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Gonadotropin Surge-Induced Upregulation of Plasminogen Activator System Components Within Bovine Periovulatory Follicular and Luteal Tissue

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GONADOTROPIN SURGE-INDUCED UPREGULATION OF PLASMINOGEN ACTIVATOR SYSTEM COMPONENTS WITHIN BOVINE PERIOVULATORY FOLLICULAR AND LUTEAL TISSUE

by

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ABSTRACT

GONADOTROPIN SURGE-INDUCED UPREGULATION OF PLASMINOGEN ACTIVATOR SYSTEM COMPONENTS WITHIN BOVINE PERIOVULATORY FOLLICULAR AND LUTEAL TISSUE

by Mark P.D. Dow

Ovulation and subsequent release of a mature oocyte is essential for establishment of pregnancy. Maintenance of pregnancy requires transformation of this ruptured follicle into a corpus luteum (CL). The preovulatory LH surge initiates both the ovulatory process and CL formation. Both the ovulatory process and CL formation feature degradation and reorganization of follicular wall and extracellular matrix (ECM) components. The plasminogen activator/plasmin system includes specific activators, inhibitors and receptors that directly and indirectly promote ECM degradation. In rodents, the gonadotropin surge induces expression of specific plasminogen activator/plasmin system components prior to ovulation. In plasminogen activator double knockout mice, ovulation rate is reduced 30%. Therefore, the plasminogen activator/plasmin system presumably plays an integral role in ovulation and CL formation in cattle. My working hypothesis is that in cattle, the gonadotropin surge up-regulates the de novo expression of plasminogen activator/plasmin system components that promote site directed degradation of the follicular wall and subsequent CL development. To test this hypothesis, I will determine the effect of a gonadotropin releasing hormone-induced gonodotropin surge on the localization, expression and activity of the plasminogen activator/plasmin system components. To date, plasminogen activator/plasmin system components have not been investigated in cattle. An increased

understanding of the mechanisms that control ovulation and CL development may lead to improved methods to promote reproductive efficiency in cattle. This study examined the effect of the preovulatory gonadotropin surge on the plasminogen activator system components in bovine preovulatory follicles and new CL collected at approximately 0, 6, 12, 18, 24 and 48 h after a GnRH-induced gonadotropin surge. Messenger RNAs for tPA, uPA, uPAR, PAI-1 and PAI-2 were all increased in a temporally specific fashion within 24 h of the gonadotropin surge. Messenger RNAs for uPA, uPAR, PAI-1 and PAI-2 remained elevated in the developing new CL. Localization of tPA and PAI-2 mRNAs were to the granulosal layer while PAI-1 mRNA was localized to the thecal layer. Both uPA and uPAR mRNAs were detected in both the granulosal and thecal layers. Activity for tPA was increased in follicular fluid and the preovulatory follicle apex and base within 12 h following the gonadotropin surge. The increase in tPA activity in the follicle base was transient, whereas increased activity in the apex was maintained through the 24 h timepoint. Activity for uPA increased in the follicle apex and base within 12 h of the gonadotropin surge and remained elevated through the time of follicular rupture. Plasmin activity in follicular fluid also increased within 12 h following the preovulatory gonadotropin surge and was greatest at 24 h. Significant plasminogen activator inhibitor activity was detected in follicle extracts, but temporal or spatial differences in plasminogen activator inhibitor activity were not detected in response to the gonadotropin surge. My results indicate that all the plasminogen activator components are upregulated in bovine preovulatory follicles following the gonadotropin surge in a cell-specific manner. Increased plasminogen activator and plasmin activity may be a contributing factor in the mechanisms of follicular rupture in cattle.

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α_2-MG	alpha-2-macroglobulin
ACTH	andrenocorticotropic hormone
ADAMTS-1	A disintegrin and metalloprotease with thrombospondin type1 motifs
AMH	anti-mullerian hormone
AP	activator protein
ATF	amino terminal fragment
bFGF	basic fibroblast growth factor
cAMP	cyclic adenosine monosphosphate
CL	corpus luteum
COX-2	cyclooxygenase-2
CRE	cAMP response element
CREB	CRE-binding protein
CS	chondroitan sulfate
DS	dextran sulfate

E_2	estradiol
ECM	extracellular matrix
EGF	epidermal growth factor
FSH	Follicle Stimulating Hormone
GAG	glycosaminoglycans
GnRH	gonadotropin releasing hormone
hCG	human chorionic gonadotropin
HGF/SF	hepatocyte growth factor/scatter factor
HS	heparin sulfate
LH	Luteinizing Hormone
LMW-uPA	low molecular weight urokinase plasminogen activator
LRP	low density lipoprotein receptor related protein
MMP	matrix metalloproteinase
MSP	macrophage-stimulating protein
OSE	ovarian surface epithelial cells

P_4	progesterone
P_4R	progesterone receptor
PAI	plasminogen activator inhibitor
PG	proteoglycans
PGE	prostaglandin E
PGF	prostaglandin F
PGHS	prostaglandin endoperioxide synthase
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol myristate acetate
PN-1	protease nexin-1
RCL	reactive center loop
scuPA	single-chained urokinase plasminogen activator
tcuPA	two-chained urokinase plasminogen activator
TGF	transforming growth factor

TNF	tumor necosis factor
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator
uPAR	uPA receptor
VLDL	very low density lipoprotein
VEGF	vascular endothelial growth factor

Chapter 1

INTRODUCTION

Follicular development, ovulation, and corpus luteum (CL) formation are required for successful spontaneous reproduction in the female. In cattle, follicular development leads to the production of a single mature dominant follicle per wave. This follicle is usually the largest in the cohort of developing follicles and the one capable of ovulation. The remaining subordinate follicles undergo atresia. The preovulatory luteinizing hormone (LH) surge triggers rupture of the dominant follicle and liberation of a mature oocyte. Cellular reorganization of the remaining follicular tissue is characteristic of CL formation. All of these processes require cellular proliferation, differentiation, and selective extracellular matrix formation and degradation. The plasminogen activator/plasmin system and the matrix metalloproteinases (MMPs) are believed to play instrumental roles in these processes.

The plasminogen activator/plasmin system includes plasminogen, two specific plasminogen activators, cell surface plasminogen activator receptors, several plasminogen activator inhibitors and several plasmin inhibitors. Plasminogen is abundant in blood and peripheral tissues and consequently, regulation of its activity occurs at several levels. The plasminogen activators, tissue (tPA) and urokinase specific (uPA), proteolytically cleave plasminogen into its active form plasmin. Activation of plasminogen occurs in the extracellular milieu. Both of the plasminogen activators and plasminogen belong to the serine proteinase family. Plasmin substrates include fibrin, fibrinogen, types III, IV, VI collagen, fibronectin, laminin, gelatin, elastin, vitronectin, and proteoglycans [1, 2]. The plasminogen activators are products of

different genes and are secreted as single chain proteins. The plasminogen activator/plasmin system also includes specific plasminogen activator inhibitors (PAI-1, PAI-2, and PAI-3). These inhibitors prevent the plasminogen activators from converting plasminogen to plasmin. Binding of plasminogen activator inhibitor to the plasminogen activators results in increased affinity of the plasminogen activator-plasminogen activator inhibitor complex for the cell surface via interaction of the proteinase/inhibitor complex with the low-density lipoprotein receptor-related protein/α_2-macroglobulin receptor (LRP/α_2-MR) [3]. Binding to these receptors induces rapid endocytotic clearance of ligand/receptor complexes. Similarly, inhibitors of plasmin also have an affinity for LRP/α_2-MR and cleared via the same mechanism. There is also a cell surface receptor for uPA (uPAR). Binding of uPA to its receptor activates a signal transduction pathway and focuses uPA directed plasmin activity at the cell surface.

Schochet (1916) [4] was the first to hypothesize that proteolytic digestion of the follicular wall leads to ovulation. The following lines of evidence suggest that the plasminogen activator/plasmin system plays a key role in the ovulatory process: 1) Plasminogen is present in follicular fluid and plasmin weakens follicular wall strips in vitro [5]. 2) Antibodies to tPA and uPA suppress ovulation in rats and sheep, respectively [6-8]. 3) tPA is expressed on the surface of the ovary just before ovulation in rats [9, 10]. Similarly in mice, uPA is elevated during the same period [11]. 4) Addition of the bacterial plasminogen activator, streptokinase, to rabbit ovaries in vitro induces ovulation in the absence of gonadotropins [12]. 5) Ovulation rate is reduced approximately 26% in transgenic mice deficient in both tPA and uPA [13]. 6) The germinal epithelium of sheep preovulatory follicles contains uPA. Removal of the

germinal epithelium inhibits ovulation [6]. 7) Plasmin is an activator of the proform of several MMPs that have also been implicated in follicular rupture.

Potenital differences exists in the regulation of plasminogen activator/plasmin system components in many species (see Table 2, page 35), it is not possible to extrapolate these results to other species. Additionally, all the species investigated to date are litter bearers, it is important to also investigate the role of the plasminogen activator/plasmin system in a monoovulatory species. How the plasminogen activator/plasmin system influences periovulatory follicular events may also provide a model system more potentially applicable to humans. Many of the experiments proposed herein cannot be completed in the human due to ethical and financial issues.

The plasminogen activator/plasmin system is implicated in many areas of reproduction including embryo implantation, spermatogenesis, fertilization, parturition, and ovulation. Within the ovary, regulation of the plasminogen activator/plasmin system was investigated in rodent species. However data from other species, particularly farm animals is lacking. Experiments in rodents suggest a requirement of the plasminogen activators for optimum ovulation rates. However, the gonadotropin surge only up-regulates one plasminogen activator (tPA or uPA) in rodents and the specific plasminogen activator affected is different depending on the species [14]. Further understanding of the regulation, localization, expression and activity of these enzymes will help clarify their potential role in ovulation. This information may ultimately be used as the model for future functional studies to test the requirement of the plasminogen activator/plasmin system for ovulation in cattle. An increased understanding of the regulation of ovarian function is required for development of improved

methods to enhance reproductive efficiency in livestock. Furthermore, similar to the bovine, data are completely lacking concerning the role of plasminogen activator/plasmin system in human reproduction. Information collected in cattle may ultimately be more relevant to the human than current information derived from rodents because human and bovine both undergo dominant follicle selection and normally only ovulate a single oocyte. In addition, altered plasminogen activator/plasmin system homeostasis is linked to Polycystic Ovarian Disease (PCOD). The symptoms of this disease include abnormal endocrine profiles, reduced cyclicity and ovulation, elevated blood levels of PAI-1 [15], and excessive adhesions coating the ovary. Elevation of PAI-1 may reduce plasmin directed proteolysis on the ovarian surface allowing adhesions on the surface to accumulate [16, 17].

The purpose of the following literature review is to 1) highlight the basic mechanisms involved in the ovulatory process and subsequent luteal formation 2) review the biochemical and physiological properties of the members of the plasminogen activator/plasmin system and to 3) describe the hormonal regulation, and key evidence supporting a role of the ovarian plasminogen activator/plasmin system during the periovulatory period. Most of the information available to date is in the rodent species. However, data available on domestic ruminants or humans will be addressed and emphasized when available.

Chapter 2

LITERATURE REVIEW

OVARIAN FOLLICULAR GROWTH, ATRESIA, OVULATION AND LUTEAL FORMATION

A primary function of the ovaries in the bovine is to produce a single mature oocyte per estrous cycle that can be fertilized successfully if mating occurs. In eutherian animals, follicles are continuously being recruited for growth, albeit only a limited number or one follicle will develop into an antral follicle, ovulate and differentiate into a CL during each cycle. In the human, the number of follicles that initiate growth in a cycle is proportional to the number of primordial follicles remaining in the ovarian pool [18]. The pituitary gonadotropins [follicle stimulating hormone (FSH) and luteinizing hormone (LH)] as well as ovarian steroids [primarily progesterone (P_4) and estradiol (E_2)] temporally coordinate follicular development, ovulation, and CL formation. Furthermore, processes including ovarian tissue remodeling, cell growth and differentiation, angiogenesis, and migration play roles in these events [19]. Evidence supports a role for the plasminogen activator/plasmin system in all of these processes.

ANATOMY OF THE DEVELOPING OVARIAN FOLLICLE

The ovary is made up of three distinct domains. The outer cortex (1) contains the germinal epithelium and follicles. The central medulla (2) contains the ovarian stroma and the hilum (3)

that connects the ovary to the mesovarium. The germinal epithelium or ovarian surface epithelium (OSE) is made up of coelomic epithelial cells that line the outside of the ovary. The function of these cells is not clear. The ovary of a normal cycling mammal includes follicles at different stages of development including primordial, primary, secondary, tertiary, preovulatory and atretic. The largest proportions of follicles in most animals are immature primordial follicles. During follicular growth, the granulosal cells surrounding the oocyte proliferate to form multiple layers. Additionally, the ovarian stromal layer that surrounds the follicle will start to form the two thecal layers. A basement membrane separates these thecal layers from granulosal cells. The thecal layer adjacent to the basement membrane (theca interna) develops from steroid producing interstitial cells in rodents, while the outer layer of fibroblast like cells integrate into the blood vessels and collagen matrix. In the tertiary follicle, fluid accumulates between many of the layers of granulosal cells, eventually occupying the majority of the follicular volume. Near the time of ovulation, the thecal tissue that is closest to the exterior of the ovary (apex) separates from the basement membrane and undergoes rapid deterioration characterized by degradation of the extracellular matrix (ECM). This decomposition leads to rupture of the follicle with subsequent release of the oocyte.

The ovarian follicle contains a number of ECM components including collagen (I, III, IV and V), laminin, fibronectin, keratin, and proteoglycans (Figure 1). Ovulation requires site directed degradation of the ECM. The most abundant ECM protein in the follicle is collagen, which provides structural integrity. The ovarian surface epithelium contains collagens I, III and V, keratin and laminin. Within the thecal layers, ECM proteins include collagens I and III. The basal lamina that separates the thecal and granulosal layers is made of collagen IV, fibronectin, laminin and heparin sulfate. The granulosal cells and follicular fluid also contains fibronectin

and proteoglycans [20-23]. Proteoglycans and their covalently bound glycoaminoglycan (GAG) side chains have been identified in the follicular fluid of several species including pigs, cows, rats and humans. The major types of GAG present in bovine follicular fluid are dermatan sulfate (DS) and chondroitan sulfate (CS), with minor amounts of heparin sulfate (HS) also present [24]. Both DS and CS are attached to a core protein whereas HS is not. The proteoglycans in follicular fluid are large, with molecular weights ranging from 7.5×10^5 to 2×10^6. They may mediate follicular fluid viscosity [24] and osmotic pressure [25]. Recently, versican, perlecan, nidogen and decorin have been identified in small antral bovine follicles [22]. Versican was localized to the thecal and the granulosal layers. Decorin, perlecan and nidogen were identified as part of the basal lamina [26].

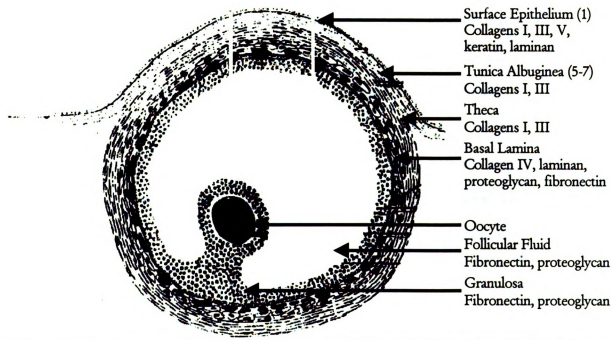


Figure 1: Anatomy of an Ovarian Follicle with ECM. Modification from Espey & Lipner [25]. ECM matrix information by Luck, McAurthor and Zhao [19-27].

Anatomy of Luteal Formation

After the preovulatory LH surge, a complex series of morphological, endocrinological and biochemical processes occur within the mature follicle that lead to CL formation. Immediately after release of the oocyte, the follicular antrum hemorrhages from breakage of blood vessels within the thecal layer. A large fibrin clot quickly forms within the ruptured follicle. This clot, however, is rapidly dissolved and removed most likely due to tPA induced plasmin formation [27]. The follicular wall undergoes a rapid involution and folding. The basement membrane disintegrates allowing previously avascular granulosal cells direct exposure to the blood supply. Additionally, there is a large infiltration of white blood cells including neutrophils, basophils, and macrophages into the area [28]. Extensive tissue and extracellular matrix remodeling, cellular migration, and cellular proliferation contribute to the formation of the vascular CL. This structure produces the necessary steroid hormones for optimization of implantation by the embryo as well as maintenance of pregnancy until the placenta can assume this role. Both granulosal and thecal cell types undergo luteinization, whereby they hypertrophy and begin to secrete large quantities progesterone. Within the CL there are two distinct types of steroidogenic cells termed large and small. The follicular lineage of both the large and small luteal cells has been controversial. However, in sheep and cattle it is predominantly believed that large luteal cells develop from granulosal cells and small luteal cells from thecal interna cells [29-32].

Much of the remaining CL tissue is composed of a complex microcirculatory bed. The formation of new capillaries during CL development results from proliferation of endothelial

cells within the thecal layer. These cells migrate towards the severed basement membrane into the granulosal layer.

INTRAFOLLICULAR REGULATION OF FOLLICLE RUPTURE

The process of ovulation in mammals is initiated by a surge release of LH from the anterior pituitary. The endocrine target for LH is the ovary, where LH initiates a series of biochemical and morphological processes including ovulation as well as luteal formation. However, the intrafollicular signaling pathways that mediate the effects of the LH surge on ovulation are not as well understood. The following section of the literature review will discuss the intrafollicular signaling pathways implicated in control of follicular rupture.

GONADOTROPIN RELEASING HORMONE (GNRH)

The release of the gonadotropins LH and FSH from the anterior pituitary has long been known to be influenced by hypothalamic GnRH. However, GnRH agonists have direct effects effects on the ovary, including the induction of ovulation in hypophysectomized rats [33, 34] and inhibition of steroidogenesis in humans [35]. Gonadotropin releasing hormone is not part of the biochemical means by which LH causes ovulation, as LH-induced ovulation is not inhibited by a GnRH antagonist in rats [36]. However, Koos and Lemaire, 1985 [37] demonstrated that a GnRH agonist can induce ovulation directly in the rat ovary and that this action can be inhibited by simultaneous treatment with a GnRH antagonist. Although GnRH receptors are found in the rat and human ovary, due to the very short half-line of GnRH (2-4 min [38]), it is unlikely that hypothalamic GnRH has a direct physiological role in ovulation. However, GnRH-like peptides are present in ovarian extracts of rat, human, cow and ewe and these peptides are hypothesized to play a paracrine role in ovarian function [39].

CYCLIC ADENOSINE MONOPHOSPHATE (CAMP)

The predominant pathway for intrafollicular action of the LH surge is through induction of cAMP and its downstream stimulation of the protein kinase A (PKA) pathway. The primary action of cAMP is to activate regulatory subunit of PKA. This activation causes dissociation of the regulatory subunit, freeing the catalytic subunit to phosphorylate proteins such as CREB that bind to DNA and modulate transcription. However, stimulation of calcium channels leading to protein kinase C activation as well as activation of tyrosine kinases have also been proposed as intracellular mediators of the ovulatory process [40-42]. Direct evidence for a role of cAMP in ovulation was revealed when several investigators demonstrated an ovulation-inducing effect of cAMP analogues, cAMP stimulators or catabolic cAMP inhibitors (phosphodiesterases) in the rabbit [43] and rat [44]. These observations support the hypothesis that cAMP is a physiological mediator of LH-induced ovulation.

PROGESTERONE

After the LH surge there are marked alterations in the preovulatory follicular steroidogenic enzymes such that a shift occurs from the final product of estradiol to that of progesterone. A growing number of studies support an intraovarian role for progesterone in the process of ovulation [45, 46]. In rats, epostane, an inhibitor of the enzyme 3 -hydroxysteroid dehydrogenase (converts pregnenolone to progesterone) was shown to inhibit ovulation in the rat and this inhibition could be reversed by progesterone administration [47]. Administration of progesterone antiserum following hCG-induced ovulation also attenuated ovulation of rat preovulatory follicles following hCG injection [48]. However, Kitai et al. [49] and Holmes et al. [50] demonstrated in perfused rabbit ovaries that an inhibitor of cholesterol side-chain cleavage or an inhibitor of 3 -hydroxysteroid dehydrogenase added to the perfusion media did

not inhibit hCG or LH induced ovulation while completely abolishing the LH induced increase in follicular progesterone levels. Additionally, FSH as well as GnRH analogs are capable of inducing ovulation by themselves in the perfused rat ovary, while inducing only nominal increases in steroidogenesis [37, 51]. Furthermore, $PGF_{2\alpha}$ added alone to the medium of perfused rabbit ovaries induces ovulation without a concomitant increase in estrogen or progesterone secretion [43]. The role of progesterone may be important in the rat than the rabbit in mediating follicular rupture.

The effects of progesterone are mediated via the intracellular progesterone receptor (P_4R) that is induced by upstream activation of the protein kinase A pathway. Induction of P_4R mRNA depends on granulosal cell differentiation in response to estradiol and a physiological amount of FSH followed by high amounts of cAMP. The A-kinase inhibitor H89 and cycloheximide but not by the estradiol antagonist, ICI 164,384 blocks the induction of P_4R mRNA by forskolin. These results indicate that phosphorylation and synthesis of some regulatory factor(s) other than or in addition to the estrogen receptor (ER) are essential for transactivation of the P_4R gene [52]. The P_4R is a member of the nuclear receptor superfamily of transcription factors. These receptors are hormonally regulated DNA-binding proteins that stimulate or suppress transcription of target genes. Ovarian binding sites for P_4 were demonstrated in human [53], cow [54], guinea pig [55] and rat [56, 57]. Immunocytochemical techniques have also identified P_4R within the ovary of human [58], chicken [59], rabbit [60] and bovine [61]. In the rat and bovine preovulatory follicles P_4R mRNA is induced in the granulosal layer in response to an ovulatory stimulus. In rats, ovarian P_4R mRNA is induced within 5 h post hCG and in cows within 6 h after a GnRH-induced gonadotropin surge [62, 63]. Treatment with an ovulatory dose of hCG also upregulates P_4R

mRNA expression by macaque granulosal cells [64]. Investigators showed that LH stimulates the PR gene in cultured rat [65, 66] and pig [67] preovulatory granulosal cells. The use of the P_4R antagonist, RU486, was shown to inhibit ovulation in the rat [45, 68, 69]. The observation that the follicles of P_4R-/- knockout mice develop to an ovulatory stage, but are unable to rupture, even in response to gonadotropin challenge, further supports the previous physiological evidence indicating that progesterone and its receptor are required for ovulation [70, 71]. Interestingly, other processes associated with the LH surge such as oocyte maturation and cumulus expansion occurred normally, but granulosal cells failed to undergo normal luteinization [71]. Further studies are required to determine the identity of PR-regulated target genes during the periovulatory period that are required for ovulation. However, two progesterone regulated proteinases identified to date are A disintegrin and metalloprotease with thrombospondin type1 motifs (ADAMTS-1) and cathepsin L. Furthermore, mRNAs encoding cathepsin L and ADAMTS-1 are reduced in the $P_4R^{-/-}$ knockout mice compared to their wild-type littermates. These novel observations indicate that these two proteinases may regulate some key step(s) controlling ovulation [72, 73].

PROSTAGLANDINS

A large body of evidence supports the hypothesis that intrafollicular prostaglandins are key regulators of the ovulatory process. Prostaglandins are potent regulatory molecules that are produced by many tissues including the ovary. During the process of ovulation, the endogenous levels of prostaglandins in the rat and rabbit follicle increase markedly and are inhibited by the prostaglandin synthase inhibitor indomethacin [74]. Furthermore, Koos et al., 1983 [75] observed a marked preovulatory increase in intrafollicular levels of prostaglandins (PGE and PGF) during in vitro perfusion of rabbit ovaries.

Systemic or local administration of cyclooxygenase inhibitors, which inhibit prostaglandin synthesis (indomethacin, aspirin), inhibit LH (or hCG)-induced ovulation in the rat, rabbit, monkeys and bovine [74, 76-78]. Furthermore, ovulation was rescued in the rabbit amd rat by the administration of exogenous prostaglandins [74, 79]. Histological examination of the corpora lutea in rabbits and marmoset monkeys treated with indomethacin confirmed the entrapment of oocytes [76, 80]. Additionally, using an ovarian perfusion system, indomethacin was shown to inhibit gonadotropin-induced ovulation of rabbit antral follicles. Addition of the prostaglandin, PGF, to the perfusion media not only reversed the inhibition of ovulation in the presence of indomethacin [43, 81], but $PGF_{2\alpha}$ stimulated ovulation in the absence of a gonadotropin stimulus [43, 81]. Similar experiments using an ovarian perfusion system showed that PGE_2 could restore ovulation in indomethacin treated rat ovaries [44].

Intrafollicular regulation of prostaglandin production is mediated primarily through regulation of the prostaglandin endoperioxide synthase (PGHS) enzyme. There are two distinct isoforms of the PGHS enzyme termed PGHS1 (or cycloxygenase-1; COX-1) and PGHS2 (or cycloxygenase-2; COX-2). Although the structural domains of the enzymes are conserved, their tissue distribution and regulation are distinct. COX-1 is constitutively expressed in many tissues and is thought to synthesize prostaglandins necessary for regular cellular processes. In contrast, COX-2 is dramatically induced during inflammatory like conditions, such as ovulation. Furthermore, COX-2 is increased in follicular cells of rats [82, 83], sheep [84] and bovine [85] by the LH surge. Ovulation is delayed in human with the use of a specific COX-2 inhibitor [86]. Furthermore, female mice carrying a null mutation for COX-2 are infertile [87] due to failures in ovulation, oocyte maturation, cumulus expansion and fertilization [88]. These processes can be restored by PGE_2, but not $PGF_{2\alpha}$ treatment at the time of ovulation

induction in the COX-2 null mutant mice [89]. In rat preovulatory follicles PGE_2 has been shown to be preferentially produced over other prostaglandins following the LH surge [90]. However, investigations looking at overriding the antiovulatory effects of indomethacin in large domestic animals including cattle, pigs and horses have suggested that $PGF_{2\alpha}$ may also be important in the ovulation cascade [78, 79, 91, 92].

Actions of PGE_2 are mediated by specific cell surface PGE_2 receptors of the EP_1, EP_2, EP_3 and EP_4 subtypes [93]. EP_2 is induced in cumulus cells of mice 8 h post hCG. Furthermore, mice deficient in EP_2 have lower number of ovulations and have poor fertilization outcome [94]. Therefore, the EP_2 receptor subtype may mediate the effects of PGE_2 on follicle rupture in mice.

DOWNSTREAM PROCESSES MEDIATED BY P_4R AND PROSTAGLANDINS

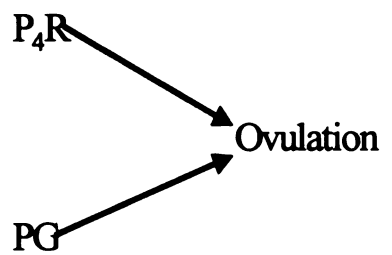


Figure 2: Diagram showing the known upstream regulators of ovulation.

Since a crucial event in follicular rupture is the weakening of the follicle wall by proteolytic enzymes, P_4R and prostaglandins likely mediate this process (Figure 2). The absence of ovulation in female mice with deficiencies in either P_4R or COX-2 support the hypothesis that both of these pathways lead to the induction of proteinases required for follicular rupture. Furthermore, mice deficient in P_4R have normal granulosa cell COX-2 mRNA induction following the gonadotropin surge. Similarly, COX-2 deficient mice have normal granulosa cell P_4R mRNA induction [95]. These results from gene targeting studies suggest that PGE_2 and P_4R act via independent pathways that likely lead to similar downstream targets (Figure 2).

Experiments by Downs and Longo [96] demonstrated that following hCG treatment, the apices of antral follicles treated with indomethacin remained thickened and tightly packed with marginal signs of disruption whereas apices of vehicle-treated animals demonstrated marked deterioration, dissociation, and thinning of tissue. Similar results were observed in the rat.

ROLE FOR PROTEINASES IN FOLLICULAR RUPTURE

In the final hours prior to follicular rupture, the follicular wall degrades at the apex (reviewed in Espey, 1978 [97]) with an eventual breakthrough at the area of the stigma [98]. Cinematographic and photographic observations of the rabbit graafian follicle clearly show these processes [99]. This thinning of the follicular wall is accompanied by a dissociation and fragmentation of the collagen fibrils [98, 100, 101]. Martin et. al. [101] concluded from scanning electron microscopic observations in the hamster that this fragmentation is due to enzymatic cleavage of the intermolecular bonds that hold the collagen fibers together, thereby allowing them to separate and give way to form the stigma. Reich et. al. (1985) was the first to correlate the ovulatory process in rats with increased ovarian collagenase activity [102]. Furthermore, increases in collagenase enzyme activity in the apex but not the base of sheep preovulatory follicles strongly support a potential key role for these class of enzymes in the proteolytic degradation of the apical follicle wall leading to ovulation [103].

