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EXPRESSION AND REGULATION OF PROTEASE NEXIN-1 AND PLASMINOGEN ACTIVATORS IN BOVINE OVARIAN FOLLICLES

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Résumé

Les changements dans la composition de la matrice extracellulaire et la membrane basale surviennent lors de la croissance folliculaire, possiblement par l'intermédiaire des cascades d'enzymes protéolytiques, incluant les activateurs du plasminogène (PA) et leurs inhibiteurs. Un tel inhibiteur est la protéine nexine-1 (PN-1), une protéine secrétée par les cellules de la granulosa (GC). Notre connaissance sur la régulation et sur l'expression des PA et la PN-1 dans les GC est très limitée. L'objectif général de cette étude était de tester l'hypothèse que l'expression de PN-1 est régulée par des gonadotrophines et les facteurs de croissance au cours de la croissance folliculaire.

L'expression des PA et de la PN-1 a été étudiée dans des GC bovine non-lutéinisée. L'activité de PA de type tissulaire (tPA) était plus élevée dans les GC issues de petits follicules comparée aux follicules plus gros, et le taux de sécrétion du PN-1 était plus élevé dans les GC de gros follicules. Dans les cellules provenant de petits follicules, les taux de sécrétion de tPA et PN-1 augmentaient en fonction du temps de culture. Dans les cellules provenant de gros follicules, l'activité de tPA augmentait de façon significative en fonction du temps de culture, alors que la sécrétion de PN-1 diminuait.

Dans les GC en culture, la FSH stimulait l'expression du gène codant pour la PN-1, ainsi que la sécrétion de PN-1. La FSH augmentait de façon dose-dépendante l'expression de l'ARNm de la tPA mais n'a pas affecté le taux de sécrétion de la protéine. L'IGF-1 a stimulé l'ARNm codant pour la PN-1, et la sécrétion d'uPA mais a diminué la sécrétion de la tPA et le taux de son ARNm. La protéine morphogénétique osseuse 7 (BMP-7) a augmenté la sécrétion de PN-1 des cellules stimulées à l'IGF-1 ou la FSH et a

augmenté la sécrétion de tPA par des cellules stimulées à l'IGF-1. Le facteur de croissance fibroblastique-2 (FGF-2) était généralement inhibiteur, diminuant la sécrétion de tPA des cellules stimulées à l'IGF-1 ou à la FSH, et diminuant la sécrétion de PN-1 des cellules stimulées à l'IGF-1. Les effets d'EGF étaient variés puisque la sécrétion de PN-1 était inhibée mais la sécrétion de tPA était augmentée.

Finalement, nous avons examiné l'expression génique de PN-1 dans les follicules à divers stades de développement. Dans les follicules périovulatoires, le taux de l'ARNm pour la PN-1 était stimulé par hCG, tout comme ceux pour les tPA et uPA. Deuxièmement, les follicules prélevés à l'abattoir ont été classifiés comme étant oestrogénique ou non-oestrogénique basé sur la concentration d'oestradiol dans le liquide folliculaire (FF). La concentration de PN-1 dans le FF et l'expression de l'ARNm de PN-1 dans les CG étaient significativement plus élevées dans les follicules possédant les concentrations d'oestradiol élevées. Finalement, la concentration de PN-1 dans le FF des follicules dominants durant la déviation de la première vague folliculaire n'a démontré aucun changement significatif, suggérant que le PN-1 n'est pas un bon indicateur du processus de la sélection folliculaire.

En résumé, l'expression et la sécrétion de PN-1 des CG bovines sont sous contrôle hormonale. Il est probable que PN-1 joue un rôle physiologique dans le remodelage tissulaire au cours de la croissance folliculaire et de la rupture de la paroi folliculaire au moment de l'ovulation.

Mots-clés : protéase nexine-1, activateur du plasminogène, follicule, cellule de la granulosa, matrice extracellulaire, remaniement tissulaire, FSH, facteur de croissance.

ABSTRACT

Understanding follicle development leads to practical control of reproduction in agriculturally important species such as cattle. Changes in the composition of the extracellular matrix (ECM) and the basement membrane occur during follicle growth, likely through proteolytic enzyme cascades, including plasminogen activators (PA) and their inhibitors. One such inhibitor is protease nexin-1 (PN-1), a granulosa cell-specific secreted protein. Regulation and expression of PN-1 and the PAs in granulosa cells is poorly understood.

The expression of PAs and PN-1 was examined in a non-luteinizing bovine granulosa cells culture model. Secreted tPA activity was higher in cultures of cells from small follicles compared to large follicles, and secreted PN-1 levels were higher in cultures of cells from large follicles. In cultures of cells from small follicles, secreted tPA and PN-1 levels increased with time of culture. In cultures of granulosa cells from large follicles, tPA activity increased significantly with time of culture, whereas PN-1 mRNA and protein levels decreased. Cell-associated uPA activity decreased with time in cells from medium and large follicles.

To study the regulation of PN-1, granulosa cells were cultured with doses of FSH and growth factors. PN-1 mRNA and protein levels and uPA secretion by cultured GCs were stimulated by FSH in a biphasic manner, with maximum levels at 1ng. FSH caused a dose-dependent increase in tPA gene expression but not secreted enzyme activity. IGF-I stimulated PN-1 and uPA secretion. However, IGF-I decreased secreted tPA activity and tPA gene. In addition, bone morphogenetic protein 7 (BMP-7) increased PN-1 secretion

in FSH- and IGF-I stimulated cells, and secreted tPA activity in IGF-I stimulated but not FSH stimulated cells. In contrast, fibroblast growth factor 2 (FGF-2) was generally inhibitory, decreasing tPA secretion in FSH- and IGF-I- stimulated cells, and decreasing PN-1 secretion in IGF-I stimulated but not FSH stimulated cells. The effects of EGF were diverse, as PN-1 secretion were inhibited, but secreted tPA activity was increased.

As PN-1 secretion differs with follicle stage, we examined PN-1 gene expression levels in follicles at defined stages in vivo. Firstly, the regulation of PN-1 gene during ovulation was measured following administration of hCG. There was an initial upregulation of gene expression, followed by a marked inhibition nearer the expected time of ovulation. Secondly, follicles collected from the abattoir were classified as nonatretic or atretic based on FF estradiol content. PN-1 protein in FF and PN-1 mRNA expression in GC was significantly higher in nonatretic than atretic follicles. In contrast, FF plasmin activity was correspondingly higher in the atretic follicles. Finally, No significant changes in PN-1 levels in FF were observed during the growth of pre-deviation follicles early in a follicle wave, suggesting PN-1 is not a good marker for the process of follicle selection. These results indicate that PN-1 may be involved in the process of atresia in nonovulatory dominant follicles and the prevention of precocious proteolysis in periovulatory follicles.

In summary, PN-1 expression and secretion from bovine granulosa cells is under hormonal regulation. PN-1 likely plays a physiological role in growing follicles and the process of follicle wall rupture at ovulation.

Key words: protease nexin-1, plasminogen activator, follicle, granulosa cell, extracellular matrix, tissue remodelling, FSH, growth factor

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LIST OF ABBREVIATIONS

ActR-II	activin type II receptor
ADAMTS-1	a disintegrin and metalloproteases with thrombospondin motifs-1
α_2-AP	α_2-antiplasmin
α2-M	α2-macroglobulin
α2-MR	α2-macroglobulin receptor
BCEC	bovine capillary endothelial cells
BMP-7	bone morphogenetic protein 7
BMP-15	bone morphogenetic protein 15
BMPR-II	BMP type II receptor
cAMP	cyclic adenosine 3',5'monophosphate
cDNA	complementary deoxyribonucleic acic
COC	cumulus-oocyte-complex
CRE	cAMP-response element
CREBP	cAMP response element-binding protein
CL	corpus luteum
DES	diethylstilbestrol
DEX	dexamethasone (glucocorticoid agonist)
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
eCG	equine chorionic gonadotropin (PMSG)

ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FF	follicular fluid
FGF-2	fibroblast growth factor 2 (bFGF)
FSH	follicle-stimulating hormone
FSHr	follicle-stimulating hormone receptor
GAG	glycosaminoglycans
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	granulosa cell
GDF-9	growth differentiation factor 9
GDN	glia-derived nexin
GnRH	gonadotropin releasing hormone
GPI anchor	glycosyl phosphatidylinositol linkage
HA	hyaluronan
HDL	high density lipoproteins
3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehidrogenase
HSPG	heparin sulphate proteoglycan perlecan
IGF-1	insulin-like growth factor 1
IGFBPs	insulin-like growth factor-binding proteins

IL-1β	interleukin-1 beta
IP3K	inositol trisphosphate 3 kinase
KL	kit ligand
KO	knock-out
LDL	low density lipoproteins
LH	luteinizing hormone
LHr	luteinizing hormone receptor
LOX	lysyl oxidase
LRP	the LDL receptor-related proteins
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MIX	1-methyl-3-isobutylxanthine
MMP	matrix metalloproteinase
MT-MMP	membrane type-matrix metalloproteinase
NF 1	nuclear factor 1
ORF	open reading frame
$P450_{arom}$	cytochrome P450 aromatase
$P450_{17OH}$	cytochrome P450 17 hydroxylase
$P450_{scc}$	cytochrome P450 side chain cleavage
PACAP	pituitary adenylate cyclase-activating polypeptide
PAI-1	plasminogen activator inhibitor-1
PAI-2	plasminogen activator inhibitor-2
PAR-1	protease activated receptor

PDGF	platelet derived growth factor
PGF2α	prostaglandin F2 alpha
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol myristate acetate
PMSG	pregnant mare serum gonadotropin (eCG)
PN-1	protease nexin-1 (Serpin-E2)
PRL	prolactin
RNA	ribonucleic acid
RIA	radioimmunoassay
SERPIN	serine protease inhibitor
Serpin-E2	serine protease inhibitor-E2 (PN-1)
SF1	steroidogenic factor 1
StAR	steroidogenic acute regulatory protein
TGFα	transforming growth factor alpha
TGF-β	transforming growth factor beta
TIMP	tissue inhibitors of metalloproteinases
TNFα	tumor necrosis factor alpha
tPA	tissue type plasminogen activator
TSG-6	tumour necrosis factor α-stimulated gene 6
uPA	urokinase type plasminogen activator
uPAR	uPA receptor
UTR	untranslated region

VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide
VLDL	the very-low-density lipoprotein

DEDICATORY

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AVANT-PROPOS (PREFACE)

Cette thèse est présentée à la Faculté des études supérieures de l'Université de Montréal pour l'obtention du grade de Philosophiae Doctor en sciences vétérinaires, option reproduction. Elle est composée d'une introduction générale, d'une revue de littérature générale; trois articles comprenant chacun: une introduction, une section matériel et méthodes, des résultats, une discussion et des références; une discussion générale, une conclusion générale ainsi que des references générales.

This thesis is presented to the Faculté des études supérieures de l'Université de Montréal for the obtention of the Philosophiae Doctor degree in veterinary sciences, option reproduction. It composes a general introduction, a general literature review; three published or submitted articles, each of which contains a specific introduction, materials and methods, results, discussion and references; a general discussion, a general conclusion and general references.

INTRODUCTION

Ovarian follicular development begins with the initial recruitment of primordial follicles into the pool of growing follicles (Fortune *et al.*, 2000). Follicles that are destined to ovulate pursue their development into the preantral and antral phases, become selected as the dominant follicle (deviation) (Ginther *et al.*, 1996; Fortune *et al.*, 2001), then undergo ovulation (Richards *et al.*, 2002) and luteinization (Murphy, 2004). However, most follicles degenerate by atresia throughout the antral phase (Markström *et al.*, 2002). The mechanisms for the recruitment, deviation and ovulation of follicles are not fully understood (Fortune *et al.*, 2001).

In cattle, follicles typically increase in size 400-fold between preantral and preovulatory stages (Lussier *et al.*, 1987). The surface area of preovulatory follicles doubles 19 times compared with primordial follicles (Rodgers *et al.*, 1999). During follicle growth, there is expansion of basal lamina and changes in composition of follicular extracellular matrix (ECM) (Rodgers *et al.*, 2003). The follicular basal lamina is formed of specialized sheets of ECM, which separates the epithelial cells (membrana granulosa) from adjoining stroma (including theca interna and externa, and vasculature). Previous studies suggest that the follicular basal lamina is extremely dynamic during follicular development; the follicular basal lamina becomes less collagenous and more laminin rich, such that it becomes more expandable to meet the requirement for follicle enlargement (Rodgers *et al.*, 2000). These changes likely occur through proteolytic enzyme cascades, such as the plasminogen activators (PA) and their inhibitors (Ny *et al.*, 2002).

Plasminogen activators are serine proteases that convert the abundant extracellular zymogen plasminogen into plasmin, an active protease that promotes degradation of components of the ECM as well as activating the matrix metalloproteinase (MMP) protease cascade (reviewed by Ny *et al.*, 2002; Liu, 2004). The PA system contains the proteolytic enzymes plasmin, tissue type (tPA) and urokinase type (uPA) plasminogen activators, and regulatory components including inhibitors, cofactors, cell surface receptors and binding proteins. The activity of proteolytic enzymes is regulated by inhibitors such as plasminogen activator inhibitor-1 (PAI-1), plasminogen activator inhibitor-2 (PAI-2), protease nexin-1 (PN-1) (Kruithof, 1988; Roberts *et al.*, 1995), and the plasmin specific inhibitor α_2-antiplasmin (α_2-AP). PAI-1, PAI-2, PN-1, and α_2-AP all belong to the serine protease inhibitor (SERPIN) superfamily (Silverman *et al.*, 2001). PN-1, also referred to as serine protease inhibitor-E2 (SERPIN-E2), and glia-derived nexin (GDN), is a secreted 43 kDa glycoprotein, and is a broad spectrum, trypsin-like inhibitor that rapidly inhibits a number of target proteases including uPA, tPA, plasmin, trypsin, and thrombin (Silverman *et al.*, 2001).

The expression and regulation of PN-1 during follicle development is poorly understood. In the ovary, PAI-1 (SERPIN-E1) mRNA and activity are predominantly synthesized by theca-interstitial cells in the rodent (Liu *et al.*, 1987b; Hägglund *et al.*, 1996), cattle (Dow *et al.*, 2002b) and monkey (Liu *et al.*, 2004). Stimulation with hCG upregulated PAI-1 expression in theca cells, and induced PAI-1 mRNA expression in GC in rats (Chun *et al.*, 1992). In contrast to PAI-1, PN-1 is exclusively expressed in GC in mammals including mice (Hägglund *et al.*, 1996), rats (Hasan *et al.*, 2002) and cattle (Bédard *et al.*, 2003). Furthermore, studies show that the level of PN-1 mRNA is high in

GC throughout the periovulatory period, and decrease in ovulated follicles of mice (Hägglund *et al.*, 1996) and rats (Hasan *et al.*, 2002). The expression of PN-1 mRNA is also demonstrated in bovine GC, and is regulated in a spatio-temporal pattern with highest steady state levels in GC of growing dominant bovine follicles compared with small follicles (Bédard *et al.*, 2003). However, the physiological role of PN-1 during follicle growth, follicle deviation and ovulation is unknown.

Ovarian follicular growth and development are integrated processes controlled by both extraovarian signals, such as gonadotropins, and intraovarian factors. Follicle-stimulating hormone (FSH) is an essential factor in the regulation of follicle development from primary follicles through to dominant preovulatory follicles. A number of growth factors are also involved in follicle development, including insulin-like growth factor-I (IGF-I), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGF) and epidermal growth factor (EGF). However, little is known that whether or not these growth factors regulate PAs and their inhibitors in GC, in particular, nothing is known about their regulation of PN-1.

Using a non-luteinizing bovine GC culture model, as well as in vivo approaches, we performed a number of studies to elucidate the regulation of PN-1 expression and secretion in ovarian GC. Our findings provided new insights on the role of PN-1 during antral follicle growth and ovulation.

LITERATURE REVIEW

FOLLICULAR DEVELOPMENT AND STEROIDOGENESIS

The release of an ovum that is ready for fertilization is one of the major functions of ovarian follicular development. Ovarian follicular growth and development are integrated processes controlled by both extraovarian signals such as gonadotropins and metabolic hormones, and intraovarian factors. Follicular development is classified into gonadotropin-independent and gonadotropin-dependent phases (Webb *et al.*, 1999).

