate the ovarian physiological and pathological processes during oocyte maturation and ovulation. With the help of many strains of gene deficient mice that lack a protease or a combination of proteases, certain proteases would be revealed by corresponding knock out mice. However, due to the complex of various proteases in the ovary, it may be even more complicated than anticipated. Further progresses could be made with the recently developed interference RNA (RNAi) technique, by which one may be able to quickly knock down expression of a protein in cultured defined cells and reveal how important the molecule is in the process (82,83).

A better understanding of these signaling networks and the related molecules activated during oocyte maturation and ovulation will provide new pharmacological opportunities for the manipulation of fertility.

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Abbreviations: ADM: adrenomedullin; AREG:amphiregulin; BMP15:bone morphogenetic protein 15; COCs:cumulus oocyte complexes; ECM:extracellular matrix; EGF:epidermal growth factor; EGFR:epidermal growth factor receptor; EREG: epiregulin; GDF9: growth differentiation factor; GVBD: germinal vesicle brick down; MMP: matrix metalloproteinase; NPPC: natriuretic peptide precursor type C; PAI-1: plasminogen activator inhibitor type-1; PCOS: polycystic ovary syndrome; PGE2: prostaglandin E2; PN-1: protease nexin-1; RIP140: nuclear receptor cofactor receptor-interacting protein; tPA: tissue-type plasminogen activator; VIP: vasoactive interstitial peptide; uPA: urokinase plasminogen activator

Key Words: Plasminogen Activator, Plasminogen Activator Inhibitor, Matrix Metalloproteinase, Protease Nexin-1, Paracrine/Autocrine Factor, Oocyte Maturation, Follicle Rupture, Review

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