While the majority of morphological and biochemical studies have focused on the collagen network surrounding the follicle (type I and III collagen), the basement membranes (type IV collagen), which separate the granulosal cells from the thecal cells and the germinal epithelium from the tunica albuginea, must also be broken down for ovulation to occur. Such a breakdown has indeed been observed with the use of histochemical techniques [104].

However, the enzymes responsible for the breakdown of the follicular basement membranes remain to be determined. Additionally, the oocyte-cumulus complex must become very loosely attached or detached from the follicle wall prior to rupture. During human in vitro fertilization, follicles are aspirated 32 to 36 hours after hCG administration and oocytes obtained. In contrast if follicles are aspirated 12 hours or less after hCG, no oocytes are obtained [105, 106].

Two families of proteinases, the plasminogen activator/plasmin system and the matrix metalloproteinases (MMP) have been implicated in follicular rupture. The remaining two sections of this literature review will focus on the biochemistry of the plasminogen activator/plasmin system and will provide direct and indirect evidence supporting a role of the plasminogen activator/plasmin system in follicular rupture and (or) luteal formation.

THE PLASMINOGEN ACTIVATOR/PLASMIN SYSTEM

Early investigation of the plasminogen activator/plasmin system focused on the traditional concepts of fibrinolysis involving tPA activation of plasminogen leading to plasmin-mediated destruction of fibrin. However, not only have multiple activators of plasminogen been identified, several cell surface receptors are now recognized to play a prominent role in local orchestration of plasmin activity. Additionally, plasmin can cleave a number of nonfibrin substrates such as transforming growth factor β (TGF-β), proenzymes, prohormones, and extracellular matrix proteins.

STRUCTURAL AND BIOCHEMICAL PROPERTIES OF PLASMINOGEN, PLASMINOGEN ACTIVATORS, AND PLASMINOGEN ACTIVATOR INHIBITORS

Plasminogen/Plasmin

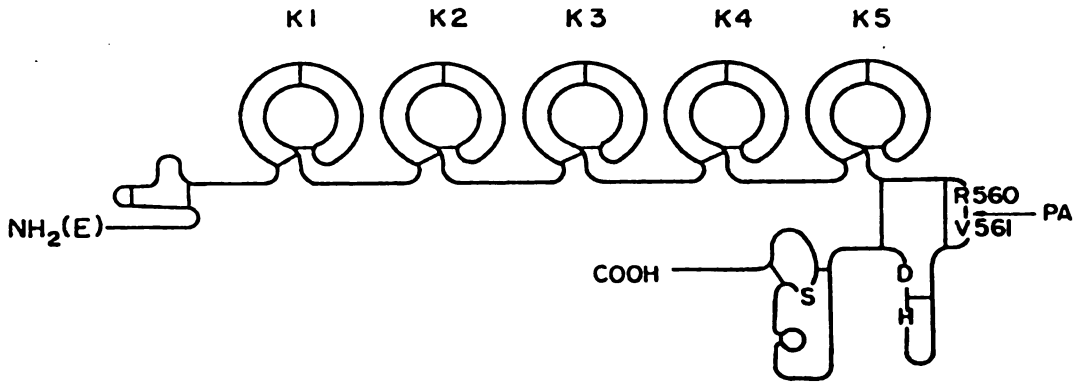


Figure 3: Diagram of the structure of plasminogen modified from Alexander and Werb, 1991 [2]. K1, K2, K3, K4, K5 represent five kringles, PA represents plasminogen activator site of cleavage. Amino acids H, S and D show active site of the enzyme.

The primary component of the plasminogen activator/plasmin system is the broad-spectrum proteinase plasmin, which is formed from the proteolytic cleavage of plasminogen (Figure 3). Plasminogen is a 92 kDa glycoprotein [107] present in plasma and other extracellular body fluids at a concentration of about 2 μM [108]. It is synthesized in the liver and brain [108-111]. Plasminogen consists of a single polypeptide chain. The N-terminal region is composed of five "kringle" domains. The kringle domains contain lysine binding sites that are important for binding to the cell surface [112]. The C-terminal region contains the catalytic serine proteinase domain. Conversion of plasminogen to plasmin occurs due to a hydrolysis of a single peptide bond between Arg_{560}-Val_{561} converting the zymogen to a two chained polypeptide held together by two disulfide bonds. The catalytic activity of plasmin is 10^4 to 10^6-fold over plasminogen. Plasmin severs peptide bonds between Lys and Arg residues. Substrates for plasmin include fibrin, fibrinogen, types III, IV, VI collagen, fibronectin, laminin, gelatin, elastin, vitronectin, and proteoglycans [1, 2]. Most other collagens are fairly resistant to

plasmin degradation. However, plasmin can convert other collagenolytic enzymes, including certain matrix metalloproteinases (MMP) to their active form. Specifically, plasmin can activate collagenase I (MMP-1), gelatinases (MMP-2 and MMP-9), and stromelysin-1 (MMP-3) [113-122]. Furthermore, due to the relatively high concentration of plasminogen in tissue and body fluids, a small increase in plasminogen activators induces a large increase in plasmin in the extracellular milieu. These proteinase families, plasmin together with the MMPs, can potentially degrade all ECM components in the ovary.

Plasmin also activates growth factors such as hepatocyte growth factor/scatter factor (HGF/SF) [123] and is involved in proteolytic processing of peptide hormones such as Anti-Mullerian hormone (AMH) [124] and ACTH [125]. Furthermore, plasmin degradation of the ECM, may release ECM-bound growth factors such as bFGF, VEGF and $TGF\beta_1$, that may mediate specific biological responses. Plasmin can also be further cleaved into polypeptides containing kringles 1-3 or 1-4 by pancreatic elastase, several MMPs [126], and by plasmin autohydrolysis. The resulting protein, termed angiostatin, is a potent inhibitor of angiogenesis [126-128].

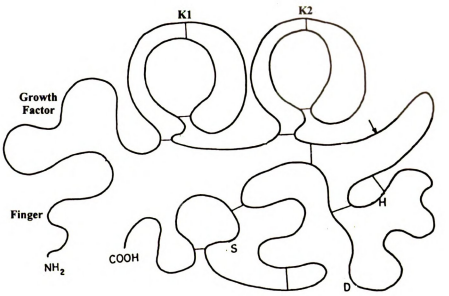


Figure 4: Diagram of tPA showing important structural domains modified from Alexander and Werb, 1991 [2]. Finger, growth factor, kringles 1 and 2 (K1, K2) and key amino acids of the acid site (H, S, D). Arrow indicates cleavage to convert sctPA to tctPA.

The first plasminogen activator identified was tPA. Tissue plasminogen activator is a 68 kDa glycoprotein [129] that is present in low levels in plasma. The structural and functional domains of tPA have been described (Figure 4). The N-terminal A chain contains a fibronectin type II domain, a growth factor domain and two two triple disulfide structures "kringles." The C-terminal B chain contains the serine proteinase domain. The growth factor domain shares homology to the receptor binding regions of TGF_{α} and EGF. Recent evidence suggests that this binding domain may be important for clearance of tPA. Tissue plasminogen activator is secreted as an active single chained form (sctPA) that is converted to an active two-chained form (tctPA) by cleavage of Arg_{275}-Ile_{276}. Additionally, the first kringle of tPA is required for tPA's ability to degrade fibronectin [2]. The activity of tctPA is 10-50

fold higher than that of the single chain form. In the presence of fibrin the activity of tPA is increased approximately 400 fold. Proteoglycans have also been shown to enhance tPA activity [130]. Although the primary substrate for tPA is plasminogen, tPA can also cleave pro-HGF/SF into active HGF/SF [131].

Urokinase Plasminogen Activator

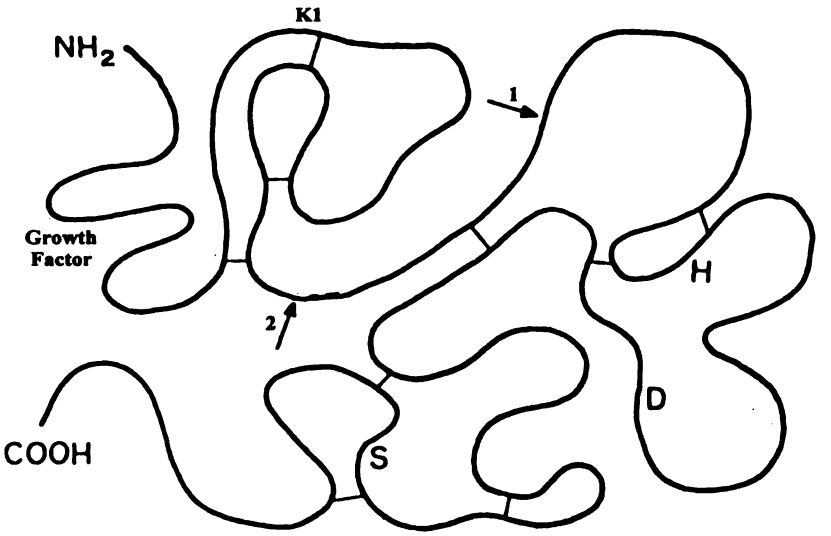


Figure 5: Diagram of uPA showing important structural domains modified from Alexander and Werb, 1991 [2]. Growth factor, kringle 1 (K1) and key amino acids of the acid site (H, S, D). Arrow 1 indicates cleavage site to convert scuPA to tcuPA. Arrow 2 indicates cleavage site separating uPA inot low molecular weight and amino terminal fragments.

Urokinase-type plasminogen activator (Figure 5) is synthesized as an inactive single-chained 54 kDa protein (scuPA; also called pro-uPA) [132]. Cleavage of the Lys_{158}-Ile_{159} peptide bond produces an active two-chained uPA held together by disulfide bonds [133]. The conversion to the two-chain form (tcuPA) is an important regulatory step. Factors that activate uPA include plasmin [134], kallikrein [133], Factor XIIa, cathepsin B [135] and MMP-3 [136]. The conversion of the single-chained form of uPA to two-chained uPA increases activity about

250-fold. Urokinase plasminogen activator has all the same structural domains as tPA except it lacks the first kringle. The C-terminal B chain contains the serine proteinase domain. The N-terminal A chains consists of a growth factor domain, a kringle domain and a interdomain linker. The growth factor domain has amino acid homology to EGF, but EGF cannot displace binding of uPA to its receptor [137]. Further proteolytic cleavage of uPA in the linker region gives rise to the growth factor domain and kringle, or amino terminal fragment (ATF), and the serine proteinase domain, or low molecular weight uPA (LMW-uPA).

Urokinase plasminogen activator can also convert the inactive proforms of HGF/SF [138] and macrophage stimulating protein (MSP) [139] to their active forms in the absence of plasminogen. It also has moderate affinity for heparin-like GAGs, mostly heparan sulfate, that appear to modulate uPA activity [140].

CONTROL OF PLASMIN ACTIVITY

The control of plasmin activity is mediated through three mechanisms: activation, inhibition and localization. Plasminogen is proteolytically cleaved between Arg_{560} and Val_{561} to generate active plasmin [141] by the plasminogen activators. Inhibition of plasmin activation by the plasminogen activators is mediated primarily by three related proteins; plasminogen activator inhibitor-1, 2 and 3 (PAI-1, PAI-2 and PAI-3). These inhibitors are members of the serine proteinase inhibitor (serpin) supergene family, that bind uPA or tPA in a 1:1 stoichiometry, and effectively block plasminogen activator activity [142]. Structurally, these serpins have an approximately 20 amino-acid-long exposed reactive center loop (RCL) [143], which inhibits their activity when inserted into the serine proteinase domain of the plasminogen activator [144].

Plasminogen Activator Inhibitor-1

Plasminogen activator inhibitor-1 is a 45 kDa single-chained protein [145] that is found in plasma at a concentration of 1 nM [108]. It is the most potent plasminogen activator inhibitor. Although synthesized in an active form, PAI-1 spontaneously converts to a more stable latent form [146]. Binding of active inhibitor to the ECM protein vitronectin stabilizes PAI-1 in its active conformation [147-151]. Plasminogen activator inhibitor-1 binds to the N-terminal somatomedin B domain (amino acids 1-44) of vitronectin. Vitronectin may also play a role in localization and concentration of active PAI-1 [152]. The PAI-1–plasminogen activator complex does not bind to vitronectin. Vitronectin also binds integrins, and accordingly both PAI-1 and integrins compete for vitronectin binding [153].

Plasminogen Activator Inhibitor-2

Plasminogen activator inhibitor-2 is undetectable in human plasma except during pregnancy. During the final stages of pregnancy, plasma PAI-2 concentrations of 100-300 ng/ml are detectable. The source of PAI-2 is the trophoblastic epithelium [154]. Additionally, PAI-2 is a major product of macrophages and monocytes in response to inflammatory conditions [155, 156] and PAI-2 has been observed in several neoplastic cells lines and malignant tissues. In monocytes, PAI-2 exists as both a nonglycosylated (46 kDa) and glycosylated (60 kDa) form. The nonglycosylated form is retained in the cytoplasm, while the more stable glycosylated form of PAI-2 is secreted to the extracellular milieu. In vivo, PAI-2 is believed to inhibit only uPA, although it also has a low affinity for tPA. In comparison to PAI-1, PAI-2 is 20-100 fold less efficient in inhibiting plasminogen activators. Recently, intracellular PAI-2 was shown to reduce TNF_{α} induced apoptosis. The mechanisms are unclear, although the region of PAI-2 responsible for this activity has been identified [157].

Plasminogen Activator Inhibitor-3

Plasminogen activator inhibitor type-3 (PAI-3; also known as protein C inhibitor) is present in high concentrations in plasma, urine and seminal fluid [158]. It is the most predominant plasminogen activator inhibitor identified bound to active uPA in biological fluids [158]. It is, however, unique from PAI-1 and PAI-2 because PAI-3 can bind and inhibit uPA reversibly [159]. Messenger RNA for PAI-3 was identified in both human and mice reproductive tracts (including the ovary), and male PAI-3 deficient knockout mice are infertile due to loss of the Sertoli cell barrier impairing spermatogenesis [160]. Recently, bovine PAI-3 was shown to be an effective inhibitor of plasmin as well as uPA [161].

Protease nexin-1

Protease nexin-I (PN-I) is a glycoprotein that is a less specific serpin than PAI-1, PAI-2 and PAI-3. It is an effective inhibitor of tcuPA and plasmin, but is slow inhibitor of tPA, and does not have affinity for scuPA. Similarly to PAI-1, PN-1 has been localized to the ECM of cultured fibroblasts [162]. Interestingly, mutant male mice deficient in PN-1 are infertile due to abnormalities in copulation plug formation [163]. Preliminary work by Bèdard et. al. [164] identified a periovulatory rise in PN-1 transcript and protein within the granulosal cells of bovine preovulatory follicles. In contrast, expression of PN-1 by granulosal cells was high during the entire periovulatory period in mouse preovulatory follicles [11].

Alpha$_2$-antiplasmin

The serpin α_2-antiplasmin is the main physiologic plasmin inhibitor. Alpha$_2$-antiplasmin is a 70 kd glycoprotein that is produced by the liver and is present in the serum at a concentration of 1 μM [108]. Alpha$_2$-antiplasmin binds to plasmin in a 1:1 stoichometry and interacts with

the region from Arg_{354} and Met_{355} to the Ser residue of the active site in plasmin [108]. Interestingly, α_2-antiplasmin is degraded by MMP-2 which may be a further means of regulating plasmin activity [165].

Alpha$_2$-macroglobulin

Alpha$_2$-macroglobulin is a key proteinase inhibitor of broad specificity. It is a large glycoprotein of approximately 725 kD, consisting of four identical chains (tetramer). The tetramer is arranged together as a pair of dimers, forming a complex with two reactive sites. Each of the reactive sites contains the sequence Arg 681-Val-Gly-Phe-Tyr-Glu-686 that acts as bait region and offers substrate specificity to many different proteins including plasmin. Alpha$_2$-macroglobulin is a "second line of defense" inhibitor and inactivates plasmin, tPA and uPA at a relatively low rates due to a low affinity for the enzymes [108]. The α_2-macroglobulin-proteinase complexes are removed by specific receptors (discussed below).

ACTIVATION AND CLEARANCE RECEPTORS FOR THE PLASMINOGEN ACTIVATOR/PLASMIN SYSTEM

Proteins at the cell surface also regulate plasmin activity. *Activation* receptors localize and, in some cases, potentiate plasminogen activation. *Clearance* receptors serve to remove plasmin and plasminogen activators from focal environments. Some activation receptors amplify plasmin activity by binding both plasminogen and plasminogen activators (coreceptors) and allowing them to interact similarly to the enzyme substrate complex. Additionally, some activation receptors protect the plasminogen activators from their inhibitors, while others simply localize plasminogen activators and hence plasmin activity to specific focal environments (e.g. cell membrane).

Plasminogen Receptors

A large number of diverse molecules have been implicated as plasminogen receptors [166]. These include gangliosides, α-enolase, adhesive glycoprotein complex IIb/IIIa, glycoprotein 330, and Heymann nephritis antigen. These receptors are found on many cell types including platelets, leukocytes, hepatocytes, neuronal cells, sarcoma cells, and endothelial cells [166]. Additionally, binding of plasminogen to these receptors does not interfere with activation of plasminogen to plasmin, and may possibly further help concentrate it by allowing plasminogen to accumulate on the cell surface.

Urokinase Plasminogen Activator Receptor

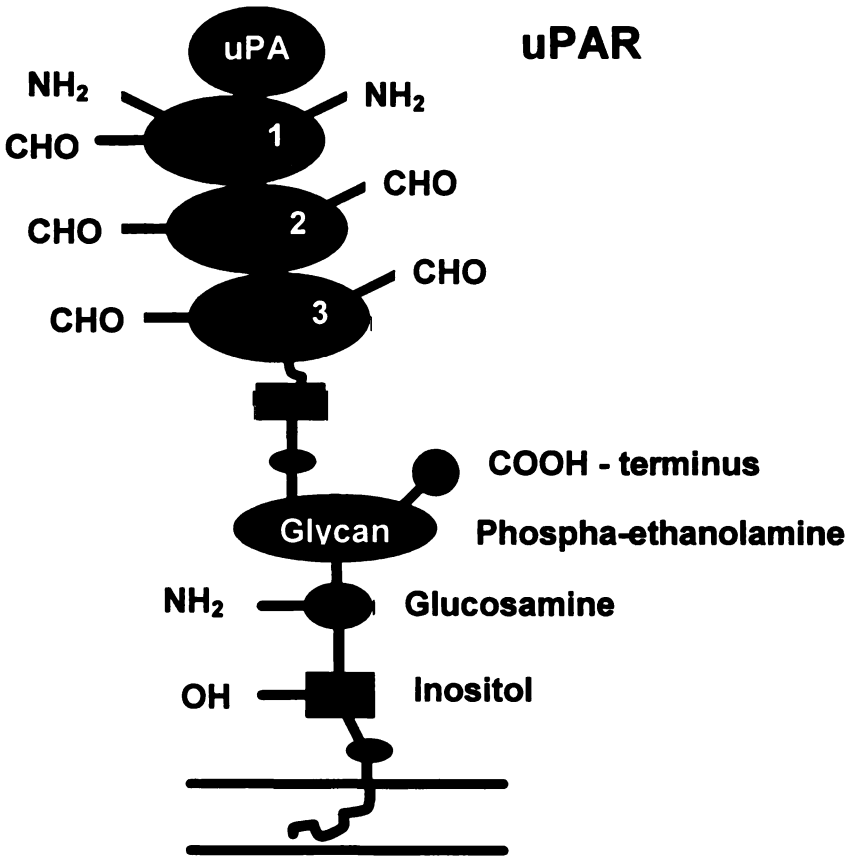


Figure 6: Diagram of the structure of uPAR modified from Wang, 2001 [175].

The uPA receptor (uPAR; Figure 6) is a highly glycosylated 55-60 kDa [167, 168] protein that is held to the cell membrane by a glycosyl-phosphatidyl inositol (GPI) anchor. Examination of the amino acid structure of uPAR shows a triple 90 amino acid repeat and hence homologous domains. One function of the uPAR is to focus and confine cell-associated uPA activity. The uPAR can also activate a cell specific signal transduction pathway. Upon binding of uPA, changes in tyrosine kinase activity and accompanying changes in gene expression were observed in monocytes, epithelial cells and ovarian cancer cell lines [169-173]. Activation of uPAR in ovarian cancer cells results in rapid c-fos expression [169, 172]. Additionally, binding of uPA to uPAR increases protein kinase C_ε activity [174] in the WISH epithelial cell line and transcription of ERK1 and ERK2 [175] in MCF-7 breast cancer cells. Upon ligand binding, uPAR can be co-immunoprecipitated with numerous tyrosine kinases including hck, fyn, lck, lyn, fgr, Jak1 and Tyk2 [176]. Activation of receptor bound uPA on the cell surface is implicated in the initiation of focal proteolytic mechanisms that permit metastasis. The uPAR is overexpressed in cancer cells of colon, breast, ovarian, lung, kidney, liver, and bone and therefore, uPAR may be a key molecule in the process of cancer invasion and metastasis [176]. Furthermore, the half-life of receptor bound uPA is extended to several hours. Cells producing uPAR may or may not produce uPA suggesting both autocrine and paracrine cell functions. Some cell types with uPAR also contain plasminogen receptors as shown in Figure 7. This may suggest a mechanism for co-localization of uPA and plasminogen resulting in enhanced plasmin formation. Both pro-uPA and uPA bind to the uPAR with similar affinity. Domain 1 of uPAR binds the growth factor domain of uPA. Additionally, the binding of scuPA to uPAR leads to a 20-fold increase in conversion of scuPA to the active tcuPA. tPA does not bind to the uPA receptor. Additionally, uPA receptors bound with uPA or scuPA ligands are not internalized but those containing uPA-PAI are endocytosed and degraded. Although the

growth factor domain has similar homology to the EGF receptor, EGF cannot displace binding of uPA to its receptor [137]. The uPAR also shares an affinity for the same domain of vitronectin as PAI-1, and hence they compete for binding. Binding of uPA to uPAR increases the receptor's affinity for vitronectin. The uPAR does not bind other ECM proteins such as fibronectin or laminin. In vitro, uPAR been shown to bind β2-integrins and may also have some affinity for β1 and β3 integrins as well. Immunocytochemistry experiments have also revealed that uPAR, both in the absence and presence of uPA, collects at specific cell-ECM contact sites, similar to clusters of integrin-actin focal contacts. Therefore, uPAR-vitronectin and/or uPAR-integrin associations may assist in the formation of these clusters.

Recently, a cellular receptor was identified that interacts with uPAR. This receptor, uPAR associated protein or uPARAP, is a transmembrane protein that is structurally related to the

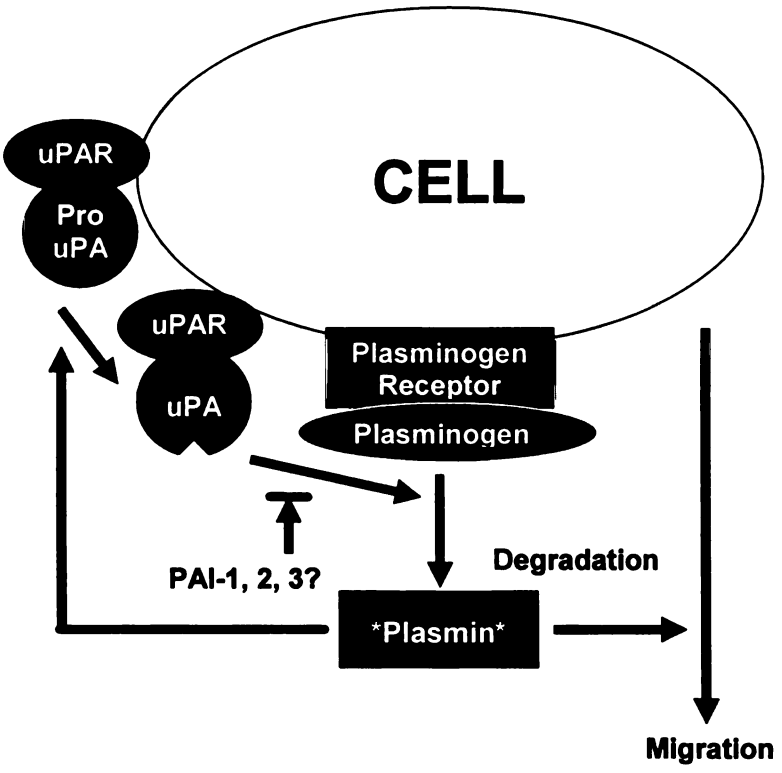


Figure 7: Diagram showing the uPAR proteolytic pathway modified from Wang, 2001 [175].

macrophage mannose receptor protein family. This family of receptors appears to function as internalization receptors [177]. Interestingly, uPARAP was shown to bind to rat collagenase 3 (MMP-13) in combination with the LRP receptor followed by internalization [178-180].

LDL-like Receptor Protein

The plasminogen activator inhibitors are also important for clearance of plasminogen activators. Upon binding of plasminogen activator to the plasminogen activator inhibitor, the plasminogen activator inhibitor undergoes a conformational change into what is called the relaxed conformation. Transformation to the relaxed conformation exposes a structural motif that binds to the multifunctional LRP clearance receptor. Binding of plasminogen activator inhibitor to the uPA-uPAR complex is also required for binding to LRP. The LRP-complexes then migrate to clathrin-coated pits and are internalized. In the early endosomes, the plasminogen activator-plasminogen activator inhibitor is targeted to lysosomes and the uPAR and (or) LRP are recycled back to the cell surface [181]. Disruption of the LRP gene compromises physiological processes that involve the plasminogen activator cascade including embryo implantation [182, 183] and cell migration. Similarly, the plasminogen activator-plasminogen activator inhibitor complex can also be internalized and degraded through the very low density lipoprotein (VLDL) receptor [184].

Co-Receptors of the Plasminogen Activator/Plasmin System

Recently, two co-receptors have been identified that simultaneously bind both plasminogen and tPA. This co-assembly mechanism both localizes and potentiates proteinase activity. Amphoterin is one co-receptor that is expressed on neuroblastoma cells [185-188]. The other co-receptor is annexin II, which was identified on human vascular endothelial cells [189]. Annexin II is a calcium-regulated phospholipid-binding protein [190, 191] that lacks a

transmembrane domain [192]. Annexin II mediated assembly of tPA and plasminogen results in a 60-fold increase in local plasmin production. Both amphoterin and annexin II can only potentiate plasmin production once since they are also substrates for plasmin.

Genetic Models for Studying the Physiological Role of the Plasminogen Activator/Plasmin System Genes

Table 1: Fertility and abnormal phenotypes of mice genetically deficient in plasminogen activator/plasmin system genees.

Gene(s)	Fertility	Abnormal Phenotypes
tPA	Normal	↓ endotoxin-induced thrombosis ↑ thrombolytic potential ↓ cerebellar granule cell migration
uPA	Normal	↓ endotoxin-induced thrombosis ↑ organ fibrin deposits
tPA/uPA	♀ reduced	↓↓ endotoxin-induced thrombosis ↑↑ thrombolytic potential ↑↑organ fibrin deposits 20% dead by 17 weeks old
plasminogen	♀ reduced/Normal	↓↓ endotoxin-induced thrombosis ↑↑ thrombolytic potential ↑↑organ fibrin deposits 20% dead by 17 weeks old
uPAR	Normal	↓↓ neutrophil recruitment
PAI-1	Normal	↑ endotoxin-induced thrombosis ↓ thrombolytic potential ↓ tumor angiogenesis
PAI-2	Normal	None detected
PAI-3	♂ reduced	None detected
α_2-antiplasmin	Normal	↑↑ endotoxin-induced thrombosis ↓↓ thrombolytic potential
Protease nexin-1	♂ reduced	None detected
α_2-macroglobulin	Normal	↓ Endotoxin lethality

The generation of null mutant mice or the identification of humans deficient in the plasminogen activator/plasmin system genes has shed important insight into the physiological roles for these proteins. To date, mice have been generated with targeted deletion of the plasminogen, tPA, uPA, uPAR, PAI-1, PAI-2, PAI-3, PN-1, α_2-antiplasmin, and α_2-macroglobulin genes. In addition, some of the above lines have been crossed to generate combination knockouts. The phenotypes of these mutant mice are outlined in Table 1.

Mice deficient in both tPA or uPA develop normally and are fertile. However, tPA deficient mice have increased endotoxin-induced thrombosis and reduced thrombolytic potential [193], and cerebellar granule cell migration is reduced by 51% [194].