In mammals, follicles develop continuously from the pool of primordial follicles throughout the reproductive lifespan of the animal. The development of follicles involves the recruitment of primordial follicles from the resting pool (follicle activation), the continued growth from primary follicles to small antral (Fortune, 2003; McNatty *et al.*, 1999; Webb *et al.*, 1999), selection of a dominant follicle (deviation) (Ginther *et al.*, 1996; Fortune *et al.*, 2001; Zeleznik, 2001; Fortune *et al.*, 2004), ovulation (Richards *et al.*, 2002) and luteinization (Murphy, 2004). Follicular development is illustrated in Figure 1.

Morphological changes in follicular development

Follicular development is morphologically characterized by an increase in the diameter of the oocyte, and a synchronous proliferation of GC, resulting in multiple layers of cells that surround each oocyte. The earliest stage of follicular growth is initially characterized by the transition of GC from flattened to cuboidal cells. This phase of

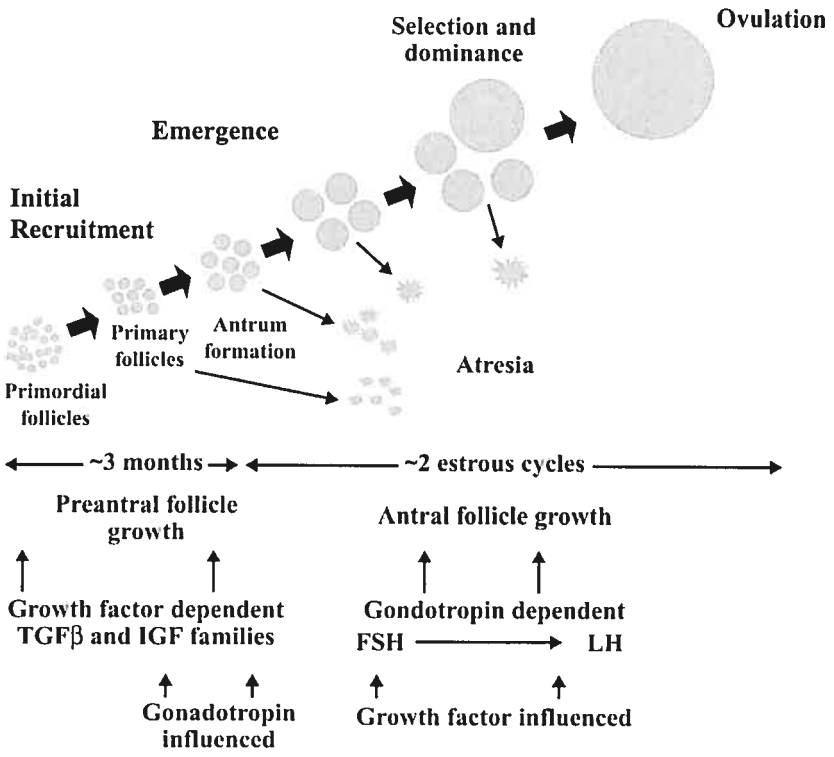

Fig 1. The follicular growth continuum

Schematic representation of the requirement for growth factors, such as the TGFß and IGF families, and gonadotropins at different stages of ovarian follicle development in cattle. Growth factors seem to be important in both the initiation of and in early follicle growth, whereas gonadotropins are essential for the final stages of follicle growth. In this regard, the dominant follicle switches its requirement from FSH to LH. There is also increasing evidence that gonadotropins can influence follicle development before antrum formation and growth factors can influence follicle development throughout the follicular growth continuum.

(Modified from Webb R. et al., 2004. J Anim Sci. 82:63-74)

preantral growth is relatively slow, comprising about 85% of the total duration of follicle growth in some species (Vanderhyden, 2002). The regulation of primordial follicle and preantral growth in cattle has been well reviewed (McNatty *et al.*, 1999; Fortune *et al.*, 2000; Fortune, 2003).

During preantral (primordial, primary, and secondary) follicular growth, although theca cells remain separated from GC by a basement membrane, they become associated with the growing follicle in this stage. Continued growth features both an increase in oocyte diameter and proliferation of granulosa cells. Granulosa cells of preantral follicles are a relatively homogenous population of proliferating cells that acquire receptors for follicle-stimulating hormone (FSH) and steroid hormones (Oxberry & Greenwald, 1982; Richards, 1975). Under the influence of FSH, cyclin D2 expression is induced in granulosa cells (Sicinski *et al.*, 1996) and the follicle continues to grow.

Transition to an antral follicle is associated with the formation of a fluid-filled cavity, and the granulosa cells differentiate into two sub-populations: cumulus granulosa cells, which are those most closely associated with the oocyte and are ovulated with it; and mural granulosa cells, which form a multi-layered wall against the basement membrane and acquire differentiated functions, including steroidogenesis (Zlotkin *et al.*, 1986) and the expression of luteinizing hormone (LH) receptors (Oxberry & Greenwald, 1982; Bortolussi *et al.*, 1979).

In cattle, follicular antrum formation begins at a diameter of 0.2 mm, and there is a large pool of mostly healthy, growing follicles from 0.2 to 2 mm in size (Lussier *et al.*, 1987). A critical physiological stage is reached at 3 to 4 mm diameter in size, when most follicles are lost by atresia (Lussier *et al.*, 1987). In the absence of sufficient FSH, or by the

natural process of follicle selection, most follicles will fail to reach ovulatory size and will undergo apoptosis or atresia (Lussier *et al.*, 1994; Gong *et al.*, 1996; Murdoch, 2000; Asselin *et al.*, 2000). Indeed, more than 99% of ovarian follicles present at birth never reach ovulation due to follicular atresia (Ireland, 1987).

Atresia is regulated by endocrine factors, notably FSH and LH, and mediated by intraovarian factors such as IGF-I (insulin-like growth factor-I), EGF (epidermal growth factor) and FGF-2 (fibroblast growth factor-2) (Markström *et al.*, 2002). The fate of follicle development versus atresia largely depends on the crosstalk between oocyte and granulosa cells and theca cells. For example, oocyte secreted factors including bone morphogenetic protein 15 (BMP-15) and growth differentiation factor 9 (GDF-9) act on the granulosa cells to enhance follicle development in mice and the inhibition of luteinization, in turn, granulosa cells produce Kit Ligand (KL) that acts through Kit receptors to promote oocyte growth (Vanderhyden, 2002; Gilchrist *et al.*, 2004).

Follicular dynamics in the cow

Follicular growth occurs in distinct waves in cattle (Ireland *et al.*, 2000; Mihm *et al.*, 2002). Early studies suggest that two waves of follicular growth occur during the cycle, the first wave emerging a few days after estrus, and the second follicular wave beginning around day 12-14 of the estrous cycle (Rajakoski, 1960). The two-wave hypothesis is not tested for more than 20 years. Studies involving measurements of follicles and steroid assays of blood and follicular fluid, lead to the conclusion that there are three follicular waves (Ireland & Roche, 1983). Monitoring of antral follicles in cattle by transrectal ultrasonic imaging technology (Pierson & Ginther, 1987) show that most (81%) estrous

cycles consist of two follicular waves (Ginther *et al.*, 1989). This technology is exploited by other groups, who find mostly (80%) three-wave cycles (Savio *et al.*, 1988; Sirois & Fortune, 1988). The number of follicle waves can change from one estrous cycle to the next in Holstein heifers (Price & Carrière, 2004). Most recently, evidence shows that numbers of antral follicles during follicular waves in cattle are highly variable among animals, very highly repeatable in individuals, and are inversely associated with serum FSH concentrations (Burns *et al.*, 2005).