Similarly, mice deficient in uPA also displayed increased endotoxin induced thrombosis, however some of these mice also developed rectal prolapse and spontaneous fibrin deposits in the intestines, liver and ulcerated skin. Additionally, macrophages collected from uPA deficient mice, but not wild-type mice fail to degrade fibrin. Not surprisingly, mice deficient in both tPA and uPA had exacerbated symptoms of the individual phenotypes and approximately 20% died at approximately 17 weeks of age. In addition, the mice lacking plasminogen activator function had reduced numbers of offspring per litter [193]. Leonardsson et. al. 1995 [13] investigated the exact cause of the reduced fertility of 25-day-old $tPA^{-/-}/uPA^{-/-}$ mice using gonadotropin-induced ovulation. Histological examination revealed that double mutant mice had an impaired ovulation mechanism, such that ovulation efficiency was reduced by 26% compared to wild-type mice. This result indicates that plasminogen activation plays a role in the ovulatory response, although neither tPA nor uPA individually or in combination is absolutely required for successful ovulation. In the mouse ovary, the loss of an individual plasminogen activator is functionally complemented by the remaining plasminogen activator. However, in the single plasminogen activator deficient mice, there is not any compensatory up-regulation of the remaining plasminogen activator [13]. Surprisingly, plasminogen null mutant mice, hence lacking active plasmin, have normal fertility and ovulation rates during spontaneous estrous cycles and in response to superovulation regimes [195]. However, the different phenotypes of the $tPA^{-/-}/uPA^{-/-}$ versus the plasminogen deficient mice may be due to some of the recently described

nonproteolytic functions of both tPA and uPA. These functions will be described in detail below.

Kim et. al. [196] reported that exogenous tPA added to cortical cultures protected the cells from zinc-induced cell death, and the addition of plasminogen activator inhibitors did not reduce this protective effect. In addition, plasmin did not provide a protective effect. This would suggest that the protective function of tPA is not related to its ability to produce plasmin.

Nonproteolytic actions of uPA were described previously, as binding of uPA to uPAR stimulation of a signal transduction pathway. Although, uPAR mutant mice are fertile and appear normal, their neutrophil recruitment in response to injury is severely compromised [197, 198]. Furthermore, the uPA ligand is not required for this function as neutrophil recruitment is normal in uPA deficient mice [198].

Mice deficient in PAI-1 are also fertile. However, they display faster clot lysis and reduced endotoxin-induced venous thrombosis, and are more susceptible to increased fibrin deposition after copper-induced arterial wall injury [199]. In addition, host mice deficient in PAI-1 displayed decreased local invasion and tumor vascularization of transplanted malignant keratinocytes [200]. In PAI-1 null mice, tumor angiogenesis was reduced 60% compared with wild-type mice using a Matrigel implant assay. Addition of exogenous PAI-1 to the assay restored angiogenesis, while mice over expressing PAI-1 had increased tumor angiogenesis 3-fold [201]. To date, PAI-1 is the only member of the plasminogen activator/plasmin system where human homozygous deficient individuals (seven) have been identified. These individuals display increased intracranial and joint bleeding after mild

trauma, prolonged surgical bleeding, severe menstrual bleeding, and frequent bruising. No other abnormalities were observed, indicating the primary function of PAI-1 is to regulate vascular fibrinolysis [202].

Knockout mice that are deficient in PAI-2 are fertile and appear completely normal. PAI-2 is a produced by macrophages, however, response to infectious challenge and (or) endotoxin challenge was not different between wild type and PAI-2 deficient mice [203].

The α_2-antiplasmin deficient mice are also fertile but exhibit increased clot lysis time and decreased endotoxin-induced thrombosis similar to the PAI-1 knockout mice. However, their bleeding times were similar to wild type mice [204]. Similarly, transgenic mice deficient in $_2$-macroglobulin did not show any defects in their viability, fertility or health. Rodents, unlike other mammals have two different types of wide spectrum proteinase inhibitors: α_2-macroglobulin and murinoglobulins. However, murinoglobulin and α_2-macroglobulin double knockout mice have a similar phenotype and exhibit normal viability, fertility and health [205].

Female mice generated with a deficiency in PN-1 were normal, but males showed a marked decrease in fertility. Further examination of the reduced fertility of the male mutant mice revealed a defect in vaginal plug formation, as semen failed to coagulate after copulation [163].

Role of the Plasminogen Activator/Plasmin System in Ovulation and Luteal Formation

A number of physiological and pathological processes require proteolytic breakdown of the ECM, including cell migration, tissue remodeling, inflammation, wound healing, angiogenesis,

neoplasia, muscle regeneration, and ovulation. Numerous roles for plasmin in ovulation are hypothesized, and plasmin may play a multifaceted role in this process. Plasmin may prevent premature fibrin (clot) formation in the preovulatory follicle, albeit fibrinogen concentrations in follicular fluid are slightly lower than serum. Near the time of ovulation, the preovulatory follicle becomes hyperemic due to breakage of blood vessels in the thecal layer. Plasmin may prevent normal fibrin formation thereby allowing the oocyte to escape [27]. Plasmin also degrades ECM components of the follicle, including collagens III and IV, as well as proteoglycans, fibronectin and laminin. The collagens are believed to provide the primary structural integrity to the follicle, while the other ECM components such as proteoglycans, laminin and fibronectin may provide further support. Additonally, plasmin's digestion of these other ECM proteins may allow other proteinases such as the collagenases, better access to the remaining collagens. Furthermore, plasmin activation of the proforms of several MMPs, specifically collagenase I (MMP-1), the gelatinases (MMP-2 & 9) and stromelysin-1 (MMP-3) may facilitate ovulation. A critical role of MMPs in follicular rupture is supported by increases in ovarian collagenase activity prior to ovulation [102, 103, 206], as well as suppression of ovulation by collagenase inhibitors [207, 208]. Plasmin and MMPs together can degrade all of the ovarian ECM that would be necessary for follicular rupture and luteal development.

Major alterations of tissue morphology are often associated with apoptosis or programmed cell death. Support for a role of apoptosis in the ovulatory process is mounting. There is significant apoptotic cell death in the OSE, thecal and granulosal cell layers, particularly near the apex of the follicle [103, 206, 209-211]. High doses of the prostaglandin inhibitor indomethacin block apoptosis in both OSE and granulosal cells [212]. Plasmin has been shown to indirectly stimulate apoptosis by cleavage of membrane bound TNF_{α}, releasing the

extracellular domain into the extracellular milieu [213]. Receptors for TNF_α are present on nearly all nucleated cells [214, 215], and binding of the extracellular domain of TNF_α to its receptor conveys an apoptotic signal [216, 217]. Recently, OSE cells were shown to secrete uPA in ewes. The OSE cells upon stimulation by estradiol and then LH, secreted uPA basally, towards the tunica albuginea. It is possible that uPA may be mediating plasmin-TNF_α induced apoptosis of follicular apical cells leading to stigma formation and ovulation [211]. Furthermore, intracellular PAI-2 may protect against TNF_α -induced apoptosis in specific cell types or regions where programmed cell death needs to be prevented [157, 218].

REGULATION OF THE PLASMINOGEN ACTIVATOR/PLASMIN SYSTEM DURING THE PERIOVULATORY PERIOD

Table 2: Ovarian Regulation of the Primary Plasminogen Activator/Plasmin System Genes

	tPA	uPA	uPAR	PAI-1	PAI-2
RNA	↑ Rat uPA*	↑ Mouse	↑ Rat	↑ Rat	↑ Mouse ↑ Human
RNA Localization	Rat GL	GL	Rat GL, TL	Rat TL	Mouse TL Human GL
Protein Localization	Rat GL Monkey GL	Sheep OSE	Rat GL, TL	Rat TL	N.D.
Activity	↑ Rat ↑ Pig	↑ Mouse ↑ Sheep	N.D.	↓ Pig	N.D.
*=controversial, see text; GL=Granulosal layer; TL=Thecal layer; N.D.=not done					

Most of the data available on the regulation of the plasminogen activator/plasmin system during the periovulatory period is from rodent species and limited data is available from other species. A summary of the data is shown in Table 2.

In the mouse, sheep and the pig only one plasminogen activator or the other is markedly increased near the time of ovulation. In the mouse, uPA is the most abundant and dramatically upregulated plasminogen activator following hCG injection [14]. In addition,

plasminogen activator activity is reduced by 90% in the ovaries of uPA null mutant mice [219]. However, a small induction of tPA mRNA [13, 219] specifically in the thecal layer [11] has been reported in mice and is in fact sufficient to support normal ovulation in uPA-deficient mutant mice [13, 219]. In contrast, nearly all of the plasminogen activator activity in pig preovulatory follicles could be neutralized by tPA antibodies [220]. Results in sheep indicate that uPA is obligatory for ovulation, as intrafollicular injection of uPA, but not tPA antibodies disrupt the ovulatory process [6]. In the rat, tPA mRNA expression is increased in response to the gonadotropin surge. However, the regulation of uPA expression during the periovulatory period in the rat is controversial. Li et. al., 1997 [221] observed a decrease in uPA mRNA and protein levels in rat preovulatory follicles after exposure to hCG. In contrast, Macchione et. al., 2000 [222] reported that both plasminogen activators are present in rat preovulatory follicles near the time of ovulation, but that the thecal and granulosal layers respond differently to the gonadotropin surge. In the above study, mRNA for tPA was increased in both the thecal and granulosal layers. However, uPA mRNA was increased in the thecal layer but decreased in the granulosal layer following exposure to the gonadotropin surge. Similar regional differences in tPA activity have also been observed in pig and rat preovulatory follicles [223, 224]. In contrast, Colgin and Murdoch, 1997 [6] observed higher levels of uPA activity in the apex versus the base of preovulatory ovine follicles and the ovarian surface epithelial cells were the primary source of the elevated uPA activity in the follicle apex [225]. Similarly, during the periovulatory period in the rat, uPAR mRNA and protein were increased in both the granulosal cells and the residual ovarian tissue [221]. Endothelial cell migration is a key component of luteal development, as capillaries must penetrate the avascular granulosal cell layer following ovulation and form the rich blood supply necessary to support luteal

development [226]. Localization of uPA to endothelial cells near the site of capillary formation in developing CL was reported previously [227].

In the rat and monkeys, PAI-1 mRNA is transiently upregulated after hCG but then subsequently declines in preovulatory follicles near the time of ovulation [223, 228]. Furthermore, high levels of tPA mRNA continue to be expressed in rat and monkey preovulatory follicles near the time of ovulation, several hours after the decline in PAI-1 mRNA. Near the time of ovulation, plasminogen activator activity in preovulatory pig follicle was increased, with a concomitant decrease in plasminogen activator inhibitor activity [220]. This suggests that a decrease in PAI-1 in the face of continued tPA activity may play a role in regulation of follicular rupture. In contrast, PAI-1 mRNA was not upregulated until after ovulation in the mouse [11, 13]. In monkeys and rats, PAI-1 mRNA was also localized primarily to the thecal layer [19], but additional expression was observed in the ovarian stroma of rat preovulatory follicles [223].

Very limited information is available regarding the temporal and cell specific regulation of PAI-2 mRNA during the periovulatory period. Messenger RNA for PAI-2 has been detected in both human and mouse ovarian tissues. Both human cumulus cells and granulosal-luteal cells collected from patients (36 h post hCG; a few h prior to ovulation) undergoing in vitro fertilization have been shown to express PAI-2 mRNA [229]. A small increase in PAI-2 mRNA abundance was also observed in mouse ovaries 4 h post hCG injection. The localization of PAI-2 mRNA in the mouse ovary was restricted primarily to a few individual cells within the thecal layer that were believed to be macrophages [13].

Plasmin has been detected previously in the follicular fluid of cattle [5] and other species including the rabbit, horse and pig [12, 220, 230, 231]. Plasmin in follicular fluid may help degrade high molecular weight proteoglycans causing a decrease in follicular fluid viscosity facilitating oocyte escape [24]. Plasmin mediated degradation of fibrinogen [232-236] may also prevent premature blood clot formation in the follicular antrum prior to rupture. Liu et. al., 1986 proposed that plasmin may assist in cumulus expansion by termination of oocyte-cumulus cell communication [237]. Thus, increased follicular fluid levels of plasmin may promote conditions that facilitate ovulatory release of the oocyte.

In sheep, intrafollicular injection of the plasmin inhibitor, α_2-antiplasmin, suppresses ovulation of preovulatory follicles [206]. Similar reduction of ovulation efficiency was observed by intrabursal injection of α_2-antiplasmin in the rat [8]. Peak plasminogen-dependent plasmin activity was detected in the stigma of rat preovulatory follicles two hours before ovulation [238].

Pathways for Regulation of Plasminogen Activator/Plasmin System Gene Expression

The pituitary polypeptide hormones LH and FSH that mediate their effect by increasing levels of cAMP regulate many biological processes including gonadotropin-induced ovulation. The LH surge is the obligatory signal required for the biochemical cascade that leads to ovulation. LH binds to a G-protein coupled receptor, which activates adenylate cyclase and increases cAMP, which in turn activates protein kinase A (PKA). FSH, LH, forskolin and bromo-cAMP induce tPA mRNA levels in rat granulosal cells up to 20-fold [9, 239]. Cyclic AMP regulation of many eukaryotic genes, including the plasminogen activators, is mediated through

a cAMP response element (CRE) in their promoters. A nuclear CRE-binding protein (CREB) promotes transcription when phosphorylated by PKA. In rats, the tPA promoter contains an exact CRE sequence, while promoters in the mouse and human differ in one central nucleotide, modifying the site to an activator protein-1 (AP-1) site. Following the conversion of the mouse and human CRE-like sequences to rat consensus CRE these promoters became cAMP responsive. In contrast, the rat promoter following conversion of the consensus CRE to the corresponding mouse and human CRE-like sequence lost the ability to efficiently respond to cAMP [240].

Primary cultures of rat granulosal cells have been extensively used to study the effects of different hormones, growth factors, etc. on tPA mRNA expression and (or) activity. Forskolin and or cell-permeable cAMP analogs stimulate tPA expression in rats granulosal cells [237] and also induce ovulation in perfused rabbit and rat ovaries [44, 241]. The latter investigators also noted that phorbol esters that activate the PKC pathway, also induce ovulation in the rabbit and rat. Stimulation of tPA synthesis by rat granulosal cells can also be induced by bFGF and EGF, suggesting other pathways may be capable of inducing tPA synthesis [242, 243]. In addition, steroid hormones such as androgens and glucocorticoids, can modulate the synthesis of tPA mRNA induced by FSH, GnRH and EGF. However, androgens and glucocorticoid treatments alone do not have any effect on tPA mRNA synthesis [244, 245].

Synthesis of PAI-1 is regulated by the many of the same factors that regulate the expression of tPA. Similarly, the PAI-1 and uPAR promoters are also regulated by the AP-1 transcription

factor and are activated in response to PKC, growth factors and cytokines [246-248]. The promoter regions of bovine tPA, uPA, uPAR, PAI-1 and PAI-2 have not been examined.

Both the P_4R and COX-2 deficient mice fail to ovulate. However, the downstream targets dependent on P_4R and COX-2 mediated prostaglandin synthesis, particularly the proteolytic enzymes necessary for ovulation, have not been determined. However, substantial evidence links both P_4R as well as prostaglandins (COX-2) to the plasminogen activator/plasmin system. Strickland and Beers, 1976 [244] observed that cultured rat granulosal cells responded to exogenous PGE_1 and PGE_2, but not $PGF_{2\alpha}$ with increased secretion of tPA. Tanaka, 1992 [249] demonstrated that epostane or indomethacin inhibit ovulation and ovarian plasminogen activator activity in a dose dependent manner in the rat model. Treatment with exogenous progesterone reversed the effects of epostane. Moreover, a specific antagonist for the P_4R, Org 31710, suppresses rat follicular plasminogen activator activity [250].

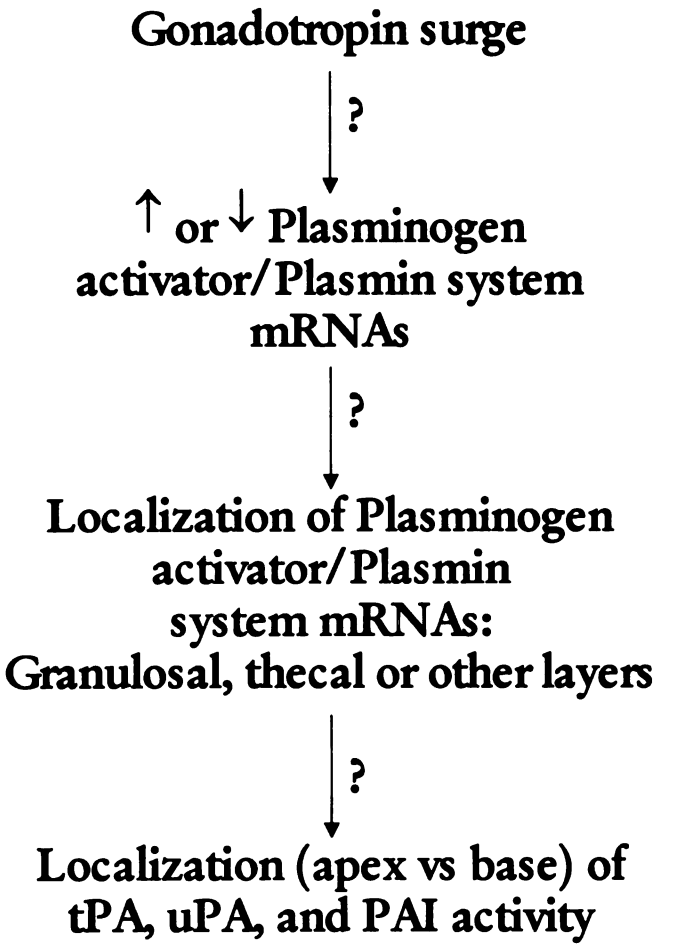


Figure 8: Diagram showing specific aims of experiments designed herein.

The plasminogen activator/plasmin system has been implicated in many areas of reproduction including embryo implantation, spermatogenesis, fertilization, parturition and ovulation. Within the ovary, regulation of the plasminogen activator/plasmin system has been primarily investigated in rodent species, however data from other species, particularly farm animals is lacking. Experiments in rodents suggest a requirement for plasminogen activator/plasmin for optimum ovulation rates. However, the gonadotropin surge only up-regulates one plasminogen activator (tPA or uPA) in rodents and it is different depending on the species (see Table 2, page 35). Further understanding of regulation, localization, expression, and activity of these enzymes will help clarify their potential role in ovulation. This information may ultimately be used as the model for future functional studies to test the requirement of the plasminogen activator/plasmin system for ovulation in

cattle. An increased understanding of the regulation of ovarian function is required for development of improved methods to enhance reproductive efficiency in livestock. Furthermore, similar to the bovine, the role of plasminogen activator/plasmin system is unclear in human reproduction. Information collected in cattle may ultimately be more relevant to the human than current information derived from rodents since human and bovine both undergo dominant follicle selection and normally only ovulate a single oocyte. In addition, altered plasminogen activator/plasmin system homeostasis is linked to Polycystic Ovarian Disease (PCOD). The symptoms of this disease include abnormal endocrine profiles, reduced cyclicity and ovulation, elevated blood levels of PAI-1 [15], and excessive adhesions coating the ovary. Elevation of PAI-1 may reduce plasmin directed proteolysis on the ovarian surface allowing adhesions on the surface to accumulate [16, 17].

Due to differences in the regulation of plasminogen activator/plasmin system components in rodents and domestic livestock, these results can not be extrapolated to other species. Additionally, since rodents are litter bearers, the role of the plasminogen activator/plasmin system in a monoovulatory species should be investigated. This will not only lead to a better understanding of how periovulatory development is influenced by the plasminogen activator/plasmin system, but may also provide a model system more potentially applicable to humans. Many of the experiments proposed herein could not be completed in the human due to ethical and financial issues. **My hypothesis is that the gonadotropin surge induces the increased expression of plasminogen activator/plasmin system components during the periovulatory period in cattle. These components direct the temporal and spatial production of plasmin that may be important for the ECM remodeling during ovulation and (or) luteal development.**

To test this hypothesis, I will investigate the effect of the gonadotropin surge on the localization and regulation of plasminogen activator/plasmin system components in bovine follicles (Figure 9). Results of experiments focused on tPA, uPA and plasmin are described in Chapter 3, whereas results of experiments focused on PAI-1 and PAI-2 are described in Chapter 4.

Chapter 3

Gonadotropin Surge-Induced Upregulation of the Plasminogen Activators (Tissue Plasminogen Activator and Urokinase Plasminogen Activator) and the Urokinase Plasminogen Activator Receptor within Bovine Periovulatory Follicular and Luteal tissue[1]

[1] This chapter has been accepted by "Biology of Reproduction."

ABSTRACT

This study examined the effect of the preovulatory gonadotropin surge on the temporal and spatial regulation of tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA) and uPA receptor (uPAR) mRNA expression and tPA, uPA and plasmin activity in bovine preovulatory follicles and new CL collected at approximately 0, 6, 12, 18, 24 and 48 h after a GnRH-induced gonadotropin surge. Messenger RNAs for tPA, uPA and uPAR were increased in a temporally specific fashion within 24 h of the gonadotropin surge. Localization of tPA mRNA was primarily to the granulosal layer while both uPA and uPAR mRNAs were detected in both the granulosal and thecal layers and adjacent ovarian stroma. Activity for tPA was increased in follicular fluid and the preovulatory follicle apex and base within 12 h following the gonadotropin surge. The increase in tPA activity in the follicle base was transient, whereas increased activity in the apex was maintained through the 24 h timepoint. Activity for uPA increased in the follicle apex and base within 12 h of the gonadotropin surge and remained elevated. Plasmin activity in follicular fluid also increased within 12 h following the preovulatory gonadotropin surge and was greatest at 24 h. Our results indicate that mRNA expression and enzyme activity for both tPA and uPA were increased in a temporally and spatially-specific manner in bovine preovulatory follicles following exposure to a gonadotropin surge. Increased plasminogen activator and plasmin activity may be a contributing factor in the mechanisms of follicular rupture in cattle.

INTRODUCTION

The preovulatory gonadotropin surge initiates the ovulatory process and subsequent corpus luteum (CL) formation. Both of these events feature extracellular matrix (ECM) degradation and tissue remodeling. Such restructuring requires the targeted action of proteolytic enzymes. One such family of enzymes implicated in above processes is the plasminogen activators. The plasminogen activator system includes plasminogen/plasmin, two specific plasminogen activators, cell surface plasminogen activator receptors and several plasminogen activator inhibitors. Plasminogen is abundant in blood and peripheral tissues and consequently, regulation of plasmin activity occurs at several levels. The plasminogen activators, tissue (tPA) and urokinase plasminogen activator (uPA), convert plasminogen into its active form plasmin in the extracellular milieu. Urokinase plasminogen activator can also bind to its cell surface receptor (uPAR) and form a stable complex for several hours [168, 251]. One function of the uPA-uPAR complex is to focus uPA directed plasmin activity at the cell surface.

Degradation of the apical follicular layers is ultimately required for oocyte escape. Ovarian targets for plasmin include fibrin, fibrinogen, types III and IV collagen, fibronectin, laminin and proteoglycans [1, 2]. In addition to a direct role in ECM degradation, plasmin can also activate the proenzyme form of several matrix metalloproteinases (MMP) implicated in follicular rupture including MMP-1, 3, and 9 [113-116, 119]. Furthermore, due to the relatively high concentration of plasminogen in tissue and body fluids, a small increase in plasminogen activator causes a large increase in plasmin in the extracellular milieu. Plasmin together with the MMP can potentially degrade all ECM components in the ovary. Therefore, regulation of plasminogen activation may be a key regulatory step in the ovulatory process.

Several lines of evidence support a potential role of the plasminogen activators and plasmin in ovulation. Plasmin can decrease the tensile strength of the bovine follicle wall [252]. Furthermore, administration of antibodies against uPA [6] or tPA [7, 8] reduces ovulation rate in sheep and rats, respectively. Prior to ovulation, tPA is the primary plasminogen activator induced in pig preovulatory follicles [224]. In contrast, uPA is the predominant plasminogen activator induced in mouse and sheep preovulatory follicles during the same time period [6, 11]. Thus, regulation of tPA and uPA in preovulatory follicles appears species specific. Furthermore, the regulation and regulatory role of the plasminogen activator system during the periovulatory period in monotocous species, such as cattle, is not understood. Therefore, our objectives were to investigate the effect of the preovulatory gonadotropin surge on the localization and regulation of mRNAs for the plasminogen activators (tPA, uPA) and the cell surface receptor for uPA (uPAR) and on tPA, uPA and plasmin activity in bovine preovulatory follicles. We observed a pronounced temporally and spatially specific increase in tPA, uPA and uPAR mRNA and tPA, uPA and plasmin activity in bovine periovulatory follicles in response to the gonadotropin surge. These results support a proposed role of gonadotropin surge-induced increases in both plasminogen activators in the ovulatory process and (or) the morphological changes associated with the ovulatory follicle-corpus luteum transition in cattle.

MATERIALS AND METHODS

Animal Care

Use of animals was approved by the All University Committee on Animal Use and Care at Michigan State University (Approval # 04/98-056-00).

Experimental Model

Follicle development and timing of the preovulatory gonadotropin surge were synchronized in Holstein cows using the Ovsynch procedure (GnRH-7d-$PGF_{2\alpha}$-36h-GnRH) [253]. Daily ultrasound analyses were performed after the first GnRH injection until the time of follicle collection to verify follicle synchrony and to exclude animals that turned over a new follicular wave prior to the second GnRH injection. Ovaries containing ovulatory follicles or new CL were collected by colpotomy at 0, 6, 12, 18, 24 and 48 hr (follicles: 0, 6, 12, 18 and 24 h; one day old CL = 48 h) after the second GnRH injection. Blood samples were collected at the time of $PGF_{2\alpha}$ injection and at the time of the second GnRH injection. Serum progesterone concentrations in these samples were measured by RIA (Diagnostic Products Corporation, Los Angeles, CA) to ensure that all animals included in the study responded to the $PGF_{2\alpha}$ injection with a decrease in serum progesterone, indicating CL regression. Intraassay and interassay coefficients of variation were 5.6 and 9.1% respectively. To verify that none of the animals included in the study exhibited a preovulatory gonadotropin surge prior to the second GnRH injection, three blood samples at 15-min intervals were collected every 8 h beginning 16 h after the $PGF_{2\alpha}$ injection until the time of ovariectomy or GnRH injection. A premature LH surge was not detected in any of the animals included in the 0 h (pre-gonadotropin surge group). In order to confirm that a gonadotropin surge was elicited by the second GnRH injection, blood samples were also collected every hour for 4 h after the second GnRH injection. In the remaining animals, a LH surge occurred only after GnRH injection, verifying control of timing of the gonadotropin surge in our model system. Concentrations of serum LH were measured by RIA [254, 255]. Intraassay and interassay coefficients of variation were 5.8 and 15.6% respectively.

Tissue Collection

For mRNA quantification and enzyme activity assays, ovaries containing the preovulatory follicle or new CL were collected at 0, 6, 12, 18, 24 and 48 h (n=5-6 each; total 35) following the second GnRH injection. Following ovariectomy, the ovulatory follicle or new CL was isolated by cutting away all remaining ovarian stroma and small follicles such that the ultrastructure at the apex of the follicle remained intact. Follicular fluid was aspirated, centrifuged, aliquoted and stored at -20°C until activity assays. Follicles were then sagitally cut in half. One half was used for total RNA isolation. For protein analysis, the remaining half was cut transversely in two equal pieces, one containing the follicle apex and one the base. New CL collected 48 h post GnRH injection were only used for mRNA analyses. Samples were frozen at -80°C within 15 min of ovariectomy. For in situ hybridization, ovaries containing the ovulatory follicles were collected at 0, 6, 12, and 24 h (n = 3 each; total 12) following GnRH injection. Ovulatory follicles were dissected from the ovary, immediately immersed in embedding medium, frozen over liquid nitrogen vapors, and stored at -80°C until sectioned.

Preparation of cDNA probes for tPA, uPA and uPAR

The cDNAs for tPA, uPA, uPAR and ribosomal protein L19 (RPL19; housekeeping gene) have all been previously cloned in the bovine. Using the reported sequences [tPA (Genbank Accession X85800); uPA (Genbank Accession X85801); uPAR (Genbank Accession S70635); RPL19 (Genbank Accession AF270675)], oligonucleotides primers were prepared and used in combination with RNA isolated from bovine corpora lutea in the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) to amplify cDNA that encode for bovine tPA (328 bp), uPA (351 bp and 210 bp), uPAR (409 bp) and RPL19 (366 bp) PCR

products were subcloned into pBluescript SK(+) vectors (Stratagene, La Jolla, CA) and identities and orientations confirmed by fluorescent dye primer sequencing. Two separate cDNA were generated for uPA for combined use for in situ hybridization analysis (see below).

Characterization of tPA, uPA, and uPAR mRNA abundance

Total RNA was isolated according to the manufacturer's instructions using the Trizol reagent (Invitrogen, Carlsbad, CA). To determine transcript size and number and to optimize specificity of hybridization conditions, approximately 15 μg pooled RNA from each sample per timepoint was subjected to Northern analysis [256]. For quantitation of tPA, uPA and uPAR mRNA abundance, 5 μg total RNA from each sample was applied in duplicate to a Zeta probe nylon membrane (BIO-RAD, Hercules, CA) using a dot blot apparatus (BIO-RAD, Hercules, CA [256]. Northern and dot blot analysis was then carried out using specific bovine tPA, uPA, uPAR or ribosomal protein L-19 (RPL19) ^{32}P-labeled cDNA probes generated by the polymerase chain reaction (PCR). RPL19 was used for normalization purposes. Each 20 μl PCR reaction included 1X PCR buffer, 2.5 mM $MgCl_2$, 1.6 μM each of dATP, dGTP, dTTP, 0.25 μM of each primer, 100 pg DNA template, 1.5 U Taq polymerase, and 0.825 μM [^{32}P]dCTP (3000 Ci/mmole; NEN® Life Science Products, Boston, MA). The amplification conditions were: 95°C for 5 min; 94°C for 0.5 min, 52°C for 1 min, 72°C 1.5 min for 40 cycles; 72°C for 10 min; hold at 4°C. After amplification, the PCR reactions were brought to 100 μl with NETS (150 mM sodium chloride; 10 mM EDTA; 50 mM Tris; 0.1% SDS) and the unincorporated ^{32}P removed by spun column chromatography through G-50 Sephadex minicolumns [256]. The membranes were incubated overnight at 42°C in 25 ml prehybridization buffer [50% formamide, 5X SSC (Saline-sodium citrate buffer; single-strength is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5X Denhardt's (single strength is 0.02%

Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 0.05 M sodium phosphate (pH 6.9), 0.1% SDS, and 250 μg/ml denatured herring sperm DNA)]. The prehybridization buffer was discarded and 25 ml of fresh hybridization buffer [50% formamide, 5X SSC, 1X Denhardt's, 0.02 M sodium phosphate, 0.1% SDS, 10% dextran sulfate, 100 μg/ml denatured herring sperm DNA and 1 x 10^6 cpm labeled probe] was added and membranes incubated overnight at 42°C. The membranes were then washed in Wash Solution I (1X SSC, 0.1% SDS, 0.1% sodium pyrophosphate) at 42°C for 15 min, followed by consecutive washes in Wash Solution II (0.1X SSC, 0.1% SDS, 0.1% sodium pyrophosphate) at 42°C and 47°C for 15 min each. Following washing, filters were exposed to a phosphoimager cassette. After exposure (2-24 h) the cassette was scanned using a phosphoimager (Biorad, Hercules, CA). After Northern analyses, size of RNA transcripts was determined based on relative migration of RNA molecular weight markers (Roche, Indianapolis, IN). After hybridization for tPA, uPA or uPAR, the membranes were then stripped and reprobed with the ^{32}P RPL19 cDNA. Preliminary experiments demonstrated that RPL19 mRNA abundance in bovine preovulatory follicles and new CL is not regulated by the gonadotropin surge ($P > 0.05$; data not shown). Relative densitometric units for tPA, uPA and uPAR were quantitated (from dot blots) and adjusted relative to RPL19 mRNA expression using Molecular Analyst Version 1.5 software (BIO-RAD, Hercules, CA). Preliminary Northern blot experiments demonstrated that hybridization and washing conditions used in subsequent dot blot analyses were specific and yielded hybridization to single transcripts of the expected size for each mRNA of interest. Preliminary experiments also demonstrated that an increase in hybridization intensity was detected following hybridization of each cDNA to increasing amounts of sample RNA (1-10 μg).

In Situ Hybridization

Follicles were cut on a Leica cryostat (W. Nuhsbaum, McHenry, IL) into 12 μm sagittal sections and mounted onto positively charged slides (Fisher Scientific, Chicago, IL). A sagittal section allows a view of the cell types contained at both the apex and the base of the follicle. Prior to hybridization, sections were prewarmed to room temperature for 10 min, fixed in 3.7% formaldehyde in PBS for 5 min, rinsed twice in 2X SSC for 2 min each, incubated in 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min, dehydrated in increasing concentrations of ethanol (70, 80, 95 and 100%) for 2 min each, delipidated in absolute chloroform for 5 min, rinsed in 100% and 95% ethanol for 2 min each and then air dried for 1 h. Hybridizations for each mRNA of interest were carried out on serial sections in triplicate using antisense and sense (negative controls) ^{35}S or ^{33}P labeled cRNA probes generated from previously described tPA, uPA and uPAR cDNAs. Both antisense and sense [^{35}S]UTP (1250 Ci/mmole, NEN® Life Science Product.; tPA) or [^{33}P]UTP; 3000 Ci/mmole; uPA and uPAR) cRNA probes were generated using linearized cDNA templates and an in vitro transcription kit (Stratagene, La Jolla, CA) according to the manufacturers directions. In order to increase sensitivity for uPA mRNA localization, two cRNA probes were generated from distinct uPA cDNAs and co-hybridized to the same follicle sections. Both uPA cDNA yielded identical results in Northern analysis (data not shown). The transcription reaction was incubated at 37°C for 1 h and template DNA was removed by incubation with 20 U RNase-free DNase (Stratagene, La Jolla, CA) at 37°C for 15 min. Following DNase treatment, the reaction was diluted to 100 μl with NETS and unincorporated radionucleotides removed as described above. Prior to hybridization, labeled probes were diluted in hybridization buffer to a concentration of 1.0 X 10^6 cpm/ml.

Hybridization buffer included 50% formamide, 0.3 M sodium chloride, 10 mM Tris (pH 8), 1 mM EDTA, 1X Denhardt's, 50 mM dithiothreitol (DTT), 0.5 mg/ml yeast tRNA and 10% dextran sulfate. Hybridizations were performed by adding 60 μl diluted probe/slide and then incubating in a humidified 55°C oven for 16 h. After hybridization, slides were washed twice by shaking in 2X SSC for 15 min at room temperature and treated with RNase-A (50 μg/ml) in 2X SSC for 1 h at 37°C. Slides were then washed at 55°C in 2X SSC containing 0.1% ß-mercaptoethanol (ßME) for 15 min, 1X SSC/0.1% ßME for 15 min, 1X SSC/50% formamide/0.1% ßME for 30 min, and twice in 0.1X SSC/0.1% ßME for 15 min. The slides were then dehydrated in increasing ethanol concentrations (60, 80, 95 and 100%), air dried for 1 h and then dipped in 50% NTB-2 emulsion (Eastman Kodak, Rochester, NY). Slides were exposed to autoradiographic emulsion for 10 (tPA) and 50 (uPA and uPAR) days at 4°C and then developed followed by counterstaining with hematoxylin and eosin. Exposure time for detection of a given mRNA of interest was the same for all timepoints. Digital bright and dark-field images were acquired on a Leica research microscope equipped with SPOT Model # 1.1.0 camera and Version 3.2.4 software (W. Nuhsbaum, McHenry, IL).

Enzyme Activity Assays

To investigate changes in tPA, uPA and plasmin activity in bovine preovulatory follicles and follicular fluid, a chromogenic assay and casein zymography were used. The chromogenic assay was used to quantitatively determine total plasminogen activator activity (free tPA and uPA) and zymography was used to individually measure tPA, uPA and plasmin activity. Enzyme activity was examined in both the apical and basal components of the preovulatory follicles. Follicles were homogenized using procedures previously described by Murdoch [103]. Briefly, the apical or basal sections of follicles were homogenized using a polytron

homogenizer (Fisher Scientific, Chicago, IL) in 10 mM calcium chloride; 0.25% Triton X-100 (800 μl). The homogenates were then centrifuged at 9,000 g for 30 min at 4°C. The supernatants were collected and frozen at -20°C until assayed. The pellets were then resuspended in 50 mM Tris; 0.15 M sodium chloride; 0.1 M calcium chloride; pH 7.6 (200 μl) and heated at 60°C for 6 min to allow for the release of proteinases from ECM. Following heat treatment, the samples were centrifuged at 27,000 g for 30 min at 4°C, and the supernatants frozen at -20°C until assayed. Preliminary experiments demonstrated that minimal plasminogen activator activity was present in the second extract collected following heat treatment. Therefore, activity in these (heated) samples was not determined.

Chromogenic Plasminogen Activator Assay

Total (free) plasminogen activator activity (tPA plus uPA) was measured using a two-step procedure described by Coleman & Green [257] with slight modifications. In the first 2 h incubation, native plasminogen activator converts exogenous plasminogen to plasmin, which is quantified after a 4 h second incubation. In the second incubation, plasmin catalyzes the hydrolysis of N_{α}-CBZ-L-lysine thiobenzyl ester hydrochloride in the presence of the chromogenic substrate 5,5'-dithiobis (2-nitrobenzoic acid) which is quantified spectrophotometrically at an absorbance of 405 nm. The omittence of plasminogen as a substrate was used a negative control. A standard curve using a plasminogen activator standard (uPA; Sigma Chemical Co., St. Louis, MO) was used to interpolate plasminogen activator activity in samples. Preliminary experiments established that an increase in plasminogen activator activity was detected with increasing amounts of sample protein (25-200 μg). Each sample (100 μg protein) was run in duplicate. All samples were run in a single assay. Plasminogen activator activity was expressed as nanounits activity per 100 μg

protein. The assay was not sufficiently sensitive to detect activity in follicular fluid. The intraassay C.V. was 8.6%.

Casein Zymography

Casein zymography was conducted as described by Roche et. al., 1983 [258] with slight modifications, to measure tPA, uPA and plasmin activity in the follicle apex and base and follicular fluid. Plasminogen free gels were used to confirm the activity (bands) detected were plasminogen dependent. Bovine brain cerebellum (a rich source of tPA [259]) and ovarian surface epithelial cell conditioned media (a source of uPA [225]) were used as standards. Follicular fluid (1 μl) or follicle homogenates (10 μg protein; apex and base) were subjected to electrophoresis at 140 V for 115 min in 10% polyacrylamide gels containing 0.2% casein (Sigma Chemical Co., St. Louis, MO), 0.1% SDS and 25 mU/ml human plasminogen (Sigma Chemical Co., St. Louis, MO). After electrophoresis, gels were washed once in 2.5% Triton X-100 for 45 min to remove SDS, and then incubated in incubation buffer (50 mM Tris, 0.1 M sodium chloride pH 7.6) at 37°C for 17 h. The incubation buffer lacked the heavy metals required for MMP-dependent caseinolytic activity. The gels were then stained using 0.05% Coomassie blue in 10% acetic acid, 45% methanol for 2 h, briefly destained in 10% acetic acid, 45% methanol and then fixed in 10% glycerol. Bands of activity were visualized as clear zones (where casein degradation occurred) across a dark background. Gels were photographed using a Gel Documentation System (BIO-RAD, Hercules, CA) and images inverted for better visual contrast. For validation purposes, equal protein amounts or volume of follicular fluid from individual samples was pooled within each timepoint, subjected to casein zymography and photographed for depiction in appropriate figures. To derive individual estimates of tPA, uPA and plasmin activity in each sample, all individual samples were run and densitometric scanning was performed. Variation between gels was adjusted relative to differences in activity

for tPA and uPA standards loaded on every gel. The intraassay (gel) and interassay (gel) C.V. were 4 and 13%, respectively. Activity for tPA and uPA was not observed when plasminogen was omitted from gels. Addition of the specific uPA inhibitor, amiloride (1 mM; Sigma Chemical Co., St. Louis, MO) to the incubation buffer attenuated bands of activity corresponding to uPA. Addition of the plasmin inhibitor aprotinin (2 μg/ml; Sigma Chemical Co., St. Louis, MO) to the incubation buffer attenuated all plasminogen activator and plasmin activity (data not shown.)

Statistical Analysis

Differences in mRNA abundance or enzyme activity were determined by one-way analysis of variance using the General Linear Models procedure of SAS (Version 8.0). Individual comparisons of mean RNA concentrations were performed using Fisher's Protected Least Significant Differences test. When heterogeneity of variance was detected, data were log transformed prior to statistical analysis.

RESULTS

Regulation of tPA, uPA and uPAR mRNA abundance during the periovulatory period

Northern analyses detected single mRNA transcripts of 2.4, 2.2 and 1.3 kb for tPA, uPA, and uPAR, respectively (Figures 9A, C and E). Relative abundance, as determined by dot blot analysis, of tPA, uPA and uPAR mRNAs were increased in bovine preovulatory follicles following the gonadotropin surge, but the temporal regulation of each plasminogen activator system component was distinct. Messenger RNA for tPA increased within 6 h after the gonadotropin surge and remained elevated through the 24 h timepoint ($P < 0.05$). However, the increase in tPA mRNA was not maintained through the ovulatory follicle-corpus luteum

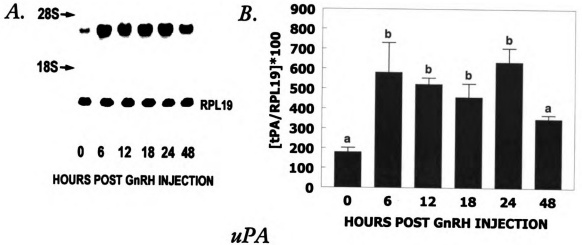
transition (48 h; Figure 9B). Relative abundance of uPA mRNA increased within 24 h following the gonadotropin surge and remained elevated in new corpora lutea (48 h; $P < 0.05$; Figure 9D). Messenger RNA for uPAR was increased at 6, 12, 24 and 48 h relative to the 0 h timepoint ($P < 0.05$). Relative levels of uPAR mRNA were most dramatically increased near the time of ovulation and the follicular-luteal transition (15 and 32-fold increase at 24 and 48 h compared to 0 h, respectively; Figure 9F).

Localization of tPA, uPA and uPAR mRNAs in bovine preovulatory follicles

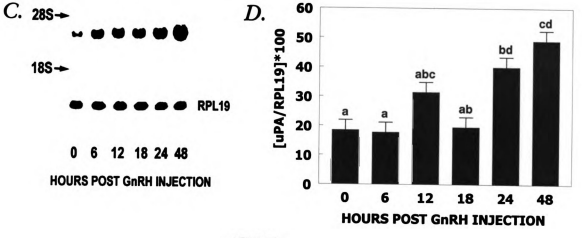
The spatial regulation of tPA, uPA and uPAR mRNA expression in response to the gonadotropin surge also was distinct. Messenger RNA for tPA was localized to the granulosal layer at all timepoints examined (Figure 10B, E, H; 0, 6, and 24 h depicted). A low level of expression was also observed in the thecal layer of follicles collected near the time of ovulation (Figure 10H; 24 h). Messenger RNA for uPA was observed in the granulosal and thecal layers at all timepoints examined (Figure 11B, E, H; 0, 6, and 24 h depicted). Unlike tPA, the localization of uPA mRNA was heterogeneous, particularly in 24 h follicles, with additional prominent hybridization signals distributed throughout the thecal layer and the adjacent ovarian stroma (Figure 11H). Messenger RNA for uPAR was localized primarily to both the granulosal and thecal cell layers of follicles collected at the 6 and 12 h timepoints (Figure 12E; 6 h depicted), but with a lower level of expression also detected in the adjacent ovarian stroma of follicles collected at the 24 h timepoint (Figure 12H). Localization of uPAR mRNA in the

Figure 9: Effect of a GnRH-induced gonadotropin surge on tPA, uPA, and uPAR mRNA abundance in bovine periovulatory follicular and luteal tissue. A) Northern analysis of tPA mRNA expression: Note hybridization to single 2.4 Kb transcript. B) Effect of the preovulatory gonadotropin surge on relative levels of tPA mRNA in bovine preovulatory follicles and new CL. C) Northern analysis of uPA mRNA expression: Note hybridization to single 2.2 Kb transcript. D) Effect of the preovulatory gonadotropin surge on relative levels of uPA mRNA in bovine preovulatory follicles and new CL. E) Northern analysis of uPAR mRNA expression: Note hybridization to single 1.3 Kb transcript. F) Effect of the preovulatory gonadotropin surge on relative levels of uPAR mRNA in bovine preovulatory follicles and new CL. Data (B, D, F) are expressed as relative units mRNA per unit RPL19 mRNA*100 (n = 5-6 per timepoint; total = 35). Due to heterogeneity of variance, values for uPA mRNA (D) were log transformed prior to analysis. Data shown as mean ± SE (tPA, uPAR) and mean ± average SE (uPA). Timepoints without a common superscript are different at $P < 0.05$.

tPA


A.
28S
18S
RPL19
0 6 12 18 24 48
HOURS POST GnRH INJECTION
B.
[tPA/RPL19]*100
900
800
700
600
500
400
300
200
100
0
a
b
b
b
b
a
0 6 12 18 24 48
HOURS POST GnRH INJECTION

uPA


C.
28S
18S
RPL19
0 6 12 18 24 48
HOURS POST GnRH INJECTION
D.
[uPA/RPL19]*100
60
50
40
30
20
10
0
a
a
abc
ab
bd
cd
0 6 12 18 24 48
HOURS POST GnRH INJECTION

uPAR

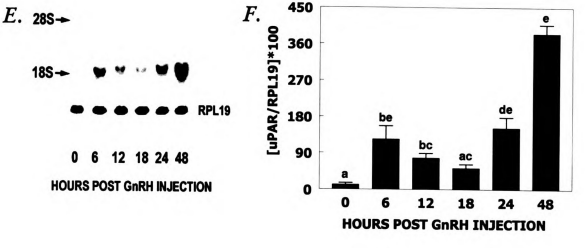

E.
28S
18S
RPL19
0 6 12 18 24 48
HOURS POST GnRH INJECTION
F.
[uPAR/RPL19]*100
450
360
270
180
90
0
a
be
bc
ac
de
e
0 6 12 18 24 48
HOURS POST GnRH INJECTION

Figure 10: In situ localization of tPA mRNA within bovine periovulatory follicles collected at 0, 6 and 24 h after GnRH injection (Magnification 120X). Representative bright-field micrographs of preovulatory follicles collected at the 0 h (A), 6 h (D), and 24 h (G) timepoints and stained with hematoxylin and eosin. Representative dark-field micrographs of the corresponding bright-field sections of preovulatory follicles collected at the 0 h (B), 6 h (E), and 24 h (H) timepoints and hybridized with a ^{35}S antisense tPA cRNA. Representative dark-field micrographs of corresponding adjacent serial sections of the same follicles collected at the 0 h (C), 6 h (F), and 24 h (I) timepoints and hybridized with a ^{35}S sense tPA cRNA (n = 3 per timepoint; total = 9). Note highest expression of tPA mRNA in granulosal layer, with additional localization in thecal layer of follicles collected at the 24 h timepoint.

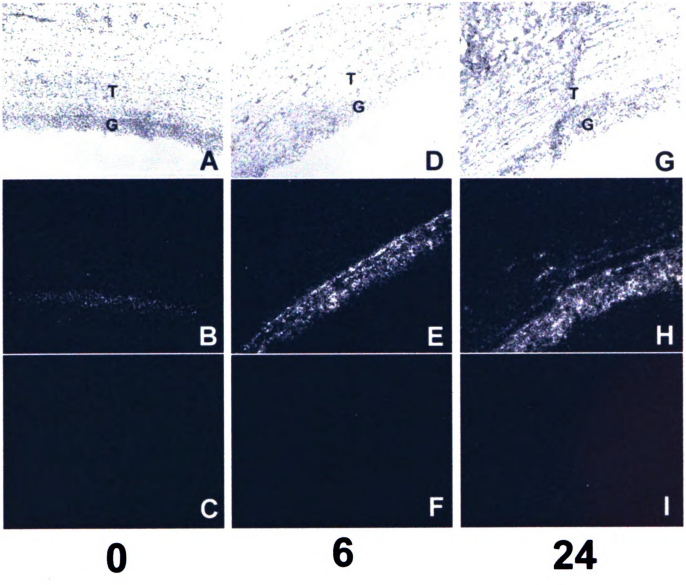

T
G
A
T
G
D
T
G
G
B
E
H
C
F
I
0
6
24

Figure 11: In situ localization of uPA mRNA within bovine periovulatory follicles collected at 0, 6 and 24 h after GnRH injection (Magnification 120X). Representative bright-field micrographs of preovulatory follicles collected at the 0 h (A), 6 h (D), and 24 h (G) timepoints and stained with hematoxylin and eosin. Representative dark-field micrographs of the corresponding bright-field sections of preovulatory follicles collected at the 0 h (B), 6 h (E), and 24 h (H) timepoints and hybridized with a ^{33}P antisense uPA cRNA. Representative dark-field micrographs of corresponding adjacent serial sections of the same follicles collected at the 0 h (C), 6 h (F), and 24 h (I) timepoints and hybridized with a ^{33}P sense uPA cRNA (n = 3 per timepoint; total = 9). Note localization of uPA mRNA to both the granulosal and thecal layers of follicles collected at the 0 and 6 h timepoints, with additional heterogeneous expression in the thecal layer and adjacent ovarian stroma of follicles collected at the 24 h timepoint.

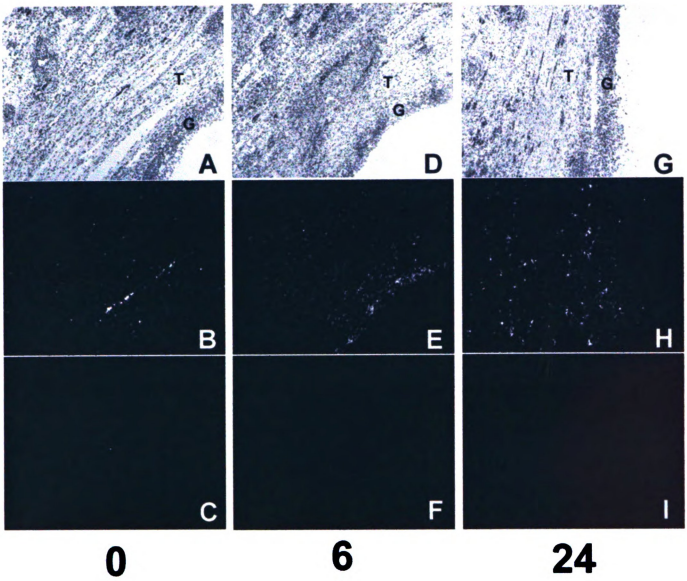
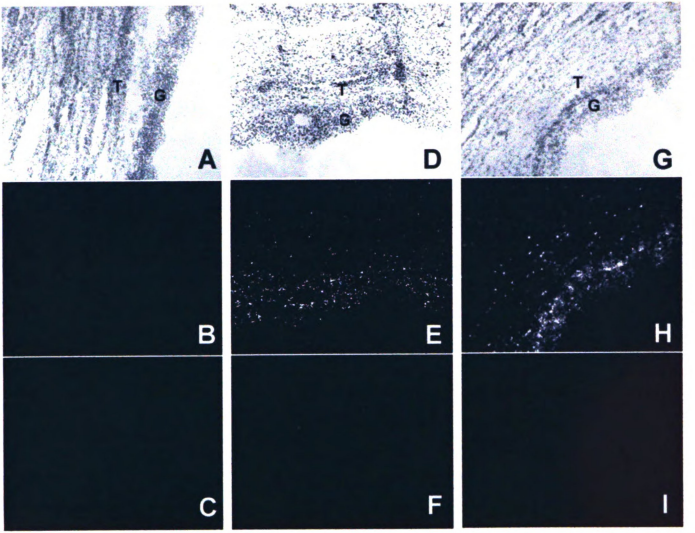

T
G
A
T
G
D
T
G
G
B
E
H
C
F
I
0
6
24

Figure 12: In situ localization of uPAR mRNA within bovine periovulatory follicles collected at 0, 6 and 24 h after GnRH injection (Magnification 120X). Representative bright-field micrographs of preovulatory follicles collected at the 0 h (A), 6 h (D), and 24 h (G) timepoints and stained with hematoxylin and eosin. Representative dark-field micrographs of the corresponding bright-field sections of preovulatory follicles collected at the 0 h (B), 6 h (E), and 24 h (H) timepoints and hybridized with a ^{33}P antisense uPAR cRNA. Representative dark-field micrographs of corresponding adjacent serial sections of the same follicles collected at the 0 h (C), 6 h (F), and 24 h (I) timepoints and hybridized with a ^{33}P sense uPAR cRNA (n = 3 per timepoint; total = 9). Note localization of uPAR mRNA primarily to the granulosal and thecal layers of follicles collected at the 6 h timepoint, with additional lower level of heterogeneous expression also detected in the adjacent ovarian stroma of follicles collected at the 24 h timepoint.


T
G
A
T
G
D
T
G
G
B
E
H
C
F
I
0
6
24

thecal layer and adjacent stroma of 24 h follicles (Figure 12H) was heterogeneous and similar to that observed for uPA (Figure 11H).

Effect of the gonadotropin surge on total plasminogen activator activity in bovine preovulatory follicles

Endogenous plasmin activity in follicular homogenates was neglible as no activity was detected in the assay when plasminogen was omitted (data not shown). Total (free) plasminogen activator activity (tPA and uPA) was significantly increased within 6 h of the gonadotropin surge in the follicle apex, but not until 18 h in the follicle base ($P < 0.05$). There was an approximately eight-fold increase in total plasminogen activator activity in the 18 h samples (apex and base) compared to samples collected before the gonadotropin surge (0 h; $P < 0.05$). Total plasminogen activator activity in both follicular regions (apex and base) subsequently decreased by the 24 h timepoint (Figure 13).

Effect of the gonadotropin surge on tPA, uPA and plasmin activity in bovine preovulatory follicles

Activity for tPA in follicle homogenates was observed as a single band that comigrated with the tPA standard (Figures 14A, C). Activity for uPA was observed as a doublet (Figures 14A, C) that comigrated with the uPA standard and plasmin activity was observed as multiple bands (Figure 14A). The uPA doublet presumably corresponds to the single and two chain forms of uPA, as both bands of activity were inhibited by amiloride (data not shown). No low molecular weight uPA activity (30-35 $M_r \times 10^{-3}$) was observed in follicular fluid or homogenates. Plasmin activity was not detected in homogenates of the follicle apex or base even when six-fold greater amounts of protein were loaded on gels (data not shown). Both tPA and uPA enzyme activity were increased within 12 h following the gonadotropin surge in both the follicle apex (Figures 14B) and base (Figure 14D). Activity for tPA was differentially

regulated in the follicle apex versus the base. Activity for tPA remained elevated in the apex but decreased to presurge (0 h) levels in the base by the 24 h timepoint (Figure 14D). Elevated uPA activity was maintained in both the apex and base through the 24 h timepoint (Figures 14B & D). In follicular fluid, tPA and plasmin activity were increased within 12 h following the gonadotropin surge and were also elevated at the 24 h timepoint (Figure 15B). Activity for uPA was not readily detectable in periovulatory follicular fluid (Figure 15A).

Figure 13: Detection of total plasminogen activator activity (tPA and uPA) in homogenates of the apex (TA) and base (TB) of bovine periovulatory follicles using a chromogenic assay. Data were expressed as nanounits PA activity/100 μg protein. Due to heterogeneity of variance data were log transformed. Data depicted as mean ± average SE (n=5-6 per timepoint). Timepoints without a common superscript are different at $P < 0.05$.

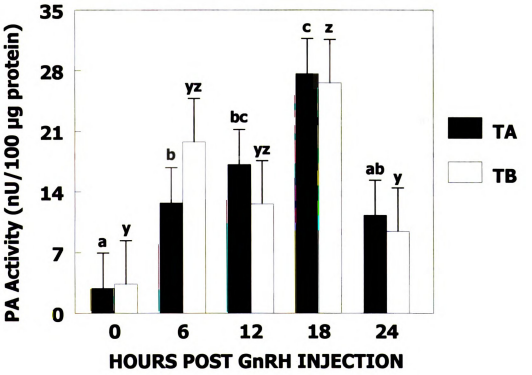
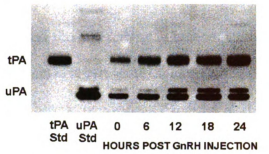
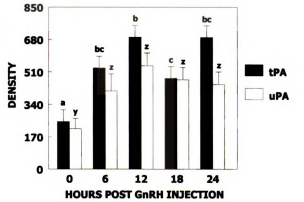


Figure 14: Detection of tPA and uPA activity in bovine periovulatory follicles by casein zymography. A) Representative zymogram demonstrating tPA and uPA activity in the apex of bovine periovulatory follicles collected at 0, 6, 12, 18 and 24 h after GnRH injection (pooled samples, 10 μg protein per timepoint). B) Densitometric analysis of tPA and uPA activity (individual samples) in apex of bovine periovulatory follicles collected at 0, 6, 12, 18 and 24 h after GnRH injection. C) Representative zymogram demonstrating tPA and uPA activity in the base of bovine periovulatory follicles collected at 0, 6, 12, 18 and 24 h after GnRH injection (pooled samples, 10 μg protein per timepoint). D) Densitometric analysis of tPA and uPA activity (individual samples) in base of bovine periovulatory follicles collected at 0, 6, 12, 18 and 24 h after GnRH injection. Note single band of tPA activity and doublet of uPA activity that comigrated with appropriate standards. Due to heterogeneity of variance data were log transformed. Data depicted as mean ± average SE (n = 5-6 per timepoint; total = 27). Timepoints without a common superscript are different at $P < 0.05$.