The appearance and regression of follicle waves is termed follicular dynamics. It is characterized by the initiation of growth of a cohort of 3-6 small antral follicles (2-4 mm), which are recruited from the pool of smaller antral follicles (<2 mm in diameter) (Lucy *et al.*, 1992). Selection is the process by which the appropriate number of follicles is selected from a cohort of growing follicles to develop to ovulatory competence. In monovular species such as cattle, a single follicle is selected to continue growth after recruitment and has the potential to achieve ovulation (Fortune, 1994). Follicular dominance is the process by which a single selected follicle exerts an inhibitory effect on the other follicles of the wave, which cease growing and undergo atresia (Lucy et al., 1992; Fortune, 1994). The dominant follicle also inhibits the recruitment of a new cohort of follicles (Ireland, 1987). Follicle waves also occur during pregnancy (Ginther *et al.*, 1989) and during the prepuberal period (Adams *et al.*, 1994). When the dominant follicle coincides with the presence of an active corpus luteum, the fate of this follicle is usually atresia, and a new follicular wave emerges. If luteal regression occurs when there is a dominant follicle present, this follicle will usually ovulate.

Follicular Steroidogenesis

Steroid production is one of the most important functions for ovarian follicles. According to the biological activity and the numbers of carbon atoms, steroid hormones can be classified into progestins, androgens and estrogens, comprising 21, 19 and 17 carbons respectively, also designated as C_{21}, C_{19} and C_{17} steroids. They comprise a ring complex, formed of three cyclohexane rings (A, B, C) and a cyclopentane ring (D) (Gore-Langton & Armstrong, 1994). Steroidogenesis involves a long and complex biosynthetic pathway. The biosynthesis of steroids is mediated by steroidogenic enzymes, with each enzyme responsible for the conversion of one steroid to another. The major enzymes include three enzyme cytochrome P450s, including P450 cholesterol side-chain cleavage (P450scc or CYP11A1), P450 17α hydroxylase ($P450_{17OH}$ or CYP17) and P450 aromatase (P450arom or CYP19A1), and two hydroxysteroid dehydrogenase (HSD) enzymes e.g. 3β-HSD (or HSD3B2) and 17β-HSD (or HSD17B1). Most of these enzymes have essential co-enzymes as electron donors or acceptors (Gore-Langton & Armstrong, 1994).

The precursor of all steroids is cholesterol. Cholesterol is imported into the cell through internalization of blood-borne lipoproteins. The predominant form used for steroidogenesis appears to be low-density-lipoprotein (LDL), which binds to the LDL receptor on follicle cells. Within the cell, cholesterol is maintained within lipid droplets as cholesterol esters. The enzyme cholesterol ester hydrolase converts the cholesterol esters to free cholesterol, which is intensely hydrophobic (Murphy & Silavin, 1989). Free cholesterol within the cytoplasm is mobilized to the mitochondria, and then internalized. This internalization of cholesterol by the mitochondria is the rate-limiting step for the general steroidogenic pathway, and is mediated by steroidogenic acute regulatory protein

(StAR) (Stocco & Clark, 1996). The major ligand-regulated step in luteal and theca cells is StAR. This protein is acutely regulated by LH in these cell types. The application of LH causes a rapid and transient production of StAR mRNA and protein. Once the protein is formed in the cytosol, it is rapidly directed to the mitochondria where it binds to a recognition site on the outer mitochrondial membrane. While StAR is bound to the mitochondria, there is a transfer of cholesterol from the cytosol to the inside of the mitochondria (Stocco *et al.*, 2001). Once inside the mitochondria, cholesterol is converted to pregnenolone by the enzyme cytochrome P450scc.

Pregnenolone is the first steroid in the pathway and is the common precursor for all species and all tissues, and from this point the converted cholesterol is committed to becoming a steroid. Pregnenolone in the microsomes can then be metabolized using two different pathways, either converting to progesterone by the enzyme 3β-HSD (Δ^4 pathway), or to 17α-hydroxypregnenolone by $P450_{17\text{-}OH}$ (Δ^5 pathway) (Gore-Langton & Armstrong, 1994). It has been suggested there are differences between species in the utilization of steroidogenic pathways, and that the Δ^5 pathway is the preferred pathway in ruminants (Zuber *et al.*, 1986). In cattle, as in other ruminants, the five separate enzymes mentioned above are required for the production of estradiol.

In bovine luteal and granulosa cells, the enzyme $P450_{17\text{-}OH}$ is not expressed, and so steroidogenesis goes through to progesterone; this progesterone is not metabolized further, and is secreted. In theca cells, however, there is abundant $P450_{17\text{-}OH}$ activity, and so pregnenolone is converted to 17α hydroxypregnenolone. This 17α-hydroxypregnenolone then undergoes sequential conversion to androstenedione by $P450_{17\text{-}OH}$ and 3β-HSD activities (Bosc & Nicolle, 1998). Bovine theca cells convert limited amounts of

androstenedione to testosterone with the enzyme 17β-HSD and both androstenedione and testosterone are secreted. A good portion of these secreted androgens are absorbed by the neighbouring granulosa cells and are further converted to estrogens. Bovine granulosa cells prefer to metabolize androstenedione to estrone by the enzyme cytochrome P450arom, and then the estrone is metabolized to estradiol by 17β-HSD (Conley & Bird, 1997). Alternatively, testosterone can be metabolized to estradiol by P450arom (Conley & Bird, 1997). The major steroidogenic pathways irrespective of species and tissues are illustrated in the Figure 2.

The expression of the steroidogenic enzymes is regulated. The enzymes expressed in luteal and theca cells are in general regulated by LH, as these cells possess LH receptors. Thus it is fairly well recognized (mainly in rodent but in some ruminant models) that LH stimulates expression/activity of P450scc, $P450_{17\text{-}OH}$ and 17β-HSD. LH also acutely upregulates StAR gene expression and LDL receptor mRNA levels (Sekar *et al.*, 2000). In granulosa cells of smaller follicles, only the FSH receptor is expressed, although both gonadotropin receptors are expressed in follicles >8mm diameter in cattle. In granulosa cells, FSH stimulates LDL receptor levels, P450scc and P450arom activity (Soumano & Price, 1997). In addition, FSH upregulates aromatase (Silva & Price, 2002) and 17β-HSD (Sahmi *et al.*, 2004). As consequences, LH stimulates progesterone secretion from luteal cells and androgen secretion from theca cells, whereas FSH stimulates progesterone and estradiol secretion from granulosa cells (Mihm & Bleach, 2003).

The gonadotropins are not the only regulators of steroidogenesis, as a number of growth factors also alter steroid production (Armstrong & Webb, 1997). Insulin and/or IGF-1 stimulate progesterone and estradiol secretion from bovine granulosa cells in vitro,

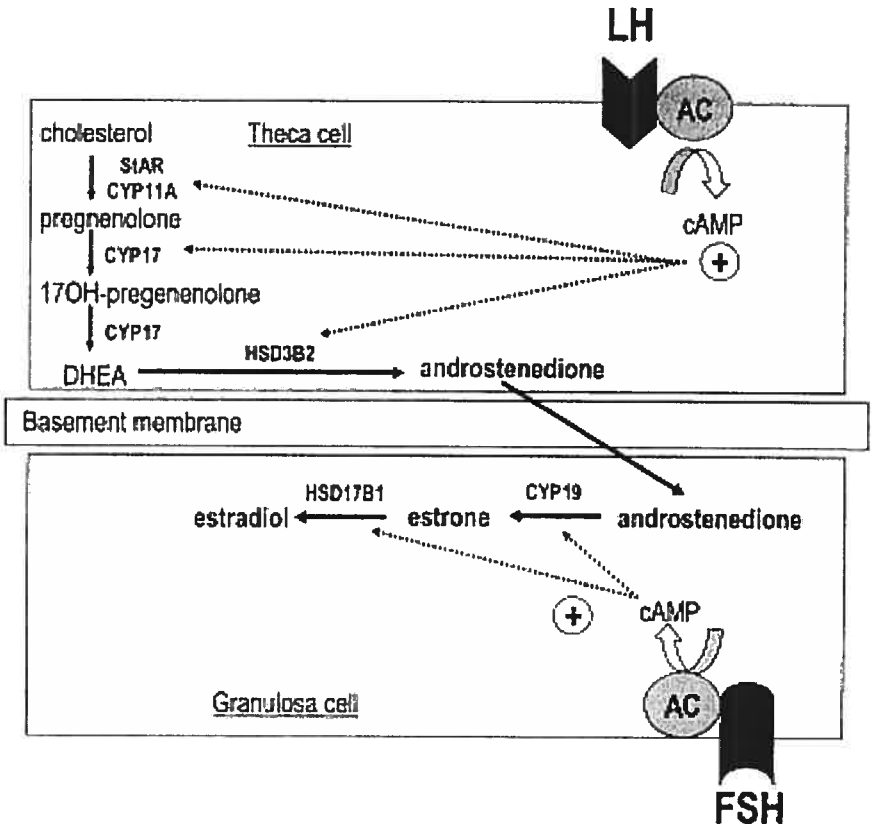

Fig. 2. Follicular phase steroid biosynthesis in the ovary with the illustration of the two-cell/two-gonadotropin theory.

(Copy from Havelock *et al.*, 2004, Mol Cell Endocrinol 228:67-78)

and increase expression of P450arom mRNA in bovine granulosa cells (Gutiérrez *et al.*, 1997; Silva & Price, 2002), and stimulate progesterone and androstenedione secretion from theca cells (Allegrucci *et al.*, 2003). Components of the insulin/IGF system also act as modulators of follicular cell responses to gonadotropins. Another major group of growth factors is the transforming growth factor-β (TGF-β) family. TGF-β enhances gonadotropin-stimulated steroidogenesis (Ke *et al.*, 2004). Two other growth factors have the opposite effect on steroidogenesis: the epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) inhibit steroidogenesis (Armstrong & Webb, 1997).

Biochemical changes in follicular development

Steroidogenic activity changes during follicle development. Estradiol concentration is a key biochemical marker for the degree of health/atresia of follicles. Data from the older literature shows that morphologically healthy ruminant follicles contain higher estradiol and lower progesterone concentrations than atretic follicles (Ireland & Roche, 1982; Sunderland *et al.*, 1994; Price *et al.*, 1995). It is now known that small follicles contain relatively little estradiol, and that follicular fluid estradiol concentrations increase with follicle size in healthy growing follicles. Estradiol concentrations decrease in subordinate follicles while the dominant follicle is growing. Once the dominant follicle reaches maximum diameter, follicular fluid estradiol concentrations fall dramatically, and decrease further once the follicle starts regressing (Price *et al.*, 1995; Mihm *et al.*, 2000).

The secretion of one steroid can be affected by a number of steps in the steroidogenic cascade, which limit or increase precursor supply. To determine which point in the pathway is responsible for increased or decreased estradiol secretion by follicles, a

number of studies have examined steroidogenic enzyme mRNA levels in bovine follicles at different stages of development (Bao & Garverick, 1998).

Preantral follicles express FSH receptor mRNA, but other aspects of the steroidogenic machinery do not appear until early antral stage. In early antral follicles, the theca cells start to express mRNA coding for LH receptors, P450scc, $P450_{17\text{-}OH}$ and 3β-HSD, thus these cells are able to make progesterone and androgens in rats (Zlotkin *et al.*, 1986). Granulosa cells continue to express only FSH receptors, and are thus steroidogenically inactive. As small antral follicles are recruited into a follicle wave, granulosa cells express P450scc and P450arom, and are thus able to synthesize pregnenolone and to convert androstenedione to estrone. They cannot in principle convert pregnenolone to progesterone as they lack 3β-HSD at this stage. The theca cells of these small recruited follicles continue to express mRNA for all thecal steroidogenic enzymes, and also start to express StAR. These cells are thus fully and actively steroidogenic (Bao *et al.*, 1998).

As a growing follicle becomes a dominant follicle, a key change occurs in granulosa cells. They start to express mRNA for LH receptors and 3β-HSD. The cells are thus able to convert pregnenolone to progesterone, and are able to respond to LH, considered to be essential for dominant follicle maturation. As the dominant follicle grows, there are also increases in mRNA for P450 arom in granulosa cells and for StAR in theca cells (Bao *et al.*, 1998). The subordinate follicles regress, and this is associated with decreases in all steroidogenic enzymes in granulosa cells. If the dominant follicle also undergoes atresia, the first changes in steroidogenesis occur as the follicle reaches the 'static' phase of its growth phase. There is a reduction in mRNA for P450scc, $P450_{17\text{-}OH}$

and LH receptor in theca cells, and P450scc in granulosa cells. These follicles secrete significantly less estradiol than growing follicles, although there are no changes in P450arom mRNA (Bao & Garverick, 1998). The decrease in estradiol secretion is most likely due to the decrease in theca $P450_{17\text{-}OH}$ and LH receptor levels, thus reducing androgen precursor supply to GC. There is no further loss of mRNAs encoding steroidogenic enzymes in the theca as the dominant follicle starts to regress, but GC suffer a loss of P450scc, P450arom, LH receptor and 3β-HSD mRNA (Bao & Garverick, 1998).

The growth of bovine follicles from primordial to preovulatory stage is characterized by an approximately 360,000-fold increase in surface area, and several hundred-fold increase in follicle size (Lussier *et al.*, 1987). Ovarian follicular growth and development involves extensive tissue remodeling (Smith *et al.*, 1999). Overall, normal ovarian function depends on cyclical remodeling of the ECM. The next section will discuss the composition, the changes and the roles of ECM in the mammalian ovary.

EXTRACELLULAR MATRIX IS DYNAMIC IN FOLLICULAR DEVELOPMENT

During follicle growth there is extensive cellular proliferation and remodeling of the ECM. This process is characterized by proliferation of GC, differentiation of the granulosa and theca compartments, and the deposition of a basement membrane separating the avascular granulosa cells from the vascularized theca layer (Ny *et al.*, 2002).

The extracellular matrix has many different roles (Rodgers *et al.*, 2000; Rodgers *et al.*, 2003; Rodgers *et al.*, 1999). First, the ECM affects cell shape and behavior, such as migration, division, differentiation, cell death and cell anchorage. All these behaviors occur

in follicle development. Second, the ECM can play a role in the fluid dynamics of a tissue, either providing osmotic forces or filtering soluble materials including nutrients and hormones. Third, the ECM can provide rigid or elastic mechanical support for tissues. Fourth, follicular growth factors can bind to the ECM directly or indirectly. For instance, FGF-2 can directly bind to the ECM, and IGF-I or activin can indirectly bind to the matrix through their binding proteins IGFBP-2 and -5, or follistatin, respectively. Collectively, the ECM defines or provides a specialized microenvironment for cells and tissues.

Collagen and laminin are structural proteins in ECM components

The ECM provides a structural tissue support, and forms barriers between tissue compartments. The matrix is known to contain three major fibre forming proteins—collagen, elastin, and fibronectin, which are interwoven in a hydrated gel formed by a network of glycosaminoglycans (GAG) domains. All of these macromolecules are locally secreted by the cells in contact with the matrix (Alberts, 1983). The collagens are ropelike, triple-stranded, helical molecules that aggregate in long cable-like fibrils or sheets in the extracellular space. Elastin molecules form an extensive cross-linked network of fibres and sheets that can stretch and recoil, imparting elasticity to the matrix. Fibronectin molecules form fibres that promote cell adhesion, and the GAGs are a heterogeneous group of long, negative charged polysaccharide chains (except for hyaluronic acid) covalently linked to protein to form proteoglycan (Alberts, 1983). Follicular growth requires the ECM to be remodeled to incorporate the increasing volume of tissue and follicular fluid. In ovarian follicles, there are a number of different compartments and extracellular matrices. These

include the follicular basal lamina, the membrana granulosa, and theca interna and externa (Rodgers *et al.*, 2000).