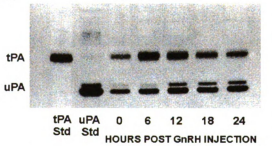
A. *Triton Apex*



B.



C. *Triton Base*



D.

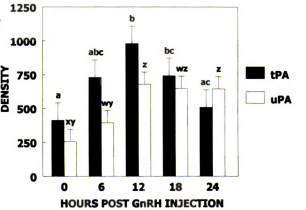
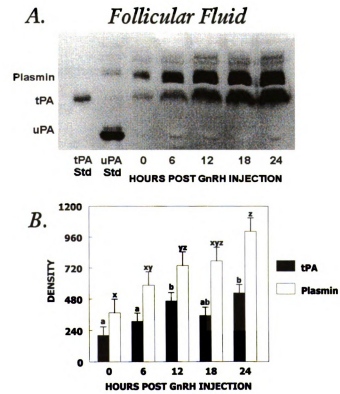


Figure 15: Detection of tPA and plasmin activity in bovine periovulatory follicular fluid by casein zymography. A) Representative zymogram demonstrating tPA and plasmin activity in follicular fluid collected from bovine periovulatory follicles at 0, 6, 12, 18 and 24 h after GnRH injection (pooled samples, 1 μl follicular fluid per timepoint). B) Densitometric analysis of tPA and plasmin activity in follicular fluid of bovine periovulatory follicles collected at 0, 6, 12, 18 and 24 h after GnRH injection (individual samples). Note single band of tPA activity and multiple bands of plasmin activity. Due to heterogeneity of variance data were log transformed. Data depicted as mean ± average SE (n = 5-6 per timepoint; total = 26). Timepoints without a common superscript are different at $P < 0.05$.



DISCUSSION

Although a potential role of the plasminogen activators in the ovulatory process has been well established, the species specific and cell specific regulation of individual components of the plasminogen activator system during the periovulatory period and their exact contribution to the ovulatory process are less understood. Our results suggest that the regulation of plasminogen activator system components (tPA, uPA and uPAR) during the periovulatory period in cattle is in many ways distinct from reports to date in other species. In the present studies, messenger RNA for tPA, uPA and uPAR in bovine preovulatory follicles was increased in response to the gonadotropin surge. In addition, tPA, uPA and plasmin activity in preovulatory follicle homogenates and (or) follicular fluid also was increased in response to the gonadotropin surge. Our results are consistent with a potential role of gonadotropin surge-induced upregulation of above plasminogen activator system components in mediating bovine follicle rupture and (or) the morphological changes associated with the ovulatory follicle-corpus luteum transition in cattle.

The observed prominent increase in mRNA abundance and enzyme activity for both tPA and uPA in bovine preovulatory follicles in response to the gonadotropin surge is in contrast to what has been observed in other species. In the mouse, sheep and the pig only one plasminogen activator or the other is markedly increased near the time of ovulation. In the mouse, uPA is the most abundant and dramatically upregulated plasminogen activator following hCG injection [14]. In addition, plasminogen activator activity is reduced by 90% in the ovaries of uPA null mutant mice [219]. However, a small induction of tPA mRNA [13, 219] specifically in the thecal layer [11] has been reported in mice and is in fact sufficient to support normal ovulation in uPA-deficient mutant mice [13, 219]. In contrast, nearly all of the

plasminogen activator activity in pig preovulatory follicles could be neutralized by tPA antibodies [220]. Results in sheep indicate that uPA is obligatory for ovulation, as intrafollicular injection of uPA, but not tPA antibodies disrupt the ovulatory process [6]. In the rat, tPA mRNA expression is increased in response to the gonadotropin surge. However, the regulation of uPA expression during the periovulatory period in the rat is controversial. Li et. al., 1997 [221] observed a decrease in uPA mRNA and protein levels in rat preovulatory follicles after exposure to hCG. In contrast, Macchione et. al., 2000 [222] reported that both plasminogen activators are present in rat preovulatory follicles near the time of ovulation, but that the thecal and granulosal layers respond differently to the gonadotropin surge. In the above study, mRNA for tPA was increased in both the thecal and granulosal layers. However, uPA mRNA was increased in the thecal layer but decreased in the granulosal layer following exposure to the gonadotropin surge. In the present studies, tPA mRNA was localized primarily to the granulosal layer of bovine follicles, with only a very low level of expression detected in the thecal layer near the time of ovulation (within 24 h after the gonadotropin surge). In situ hybridization experiments did not reveal evidence of differential regulation of uPA mRNA in the granulosal and thecal layers of bovine follicles in response to the gonadotropin surge. Thus, although the temporal regulation of tPA and uPA mRNAs was clearly distinct, steady state mRNA abundance for both plasminogen activators was clearly increased in bovine follicles following the gonadotropin surge.

Enzyme activity for both tPA and uPA were also increased in bovine follicles following exposure to the gonadotropin surge. The mechanisms that spatially regulate proteolysis of the preovulatory follicle wall and preferentially direct extracellular matrix degradation to the follicle apex are not clear. In the present study, spatial regulation of plasminogen activator activity by

the preovulatory gonadotropin surge was examined by analysis of tPA and uPA activity within samples collected from the preovulatory follicle apex (the site of ovulation) versus the base. In the follicle apex, both tPA and uPA activity were increased and remained elevated through the 24 h timepoint. In the follicle base, uPA activity also remained elevated through the 24 h timepoint. In contrast, tPA activity in the follicle base peaked within 12 h following the gonadotropin surge, but then decreased to presurge levels by 24 h. The mechanisms responsible for the differential regulation of tPA activity in the preovulatory follicle apex versus the base are not clear, but similar regional differences in tPA activity have also been observed in pig and rat preovulatory follicles [223, 224]. In contrast, Colgin and Murdoch, 1997 [6] observed higher levels of uPA activity in the apex versus the base of preovulatory ovine follicles and the ovarian surface epithelial cells were the primary source of the elevated uPA activity in the follicle apex [225]. Activity of uPA increased in bovine follicles following the gonadotropin surge, but unlike the differential temporal regulation of tPA, uPA activity in the follicle apex versus the base was not evident.

Measurement of net plasminogen activator activity (tPA plus uPA) in follicle homogenates using the chromogenic assay revealed a slightly different temporal regulation of plasminogen activator activity than when activity of individual enzymes was quantified using casein zymography. The initial increase in total plasminogen activator activity in response to the gonadotropin surge was transient and was not maintained through the 24 h timepoint. In the chromogenic assay, net plasminogen activator activity is quantified indirectly in a two-step reaction. Plasmin activity is measured following conversion of exogenous plasminogen to plasmin via the plasminogen activators present in samples. Differences in levels of endogenous proteinase inhibitors with affinity for plasmin, such as α_2-antiplasmin, α_2-macroglobulin or

protease-nexin-1 in samples assayed could influence and indirectly reduce indirect measurements of total plasminogen activator activity using the chromogenic assay. High levels of α_2-macroglobulin mRNA and protein are induced by the preovulatory gonadotropin surge in the rat [260, 261]. Levels of endogenous proteinase inhibitors dramatically influence activity of the plasminogen activators and plasmin in the extracellular milieu. Further studies will be required to characterize gonadotropin surge induced changes in the plasminogen activator inhibitors and proteinase inhibitors with an affinity for plasmin. Such information will provide a more complete understanding of the complex regulation of the plasminogen activator system during the periovulatory period and facilitate elucidation of the potential role of the individual components of the plasminogen activator system in follicle rupture and subsequent luteal formation.

One key role of the cell surface receptor for uPA (uPAR) is to localize pericellular plasmin activity. Our results indicate that uPAR mRNA abundance was increased in a cell specific manner in response to the preovulatory gonadotropin surge. A transient increase in uPAR mRNA abundance was detected at the 6 and 12 h timepoints and levels were subsequently increased again at 24 and 48 h. Messenger RNA for uPAR was localized primarily to the granulosal and thecal layers at the earlier timepoints (6 and 12 h), but a lower level of heterogeneous expression was also detected in the adjacent ovarian stroma of follicles collected at the 24 h timepoint. Similarly, during the periovulatory period in the rat, uPAR mRNA and protein are increased in both the granulosal cells and the residual ovarian tissue [221]. Interestingly, I observed a heterogeneous localization of both uPA and uPAR mRNAs within the thecal layer and adjacent ovarian stroma near the time of ovulation. Although unable to determine conclusively in present experiments using in situ hybridization, it will be

of interest to further define the specific cell types in the thecal layer and adjacent stroma with intense expression of uPA and uPAR. Colocalization of uPA and uPAR has been observed previously in migrating endothelial cells [262], and infiltrating white blood cells [263]. Endothelial cell migration is a key component of luteal development, as capillaries must penetrate the avascular granulosal cell layer following ovulation and form the rich blood supply necessary to support luteal development [226]. Localization of uPA to endothelial cells near the site of capillary formation in developing CL has been reported previously [227].

I also detected an increase in plasmin activity in bovine follicular fluid collected after the gonadotropin surge. Multiple bands of plasmin activity (of similar Mr) have been observed in mouse ovarian homogenates [219] and human blood plasma [258]. The gonadotropin surge induced increase in plasmin activity in follicular fluid can most likely be attributed to the observed increase in follicular fluid levels of tPA and enhanced activation of ubiquitous plasminogen in follicular fluid. Plasmin has been detected previously in the follicular fluid of cattle [5] and other species including the rabbit, horse and pig [12, 220, 230, 231]. Plasmin in follicular fluid may help degrade high molecular weight proteoglycans causing a decrease in follicular fluid viscosity facilitating oocyte escape [24]. Plasmin mediated degradation of fibrinogen [232-236] may also prevent premature blood clot formation in the follicular antrum prior to rupture. Liu et. al., 1986 proposed that plasmin may assist in cumulus expansion by termination of oocyte-cumulus cell communication [237]. Thus, increased follicular fluid levels of plasmin may promote conditions that facilitate ovulatory release of the oocyte.

Plasmin may also play an important role in mediating the extracellular matrix degradation required for follicle rupture in cattle. In sheep, intrafollicular injection of the plasmin inhibitor,

α_2-antiplasmin, suppresses ovulation of preovulatory follicles [206]. Similar reduction of ovulation efficiency was observed by intrabursal injection of α_2-antiplasmin in the rat [8]. Plasmin can directly degrade basement membrane ECM components including collagen IV, proteoglycans, laminin and fibronectin [1, 2]. Interestingly, peak plasminogen-dependent plasmin activity has been detected in the stigma of rat preovulatory follicles two hours prior to ovulation [238]. However, the plasmin-insensitive type I and III collagens of the thecal layer and tunica albuginea must also be degraded prior to ovulation. Here, plasmin may play a key role in activation of other extracellular matrix degrading enzymes, such as interstitial collagenase (MMP-1) [264], that degrade type I and III collagen and may be crucial for ovulation. Messenger RNA for MMP-1 is increased in bovine preovulatory follicles after exposure to the gonadotropin surge [265], but a role for plasmin in activation of pro-MMP-1 during the periovulatory period in cattle remains to be established.

In summary, I have demonstrated that both plasminogen activators (tPA and uPA) as well as the cell surface receptor for uPA (uPAR) are upregulated in bovine preovulatory follicles following the gonadotropin surge and in a temporally and spatially specific manner. These results support a potential role of tPA, uPA and uPAR during the periovulatory period, although more investigation will be required to determine the requirement of above plasminogen activator system components for ovulation and (or) luteal formation in the bovine.

ACKNOWLEDGEMENTS

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Chapter 4

Gonadotropin Surge-Induced Upregulation of Messenger RNA for Plasminogen Activator Inhibitors 1 and 2 within Bovine Periovulatory Follicular and Luteal tissue[2]

[2] This chapter has been provisionally accepted by "Reproduction."

SUMMARY

The serine proteinases, tissue-type (tPA) and urokinase plasminogen activator (uPA), are implicated in the ovulatory processes via their ability to convert plasminogen to its active form plasmin. One mechanism for regulation of plasmin-directed ovarian extracellular matrix remodeling during follicle rupture and corpus luteum (CL) formation is through inhibition of plasminogen activation by the plasminogen activator inhibitors (PAI-1 and PAI-2). This study examined the effect of the preovulatory gonadotropin surge on the temporal and spatial regulation of PAI-1 and 2 mRNA expression and plasminogen activator inhibitor activity in bovine preovulatory follicles and new CL collected at 0, 6, 12, 18, 24 and 48 h (CL) after a GnRH-induced gonadotropin surge. Both PAI-1 and PAI-2 mRNAs were dramatically upregulated following the gonadotropin surge, with the highest levels of expression observed in follicles collected near the time of ovulation (24 h) and in CL (48 h). Localization of PAI-1 mRNA was primarily to the thecal layer of preovulatory follicles. In contrast, PAI-2 mRNA was localized specifically to the granulosal layer. Significant plasminogen activator inhibitor activity was detected in follicle extracts, but temporal or spatial differences in plasminogen activator inhibitor activity were not detected in response to the gonadotropin surge. Our results indicate that PAI-1 and PAI-2 mRNAs are upregulated in bovine preovulatory follicles following the gonadotropin surge in a cell-specific manner. Regulation of PAI-1 and PAI-2 may help control plasminogen activator activity associated with ovulation and (or) early CL formation.

INTRODUCTION

The oocyte must be liberated from the preovulatory follicle in order for fertilization and subsequent pregnancy to occur. The gonadotropin surge initiates the production of proteinases that mediate the extracellular matrix (ECM) degradation and cellular remodeling required for follicular rupture and corpus luteum formation. One such family of proteinases implicated in the above processes are the plasminogen activators. The plasminogen activators consist of two enzymes, tPA and uPA, that convert ubiquitous plasminogen into the broad-spectrum serine proteinase plasmin. One mechanism for the temporal and spatial regulation of plasminogen activator activity is through the action of two members of the serine protease inhibitor (Serpin) gene family, plasminogen activator inhibitor 1 and 2 (PAI-1 and PAI-2). Both inhibitors bind to uPA or tPA in a 1:1 stoichiometry in vitro. Structurally, both serpins have an approximately 20 amino-acid-long exposed reactive center loop (RCL) which when inserted into the active site of the plasminogen activator, inhibits their activity [142]. In vivo, PAI-1 is an efficient inhibitor of both tPA and uPA, while PAI-2 is believed to inhibit primarily uPA [266, 267]. Plasminogen activator inhibitor-2 exists as both an intracellular nonglycoslyated form and a secreted glycosylated form with different biological activities [268]. Furthermore, PAI-2 is 20-100 fold less efficient in inhibiting plasminogen activator activity than is PAI-1 [269].

Evidence indicates that PAI-1 may play a key role in temporal and spatial regulation of plasminogen activator activity in rat preovulatory follicles during the periovulatory period. Messenger RNA for tPA and PAI-1 show parallel increases until just prior to the time of ovulation, when PAI-1 mRNA abundance plummets but tPA mRNA remains elevated. The spatial localization of tPA and PAI-1 are also distinct in rat preovulatory follicles. Activity for tPA is primarily associated with granulosal cells, while PAI-1 immunoreactivity is

predominantly concentrated in the thecal cells of preovulatory follicles collected near the time of ovulation [19, 223]. This differential regulation of tPA and PAI-1 may provide a temporal and spatial window of elevated tPA activity that may be important for mediating the ovulatory process in rats. Even less information is available about the intrafollicular localization and regulation of PAI-2 mRNA during the periovulatory period.

To date, the periovulatory regulation and potential role of plasminogen activator inhibitors (PAI-1 and PAI-2) in the regulation of the ovarian ECM remodeling characteristic of follicle rupture and CL formation are not completely understood. Therefore, our objective was to determine the effect of the preovulatory gonadotropin surge on localization and regulation of PAI-1 and PAI-2 mRNAs and plasminogen activator inhibitor activity in bovine periovulatory follicular and luteal tissue.

MATERIALS AND METHODS

Animal Care

Mature Holstein cows (*bos taurus*; ≥ 2 years old) were fed a balanced corn silage diet and housed at the Michigan State University Beef Cattle Research Center during the course of the experiments. All experiments were approved by the All University Committee on Animal Use and Care at Michigan State University (Approval # 04/98-056-00).

Experimental Model

Follicle development and timing of the preovulatory gonadotropin surge were synchronized in Holstein cows using the Ovsynch procedure (GnRH-7d-$PGF_{2\alpha}$-36h-GnRH) [253]. Daily ultrasound analyses were performed after the first GnRH injection until the time of follicle collection to verify follicle synchrony and to exclude animals that turned over a new follicular

wave prior to the second GnRH injection. Average time of ovulation is approximately 28 h (range 24-32) after the 2nd GnRH injection [270]. Ovaries containing ovulatory follicles or new CL were collected by colpotomy (under epidural anesthesia) at 0, 6, 12, 18, 24 and 48 hr (CL) after the second GnRH injection. Blood samples were collected at the time of $PGF_{2\alpha}$ injection and at the time of the second GnRH injection. Serum progesterone concentrations in these samples were measured by RIA (Diagnostic Products Corporation, Los Angeles, CA) to ensure that all animals included in the study responded to the $PGF_{2\alpha}$ injection with a decrease in serum progesterone below 1 ng/ml, indicating CL regression. Intra- and inter-assay coefficients of variation were 5.6 and 9.1% respectively. To verify that none of the animals included in the study exhibited a preovulatory gonadotropin surge prior to the second GnRH injection, three blood samples at 15-min intervals were collected every 8 h beginning 16 h after the $PGF_{2\alpha}$ injection until the time of ovariectomy or GnRH injection. A premature LH surge was not detected in any of the animals included in the 0 h (pre-gonadotropin surge group). In order to confirm that a gonadotropin surge was elicited by the second GnRH injection, blood samples were also collected every hour for 4 h after the second GnRH injection. In the remaining animals, the LH surge occurred only after GnRH injection, verifying control of timing of the gonadotropin surge in our model system. Concentrations of serum LH were measured by RIA [254, 255]. Intra- and inter-assay coefficients of variation were 5.8 and 15.6% respectively.

Tissue Collection

For mRNA quantification and proteinase inhibitor activity assay, ovaries containing the ovulatory follicle or new CL were collected at 0, 6, 12, 18, 24 and 48 h (n = 5 - 6 each) following the second GnRH injection. Following ovariectomy, the ovulatory follicle or new

CL was isolated by cutting away all remaining ovarian stroma and small follicles such that the ultrastructure at the apex of the follicle remained intact. Follicles were then transversely cut in half. One half was used for total RNA isolation. For protein analysis, the remaining half was cut sagitally in two equal pieces, one containing the follicle apex and one the base. New CL collected 48 h post GnRH injection were only used for mRNA analyses. Samples were frozen at -80°C within 15 min of ovariectomy. For in situ hybridization, ovaries containing the ovulatory follicles were collected at 0, 6 and 24 h (n = 3 each) following GnRH injection. Ovulatory follicles were dissected from the ovary, immediately immersed in embedding medium, frozen over liquid nitrogen vapors, and stored at -80°C until sectioned.

Preparation of cDNA probes for PAI-1 and PAI-2

The nucleotide sequence of bovine PAI-1 has been reported. Using the reported sequence, oligonucleotides primers were prepared and used in combination with RNA isolated from bovine corpora lutea in the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) to amplify a 491 bp cDNA that encoded for bovine PAI-1. To obtain a bovine PAI-2 cDNA, a set of degenerate primers (CTCCTCRTTCACATCCAC and GCTTTATCCTTTCCGTGTMAA; R = G + A; M = A + C) were designed based on nucleotide sequence of PAI-2 from human, rat and mouse. Using these primers, a 442 bp PAI-2 cDNA was amplified by RT-PCR from bovine luteal RNA. PCR products (PAI-1 and PAI-2) were subcloned into pBluescript SK(+) vectors (Stratagene, La Jolla, CA) and their identities and orientations confirmed by fluorescent dye terminator sequencing. The partial bovine PAI-2 cDNA (Genbank accession # AF416234) shared 84% identity with human PAI-2 [271]. In addition, a shorter PAI-2 cDNA (247 bp) was amplified from the

above PAI-2 cDNA using an internal primer and one original PAI-2 primer and subcloned and sequenced as described above.

Characterization of PAI-1 and PAI-2 mRNA abundance

Total RNA was isolated according to the manufacturer's instructions using the Trizol reagent (Invitrogen, Carlsbad, CA). To determine transcript size and number and to optimize specificity of hybridization conditions, approximately 15 μg pooled RNA from each sample per timepoint was subjected to Northern analysis [256]. For quantitation of PAI-1 and PAI-2 mRNA abundance, 5 μg total RNA from each sample was applied in duplicate to a Zeta probe nylon membrane (Bio-rad, Hercules, CA) using a dot blot apparatus (Bio-rad, Hercules, CA [256]. Northern and dot blot analysis was then carried out using specific bovine PAI-1, PAI-2 or ribosomal protein L-19 (RPL19) ^{32}P-labeled cDNA probes generated by the polymerase chain reaction (PCR). RPL19 was used for normalization purposes. Each 20 μl PCR reaction included 1X PCR buffer, 2.5 mM $MgCl_2$, 1.6 μM each of dATP, dGTP, dTTP, 0.25 μM of each primer, 100 pg DNA template, 1.5 U Taq polymerase, and 0.825 μM [^{32}P]dCTP (3000 Ci mM; NEN® Life Science Products, Boston, MA). The amplification conditions were: 95°C for 5 min; 94°C for 0.5 min, 52°C for 1 min, 72°C 1.5 min for 40 cycles; 72°C for 10 min; hold at 4°C. After amplification, the PCR reactions were brought to 100 μl with NETS [150 mM NaCl; 10 mM EDTA; 50 mM Tris; 0.1% SDS (w/v)] and the unincorporated ^{32}P removed by spun column chromatography through G-50 Sephadex minicolumns [256]. The membranes were incubated overnight at 42°C in 25 ml prehybridization buffer [50% formamide (v/v), 5X SSC (Saline-sodium citrate buffer; single-strength is 0.15 mM NaCl and 0.015 M sodium citrate, pH 7.0), 5X Denhardt's (single strength is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA; all v/w), 0.05 M sodium phosphate (pH 6.9), 0.1% SDS, and 250 μg/ml denatured herring sperm DNA]. The prehybridization buffer was discarded and 25 ml of fresh

hybridization buffer [50% formamide, 5X SSC, 1X Denhardt's, 0.02 M sodium phosphate, 0.1% SDS, 10% dextran sulfate, 100 μg/ml denatured herring sperm DNA ml and 1 x 10^6 cpm labeled probe] was added and membranes incubated overnight at 42°C. The membranes were then washed in 1X SSC, 0.1% SDS, 0.1% sodium pyrophosphate (w/v) at 42°C for 15 min, followed by consecutive washes in 0.1X SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 42°C and 47°C for 15 min each. Following washing, filters were exposed to a phosphoimager cassette. After exposure (2-24 h) the cassette was scanned using a phosphoimager (Biorad, Hercules, CA). After Northern analyses, size of RNA transcripts was determined based on relative migration of RNA molecular weight markers (Roche, Indianapolis, IN). After hybridization for PAI-1 or PAI-2, the membranes were then stripped and reprobed with the ^{32}P RPL19 cDNA. Preliminary experiments demonstrated that RPL19 mRNA abundance in bovine preovulatory follicles and new CL is not regulated by the gonadotropin surge ($P > 0.05$; data not shown). Relative densitometric units for PAI-1 and PAI-2 were quantitated and adjusted relative to RPL19 mRNA expression using Molecular Analyst Version 1.5 software (Bio-rad, Hercules, CA). Preliminary Northern blot experiments demonstrated that hybridization and washing conditions used in subsequent dot blot analyses were specific and yielded hybridization to single transcripts of the expected size for each mRNA of interest. Preliminary experiments also demonstrated that an increase in hybridization intensity was detected following hybridization of each cDNA to increasing amounts of sample RNA (1-10 μg).

In Situ Hybridization

Follicles were cut on a Leica cryostat (W. Nuhsbaum, McHenry, IL) into 12 μm transverse sections and mounted onto positively charged slides (Fisher Scientific, Chicago, IL). A

transverse section allows a view of the cell types contained at both the apex and the base of the follicle. Prior to hybridization, sections were prewarmed to room temperature for 10 min, fixed in 3.7% formaldehyde (v/v) in PBS for 5 min, rinsed twice in 2X SSC for 2 min each, incubated in 0.25% acetic anhydride (v/v) in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min, dehydrated in increasing concentrations of ethanol (70, 80, 95 and 100%; all v/v) for 2 min each, delipidated in absolute chloroform for 5 min, rinsed in 100% and 95% ethanol for 2 min each and then air dried for 1 h. Hybridizations for each mRNA of interest were carried out on serial sections in triplicate using antisense and sense (negative controls) ^{35}S or ^{33}P labeled cRNA probes generated from previously described PAI-1 and PAI-2 cDNAs. Both antisense and sense [^{35}SJUTP (1250 Ci mM, NEN® Life Science Product.; PAI-1) or [^{33}PJUTP; 3000 Ci mM; PAI-2) cRNA probes were generated using linearized cDNA templates and an in vitro transcription kit (Stratagene, La Jolla, CA) according to the manufacturers directions. Plasmid DNA containing the shorter (247 bp) PAI-2 cDNA was used as template for cRNA synthesis and hybridized to follicle sections. Both PAI-2 cDNA yielded identical results in Northern analysis (data not shown). The transcription reaction was incubated at 37°C for 1 h and template DNA was removed by incubation with 20 U RNase-free DNase (Stratagene, La Jolla, CA) at 37°C for 15 min. Following DNase treatment, the reaction was diluted to 100 µl with NETS and unincorporated radionucleotides removed as described above. Prior to hybridization, labeled probes were diluted in hybridization buffer to a concentration of 1.0 x 10^6 cpm/ml. Hybridization buffer included 50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8), 1 mM EDTA, 1X Denhardt's, 50 mM dithiothreitol (DTT), 0.5 mg/ml yeast tRNA and 10% dextran sulfate. Hybridizations were performed by adding 60 µl diluted probe per slide and then incubating in a humidified 55°C oven for 16 h. After hybridization, slides were washed twice by shaking in 2X SSC for 15 min at room temperature and treated with RNase-A (50 µg/

ml in 2X SSC) for 1 h at 37°C. Slides were then washed at 55°C in 2X SSC containing 0.1% ß-mercaptoethanol (ßME; v/v) for 15 min, 1X SSC/0.1% ßME for 15 min, 1X SSC/50% formamide/0.1% ßME for 30 min, and twice in 0.1X SSC/0.1% ßME for 15 min. The slides were then dehydrated in increasing ethanol concentrations (60, 80, 95 and 100%), air dried for 1 h and then dipped in 50% NTB-2 emulsion (Eastman Kodak, Rochester, NY). Slides were exposed to autoradiographic emulsion for either 10 days (PAI-1) or 50 days (PAI-2) at 4°C and then developed followed by counterstaining with hematoxylin and eosin. Exposure time for detection of a given mRNA of interest was the same for all timepoints. Digital bright and dark-field images were acquired on a Leica research microscope equipped with SPOT Model # 1.1.0 camera and Version 3.2.4 software (W. Nuhsbaum, McHenry, IL).

Follicle Homogenization Procedure and Chromogenic Plasminogen Activator Inhibitor Assay

Follicles were homogenized using procedures previously described by Murdoch [103]. Briefly, the apical or basal sections of follicles were homogenized using a polytron homogenizer (Fisher Scientific, Chicago, IL) in 800 µl of 10 mM calcium chloride; 0.25% Triton X-100 (v/v). The homogenates were then centrifuged at 9000 g for 30 min at 4°C and supernatants collected and frozen at -20°C until assayed.

Two chromogenic assays were required to quantitatively determine the levels of plasminogen activator inhibitor (PAI-1 and PAI-2) activity present in above samples. In the first assay, *endogenous* plasminogen activator activity (tPA plus uPA) was measured in the samples. We have reported changes in *endogenous* plasminogen activator activity previously [272]. In the second assay, a fixed amount of *exogenous* uPA was added to the samples and allowed to react with the plasminogen activator inhibitors. Then the *residual* plasminogen activator activity

was determined and the plasminogen activator inhibitor activity expressed as the percent of plasminogen activator activity (endogenous and exogenous) quenched in each sample. In both assays the *endogenous* or *residual* plasminogen activator activity was quantified using a procedure described by Coleman & Green [257]. The ommittence of plasminogen as a substrate was used a negative control. Standard curves using a plasminogen activator standard (uPA; Sigma Chemical Co., St. Louis, MO) were used to interpolate plasminogen activator activity (endogenous or residual) in samples. Preliminary experiments established that an increase in plasminogen activator and plasminogen activator inhibitor activity was detected with increasing amounts of sample protein (25-200 μg). Each sample was run in duplicate for both assays and all samples were run in a single assay. The intra-assay C.V. was 8.6%.