The follicular basal lamina is formed of specialized sheets of ECM, which separate the epithelial cells (membrana granulosa) from adjoining stroma (including theca interna and externa, and vasculature). The basal lamina is composed of a lattice-type network of collagen type IV interwound with a network of laminin. This structure is stabilized by the binding of entactin or nidogen to the collagen and laminin. The heparin sulphate proteoglycan perlecan (HSPG) and other molecules such as fibronectin are associated with the collagen type IV-laminin backbone (Rodgers *et al.*, 2003). Furthermore, each molecule of collagen type IV comprises three α chains; there are six different chains (α1- α6) of collagen type IV (Rodgers *et al.*, 1998). Similarly, each laminin molecule is composed of three chains, the α, β, & γ chain, there are five different α chains, three β chains and one γ chain, thus, many potential different combinations of collagen type IV and laminin are possible, and many of these combinations have been observed in nature (van Wezel *et al.*, 1998). The ECM undergoes cyclic changes in its composition (Greenwald, 1994); (Monniaux *et al.*, 1997). Interestingly, the composition of follicular basal lamina changes during follicle development. Cyclic expression patterns of the mRNA encoding type III, IV, and VI collagens as well as the proteoglycans have been observed in mouse ovary, suggesting that the ovarian ECM changes during follicular growth (Oksjoki *et al.*, 1999). For instance, in cattle collagen type IV α3, α4, α5 and α6 levels are lower in antral follicles compared to primordial and preantral follicles, and laminin α1, β2 and γ1 are higher in antral follicles than in primordial and preantral follicles (Rodgers *et al.*, 2003). Thus, during follicle development, the follicular basal lamina becomes less collagenous and more

laminin rich, such that it becomes more expandable to meet the requirement for follicle enlargement. In addition, perlecan and nidogen are absent in primordial follicles, but become components of the follicular basal lamina in antral follicles, and atretic but not healthy antral follicle express laminin α2 (van Wezel *et al.*, 1998). In the sheep, laminin 1 (α1β1γ1 structure) and different collagen I chains have been immunolocalized in the basal lamina, and the levels of type I collagen increase in granulosa layers during terminal follicular growth (Huet *et al.*, 1997). All these studies suggest that the follicular basal lamina is extremely dynamic during follicular development (Rodgers *et al.*, 2000).

ECM components in theca layers (named the theca matrix) slightly differ from those in the follicular basal lamina. Even though laminin 1 components (α1 or β2 or γ1) (van Wezel *et al.*, 1998) and collagen IV α1, α2 (Rodgers *et al.*, 1998) are present in bovine follicles throughout the theca interna, other structural proteins such as collagen type I (2 α1 and 1 α2) (Luck *et al.*, 1995; Zhao & Luck, 1995) and collagen type VI (Iwahashi *et al.*, 2000) have been identified in the theca interna.

Other ECM components

Apart from collagen and laminin described above, there are a number of other extracellular matrix proteins in ovarian follicles such as gelatin, elastin, fibronectin, integrins, vitronectin, and proteoglycans including versican, hyaluronan.

Gelatin contains a large number of glycine, proline and 4-hydroxyproline residues. Gelatin is a heterogeneous mixture of single or multi-stranded polypeptides, each with extended left-handed proline helix conformations and containing between 300 - 4000 amino acids (Alberts, 1983). Type I collagen can be used as a source for gelatin. There are

two types of gelatin, acid pretreatment (type A gelatin) and alkaline treatment (type B gelatin) dependent on whether or not the preparation involves an alkaline pretreatment.

Elastin molecules form an extensive cross-linked network of fibres and sheets that can stretch and recoil, imparting elasticity to the matrix (Alberts, 1983). Elastin and collagen forms a cross-link structure in the ECM. Lysyl oxidase (LOX) initiates cross-link formation of the collagen and elastin, and therefore has a crucial role in the regulation the formation and maintenance of the ECM in the ovary (Henmi *et al.*, 2001; Harlow *et al.*, 2003).

Fibronectin is a common ECM compound in stroma and it is important for cell migration, which occur in theca expansion during follicular development (Rodgers *et al.*, 2003). Due to alternative splicing of mRNA at three separate sites, there are at least 20 different isoforms of fibronectin in humans (De Candia & Rodgers, 1999). Fibronectin exists as a homo- or heterodimer of these splice variants, a number of the splice variants are expressed in bovine follicles in vivo (De Candia & Rodgers, 1999). Fibronectin is mitogenic in granulosa cells in vitro (Colman-Lerner *et al.*, 1999), and fibronectin synthesis by granulosa cells can be upregulated by FGF-2 (Rodgers *et al.*, 1996). Further experiments are needed to assess the physiological importance of the different fibronectin isoforms, as well as the respective roles of the soluble forms present in follicular fluid and the insoluble forms deposited in basal lamina and on cell membranes.

Integrins are ECM receptors on the cell surface. Cells interact with the matrix through cell-surface adhesion receptors including the integrins. Integrins are heterodimeric glycoproteins composed of α and β subunits. Over 17 α and 8 β subunits can make over 23 different heterodimeric combinations (Belkin & Stepp, 2000). Only a few integrins have

been localized to granulosa cells: α6β1 in non-luteinized granulosa cells (Fujiwara *et al.*, 1997; Le Bellego *et al.*, 2002) and α2 and α5 in luteinizing cells (Yamada *et al.*, 1999). Moreover, the α6β1integrin serves as a laminin receptor, the α5β1integrin serves as a fibronectin receptor, and the α2β1 integrin serves as a collagen type I receptor in various species such as mouse (Fujiwara *et al.*, 1998), human (Nardo *et al.*, 2003; Yamada *et al.*, 1999), and sheep (Le Bellego *et al.*, 2002).

Versican (also named chondroitin sulfate proteoglycan-2, CSPG2) is a soluble ECM molecule. Granulosa cells in antral follicles are bathed in follicular fluid containing proteoglycans. Proteoglycans consist of a core protein with attached GAGs. Belonging to the proteoglycan family, versican is likely to be synthesized in the granulosa cells (and also theca). Versican was identified in human follicular fluid (Eriksen *et al.*, 1999) and in all follicular layers in small bovine follicles (McArthur *et al.*, 2000), and may participate in cell-matrix and cell-cell interactions. Versican plays a key role in cumulus oocyte expansion and fertility (Russell *et al.*, 2003), together with tumour necrosis factor α-stimulated gene 6 (TSG-6) (Mukhopadhyay *et al.*, 2001; Ochsner *et al.*, 2003), inter-α-trypsin inhibitor (Carrette *et al.*, 2001; Ochsner *et al.*, 2003), and hyaluronan (Mahoney *et al.*, 2001). There are four isoforms of versican (V0, V1, V2, and V3), of which V0 and V1 expression is localized to granulosa cells (Russell *et al.*, 2003). Versican V0 and V1 mRNA are differentially expressed in GC of actively growing dominant follicles compared to small follicles (2-4mm) in cattle (Fayad *et al.*, 2004b). In addition, a disintegrin and metalloproteases with thrombospondin motifs (ADAMTS-1) can proteolytically cleave versican (Sandy, 2001).

Hyaluronan (HA), a glycosaminoglycan polymer, is synthesized by cumulus cells surrounding oocytes before ovulation. Along with other factors, hyaluronan makes a gelatinous matrix, and plays a role in cumulus expansion (Salustri *et al.*, 1999; Mahoney *et al.*, 2001).

Interaction of ECM proteins and granulosa cell function

In terms of their origins, most components of the follicular basal lamina are probably synthesized by GC. Both fibronectin and laminin γ1 chain are expressed by GC in rats (Carnegie, 1990) and cows (Zhao & Luck, 1995). Luteinized granulosa cells in culture express ECM proteins (collagen I and collagen IV) and their regulators, matrix metalloproteinase 9 (MMP-9) and tissue inhibitors of metalloproteinases (TIMP-1) (Zhao & Luck, 1996). Bovine GC in culture can also synthesize a basal lamina containing collagen IV and fibronectin (Rodgers *et al.*, 1995; Rodgers *et al.*, 1996), providing a possible model to study the origin of ECM proteins and as well as the interaction of ECM proteins and GC function. Furthermore, TGFα stimulates the production and deposition of fibronectin by chicken GC (Asem & Novero, 1994).