Statistical Analysis

Differences in mRNA abundance or plasminogen activator inhibitor activity were determined by one-way analysis of variance (ANOVA) using the General Linear Models procedure of SAS (Version 8.0). For plasminogen activator inhibitor activity, percentages were arcsin transformed prior to statistical analysis. Individual comparisons of mean RNA concentrations or plasminogen activator inhibitor activity were performed using Fisher's Protected Least Significant Differences test. When heterogeneity of variance was detected, data were log transformed prior to statistical analysis.

RESULTS

Regulation of PAI-1 and PAI-2 mRNA abundance during the periovulatory period

Messenger RNA for PAI-1 was detected as a single transcript of 2.9 kb (Figure 16A). Messenger RNA for PAI-1 increased following the gonadotropin surge (Figures 16B; $P < 0.05$), with the highest mRNA abundance detected in follicles collected near the time of ovulation (24 h) and in early CL (48 h). There was also a transient increase in PAI-1 mRNA in follicles collected at the 6 h timepoint ($P < 0.05$), but then expression declined to presurge levels by 12 h (Figure 16B).

The gonadotropin surge also upregulated PAI-2 mRNA in bovine periovulatory follicular and luteal tissue. A predominant transcript of 1.9 kb for PAI-2 was detected by Northern analysis (Figure 17A). Relative levels of PAI-2 mRNA were transiently increased at the 6 hr timepoint ($P < 0.05$), but then expression declined to presurge levels in follicles collected at the 12 h timepoint. Subsequently, PAI-2 mRNA abundance also increased in 24 h follicles and was further increased in new CL (48 h; $P < 0.05$; Figure 17B) collected after GnRH injection.

Localization of PAI-1 and PAI-2 mRNAs in bovine preovulatory follicles

The cell-specific regulation of PAI-1 and PAI-2 mRNA expression in response to the gonadotropin surge was distinct. Messenger RNA for PAI-1 was detected in the thecal layer at all timepoints examined (Figures 18D, E and F; 0, 6 and 24 h depicted). However, a low level of expression was also observed in the granulosal layer in follicles collected near the time

Figure 16: Effect of a GnRH-induced gonadotropin surge on PAI-1 mRNA abundance in bovine periovulatory follicular and luteal tissue. A) Northern analysis of PAI-1 mRNA expression: Note hybridization to single 2.9 Kb transcript. B) Effect of the preovulatory gonadotropin surge on relative levels of PAI-1 mRNA in bovine preovulatory follicles and new CL. Data (B) are expressed as relative units PAI-1 mRNA per unit RPL19 mRNA*100. Data shown as mean ± SE. Timepoints without a common superscript are different at $P < 0.05$.

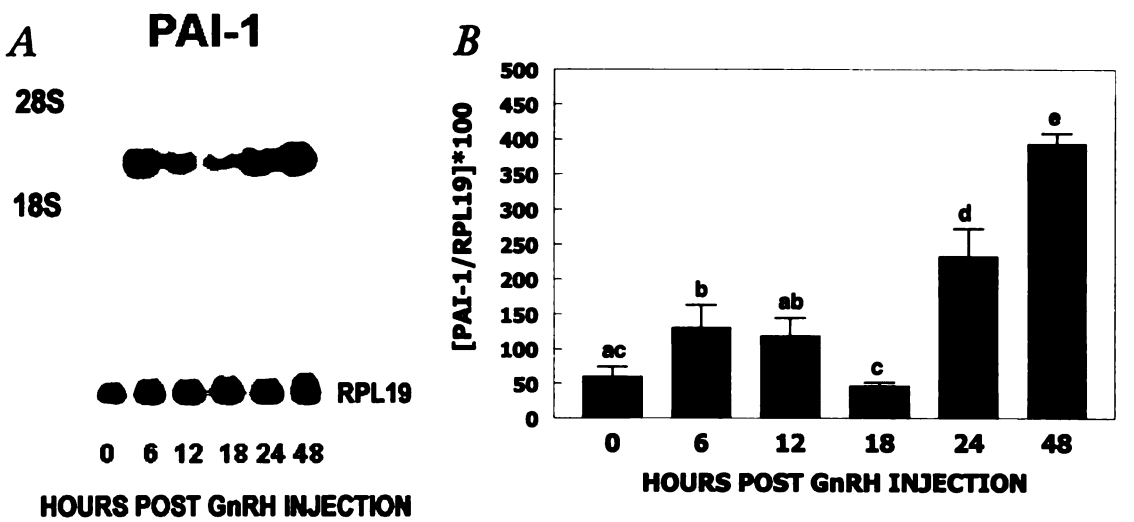

Figure 17: Effect of a GnRH-induced gonadotropin surge on PAI-2 mRNA abundance in bovine periovulatory follicular and luteal tissue. A) Northern analysis of PAI-2 mRNA expression: Note hybridization predominantly to a single 1.9 Kb transcript. B) Effect of the preovulatory gonadotropin surge on relative levels of PAI-2 mRNA in bovine preovulatory follicles and new CL. Data (B) are expressed as relative units PAI-2 mRNA per unit RPL19 mRNA*100. Due to heterogeneity of variance, values for PAI-2 mRNA were log transformed prior to analysis. Data shown as mean ± average SE. Timepoints without a common superscript are different at $P < 0.05$.

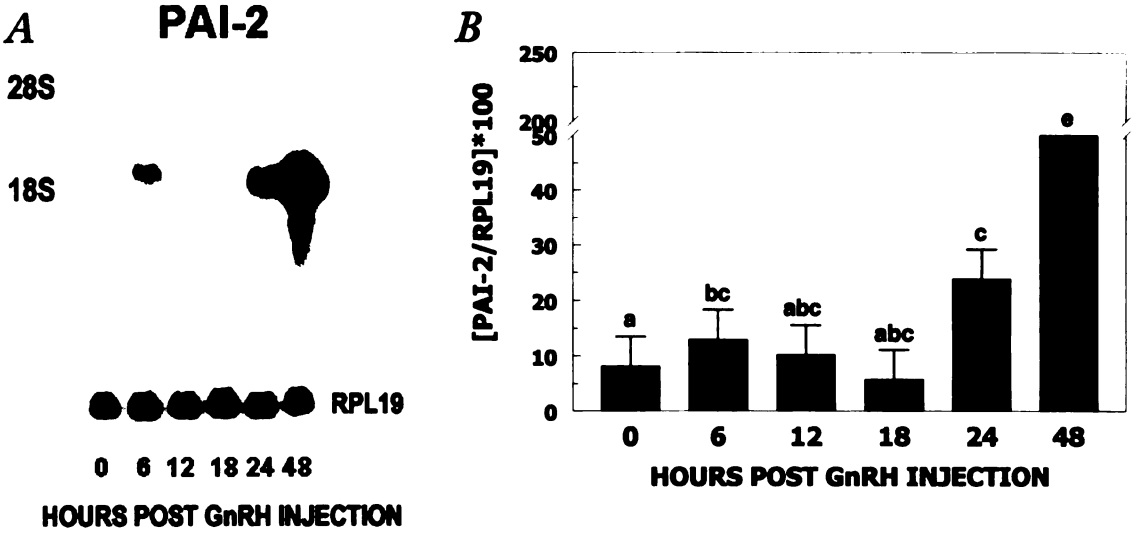

Figure 18: In situ localization of PAI-1 mRNA within bovine periovulatory follicles collected at 0, 6 and 24 h after GnRH injection. Representative bright-field micrographs of preovulatory follicles collected at the 0 h (A), 6 h (B), and 24 h (C) timepoints and stained with hematoxylin and eosin. Representative dark-field micrographs of the corresponding bright-field sections of preovulatory follicles collected at the 0 h (D), 6 h (E), and 24 h (F) timepoints and hybridized with a ^{35}S antisense PAI-1 cRNA. Representative dark-field micrographs of corresponding adjacent serial sections of the same follicles collected at the 0 h (G), 6 h (H), and 24 h (I) timepoints and hybridized with a ^{35}S sense PAI-1 cRNA (n = 3 per timepoint; total = 9). Note highest expression of PAI-1 mRNA in thecal layer, with additional localization in granulosa cell layer of follicles collected at the 24 h timepoint. Bar = 250 μM.

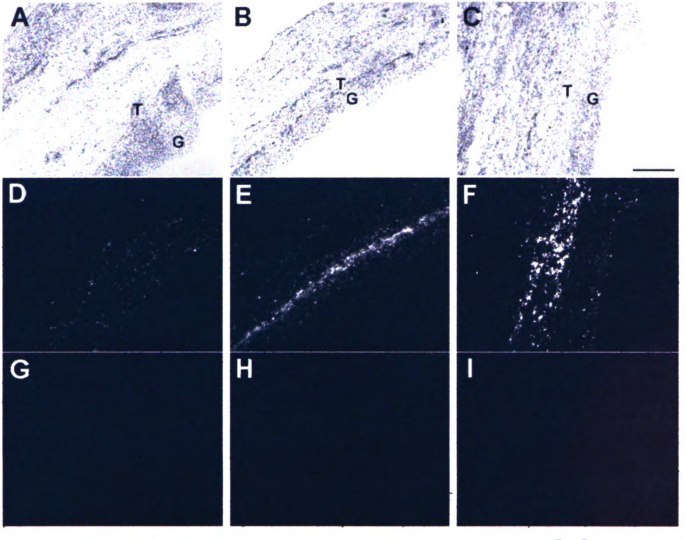
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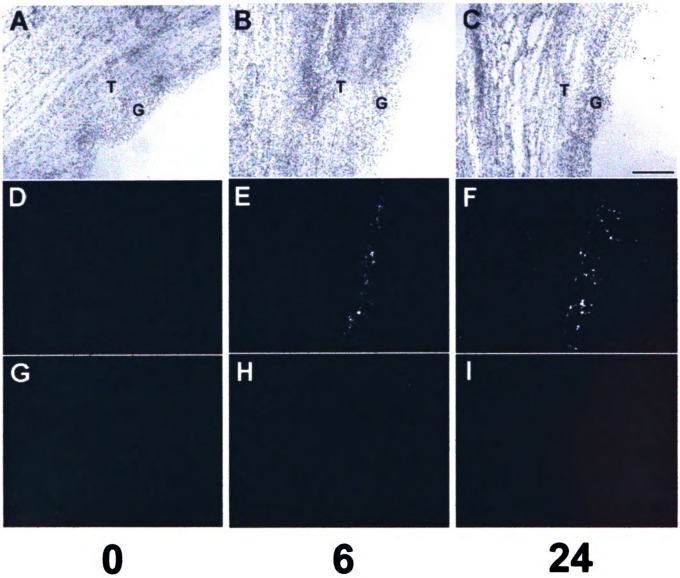
Figure 19: In situ localization of PAI-2 mRNA within bovine periovulatory follicles collected at 0, 6 and 24 h after GnRH injection. Representative bright-field micrographs of preovulatory follicles collected at the 0 h (A), 6 h (B), and 24 h (C) timepoints and stained with hematoxylin and eosin. Representative dark-field micrographs of the corresponding bright-field sections of preovulatory follicles collected at the 0 h (D), 6 h (E), and 24 h (F) timepoints and hybridized with ^{33}P antisense PAI-2 cRNAs. Representative dark-field micrographs of corresponding adjacent serial sections of the same follicles collected at the 0 h (G), 6 h (H), and 24 h (I) timepoints and hybridized with ^{33}P sense PAI-2 cRNAs (n = 3 per timepoint; total = 9). Note localization of PAI-2 mRNA to the granulosa cell layer of follicles collected at the 6 and 24 h timepoints. Bar = 250 μM.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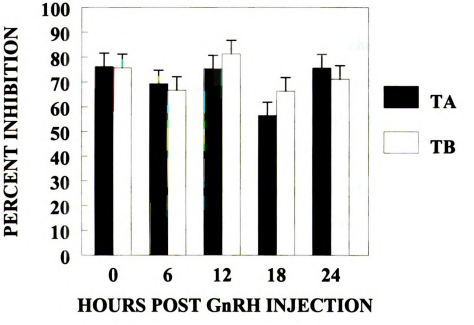
of ovulation (Figure 18F; 24 h). In contrast, PAI-2 mRNA expression was localized specifically to the granulosal layer of preovulatory follicles collected at the 6 and 24 h timepoints (Figure 19E & F). Significant expression in the thecal layer and adjacent ovarian stroma was not detected.

Effect of the gonadotropin surge on PAI activity in bovine preovulatory follicles

Preovulatory follicle homogenates (apex and base) dramatically attenuated activity of exogenous uPA, indicating the presence of endogenous plasminogen activator inhibitor activity. However, levels of plasminogen activator inhibitor activity in the preovulatory follicle apex and base were not regulated by the gonadotropin surge (Figure 20; $P > 0.05$).

Figure 20: Detection of total plasminogen activator inhibitor activity (PAI-1 and PAI-2) in homogenates of the apex (TA) and base (TB) of bovine periovulatory follicles using a chromogenic assay. Data were expressed as percentage plasminogen activator inhibitor activity/100 μg protein. Due to heterogeneity of variance data were log transformed. Percentages were arcsin transformed prior to statistical analysis. Data depicted as mean ± average SE (n=5-6 per timepoint). Plasminogen activator inhibitor activity in the preovulatory follicle apex and base were not regulated in response to the gonadotropin surge (P >0.05).



DISCUSSION

Controlling and focusing extracellular proteolytic activity is essential for many reproductive processes including ovulation. We have previously shown that there is a dramatic preovulatory rise in both mRNA and activity for tPA and uPA in bovine preovulatory follicles [272-274]. The induction of plasminogen activator inhibitors is believed to be one of the key means for regulation of plasmin mediated proteolysis initiated by the plasminogen activators. However, the regulation of the plasminogen activator inhibitors in bovine preovulatory follicles during the periovulatory period has not been reported. Our results here clearly show that PAI-1 and PAI-2 mRNAs were upregulated in a cell specific fashion in response to the gonadotropin surge, but temporal changes in plasminogen activator inhibitor activity were not detected. Furthermore, the regulation of PAI-1 and PAI-2 mRNAs and plasminogen activator inhibitor activity observed are distinct from reports in other species to date.

I observed a transient increase in PAI-1 mRNA abundance within 6 h following the gonadotropin surge, with increased expression detected near the time of ovulation (24 h follicles) and in new CL. This is in contrast to reported changes in PAI-1 mRNA in rat and monkey preovulatory follicles in response to hCG. In the above species, PAI-1 mRNA is transiently upregulated but then subsequently declines in preovulatory follicles near the time of ovulation [217, 222]. Furthermore, high levels of tPA mRNA continue to be expressed in rat and monkey preovulatory follicles near the time of ovulation, several hours after the decline in PAI-1 mRNA. Near the time of ovulation, plasminogen activator activity in preovulatory pig follicle increases, with a concomitant decrease in plasminogen activator inhibitor activity [220]. This suggests that a decrease in PAI-1 in the face of continued tPA activity may play a role in regulation of follicular rupture. In contrast, PAI-1 mRNA is not upregulated until after

ovulation in the mouse [11, 13]. In the present studies, PAI-1 mRNA was localized primarily to the thecal layer, with a lower level of expression observed in the adjacent granulosal layer of preovulatory follicles collected at the 24 h timepoint. In monkeys and rats, PAI-1 mRNA was also localized primarily to the thecal layer [19], but additional expression has also been observed in the ovarian stroma of rat preovulatory follicles [223].

I also observed a significant increase in PAI-2 mRNA in bovine preovulatory follicles near the time of follicular rupture (24 h timepoint) and PAI-2 mRNA was localized specifically to the granulosal layer. Very limited information is available regarding the temporal and cell specific regulation of PAI-2 mRNA during the periovulatory period in other species. Messenger RNA for PAI-2 has been previously detected in both human and mouse ovarian tissues. Both human cumulus cells and granulosal-luteal cells collected from patients (36 h post hCG; a few h prior to ovulation) undergoing in vitro fertilization have been shown to express PAI-2 mRNA [229]. A small increase in PAI-2 mRNA abundance was also observed in mouse ovaries 4 h post hCG injection. The localization of PAI-2 mRNA in the mouse ovary was distinct from that observed in cattle and restricted primarily to a few individual cells within the thecal layer that were believed to be macrophages [13].

Plasminogen activator inhibitor-2 is a major product of macrophages and monocytes in response to inflammatory conditions [155, 156]. Furthermore, the two different forms of PAI-2 (extracellular and intracellular) in monocytes likely serve distinct functions. The extracellular form has been shown to inhibit uPA activity, while the predominant intracellular form inhibits TNF_{α} directed apoptosis [157, 218]. Plasmin production at the site of follicle rupture in sheep preovulatory follicles has been shown to facilitate liberation of membrane

anchored TNF_{α} and subsequent ovarian surface epithelial cell apoptosis [206, 209, 210, 275, 276]. Although the presence of the two forms of PAI-2 in bovine granulosal cells has not been established, it is interesting to hypothesize that intracellular PAI-2 may serve a protective role against TNF_{α} directed apoptosis in the granulosal layer of bovine preovulatory follicles.

Formation of the CL involves dramatic biochemical and morphological processes including luteinization, angiogenesis and cellular proliferation and migration. In the present studies, the highest relative abundance of PAI-1 and PAI-2 mRNA was observed in tissue collected during CL formation. Furthermore, it is possible that the increased abundance of PAI-1 and PAI-2 mRNA detected near the time of ovulation may in fact be more relevant to regulation of luteal development. In the rat, PAI-1 mRNA is localized adjacent to uPA-expressing capillaries of the early CL, and it thought to play an important role in the regulation of angiogenesis [227]. Studies using PAI-1 deficient mice have shown the absolute requirement for PAI-1 during tumor-induced angiogenesis [200, 277]. The role of PAI-2 [intracellular and (or) extracellular] during CL formation is unclear. More investigation will be critical to understanding the exact role of PAI-1 and PAI-2 during the periovulatory period in cattle.

Significant plasminogen activator inhibitor activity (PAI-1 plus PAI-2) was detected in the apex and base of bovine preovulatory follicles. However, no temporally or spatially significant changes in plasminogen activator inhibitor activity were detected in response to the gonadotropin surge. I have observed elevated tPA mRNA and activity during the periovulatory period in bovine preovulatory follicles [272, 273]. Furthermore, tPA activity is spatially regulated in bovine preovulatory follicles following the gonadotropin surge. In the follicle apex, tPA activity is increased in response to the gonadotropin surge and remains

elevated through the 24 h timepoint. In contrast, tPA activity in the follicle base peaks within 12 h following the gonadotropin surge, but then decreases to presurge levels by 24 h [272]. Interestingly, I did not detect a differential rise in plasminogen activator inhibitor activity in the base of bovine preovulatory follicles near the time of follicular rupture. Therefore, the differential upregulation of tPA activity in the follicular apex versus the base is not like likely due to regional differences in upregulation of the plasminogen activator inhibitor activity in response to the gonadotropin surge. This difference in the regulation of plasminogen activator (tPA) and plasminogen activator inhibitor levels may provide a temporal and spatial window of increased plasminogen activator activity that may help facilitate follicular rupture.

In summary, I have demonstrated that PAI-1 and PAI-2 mRNAs are upregulated in bovine preovulatory follicles in response to the preovulatory gonadotropin surge with maximal expression during CL formation. Furthermore, the temporal and spatial regulation of PAI-1 and PAI-2 mRNAs in bovine preovulatory follicles in response to the gonadotropin surge was distinct from reports to date in other species. Further investigation will be required to elucidate the precise physiological role of PAI-1 and PAI-2 during the periovulatory period in cattle.

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Chapter 5

Summary: Gonadotropin Surge-Induced Upregulation of Plasminogen Activator System Components within Bovine Periovulatory Follicular and Luteal tissue

These studies evaluated the effect of the gonadotropin surge on the plasminogen activator system components on within bovine periovulatory follicular and luteal tissue. A summary is shown in Table 3.

It is clear from these studies that the regulation of the plasminogen activator system components is different than observed in other species. Periovulatory follicles of the rat, mouse, pig and sheep have only one plasminogen activator strongly upregulated in by gonadotropin surge. Here I have clearly established by both mRNA and enzyme activity that both tPA and uPA are increased in bovine periovulatory follicles by the gonadotropin surge.

It is also interesting that both the temporal regulation of tPA mRNA and spatial regulation of tPA enzyme activity suggests that this enzyme could be important for follicular rupture in the cow. Messenger RNA for tPA is upregulated by the gonadotropin surge and remains elevated at the time of follicular rupture and then mRNA is decreased following ovulation. Activity for tPA also was transiently elevated in the base but remained elevated at the apex (site of degradation and rupture). Elevated tPA activity may be important for plasminogen dependent plasmin formation at the apical region of the follicle. This plasmin could directly via degradation of follicle ECM components lead to ovulation. However, collagens I and III provide the greatest structural support of the follicle, and these collagens are not sensitive to plasmin degradation. However, plasmin can activate the proforms of several MMPs, perhaps most importantly MMP-1 that can degrade collagens I and III. Therefore, elevated production of plasmin directed by increased tPA activity may induce ovulation indirectly by increased MMP activity.

Table 3: Summary of the Gonadotropin Surge-Induced Upregulation of the Plasminogen Activator System Components within Bovine Periovulatory Follicular and Luteal Tissue

Name	**Abbreviation**	**Effect of preovulatory LH surge on mRNA expression at 6, 12, 18, 24, and 48 h post GnRH vs 0 h timepoint**	**Intrafollicular localization**	**Effect of preovulatory LH surge on activity in follicular fluid or follicle extracts**
Tissue plasminogen activator	tPA	increased at 6, 12, 18 & 24 h	granulosal layer (6 &12 h), thecal and granulosal layers (24h)	Apex: Increased at 6, 12, 18 and 24 h Base: Increased at 12 and 18 h FF: Increased at 12 and 24 h
Urokinase plasminogen activator	uPA	increased at 24 & 48 h	granulosal and thecal layer	Apex: Increased at 6, 12, 18 and 24 h Base: Increased at 12, 18 and 24 h FF: not detected
Urokinase plasminogen activator receptor	uPAR	increased at 6, 24 and 48h	granulosal layer and thecal layer	not applicable
Plasmin	Plasmin	Not applicable	Not applicable	Apex: Not detected Base: Not detected FF: Increased at 12 and 24 h
Plasminogen activator inhibitor-1	PAI-1	increased at 6, 24 & 48h	thecal layer (6 &12 h), thecal and granulosal layers (24h)	PAI Activity (PAI-1 and PAI-2) Apex: Not regulated Base: Not regulated
Plasminogen activator inhibitor-2	PAI-2	increased at 6, 24 & 48 h	granulosal layer	

The LH surge induced upregulation of uPA is the primary mechanism for plasmin generation in mouse and sheep ovulatory follicles. In the cow, the gonadotropin surge also upregulates uPA in the ovulatory follicle near the time of ovulation. However, unlike tPA, uPA activity is not spatially regulated in the ovulatory follicle suggesting that it may not be important for the degradation of the apical follicle wall leading to ovulation. This is in contrast with the sheep, where uPA is secreted by the OSE cells just at the apex of the follicle near the time of ovulation [6]. In these studies, the number of bovine OSE cells that cover the apex of the follicle is relatively small compared to thecal and granulosal cells present in the homogenate. Therefore, it is possible that the techniques used were not sensitive enough to detect differences in a small upregulation of uPA from a limited number of OSE cells. However, the temporal regulation of both uPA and its cell surface receptor, uPAR, in bovine follilcles suggest that they may play roles in the ovulatory process and (or) corpus luteum formation. Both uPA and its receptor have been shown to be important for cell migration and angiogenesis. Colocalization of uPA and uPAR has been observed previously in migrating endothelial cells [262], and infiltrating white blood cells [263]. Endothelial cell migration is a key component of luteal development and localization of uPA to endothelial cells near the site of capillary formation in developing CL has been reported [227]. I was unable, using the techniques utilized here, to co-localize mRNA for both uPA and its receptor. However, future immunocytochemistry on follicle sections could be done to identify the colocalization of these two proteins.

I also detected an increase in plasmin activity in bovine follicular fluid collected after the gonadotropin surge. The gonadotropin surge induced increase in plasmin activity in follicular fluid can most likely be attributed to the observed increase in follicular fluid levels of tPA and

enhanced activation of ubiquitous plasminogen in follicular fluid. Plasmin in follicular fluid may 1) help degrade high molecular weight proteoglycans causing a decrease in follicular fluid viscosity facilitating oocyte escape [24] (2) degrade fibrinogen and prevent premature blood clot formation in the follicular antrum prior to rupture [232-236]. Future experiments with intrafollicular injection of tPA antibodies and (or) antiplasmin into ovulatory follicles post the LH surge may provide evidence supporting role of plasmin in follicular rupture in the bovine.

Since the regulation of the plasminogen activators (tPA and uPA) is different than other species to date, it was not unexpected that plasminogen activator inhibitors (PAI-1 and PAI-2) were also differentially regulated. Here I have demonstrated that PAI-1 and PAI-2 mRNAs are upregulated in bovine preovulatory follicles in response to the preovulatory gonadotropin surge with high levels of expression in ovulatory follicles near the time of ovulation and in early corpora lutea. Significant plasminogen activator inhibitor activity (PAI-1 plus PAI-2) was detected in the apex and base of bovine preovulatory follicles. However, no temporally or spatially significant changes in plasminogen activator inhibitor activity were detected in response to the gonadotropin surge. The technique used to measure PAI activity is a measurement of free PAI activity. Therefore, in both plasminogen activator increases in parallel with plaminogen activity inhibitor, then no net increase in free plasminogen activator inhibitor activity would be detected. Interestingly, I did not detect a differential rise in plasminogen activator inhibitor activity in the base of bovine preovulatory follicles near the time of follicular rupture. Therefore, the differential upregulation of tPA activity in the follicular apex versus the base is not like likely due to regional differences in upregulation of the plasminogen activator inhibitor activity in response to the gonadotropin surge.

Furthermore, the two different forms of PAI-2 (extracellular and intracellular) in monocytes likely serve distinct functions. The extracellular form has been shown to inhibit uPA activity, while the predominant intracellular form inhibits TNF_{α} directed apoptosis [157, 218]. Plasmin production at the site of follicle rupture in sheep preovulatory follicles has been shown to facilitate liberation of membrane anchored TNF_{α} and subsequent ovarian surface epithelial cell apoptosis [206, 209, 210, 275, 276]. Although the presence of the two forms of PAI-2 in bovine granulosal cells has not been established, it is interesting to hypothesize that intracellular PAI-2 may serve a protective role against TNF_{α} directed apoptosis in the granulosal layer of bovine preovulatory follicles. Furthermore, the marked increase in PAI-2 mRNA abundance observed in the early corpus luteum suggests an important role of protein during the ovulatory follicle corpus luteum transition. Future localization experiments using PAI-2 specific antibodies may further clarify the role of PAI-2 (extracellular and intracellular) in the bovine ovulatory follicle and (or) corpus luteum.

In summary, the gonadotropin surge clearly regulates the plamininogen activator/plasmin system differently in the in bovine preovulatory follicles and (or) corpus luteum than in other species. Perhaps this knowledge will lead to better understanding of the ovulatory process and (or) corpus luteum formation in the cow and other monoovulory species in the future.

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BIBLIOGRAPHY

1. Mignatti P, Robbins E, Rifkin DB. Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell 1986; 47: 487-498.

2. Alexander CM, Werb Z. Extracellular Matrix Degradation. In: Hay ED (ed.) Cell Biology of the Extracellular Matrix, 2nd ed. New York: Plenum Press; 1991: 255-302.

3. Bu G, Williams S, Strickland DK, Schwartz AL. Low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor is an hepatic receptor for tissue-type plasminogen activator. Proceedings of the Natl Academy of Science USA 1992; 89: 7427-7431.

4. Schochet SSA. A suggestion as to the process of ovulation and ovarian cyst formation. Anat Rec 1916; 10: 447-457.

5. Beers WH. Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. Cell 1975; 6: 379-386.

6. Colgin DC, Murdoch WJ. Evidence for a role of the ovarian surface epithelium in the ovulatory mechanism of the sheep: secretion of urokinase-type plasminogen activator. Anim Reprod Sci 1997; 47: 197-204.

7. Reich R, Miskin R, Tsafriri A. Follicular plasminogen activator: involvement in ovulation. Endocrinology 1985; 116: 516-521.

8. Tsafriri A, Bicsak TA, Cajander SB, Ny T, Hsueh AJ. Suppression of ovulation rate by antibodies to tissue-type plasminogen activator and alpha 2-antiplasmin. Endocrinology 1989; 124: 415-421.

9. Canipari R, Strickland S. Plasminogen activator in the rat ovary. Production and gonadotropin regulation of the enzyme in granulosa and thecal cells. J Biol Chem 1985; 260: 5121-5125.

10. Liu Y-X, Cajander S, Ny T, Kristensen PJW, Hsueh AJW. Gonadotropin regulation of tissue-type and urokinase-type plasminogen activator in the rat granulosa and theca-interstitial cells during periovulatory period and plasminogen activator and their inhibitor in rat granulosa and theca-interstitial cells during preovulatory phases. Mol Cell Endocrinol 1987; 54: 221-229.