In vitro studies show that the ECM modulates GC function in various mammalian species. For example, ECM stimulates bovine GC proliferation and progesterone secretion in response to FSH (Savion *et al.*, 1981). Similarly, rat and human luteinized GC require ECM in order to retain their structural and functional characteristics in culture (Amsterdam *et al.*, 1998). To test the role of ECM proteins in GC survival, proliferation and steroidogenesis, Huet and colleagues carry out an experiment in which various pure ECM components (type I collagen, fibronectin and laminin) are added to ovine GC in vitro. They

observe that collagen I is able to maintain estradiol secretion in GC derived from large follicles (4-7 mm in diameter), whereas fibronectin and laminin dramatically increase the proliferation rate and enhance survival of GC from both small (1-3 mm) and large follicles (Huet *et al.*, 2001). In addition, the authors also report that heparin treatment changes cell morphology (induces cell rounding), reduces cell proliferation, enhances estradiol but inhibits progesterone secretion (Huet *et al.*, 2001). However, it remains to be determined whether changes in GC function resulting from heparin treatment are directed by the change in cell shape, or involve other mechanisms. One possible explanation is that the addition of excess heparin to the cultured cells likely disturbs the action of endogenous heparin-binding growth factors such as FGF-2, by regulating its bioavailability (Ruoslahti & Yamaguchi, 1991). Immunostaining studies show that laminin and fibronectin are mainly localized to vascular walls, the outer layer of GC, and the basement membrane of the rat ovary (Akkoyunlu *et al.*, 2003). Taken together, these data indicate ECM proteins, together with growth factors, are involved in follicle development during the estrous (menstrual) cycle. In conclusion, the ECM influences basic cellular process such as proliferation, differentiation, migration and adhesion, are involved in the control of ovarian follicular development, and modulate interactions between growing follicles and surrounding connective tissue.

PROTEASES AND THEIR INHIBITORS REGULATE ECM REMODELING

Proteases and their inhibitors are regulators of ECM remodeling during follicular development. Based on their evolutionary structure, proteases and their inhibitors can be

classified into three groups (three proteolytic systems), the plasminogen activator (PA) system (Liu, 1999; Liu *et al.*, 2004), the matrix metalloproteinase (MMP) system (Ny *et al.*, 2002; Curry & Osteen, 2003) and the cysteine protease system (Sriraman & Richards, 2004). This review will focus on the plasminogen activator system (see the PA section in detail), and describe briefly the MMP system and cysteine protease system.

The MMP system

Currently, the MMP family encompasses at least 25 related proteolytic enzymes that include four broad classes: collagenases, gelatinases, stromelysins, and membrane type enzymes (MT-MMPs) (reviewed by Curry & Osteen, 2003). Common features of the MMP family include: 1) the presence of zinc in the active site of the catalytic domain; 2) synthesis of the MMPs as preproenzymes that are secreted in an inactive form; 3) activation of the latent zymogen in the extracellular space; 4) recognition and cleavage of the ECM by the catalytic domain of the enzyme; and 5) inhibition of enzyme action by metalloproteinase inhibitors in the extracellular environment (Curry & Osteen, 2003). Although similarities exist in the structure of the MMPs, there are also distinct differences in the recognition and specificity for the ECM components (Nagase & Woessner, 1999); (Murphy *et al.*, 1999b). For instance, collagenases (MMP-1, MMP-8, MMP-13) cleave fibrillar collagens such as collagen types I, II, III, V, and XI, as well as nonfibrillar collagens. Cleavage of the triple helical collagen by collagenases results in denaturation of collagen molecules into gelatin by changing the stability and solubility of collagen. The gelatinases (MMP-2 and MMP-9) contain a fibronectin-like sequence within their catalytic domain, which results in a potent ability for these MMPs to bind and cleave gelatin. The

stromelysins (MMP-3, MMP-7, MMP-10 and MMP-11) act on a diverse array of ECM substrates, including collagen type IV, laminin, and fibronectin. The MT-MMPs (MT1 or MMP-14, MT-2 or MMP-15, MT-3 or MMP-16, and MT-4 or MMP-17) contain a transmembrane domain near their C-terminal region, and their extracellular region contains the catalytic domain (Curry & Osteen, 2003). One important role of the MT-MMPs is activation of MMP-2 (Strongin *et al.*, 1995). In addition to degrading ECM, MMPs and especially stromelysins exhibit activity toward other MMPs, growth factors, and cytokines such as IGF binding proteins, epidermal growth factor (EGF), TNF-α, and substance P (Sternlicht & Werb, 2001), and subsequently modulate cell growth. The regulatory action on cell growth occurs either directly by controlling cell-matrix interactions or indirectly by controlling growth factor bioavailability.

Apart from the MMPs discussed above, there are a number of family members that are classified outside of the four broad classes of MMPs. ADAMTS-1 is a member of the ADAMTS family of metalloproteinases (termed the adamalysins, a different metalloproteinase family) that degrades members of the lectican family of proteoglycans (Kuno & Matsushima, 1998). In the ovary, ADAMTS-1 is involved in ovulation (Robker *et al.*, 2000a).

MMP activity in the extracellular environment is rigorously controlled by MMP inhibitors. Two major classes of MMP inhibitors are generally distinguished, serum-borne and tissue-derived inhibitors (reviewed by Gomez *et al.*, 1997; Brew *et al.*, 2000). Alpha 2-macroglobulin (α2-M), a 720 kDa tetrameric glycoprotein, belongs to serum-borne inhibitor of metalloproteinases, and α2-M is present in human follicular fluid whereas α2-M mRNA is virtually undetectable in GC (Curry *et al.*, 1990). The tissue inhibitors of

metalloproteinase (TIMP) are locally produced and specifically inhibit MMPs, and are highly expressed and hormonally regulated in reproductive tissues. The ability of TIMPs to inhibit MMP action occurs through the interaction of the N-terminal domain of TIMP with the active site on the catalytic domain and the substrate-binding groove of the MMP. TIMPs act selectively on different MMPs (Gomez *et al.*, 1997). Currently, there are four TIMP members. TIMP-1 is a secreted glycoprotein, which preferentially binds to MMP-9 but cannot act on MT1-MMP (MMP-14). TIMP-2 is also a secreted glycoprotein, and has a high affinity for MMP-2 (Gomez *et al.*, 1997). Unlike TIMP-1 or TIMP-2, TIMP-3 is secreted and then bound to the ECM, and has been suggested to act as an additional regulatory stop point as opposed to being free in the extracellular fluid. TIMP-3 also exhibits a differential preference for the MMPs, having a high affinity for MMP-9 and being able to inhibit MT1-MMP (Leco *et al.*, 1994). TIMP-4 is cloned and expressed in reproductive tissues (Leco *et al.*, 1997). TIMP-4 also acts on numerous MMPs (Stratmann *et al.*, 2001), suggesting that this TIMP may be a good non-specific inhibitor for all classes of MMPs.

The regulation of MMP and TIMP synthesis and activation in ovarian follicular development and ovulation are reviewed (Smith *et al.*, 1999; Curry & Osteen, 2003). Most studies in different species show that MMP and TIMP mRNA levels or protein activities are increased during ovulation (Smith *et al.*, 1999). For example, in the rat, mRNA levels of MMP-1, MMP-2 (Reich *et al.*, 1991), MMP-13 (Balbin *et al.*, 1996) and MT1-MMP (Jo *et al.*, 2002b) all increase after the endogenous LH surge or hCG administration, similar to previous reports for enzyme activity (Smith *et al.*, 1999). The gene expression of TIMP-1 and TIMP-3 also increases (Reich *et al.*, 1991), whereas TIMP-4 expression decreases in

rat ovarian follicles during ovulation (Simpson *et al.*, 2003). Although there are many similarities in expression patterns of MMPs between the rat and mouse, there are differences in MMP-2 and MMP-9 expression. In the rat, MMP-9 mRNA does not change, whereas MMP-2 mRNA increase during ovulation (Curry *et al.*, 2000). In contrast to the rat, MMP-9 mRNA increases after LH stimulus in the mouse, whereas MMP-2 is unchanged (Robker *et al.*, 2000b). After investigation of eleven different MMPs during the periovulatory period in mice ovaries (Hägglund *et al.*, 1999), only MMP-19 mRNA increases. The other MMP members are either unchanged or undetectable. TIMP-1 and TIMP-3 mRNA expression increases in the mouse, whereas TIMP-2 is unchanged as in the rat (Hägglund *et al.*, 1999; Inderdeo *et al.*, 1996). The parallel up-regulation of MMPs and their inhibitors is postulated to maintain proteolytic homeostasis (Nagase & Woessner, 1999), and therefore act to regulate the location and extent of ECM remodeling of the follicular apex during ovulation.