11. Hagglund AC, Ny A, Liu K, Ny T. Coordinated and cell-specific induction of both physiological plasminogen activators creates functionally redundant mechanisms for plasmin formation during ovulation. Endocrinology 1996; 137: 5671-5677.

12. Yoshimura Y, Santulli R, Atlas SJ, Fujii S, Wallach EE. The effects of proteolytic enzymes on in vitro ovulation in the rabbit. Am J Obstet Gynecol 1987; 157: 468-475.

13. Leonardsson G, Peng XR, Liu K, Nordstrom L, Carmeliet P, Mulligan R, Collen D, Ny T. Ovulation efficiency is reduced in mice that lack plasminogen activator gene function: functional redundancy among physiological plasminogen activators. Proceedings of the Natl Academy of Science USA 1995; 92: 12446-12450.

14. Canipari R, ML OC, Meyer G, Strickland S. Mouse ovarian granulosa cells produce urokinase-type plasminogen activator, whereas the corresponding rat cells produce tissue-type plasminogen activator. J Cell Biol 1987; 105: 977-981.

15. Atiomo WU, Bates SA, Condon JE, Shaw S, West JH, Prentice AG. The plasminogen activator system in women with polycystic ovary syndrome. Fertil Steril 1998; 69: 236-241.

16. Raj SG, Thompson IE, Berger MJ, Taymor ML. Clinical aspects of the polycystic ovary syndrome. Obstet Gynecol 1977; 49: 552-556.

17. Shearman RP, Cox RI. The enigmatic polycystic ovary. Obstet Gynecol Surv 1966; 21: 1-33.

18. Mishell's Textbook of Infertility, Contraception, and Reproduction Endocrinology. Malden: Blackwell Science, Inc.; 1997.

19. Liu YX. Regulation of the plasminogen activator system in the ovary. Biological Signals and Receptors 1999; 8: 160-177.

20. Luck MR. The gonadal extracellular matrix. In: Oxford Reviews of Reproductive Biology, vol. 16; 1994: 34-85.

21. Luck MR, Zhao Y, Silvester LM. Identification and localization of collagen types I and IV in the ruminant follicle and corpus luteum. J Reprod Fertil Suppl 1995; 49: 517-521.

22. McArthur ME, Irving-Rodgers HF, Byers S, Rogers RJ. Identification and immunolocalization of decorin, versican, perlecan, nidogen, and chondroitan sulfate proteoglycans in bovine small-antral ovarian follicles. Biology of Reproduction 2000; 63: 913-924.

23. Zhao Y, Luck MR. Gene expression and protein distribution of collagen, fibronectin and laminin in bovine follicles and corpora lutea. J Reprod Fertil 1995; 104: 115-123.

24. Zachariae F. Acid mucopolysaccharides in the ovary and their role in the mechanism of ovulation. Acta Endocrin 1959; 47: 1-65.

25. Mueller PL, Schreiber JR, Lucky AW, Schulman JD, Rodbard D, Ross GT. Follicle-stimulating hormone stimulates ovarian synthesis of proteoglycans in the estrogen-stimulated hypophysectomized immature female rat. Endocrinology 1978; 102: 824-831.

26. Espey LL, Lipner H. Ovulation. In: Knobil E, Neill JD (eds.), Physiology of Reproduction 2nd Edition. New York: Raven Press; 1994: 725-780.

27. Saksela O, Rifkin DB. Cell-associated plasminogen activation: regulation and physiological functions. Annu Rev Cell Biol 1988; 4: 93-126.

28. Brannstrom M, Friden B. Immune regulation of corpus luteum function. Semin Reprod Endocrinol 1997; 15: 363-370.

29. O'Shea JD, Rodgers RJ, Wright PJ. Cellular composition of the sheep corpus luteum in the mid- and late luteal phases of the oestrous cycle. J Reprod Fertil 1986; 76: 685-691.

30. Rodgers RJ, O'Shea JD, Findlay JK. Do small and large luteal cells of the sheep interact in the production of progesterone? J Reprod Fertil 1985; 75: 85-94.

31. O'Shea JD, Cran DG, Hay MF. Fate of the theca interna following ovulation in the ewe. Cell Tissue Res 1980; 210: 305-319.

32. O'Shea JD, Cran DG, Hay MF. The small luteal cell of the sheep. J Anat 1979; 128: 239-251.

33. Ekholm C, Hillensjo T, Isaksson O. Gonadotropin releasing hormone agonists stimulate oocyte meiosis and ovulation in hypophysectomized rats. Endocrinology 1981; 108: 2022-2024.

34. Corbin A, Bex FJ. Luteinizing hormone releasing hormone agonists induce ovulation in hypophysectomized proestrous rats: direct ovarian effect. Life Sci 1981; 29: 185-192.

35. Uemura T, Namiki T, Kimura A, Yanagisawa T, Minaguchi H. Direct effects of gonadotropin-releasing hormone on the ovary in rats and humans. Horm Res 1994; 41: 7-13.

36. Dekel N, Sherizly I, Tsafriri A, Naor Z. A comparative study of the mechanism of action of luteinizing hormone and a gonadotropin releasing hormone analog on the ovary. Biology of Reproduction 1983; 28: 161-166.

37. Koos RD, LeMaire WJ. The effects of a gonadotropin-releasing hormone agonist on ovulation and steroidogenesis during perfusion of rabbit and rat ovaries in vitro. Endocrinology 1985; 116: 628-632.

38. Williams Textbook of Endocrinology. Philadelphia: W.B. Saunders Company; 1998.

39. Behrman HR, Aten RF, Ireland JJ, Milvae RA. Characteristics of an antigonadotrophic GnRH-like protein in the ovaries of diverse mammals. J Reprod Fertil Suppl 1989; 37: 189-194.

40. Shimamoto T, Yamoto M, Nakano R. The role of tyrosine kinase in gonadotropin-induced ovulation in the rat ovary. Eur J Endocrinol 1998; 138: 594-600.

41. Leung PC, Steele GL. Intracellular signaling in the gonads. Endocr Rev 1992; 13: 476-498.

42. Shimamoto T, Yamoto M, Nakano R. Possible involvement of protein kinase C in gonadotropin-induced ovulation in the rat ovary. Endocrinology 1993; 133: 2127-2132.

43. Holmes PV, Janson PO, Sogn J, Kallfelt B, LeMaire WJ, Ahren KB, Cajander S, Bjersing L. Effects of PGF2 alpha and indomethacin on ovulation and steroid production in the isolated perfused rabbit ovary. Acta Endocrinol (Copenh) 1983; 104: 233-239.

44. Brannstrom M, Koos RD, Le Maire WJ, Janson PO. Cyclic adenosine 3',5'-monophosphate-induced ovulation in the perfused rat ovary and its mediation by prostaglandins. Biology of Reproduction 1987; 37: 1047-1053.

45. Lipner H, Greep RO. Inhibition of steroidogenesis at various sites in the biosynthetic pathway in relation to induced ovulation. Endocrinology 1971; 88: 602-607.

46. Lipner H, Wendelken L. Inhibition of ovulation by inhibition of steroidogenesis in immature rats. Proc Soc Exp Biol Med 1971; 136: 1141-1145.

47. Snyder BW, Beecham GD, Schane HP. Inhibition of ovulation in rats with epostane, an inhibitor of 3 beta-hydroxysteroid dehydrogenase. Proc Soc Exp Biol Med 1984; 176: 238-242.

48. Mori T, Suzuki A, Nishimura T, Kambegawa A. Inhibition of ovulation in immature rats by anti-progesterone antiserum. J Endocrinol 1977; 73: 185-186.

49. Kitai H, Santulli R, Wright KH, Wallach EE. Examination of the role of calcium in ovulation in the in vitro perfused rabbit ovary with use of ethyleneglycol-bis(beta-aminoethyl ether)-n,n'-tetraacetic acid and verapamil. Am J Obstet Gynecol 1985; 152: 705-708.

50. Holmes PV, Sogn J, Schillinger E, Janson PO. Effects of high and low preovulatory concentrations of progesterone on ovulation from the isolated perfused rabbit ovary. J Reprod Fertil 1985; 75: 393-399.

51. Shaykh M, LeMaire WJ, Papkoff H, Curry TE, Sogn JH, Koos RD. Ovulations in rat ovaries perfused in vitro with follicle-stimulating hormone. Biology of Reproduction 1985; 33: 629-636.

52. Clemens JW, Robker RL, Kraus WL, Katzenellenbogen BS, Richards JS. Hormone induction of progesterone receptor (PR) messenger ribonucleic acid and activation of PR promoter regions in ovarian granulosa cells: evidence for a role of cyclic adenosine 3',5'-monophosphate but not estradiol. Mol Endocrinol 1998; 12: 1201-1214.

53. Jacobs BR, Suchocki S, Smith RG. Evidence for a human ovarian progesterone receptor. Am J Obstet Gynecol 1980; 138: 332-336.

54. Jacobs BR, Smith RG. Evidence for a receptor-like protein for progesterone in bovine ovarian cytosol. Endocrinology 1980; 106: 1276-1282.

55. Pasqualini JR, Nguyen BL. Progesterone receptors in the fetal uterus and ovary of the guinea pig: evolution during fetal development and induction and stimulation in estradiol-primed animals. Endocrinology 1980; 106: 1160-1165.

56. Schreiber JR, Hsueh JW. Progesterone "receptor" in rat ovary. Endocrinology 1979; 105: 915-919.

57. Naess O. Characterization of cytoplasmic progesterone receptors in rat granulosa cells. Evidence for nuclear translocation. Acta Endocrinol (Copenh) 1981; 98: 288-294.

58. Press MF, Greene GL. Localization of progesterone receptor with monoclonal antibodies to the human progestin receptor. Endocrinology 1988; 122: 1165-1175.

59. Isola J, Korte JM, Tuohimaa P. Immunocytochemical localization of progesterone receptor in the chick ovary. Endocrinology 1987; 121: 1034-1040.

60. Korte JM, Isola JJ. An immunocytochemical study of the progesterone receptor in rabbit ovary. Mol Cell Endocrinol 1988; 58: 93-101.

61. Rueda BR, Hendry IR, Hendry IW, Stormshak F, Slayden OD, Davis JS. Decreased progesterone levels and progesterone receptor antagonists promote apoptotic cell death in bovine luteal cells. Biol Reprod 2000; 62: 269-276.

62. Park OK, Mayo KE. Transient expression of progesterone receptor messenger RNA in ovarian granulosa cells after the preovulatory luteinizing hormone surge. Mol Endocrinol 1991; 5: 967-978.

63. Cassar CA, Dow MPD, Pursley RJ, Smith GW. Effect of the preovulatory LH surge on bovine follicular progesterone receptor mRNA expression. Domestic Animal Endocrinology 2001; Submitted 8/01.

64. Chaffin CL, Stouffer RL, Duffy DM. Gonadotropin and steroid regulation of steroid receptor and aryl hydrocarbon receptor messenger ribonucleic acid in macaque granulosa cells during the periovulatory interval. Endocrinology 1999; 140: 4753-4760.

65. Natraj U, Richards JS. Hormonal regulation, localization, and functional activity of the progesterone receptor in granulosa cells of rat preovulatory follicles. Endocrinology 1993; 133: 761-769.

66. Park-Sarge OK, Mayo KE. Regulation of the progesterone receptor gene by gonadotropins and cyclic adenosine 3',5'-monophosphate in rat granulosa cells. Endocrinology 1994; 134: 709-718.

67. Iwai M, Yasuda K, Fukuoka M, Iwai T, Takakura K, Taii S, Nakanishi S, Mori T. Luteinizing hormone induces progesterone receptor gene expression in cultured porcine granulosa cells. Endocrinology 1991; 129: 1621-1627.

68. Uilenbroek JT. Hormone concentrations and ovulatory response in rats treated with antiprogestagens. J Endocrinol 1991; 129: 423-429.

69. van der Schoot P, Bakker GH, Klijn JG. Effects of the progesterone antagonist RU486 on ovarian activity in the rat. Endocrinology 1987; 121: 1375-1382.

70. Lydon JP, DeMayo FJ, Conneely OM, O'Malley BW. Reproductive phenotpes of the progesterone receptor null mutant mouse. J Steroid Biochem Mol Biol 1996; 56: 67-77.

71. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Jr., Shyamala G, Conneely OM, O'Malley BW. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev 1995; 9: 2266-2278.

72. Robker RL, Russell DL, Espey LL, Lydon JP, O'Malley BW, Richards JS. Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. Proc Natl Acad Sci U S A 2000; 97: 4689-4694.

73. Robker RL, Russell DL, Yoshioka S, Sharma SC, Lydon JP, O'Malley BW, Espey LL, Richards JS. Ovulation: a multi-gene, multi-step process. Steroids 2000; 65: 559-570.

74. LeMaire WJ, Clark MR, Chainy GBN, Marsh JM. The role of prostaglandins in the mechanism of ovulation. In: Tozzini RI, Reaves G, Pineda RL (eds.), International Symposium on the endocrine physiopathology of the ovary. Amsterdam: Elsevier/North Holland; 1980: 207-217.

75. Koos RD, Clark MR, Janson PO, Ahren KE, LeMaire WJ. Prostaglandin levels in preovulatory follicles from rabbit ovaries perfused in vitro. Prostaglandins 1983; 25: 715-724.

76. Maia H, Barbosa I, Coutinho EM. Inhibition of ovulation in marmoset monkeys by indomethacin. Fertil Steril 1978; 29: 565-570.

77. Wallach EE, Cruz A, Hunt J, Wright KH, Stevens VC. The effect of indomethacin of HMG-HCG induced ovulation in the phesus monkey. Prostaglandins 1975; 9: 645-658.

78. Algire JE, Srikandakumar A, Guilbault LA, Downey BR. Preovulatory changes in follicular prostaglandins and their role in ovulation in cattle. Can J Vet Res 1992; 56: 67-69.

79. Downey BR, Ainsworth L. Reversal of indomethacin blockade of ovulation in gilts by prostaglandins. Prostaglandins 1980; 19: 17-22.

80. O'Grady JP, Caldwell BV, Auletta FJ, Speroff L. The effects of an inhibitor of prostaglandin synthesis (indomethacin) on ovulation, pregnancy, and pseudopregnancy in the rabbit. Prostaglandins 1972; 1: 97-106.

81. Hamada Y, Bronson RA, Wright KH, Wallach EE. Ovulation in the perfused rabbit ovary: the influence of prostaglandins and prostaglandin inhibitors. Biology of Reproduction 1977; 17: 58-63.

82. Sirois J, Richards JS. Purification and characterization of a novel, distinct isoform of prostaglandin endoperoxide synthase induced by human chorionic gonadotropin in granulosa cells of rat preovulatory follicles. J Biol Chem 1992; 267: 6382-6388.

83. Sirois J, Simmons DL, Richards JS. Hormonal regulation of messenger ribonucleic acid encoding a novel isoform of prostaglandin endoperoxide H synthase in rat preovulatory follicles. Induction in vivo and in vitro. J Biol Chem 1992; 267: 11586-11592.

84. Murdoch WJ, McCormick RJ. Dose-dependent effects of indomethacin on ovulation in the sheep: relationship to follicular prostaglandin production, steroidogenesis, collagenolysis, and leukocyte chemotaxis. Biol Reprod 1991; 45: 907-911.

85. Sirois J. Induction of prostaglandin endoperoxide synthase-2 by human chorionic gonadotropin in bovine preovulatory follicles in vivo. Endocrinology 1994; 135: 841-848.

86. Pall M, Friden BE, Brannstrom M. Induction of delayed follicular rupture in the human by the selective COX-2 inhibitor rofecoxib: a randomized double-blind study. Hum Reprod 2001; 16: 1323-1328.

87. Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffee BD, Covington MB, Contel NR, Eng VM, Collins RJ, Czerniak PM, et al. Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. Nature 1995; 378: 406-409.

88. Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 1997; 91: 197-208.

89. Davis BJ, Lennard DE, Lee CA, Tiano HF, Morham SG, Wetsel WC, Langenbach R. Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1beta. Endocrinology 1999; 140: 2685-2695.

90. Brown CG, Poyser NL. Studies on ovarian prostaglandin production in relation to ovulation in the rat. J Reprod Fertil 1984; 72: 407-414.

91. Srikandakumar A, Downey BR. Induction of ovulation in gilts with closprostenol. Theriogenology 1989; 32: 445-449.

92. Savage NC, Liptrap RM. Induction of ovulation in cyclic mares by administration of a synthetic prostaglandin, fenprostalene, during oestrus. J Reprod Fertil Suppl 1987; 35: 239-243.

93. Coleman RA, Smith WL, Narumiya S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 1994; 46: 205-229.

94. Hizaki H, Segi E, Sugimoto Y, Hirose M, Saji T, Ushikubi F, Matsuoka T, Noda Y, Tanaka T, Yoshida N, Narumiya S, Ichikawa A. Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP(2). Proc Natl Acad Sci U S A 1999; 96: 10501-10506.

95. Richards JS, Russell DL, Robker RL, Dajee M, Alliston TN. Molecular mechanisms of ovulation and luteinization. Mol Cell Endocrinol 1998; 145: 47-54.

96. Downs SM, Longo FJ. Effects of indomethacin on preovulatory follicles in immature, superovulated mice. Am J Anat 1982; 164: 265-274.

97. Espey LL. Ovulation. In: Jones RE (ed.) The vertebrate ovary. New York: Plenum Press; 1978: 503-532.

98. Espey LL. Ultrastructure of the apex of the rabbit Graafian follicle during the ovulatory process. Endocrinology 1962; 81: 267-276.

99. Lofman CO, Janson PO, Kallfelt BJ, Ahren K, LeMaire WJ. The study of ovulation in the isolated perfused rabbit ovary. II. Photographic and cinematographic observation. Biology of Reproduction 1982; 26: 467-473.

100. Bjersing L, Cajander S. Ovulation and the mechanism of follicle rupture. III. Transmission electron microscopy of rabbit germinal epithelium prior to induced ovulation. Cell Tissue Res 1974; 149: 313-327.

101. Martin GG, Miller Walker C. Visualization of the three-dimensional distribution of collagen fibrils over preovulatory follicles in the hamster. J Exp Zool 1983; 225: 311-319.

102. Reich R, Tsafriri A, Mechanic GL. The involvement of collagenolysis in ovulation in the rat. Endocrinology 1985; 116: 522-527.

103. Murdoch WJ, McCormick RJ. Enhanced degradation of collagen within apical vs. basal wall of ovulatory ovine follicle. American Journal of Physiology 1992; 263: E221-225.

104. Palotie A, Peltonen L, Foidart JM, Rajaniemi H. Immunohistochemical localization of basement membrane components and interstitial collagen types in preovulatory rat ovarian follicles. Coll Relat Res 1984; 4: 279-287.

105. Asch RH, Li HP, Yovich JL, Katayama KP, Balmaceda JP, Rojas FJ, Stone SC. Failed oocyte retrieval after lack of human chorionic gonadotropin administration in assisted reproductive technology. Fertil Steril 1992; 58: 361-365.

106. Sauer MV, Paulson RJ. Mishaps and misfortunes: complications that occur in oocyte donation. Fertil Steril 1994; 61: 963-965.

107. Wallen P. Biochemistry of plasminogen. In: Kline DL (ed.) Redd, K.N.N. Boca Raton: CRC Press; 1980: 2-25.

108. Bachmann F. Fibrinolysis. In: Verstraete M, Vermylen J, Lijnen HR, Arnout J (eds.), Thrombosis and Haemostasis. Leuven: International Soc. on Thrombosis and Haemostasis and Leuven University Press; 1987: 227-265.

109. Raum D, Marcus D, Alper CA, Levey R, Taylor PD, Starzl TE. Synthesis of human plasminogen by the liver. Science 1980; 208: 1036-1037.

110. Sappino AP, Madani R, Huarte J, Belin D, Kiss JZ, Wohlwend A, Vassalli JD. Extracellular proteolysis in the adult murine brain. J Clin Invest 1993; 92: 679-685.

111. Tsirka SE, Rogove AD, Bugge TH, Degen JL, Strickland S. An extracellular proteolytic cascade promotes neuronal degeneration in the mouse hippocampus. J Neurosci 1997; 17: 543-552.

112. Hajjar KA. The endothelial cell tissue plasminogen activator receptor. Specific interaction with plasminogen. J Biol Chem 1991; 266: 21962-21970.

113. DeClerck YA, Laug WE. Cooperation between matrix metalloproteinases and the plasminogen activator-plasmin system in tumor progression. Enzyme Protein 1996; 49: 72-84.

114. Makowski GS, Ramsby ML. Binding of latent matrix metalloproteinase 9 to fibrin: activation via a plasmin-dependent pathway. Inflammation 1998; 22: 287-305.

115. Lijnen HR, Van Hoef B, Lupu F, Moons L, Carmeliet P, Collen D. Function of the plasminogen/plasmin and matrix metalloproteinase systems after vascular injury in mice with targeted inactivation of fibrinolytic system genes. Arterioscler Thromb Vasc Biol 1998; 18: 1035-1045.

116. Murphy G, Stanton H, Cowell S, Butler G, Knauper V, Atkinson S, Gavrilovic J. Mechanisms for pro matrix metalloproteinase activation. APMIS 1999; 107: 38-44.

117. Ramos DeSimone N, Hahn Dantona E, Sipley J, Nagase H, French DL, Quigley JP. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. J Biol Chem 1999; 274: 13066-13076.

118. Okumura Y, Sato H, Seiki M, Kido H. Proteolytic activation of the precursor of membrane type 1 matrix metalloproteinase by human plasmin. A possible cell surface activator. FEBS Lett 1997; 402: 181-184.

119. Mazzieri R, Masiero L, Zanetta L, Monea S, Onisto M, Garbisa S, Mignatti P. Control of type IV collagenase activity by components of the urokinase-plasmin system: a regulatory mechanism with cell-bound reactants. EMBO J 1997; 16: 2319-2332.

120. Baramova EN, Bajou K, Remacle A, C LH, Krell HW, Weidle UH, Noel A, Foidart JM. Involvement of PA/plasmin system in the processing of pro-MMP-9 and in the second step of pro-MMP-2 activation. FEBS Lett 1997; 405: 157-162.

121. Mignatti P, Rifkin DB. Plasminogen activators and matrix metalloproteinases in angiogenesis. Enzyme Protein 1996; 49: 117-137.

122. Hahn Dantona E, Ramos DeSimone N, Sipley J, Nagase H, French DL, Quigley JP. Activation of proMMP-9 by a plasmin/MMP-3 cascade in a tumor cell model. Regulation by tissue inhibitors of metalloproteinases. Annals of the New York Academy of Science 1999; 878: 372-387.

123. Ried S, Jager C, Jeffers M, Vande Woude GF, Graeff H, Schmitt M, Lengyel E. Activation mechanisms of the urokinase-type plasminogen activator promoter by hepatocyte growth factor/scatter factor. J Biol Chem 1999; 274: 16377-16386.

124. Pepinsky RB, Sinclair LK, Chow EP, Mattaliano RJ, Manganaro TF, Donahoe PK, Cate RL. Proteolytic processing of mullerian inhibiting substance produces a transforming growth factor-beta-like fragment. J Biol Chem 1988; 263: 18961-18964.

125. Granelli-Piperno A, Reich E. Plasminogen activators of the pituitary gland: enzyme characterization and hormonal modulation. J Cell Biol 1983; 97: 1029-1037.

126. Patterson BC, Sang QA. Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9). J Biol Chem 1997; 272: 28823-28825.

127. Hu GF. Limited proteolysis of angiogenin by elastase is regulated by plasminogen. J Protein Chem 1997; 16: 669-679.

128. Cornelius LA, Nehring LC, Harding E, Bolanowski M, Welgus HG, Kobayashi DK, Pierce RA, Shapiro SD. Matrix metalloproteinases generate angiostatin: effects on neovascularization. J Immunol 1998; 161: 6845-6852.

129. Bykowska K, Levin EG, Rijken DC, Loskutoff DJ, Collen D. Characterization of a plasminogen activator secreted by cultured bovine aortic endothelial cells. Biochim Biophys Acta 1982; 703: 113-115.

130. Andrade Gordon P, Strickland S. Interaction of heparin with plasminogen activators and plasminogen: effects on the activation of plasminogen. Biochemistry 1986; 25: 4033-4040.

131. Mars WM, Zarnegar R, Michalopoulos GK. Activation of hepatocyte growth factor by the plasminogen activators uPA and tPA. Am J Pathol 1993; 143: 949-958.

132. White WF, Barlow GH, Mozen MM. The isolation and characterization of plasminogen activators (urokinase) from human urine. Biochemistry 1966; 5: 2160-2169.

133. Ichinose A, Fujikawa K, Suyama T. The activation of pro-urokinase by plasma kallikrein and its inactivation by thrombin. J Biol Chem 1986; 261: 3486-3489.

134. Dano K, Andreasen PA, Grondahl Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 1985; 44: 139-266.

135. Kobayashi H, Schmitt M, Goretzki L, Chucholowski N, Calvete J, Kramer M, Gunzler WA, Janicke F, Graeff H. Cathepsin B efficiently activates the soluble and the tumor cell receptor-bound form of the proenzyme urokinase-type plasminogen activator (Pro-uPA). J Biol Chem 1991; 266: 5147-5152.

136. Orgel D, Schroder W, Hecker Kia A, Weithmann KU, Kolkenbrock H, Ulbrich N. The cleavage of pro-urokinase type plasminogen activator by stromelysin-1. Clin Chem Lab Med 1998; 36: 697-702.

137. Appella E, Robinson EA, Ullrich SJ, Stoppelli MP, Corti A, Cassani G, Blasi F. The receptor-binding sequence of urokinase. A biological function for the growth-factor module of proteases. J Biol Chem 1987; 262: 4437-4440.

138. Naldini L, Vigna E, Bardelli A, Follenzi A, Galimi F, Comoglio PM. Biological activation of pro-HGF (hepatocyte growth factor) by urokinase is controlled by a stoichiometric reaction. J Biol Chem 1995; 270: 603-611.

139. Andreasen PA, Egelund R, Petersen HH. The plasminogen activation system in tumor growth, invasion, and metastasis. Cell Mol Life Sci 2000; 57: 25-40.

140. Kjellen L, Lindahl U. Proteoglycans: structures and interactions [published erratum appears in Annu Rev Biochem 1992;61:following viii]. Annu Rev Biochem 1991; 60: 443-475.

141. Robbins KC, Summaria L, Hsieh B, Shah RJ. The peptide chains of human plasmin. Mechanism of activation of human plasminogen to plasmin. J Biol Chem 1967; 242: 2333-2342.

142. Loskutoff DJ, Sawdey M, Mimuro J. Type I plasminogen activator inhibitor. In: Coller BS (ed.) Progress in Haemostasis and Thrombosis. Philadelphia: Saunders; 1988: 87-115.

143. Aertgeerts K, De Bondt HL, De Ranter C, Declerck PJ. A model of the reactive form of plasminogen activator inhibitor-1. J Struct Biol 1994; 113: 239-245.

144. Lawrence DA, Ginsburg D, Day DE, Berkenpas MB, Verhamme IM, Kvassman JO, Shore JD. Serpin-protease complexes are trapped as stable acyl-enzyme intermediates. J Biol Chem 1995; 270: 25309-25312.

145. van Mourik JA, Lawrence DA, Loskutoff DJ. Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. J Biol Chem 1984; 259: 14914-14921.

146. Loskutoff DJ, Sawdey M, Mimuro J. In: Coller BS (ed.) Progress in Hemostasis and Thrombosis. Philadelphia: W.B. Saunders Company; 1989: 87-115.

147. Seiffert D, Loskutoff DJ. Evidence that type 1 plasminogen activator inhibitor binds to the somatomedin B domain of vitronectin. J Biol Chem 1991; 266: 2824-2830.

148. Ehrlich HJ, Gebbink RK, Keijer J, Linders M, Preissner KT, Pannekoek H. Alteration of serpin specificity by a protein cofactor. Vitronectin endows

plasminogen activator inhibitor 1 with thrombin inhibitory properties. J Biol Chem 1990; 265: 13029-13035.

149. Mimuro J, Loskutoff DJ. Binding of type 1 plasminogen activator inhibitor to the extracellular matrix of cultured bovine endothelial cells. J Biol Chem 1989; 264: 5058-5063.

150. Mimuro J, Loskutoff DJ. Purification of a protein from bovine plasma that binds to type 1 plasminogen activator inhibitor and prevents its interaction with extracellular matrix. Evidence that the protein is vitronectin. J Biol Chem 1989; 264: 936-939.

151. Declerck PJ, De Mol M, Alessi MC, Baudner S, Paques EP, Preissner KT, Muller Berghaus G, Collen D. Purification and characterization of a plasminogen activator inhibitor 1 binding protein from human plasma. Identification as a multimeric form of S protein (vitronectin). J Biol Chem 1988; 263: 15454-15461.

152. Lupu F, Bergonzelli GE, Heim DA, Cousin E, Genton CY, Bachmann F, Kruithof EK. Localization and production of plasminogen activator inhibitor-1 in human healthy and atherosclerotic arteries. Arterioscler Thromb 1993; 13: 1090-1100.

153. Chavakis T, Kanse SM, Yutzy B, Lijnen HR, Preissner KT. Vitronectin concentrates proteolytic activity on the cell surface and extracellular matrix by trapping soluble urokinase receptor-urokinase complexes. Blood 1998; 91: 2305-2312.

154. Astedt B, Hagerstrand I, Lecander I. Cellular localisation in placenta of placental type plasminogen activator inhibitor. Thromb Haemost 1986; 56: 63-65.

155. Schwartz BS, Monroe MC, Levin EG. Increased release of plasminogen activator inhibitor type 2 accompanies the human mononuclear cell tissue factor response to lipopolysaccharide. Blood 1988; 71: 734-741.

156. Gyetko MR, Shollenberger SB, Sitrin RG. Urokinase expression in mononuclear phagocytes: cytokine-specific modulation by interferon-gamma and tumor necrosis factor-alpha. Journal of Leukocyte Biology 1992; 51: 256-263.

157. Dickinson JL, Norris BJ, Jensen PH, Antalis TM. The C-D interhelical domain of the serpin plasminogen activator inhibitor-type 2 is required for protection against TNF-α induced apoptosis. Cell Death and Differentiation 1998; 5: 163-171.

158. Espana F, Estelles A, Fernandez PJ, Gilabert J, Sanchez Cuenca J, Griffin JH. Evidence for the regulation of urokinase and tissue type plasminogen activators by the serpin, protein C inhibitor, in semen and blood plasma. Thromb Haemost 1993; 70: 989-994.

159. Schwartz BS, Espana F. Two distinct urokinase-serpin interactions regulate the initiation of cell surface-associated plasminogen activation. J Biol Chem 1999; 274: 15278-15283.

160. Uhrin P, Dewerchin M, Hilpert M, Chrenek P, Schofer C, Zechmeister-Machhart M, Kronke G, Vales A, Carmeliet P, Binder BR, Geiger M. Disruption of the protein C inhibitor gene results in impaired spermatogenesis and male infertility. J Clin Invest 2000; 106: 1531-1539.

161. Yuasa H, Tanaka H, Hayashi T, Wakita T, Nakamura H, Nishioka J, Kawarada Y, Suzuki K. Bovine protein C inhibitor has a unique reactive site and can transiently inhibit plasmin. Thromb Haemost 2000; 83: 262-267.

162. Farrell DH, Wagner SL, Yuan RH, Cunningham DD. Localization of protease nexin-1 on the fibroblast extracellular matrix. J Cell Physiol 1988; 134: 179-188.

163. Murer V, Spetz JF, Hengst U, Altrogge LM, de Agostini A, Monard D. Male fertility defects in mice lacking the serine protease inhibitor protease nexin-1. Proceedings of the Natl Academy of Science USA 2001; 98: 3029-3033.

164. B\dard J, Brule S, Price C, Silversides DW, Lussier JG. Bovine Serpin-1: A gene differentially expressed during follicular development that is potentially involved in follicular dominance. Biology of Reproduction 2001; 64: Abstract 474.

165. Lijnen HR, Van Hoef B, Collen D. Inactivation of the serpin alpha(2)-antiplasmin by stromelysin-1. Biochim Biophys Acta 2001; 1547: 206-213.

166. Hajjar KA, Nachman RL. The human endothelial cell plasmin-generating system. In: Colman RW, Hirsh J, Marder VJ, Salzman EW (eds.), Hemostasis and Thrombosis: Basic Principles and Clinical Practice 3rd Edition. Philadelphia: Lippincott Company; 1994: 823-836.

167. Nielsen LS, Kellerman GM, Behrendt N, Picone R, Dano K, Blasi F. A 55,000-60,000 Mr receptor protein for urokinase-type plasminogen activator. Identification in human tumor cell lines and partial purification. J Biol Chem 1988; 263: 2358-2363.

168. Roldan AL, Cubellis MV, Masucci MT, Behrendt N, Lund LR, Dano K, Appella E, Blasi F. Cloning and expression of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plasmin dependent proteolysis [published erratum appears in EMBO J 1990 May;9(5):1674]. EMBO J 1990; 9: 467-474.

169. Dumler I, Petri T, Schleuning WD. Interaction of urokinase-type plasminogenactivator (u-PA) with its cellular receptor (u-PAR) induces phosphorylation on tyrosine of a 38 kDa protein. FEBS Lett 1993; 322: 37-40.

170. Resnati M, Guttinger M, Valcamonica S, Sidenius N, Blasi F, Fazioli F. Proteolytic cleavage of the urokinase receptor substitutes for the agonist-induced chemotactic effect. EMBO J 1996; 15: 1572-1582.

171. Bohuslav J, Horejsi V, Hansmann C, Stockl J, Weidle UH, Majdic O, Bartke I, Knapp W, Stockinger H. Urokinase plasminogen activator receptor, beta 2-integrins, and Src-kinases within a single receptor complex of human monocytes. J Exp Med 1995; 181: 1381-1390.

172. Dumler I, Petri T, Schleuning WD. Induction of c-fos gene expression by urokinase-type plasminogen activator in human ovarian cancer cells. FEBS Lett 1994; 343: 103-106.

173. Mirshahi SS, Lounes KC, Lu H, Pujade Lauraine E, Mishal Z, Benard J, Bernadou A, Soria C, Soria J. Defective cell migration in an ovarian cancer cell line is associated with impaired urokinase-induced tyrosine phosphorylation. FEBS Lett 1997; 411: 322-326.

174. Busso N, Masur SK, Lazega D, Waxman S, Ossowski L. Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. J Cell Biol 1994; 126: 259-270.

175. Nguyen DHD, Hussaini IM, Gonias SL. Binding of urokinase-type plasminogen activator to its receptor in MCF-7 cells activates extracellular siganl-regulated kinase 1 and 2 which is required for increased cellular motility. J Biol Chem 1998; 273: 8502-8507.

176. Wang Y. The role and regulation of urokinase-type plasminogen activator receptor gene expression in cancer invasion and metastasis. Med Res Rev 2001; 21: 146-170.

177. Taylor ME. Evolution of a family of receptors containing multiple C-type carbohydrate-recognition domains. Glycobiology 1997; 7: v-viii.

178. Wu K, Yuan J, Lasky LA. Characterization of a novel member of the macrophage mannose receptor type C lectin family. J Biol Chem 1996; 271: 21323-21330.

179. Barmina OY, Walling HW, Fiacco GJ, Freije JM, Lopez-Otin C, Jeffrey JJ, Partridge NC. Collagenase-3 binds to a specific receptor and requires the low density lipoprotein receptor-related protein for internalization. J Biol Chem 1999; 274: 30087-30093.

180. Engelholm LH, Nielsen BS, Dano K, Behrendt N. The urokinase receptor associated protein (uPARAP/endo180): a novel internalization receptor connected to the plasminogen activation system. Trends Cardiovasc Med 2001; 11: 7-13.

181. Czekay RP, Kuemmel TA, Orlando RA, Farquhar MG. Direct Binding of Occupied Urokinase Receptor (uPAR) to LDL Receptor-related Protein Is

Required for Endocytosis of uPAR and Regulation of Cell Surface Urokinase Activity. Mol Biol Cell 2001; 12: 1467-1479.

182. Herz J, Couthier DE, Hammer RE. Correction: LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation [letter]. Cell 1993; 73: 428.

183. Herz J, Clouthier DE, Hammer RE. LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation [published erratum appears in Cell 1993 May 7;73(3):428]. Cell 1992; 71: 411-421.

184. Argraves KM, Battey FD, MacCalman CD, McCrae KR, Gafvels M, Kozarsky KF, Chappell DA, Strauss JF, Strickland DK. The very low density lipoprotein receptor mediates the cellular catabolism of lipoprotein lipase and urokinase-plasminogen activator inhibitor type I complexes. J Biol Chem 1995; 270: 26550-26557.

185. Parkkinen J, Rauvala H. Interactions of plasminogen and tissue plasminogen activator (t-PA) with amphoterin. Enhancement of t-PA-catalyzed plasminogen activation by amphoterin. J Biol Chem 1991; 266: 16730-16735.

186. Merenmies J, Pihlaskari R, Laitinen J, Wartiovaara J, Rauvala H. 30-kDa heparin-binding protein of brain (amphoterin) involved in neurite outgrowth. Amino acid sequence and localization in the filopodia of the advancing plasma membrane. J Biol Chem 1991; 266: 16722-16729.

187. Rauvala H, Pihlaskari R. Isolation and some characteristics of an adhesive factor of brain that enhances neurite outgrowth in central neurons. J Biol Chem 1987; 262: 16625-16635.

188. Rauvala H, Merenmies J, Pihlaskari R, Korkolainen M, Huhtala ML, Panula P. The adhesive and neurite-promoting molecule p30: analysis of the amino-terminal sequence and production of antipeptide antibodies that detect p30 at the surface of neuroblastoma cells and of brain neurons. J Cell Biol 1988; 107: 2293-2305.

189. Hajjar KA, Jacovina AT, Chacko J. An endothelial cell receptor for plasminogen/tissue plasminogen activator. I. Identity with annexin II. J Biol Chem 1994; 269: 21191-21197.

190. Raynal P, Pollard HB. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochim Biophys Acta 1994; 1197: 63-93.

191. Swairjo MA, Seaton BA. Annexin structure and membrane interactions: a molecular perspective. Annu Rev Biophys Biomol Struct 1994; 23: 193-213.

192. Huang KS, Wallner BP, Mattaliano RJ, Tizard R, Burne C, Frey A, Hession C, McGray P, Sinclair LK, Chow EP, et al. Two human 35 kd inhibitors of phospholipase A2 are related to substrates of pp60v-src and of the epidermal growth factor receptor/kinase. Cell 1986; 46: 191-199.

193. Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord JJ, Collen D, Mulligan RC. Physiological consequences of loss of plasminogen activator gene function in mice. Nature 1994; 368: 419-424.

194. Seeds NW, Basham ME, Haffke SP. Neuronal migration is retarded in mice lacking the tissue plasminogen activator gene. Proc Natl Acad Sci U S A 1999; 96: 14118-14123.

195. Ny A, Leonardsson G, Hagglund AC, Hagglof P, Ploplis VA, Carmeliet P, Ny T. Ovulation in plasminogen-deficient mice. Endocrinology 1999; 140: 5030-5035.

196. Kim YH, Park JH, Hong SH, Koh JY. Nonproteolytic neuroprotection by human recombinant tissue plasminogen activator. Science 1999; 284: 647-650.

197. Waltz DA, Fujita RM, Yang X, Natkin L, Zhuo S, Gerard CJ, Rosenberg S, Chapman HA. Nonproteolytic role for the urokinase receptor in cellular migration in vivo. Am J Respir Cell Mol Biol 2000; 22: 316-322.

198. Gyetko MR, Sud S, Kendall T, Fuller JA, Newstead MW, Standiford TJ. Urokinase receptor-deficient mice have impaired neutrophil recruitment in response to pulmonary Pseudomonas aeruginosa infection. J Immunol 2000; 165: 1513-1519.

199. Carmeliet P, Stassen JM, Schoonjans L, Ream B, van den Oord JJ, De Mol M, Mulligan RC, Collen D. Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, and thrombolysis. J Clin Invest 1993; 92: 2756-2760.

200. Bajou K, Noel A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, Skobe M, Fusenig NE, Carmeliet P, Collen D, Foidart JM. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat Med 1998; 4: 923-928.

201. McMahon GA, Petitclerc E, Stefansson S, Smith E, Wong MK, Westrick RJ, Ginsburg D, Brooks PC, Lawrence DA. Plasminogen activator inhibitor-1 regulates tumor growth and angiogenesis. J Biol Chem 2001; 276: 33964-33968.

202. Fay WP, Parker AC, Condrey LR, Shapiro AD. Human plasminogen activator inhibitor-1 (PAI-1) deficiency: characterization of a large kindred with a null mutation in the PAI-1 gene. Blood 1997; 90: 204-208.

203. Dougherty KM, Pearson JM, Yang AY, Westrick RJ, Baker MS, Ginsburg D. The plasminogen activator inhibitor-2 gene is not required for normal murine

development or survival. Proceedings of the Natl Academy of Science USA 1999; 96: 686-691.

204. Lijnen HR, Okada K, Matsuo O, Collen D, Dewerchin M. Alpha2-antiplasmin gene deficiency in mice is associated with enhanced fibrinolytic potential without overt bleeding. Blood 1999; 93: 2274-2281.

205. Umans L, Serneels L, Overbergh L, Stas L, Van Leuven F. alpha2-macroglobulin- and murinoglobulin-1- deficient mice. A mouse model for acute pancreatitis. Am J Pathol 1999; 155: 983-993.

206. Murdoch WJ. Regulation of collagenolysis and cell death by plasmin within the formative stigma of preovulatory ovine follicles. Journal Reproduction and Fertility 1998; 113: 331-336.

207. Butler TA, Zhu C, Mueller RA, Fuller GC, Lemaire WJ, Woessner JF, Jr. Inhibition of ovulation in the perfused rat ovary by the synthetic collagenase inhibitor SC 44463. Biol Reprod 1991; 44: 1183-1188.

208. Brannstrom M, Woessner JF, Jr., Koos RD, Sear CH, LeMaire WJ. Inhibitors of mammalian tissue collagenase and metalloproteinases suppress ovulation in the perfused rat ovary. Endocrinology 1988; 122: 1715-1721.

209. Murdoch WJ. Endothelial cell death in preovulatory ovine follicles: possible implication in the biomechanics of rupture. Journal of Reproduction and Fertility 1995; 105: 161-164.

210. Murdoch WJ, Colgin DC, Ellis JA. Role of tumor necrosis factor-alpha in the ovulatory mechanism of ewes. Journal of Animal Science 1997; 75: 1601-1605.

211. Murdoch WJ, Van Kirk EA, Murdoch J. Plasmin cleaves tumor necrosis factor alpha exodomain from sheep follicular endothelium: implication in the ovulatory process. Biology of Reproduction 1999; 60: 1166-1171.

212. Murdoch WJ, Lund SA. Prostaglandin-Independent anovulatory mechanism of indomethacin action: Inhibition of tumor necrosis factor α–induced sheep ovarian cell apoptosis. Biology of Reproduction 1999; 61: 1655-1659.

213. Perona JJ, Craik CS. Structural basis of substrate specificity in the serine proteases. Protein Sci 1995; 4: 337-360.

214. Vilcek J, Lee TH. Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. J Biol Chem 1991; 266: 7313-7316.

215. Terranova PF. Potential roles of tumor necrosis factor-alpha in follicular development, ovulation, and the life span of the corpus luteum. Domestic Animal Endocrinology 1997; 14: 1-15.

216. Steller H. Mechanisms and genes of cellular suicide. Science 1995; 267: 1445-1449.

217. Larrick JW, Wright SC. Cytotoxic mechanism of tumor necrosis factor-alpha. FASEB J 1990; 4: 3215-3223.

218. Dickinson JL, Bates EJ, Ferrante A, Antalis TM. Plasminogen activator inhibitor type 2 inhibits tumor necrosis factor alpha-induced apoptosis. Evidence for an alternate biological function. Journal Biological Chemistry 1995; 270: 27894-27904.

219. Ny A, Nordstrom L, Carmeliet P, Ny T. Studies of mice lacking plasminogen activator gene function suggest that plasmin production prior to ovulation exceeds the amount needed for optimal ovulation efficiency. European Journal of Biochemistry 1997; 244: 487-493.

220. Politis I, Srikandakumar A, Turner JD, Tsang BK, Ainsworth L, Downey BR. Changes in and partial identification of the plasminogen activator and plasminogen activator inhibitor systems during ovarian follicular maturation in the pig. Biology of Reproduction 1990; 43: 636-642.

221. Li M, Karakji EG, Xing R, Fryer JN, Carnegie JA, Rabbani SA, Tsang BK. Expression of urokinase-type plasminogen activator and its receptor during ovarian follicular development. Endocrinology 1997; 138: 2790-2799.

222. Macchione E, Epifano O, Stefanini M, Belin D, Canipari R. Urokinase redistribution from the secreted to the cell-bound fraction in granulosa cells of rat preovulatory follicles. Biology of Reproduction 2000; 62: 895-903.

223. Peng XR, Hsueh AJ, Ny T. Transient and cell-specific expression of tissue-type plasminogen activator and plasminogen-activator-inhibitor type 1 results in controlled and directed proteolysis during gonadotropin-induced ovulation. European Journal of Biochemistry 1993; 214: 147-156.

224. Smokovitis A, Kokolis N, Alexaki-Tzivanidou E. The plasminogen activator activity is markedly increased mainly at the area of the rupture of the follicular wall at the time of ovulation. Animal Reproduction Science 1988; 16: 285-294.

225. Murdoch J, Van Kirk EA, Murdoch WJ. Hormonal control of urokinase plasminogen activator secretion by sheep ovarian surface epithelial cells. Biology of Reproduction 1999; 61: 1487-1491.

226. Smith MF, McIntush EW, Smith GW. Mechanisms associated with corpus luteum development. Journal of Animal Science 1994; 72: 1857-1872.

227. Bacharach E, Itin A, Keshet E. In vivo patterns of expression of urokinase and its inhibitor PAI-1 suggest a concerted role in regulating physiological angiogenesis. Proceedings of the National Academy of Science USA 1992; 89: 10686-10690.

228. Liu YX, Peng XR, Ny T. Tissue-specific and time-coordinated hormone regulation of plasminogen-activator-inhibitor type I and tissue-type plasminogen activator in the rat ovary during gonadotropin-induced ovulation. European Journal of Biochemistry 1991; 195: 549-555.

229. Piquette GN, Crabtree ME, el-Danasouri I, Milki A, Polan ML. Regulation of plasminogen activator inhibitor-1 and -2 messenger ribonucleic acid levels in human cumulus and granulosa-luteal cells. Journal of Clinical Endocrinology and Metabolism 1993; 76: 518-523.

230. Yamada M, Horiuchi T, Oribe T, Yamamoto S, Matsushita H, Gentry PA. Plasminogen activator activity in the bovine oocyte-cumulus complex and early embryo. J Vet Med Sci 1996; 58: 317-322.

231. Yamada M, Gentry PA. The hemostatic profile of equine ovarian follicular fluid. Thromb Res 1995; 77: 45-54.

232. Reganon E, Aznar J, Vila V. Degradation of human fibrinogen by plasmin: isolation and partial characterization of an early degradation product. Haemostasis 1978; 7: 26-34.

233. Camiolo SM, Thorsen S, Astrup T. Fibrinogenolysis and fibrinolysis with tissue plasminogen activator, urokinase, streptokinase-activated human globulin, and plasmin. Proc Soc Exp Biol Med 1971; 138: 277-280.

234. Beck EA, Jackson DP. Studies on the degradation of human fibrinogen by plasmin and trypsin. Thromb Diath Haemorrh 1966; 16: 526-540.

235. Fletcher AP, Alkjaersig N, Fisher S, Sherry S. The proteolysis of fibrinogen by plasmin: the identification of thrombin-clottable fibrinogen derivatives which polymerize abnormally. J Lab Clin Med 1966; 68: 780-802.

236. Backova IP, Budzinskii AZ. [Fractionation and properties of early products of proteolysis of fibrinogen with the use of plasmin]. Biokhimiia 1965; 30: 322-326.

237. Liu YX, Ny T, Sarkar D, Loskutoff D, Hsueh AJ. Identification and regulation of tissue plasminogen activator activity in rat cumulus-oocyte complexes. Endocrinology 1986; 119: 1578-1587.

238. Akazawa K, Mori N, Kosugi T, Matsuo O, Mihara H. Localization of fibrinolytic activity in ovulation of the rat follicle as determined by the fibrin slide method. Jpn J Physiol 1983; 33: 1011-1018.

239. Ny T, Bjersing L, Hsueh AJ, Loskutoff DJ. Cultured granulosa cells produce two plasminogen activators and an antiactivator, each regulated differently by gonadotropins. Endocrinology 1985; 116: 1666-1668.

240. Holmberg M, Leonardsson G, Ny T. The species-specific differences in the cAMP regulation of the tissue-type plasminogen activator gene between rat, mouse and human is caused by a one-nucleotide substitution in the cAMP-responsive element of the promoters. European Journal of Biochemistry 1995; 231: 466-474.

241. Holmes PV, Hedin L, Janson PO. The role of cyclic adenosine 3',5'-monophosphate in the ovulatory process of the in vitro perfused rabbit ovary. Endocrinology 1986; 118: 2195-2202.

242. Galway AB, Oikawa M, Ny T, Hsueh AJ. Epidermal growth factor stimulates tissue plasminogen activator activity and messenger ribonucleic acid levels in cultured rat granulosa cells: mediation by pathways independent of protein kinases-A and -C. Endocrinology 1989; 125: 126-135.

243. LaPolt PS, Yamoto M, Veljkovic M, Sincich C, Ny T, Tsafriri A, Hsueh AJ. Basic fibroblast growth factor induction of granulosa cell tissue-type plasminogen activator expression and oocyte maturation: potential role as a paracrine ovarian hormone. Endocrinology 1990; 127: 2357-2363.

244. Strickland S, Beers WH. Studies on the role of plasminogen activator in ovulation. In vitro response of granulosa cells to gonadotropins, cyclic nucleotides, and prostaglandins. J Biol Chem 1976; 251: 5694-5702.

245. Jia XC, Ny T, Hsueh AJ. Synergistic effect of glucocorticoids and androgens on the hormonal induction of tissue plasminogen activator activity and messenger ribonucleic acid levels in granulosa cells. Mol Cell Endocrinol 1990; 68: 143-151.

246. Muegge K, Vila M, Gusella GL, Musso T, Herrlich P, Stein B, Durum SK. Interleukin 1 induction of the c-jun promoter. Proceedings of the Natl Academy of Science USA 1993; 90: 7054-7058.

247. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1991; 1072: 129-157.

248. Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf HJ, Jonat C, Herrlich P, Karin M. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell 1987; 49: 729-739.

249. Tanaka N, Espey LL, Stacy S, Okamura H. Epostane and indomethacin actions on ovarian kallikrein and plasminogen activator activities during ovulation in the gonadotropin-primed immature rat. Biol Reprod 1992; 46: 665-670.

250. Pall M, Mikuni M, Mitsube K, Brannstrom M. Time-dependent ovulation inhibition of a selective progesterone-receptor antagonist (Org 31710) and effects on ovulatory mediators in the in vitro perfused rat ovary. Biol Reprod 2000; 63: 1642-1647.

251. Barnathan ES, Kuo A, Rosenfeld L, Kariko K, Leski M, Robbiati F, Nolli ML, Henkin J, Cines DB. Interaction of single-chain urokinase-type plasminogen activator with human endothelial cells. J Biol Chem 1990; 265: 2865-2872.

252. Beers WH, Strickland S, Reich E. Ovarian plasminogen activator: relationship to ovulation and hormonal regulation. Cell 1975; 6: 387-394.

253. Pursley JR, Kosorok MR, Wiltbank MC. Reproductive management of lactating dairy cows using synchronization of ovulation. Journal of Dairy Science 1997; 80: 301-306.

254. Jimenez Krassel F, Binelli M, Tucker HA, Ireland JJ. Effect of long-term infusion with recombinant growth hormone-releasing factor and recombinant bovine somatotropin on development and function of dominant follicles and corpora lutea in Holstein cows. Journal of Dairy Science 1999; 82: 1917-1926.

255. Matteri RL, Roser JF, Baldwin DM, Lipovetsky V, Papkoff H. Characterization of a monoclonal antibody which detects luteinizing hormone from diverse mammalian species. Domestic Animal Endocrinology 1987; 4: 157-165.

256. Sambrook J, Fritsch EF, Maniatis T. Extraction, Purification, and Analysis of Messenger RNA from Eukaryotic Cells. In: Nolan C (ed.) Molecular Cloning: A Laboratory Manual, vol. 1, 2 ed. Plainview: Cold Spring Harbor Laboratory Press; 1989: 7.53-57.55.

257. Coleman PL, Green GD. A sensitive, coupled assay for plasminogen activator using a thiol ester substrate for plasmin. Annals of the New York Academy of Science 1981; 370: 617-626.

258. Roche PC, Campeau JD, Shaw ST, Jr. Comparative electrophoretic analysis of human and porcine plasminogen activators in SDS-polyacrylamide gels containing plasminogen and casein. Biochim Biophys Acta 1983; 745: 82-89.

259. Thewke DP, Seeds NW. The expression of mRNAs for hepatocyte growth factor/scatter factor, its receptor c-met, and one of its activators tissue-type plasminogen activator show a systematic relationship in the developing and adult cerebral cortex and hippocampus. Brain Res 1999; 821: 356-367.

260. Gaddy-Kurten D, Richards JS. Regulation of alpha 2-macroglobulin by luteinizing hormone and prolactin during cell differentiation in the rat ovary. Mol Endocrinol 1991; 5: 1280-1291.

261. Gaddy-Kurten D, Hickey GJ, Fey GH, Gauldie J, Richards JS. Hormonal regulation and tissue-specific localization of alpha 2-macroglobulin in rat ovarian follicles and corpora lutea. Endocrinology 1989; 125: 2985-2995.

262. Pepper MS, Sappino AP, Stocklin R, Montesano R, Orci L, Vassalli JD. Upregulation of urokinase receptor expression on migrating endothelial cells. J Cell Biol 1993; 122: 673-684.

263. Pedersen TL, Plesner T, Horn T, Hoyer-Hansen G, Sorensen S, Hansen NE. Subcellular distribution of urokinase and urokinase receptor in human neutrophils determined by immunoelectron microscopy. Ultrastruct Pathol 2000; 24: 175-182.

264. Werb Z, Mainardi CL, Vater CA, Harris ED, Jr. Endogenous activiation of latent collagenase by rheumatoid synovial cells. Evidence for a role of plasminogen activator. N Engl J Med 1977; 296: 1017-1023.

265. Bakke LJ, Dow MPD, Pursley RJ, Smith GW. Differential upregulation of interstitial collagenase (MMP-1) mRNA transcripts following the LH surge in bovine periovulatory follicular and luteal tissue. Serono Symposia USA, XIIIth Ovarian Workshop, Madison, WI. 2000; Abstract.

266. Holmberg L, Lecander I, Persson B, Astedt B. An inhibitor from placenta specifically binds urokinase and inhibits plasminogen activator released from ovarian carcinoma in tissue culture. Biochimica Biophysica Acta 1978; 544: 128-137.

267. Lecander I, Roblin R, Astedt B. Differential inhibition of two molecular forms of melanoma cell plasminogen activator by a placental inhibitor. British Journal Haematology 1984; 57: 407-412.

268. Kruithof EK, Tran Thang C, Gudinchet A, Hauert J, Nicoloso G, Genton C, Welti H, Bachmann F. Fibrinolysis in pregnancy: a study of plasminogen activator inhibitors. Blood 1987; 69: 460-466.

269. Kruithof EK, Vassalli JD, Schleuning WD, Mattaliano RJ, Bachmann F. Purification and characterization of a plasminogen activator inhibitor from the histiocytic lymphoma cell line U-937. Journal Biological Chemistry 1986; 261: 11207-11213.

270. Pursley JR, Mee MO, Wiltbank MC. Synchronization of ovulation in dairy cows using PGF2 alpha and GnRH. Theriogenology 1995: 915-923.

271. Schleuning WD, Medcalf RL, Hession C, Rothenbuhler R, Shaw A, Kruithof EK. Plasminogen activator inhibitor 2: regulation of gene transcription during phorbol ester-mediated differentiation of U-937 human histiocytic lymphoma cells. Molecular and Cellular Biology 1987; 7: 4564-4567.

272. Dow MPD, Pursley RJ, Smith GW. LH surge-induced upregulation of the plasminogen activator and plasmin activity with bovine preovulatory follicles. Biology of Reproduction 2001; 62: Abstract 140.

273. Dow MPD, Bakke LJ, Pursley RJ, Smith GW. The LH surge upregulates expression of tissue-type plasminogen activator mRNA in bovine preovulatory follicles. Biology of Reproduction 1999; 60: Abstract 96.

274. Dow MPD, Pursley RJ, Smith GW. LH surge-induced upregulation of urokinase plasminogen activator and its receptor mRNA expression within bovine periovulatory follicular and luteal tissue. In: Serono Symposia USA, XIIIth Ovarian Workshop; 2000; Madison, WI.

275. Murdoch WJ. Plasmin-tumour necrosis factor interaction in the ovulatory process. Journal of Reproduction and Fertility 1999; 54: 353-358.

276. Murdoch WJ. Programmed cell death in preovulatory ovine follicles. Biology of Reproduction 1995; 53: 8-12.

277. Gutierrez LS, Schulman A, Brito-Robinson T, Noria F, Ploplis VA, Castellino FJ. Tumor development is retarded in mice lacking the gene for urokinase-type plasminogen activator or its inhibitor, plasminogen activator inhibitor-1. Cancer Research 2000; 60: 5839-5847.