There are potentially important species differences in mRNA expression patterns of the MMP system during ovulation. In the rat, MT1-MMP mRNA levels increase (Jo *et al.*, 2002a), but TIMP-2 mRNA levels do not change (Curry & Nothnick, 2000). In cattle, the expression of mRNA for MT1-MMP and TIMP-2 increases at 24 h and 6 h after the preovulatory LH surge, respectively (Bakke *et al.*, 2002). However, MMP-2 mRNA in follicular tissue and enzyme activity in follicular fluid is unchanged. The MT1-MMP mRNA is localized primarily to the theca layer before the gonadotropin surge but is expressed in GC at 12 h and 24 h after the surge (Bakke *et al.*, 2002), suggesting that this enzyme is required more for follicle wall degradation in bovine. In sheep, MMP-2 appears a pivotal MMP member in ovulation, as the injection of MMP-2 antibody into the antral

cavity of preovulatory follicles in the ewe result in the formation of luteinized, unruptured follicles (Gottsch *et al.*, 2002).

There is considerable evidence that the MMPs and TIMPs function at earlier stages of follicular growth. In the neonatal rat ovary, MMP-2 is immunostained in GC and the surface epithelium, whereas MMP-9 is absent at earlier stages of follicular development (Bagavandoss, 1998). In the same study, follicular growth induced by PMSG increases the cellular expression of MMP-2 and MMP-9, which is consistent with protein expression patterns (Bagavandoss, 1998). The cellular activity of the MMPs measured by in-situ zymography demonstrate a pattern of gelatinolytic activity that corresponds with the localization of MMP-2 and MMP-9 mRNA around developing follicles (Curry *et al.*, 2001). MMP-2 and MMP-9 activity also increases during follicular growth in sheep, pigs, cattle, horses and humans (reviewed by Curry & Osteen, 2003).

The emerging data for TIMP mRNA expression patterns during follicular growth suggest that the expression of TIMP-1 parallels changes in MMPs. Curry and Osteen (2003) summarized that TIMP-1 mRNA levels increase after PMSG administration, but TIMP-2 mRNA does not change, and TIMP-3 mRNA level even decline slightly in the rat (Curry & Osteen, 2003). However, the mRNA and protein for TIMP-4 increase after PMSG treatment (Simpson *et al.*, 2003). Thus, the parallel regulation of MMPs and TIMP-1 and TIMP-4, as well as the basal expression of TIMP-2, and TIMP-3, may act to maintain a proteolytic balance during follicular growth that provides localized control of ECM degradation, thereby regulating the location and extent of follicular remodeling (Curry & Osteen, 2003).

In summary, the MMPs would facilitate remodeling of the granulosa cell basement membrane and the theca ECM and allow follicular expansion. The TIMPs may provide control for the location and extent of MMP action. It is readily clear that the MMPs and TIMPs are in the appropriate cellular compartments, and are regulated by the hormonal signals that regulate follicular development and atresia.

The cysteine proteases

There are limited studies on cysteine proteases compared to other major protease families. Cysteine proteases are lysosomal enzymes that act at acid pH, whereas most serine proteases and the metalloproteinases act at neutral pH. The best-known cysteine proteases are cathepsin B and cathepsin L (Salamonsen, 1999). Cathepsin L is a lysosomal cysteine protease expressed in many endocrine tissues and cell types including granulosa cells (Oksjoki *et al.*, 2001; Robker *et al.*, 2000a). Cathepsin L is a secreted protein indicating that this protease functions at both intracellular and extracellular sites (Ishidoh & Kominami, 1998). In the ovary, cathepsin L is expressed in GC of follicles at different stages of growth suggesting that this protease may play diverse roles in this tissue. Along with other proteases, cathepsin L may impact the extensive remodeling of the ECM during ovulation (Robker *et al.*, 2000a). Specifically, since cathepsin L is activated when complexed with glycosaminoglycans (GAG) present in follicular fluid, and since it can degrade collagen (I and IV), elastin and fibronectin, cathepsin L is likely a modifier of ECM in preovulatory follicles (Salustri *et al.*, 1999; Robker *et al.*, 2000a). Although no endogenous cysteine protease inhibitor has been identified, TIMP-1 forms a complex with procathepsin L in steroidogenic cells, including Leydig cells and ovarian GC, indicating

that the complex is a potent activator of steroidogenesis and may regulate germ cell development in both males and females (Boujrad *et al.*, 1995).

PLASMINOGEN ACTIVATOR SYSTEM

Plasminogen activators (PA) are serine proteases that convert the abundant extracellular zymogen plasminogen into plasmin, an active protease that, directly or indirectly, promotes degradation of all components of the ECM (Blasi *et al.*, 1987). Initially, plasmin degrades fibrin into soluble products in the circulation (reviewed by (Lijnen, 2002). In addition, there are several other ECM proteins that are degraded by plasmin, including gelatin, fibrinogen, type IV collagen, fibronectin, laminin, elastin and proteoglycans (reviewed by Liu *et al.*, 2004). The targeted ECM degradation generated by PAs affects a wide variety of physiological and pathological processes (Liu, 1999), including ovulation, luteal regression, sperm maturation, fertilization, embryo implantation and uterus involution (Sappino *et al.*, 1989).

The PA system is proposed to activate pro-MMPs at the cell surface (Murphy *et al.*, 1999a). Thus, the activation of MMPs can be regulated by a balance between serine proteases such as uPA, and the plasminogen activator inhibitors (Nagase & Woessner, 1999; Murphy *et al.*, 1999b).

The PA system contains not only the proteolytic enzymes, but also regulatory components including inhibitors, cofactors, cell surface receptors and binding proteins (reviewed by Liu, 1999; Ny *et al.*, 2002; Liu, 2004). Two forms of plasminogen activator, tissue type (tPA) and urokinase type (uPA), are characterized in mammals (Macchione *et*

al., 2000). Plasminogen activation mediated by tPA plays an important role in the dissolution of fibrin in the circulation (Carmeliet & Collen, 1998), whereas the main role of uPA appears to be in pericellular proteolysis via the degradation of matrix components or via activation of latent proteinases or growth factors (Lijnen, 2002). A cell surface uPA binding protein, the uPA receptor (uPAR) is identified in some cell types (Andreasen *et al.*, 1990; Vassalli & Pepper, 1994). Urokinase PA binds to its specific cellular receptor resulting in enhanced activation of cell-bound plasminogen, hence, the proteolytic activity becomes localized to the cell surface or to the vicinity of these cells (Ny *et al.*, 2002). Other identified proteins of the PA system include the specific and fast-acting plasminogen activator inhibitor-1 (PAI-1), plasminogen activator inhibitor-2 (PAI-2), the broad inhibitor protease nexin-1 (PN-1) (Kruithof, 1988; Roberts *et al.*, 1995), and the plasmin inhibitor α_2-antiplasmin (α_2-AP). Inhibition of the PA system may occur either at the level of the PA by specific PAIs, or at the level of plasmin, mainly by α_2-AP (Lijnen, 2002). PAI-1, PAI-2, PN-1, and α_2-AP all belong to the **SER**ine **P**rotease **IN**hibitor superfamily, termed **SERPIN** (Potempa *et al.*, 1994). The Serpins are a huge superfamily of proteins that fold into a conserved structure and employ a unique suicide substrate-like inhibitory mechanism. A Serpin nomenclature committee re-named PAI-1, PAI-2, PN-1, and α_2-AP as SERPIN-E1, SERPIN-B2, SERPIN-E2, and SERPIN-F2, respectively (Silverman *et al.*, 2001).

Plasminogen

The proteases of the PA system are characterized by their reactive pocket, which contains the catalytic triad of histidine (His), asparagine (Asp) and serine (Ser) residues

(Kraut, 1977). Plasminogen is a single-chain glycoprotein containing 790 amino acids with a molecular weight of approximately 92 kDa. Native plasminogen is referred to as Glu-plasminogen since it has a glutamic acid at its aminoterminus (N-terminus). The Glu-plasminogen is cleaved by plasmin at Lys76-Lys77 to produce Lys-plasminogen (Wiman & Wallen, 1977). Both the Glu- and the Lys-plasminogen are activated to plasmin by a single PA-catalyzed cleavage at the Arg560-Val561 bond, which results in a two-chain protein held by two disulfide bonds (Sottrup-Jensen *et al.*, 1975). Because plasminogen is present in all body fluids, plasminogen activation needs to be restricted, as loss of exquisite control of plasminogen activation leads to extensive and often destructive degradation of ECM.

Plasmin

Plasmin is a protease with a broad trypsin-like activity. Plasmin can directly degrade basement membrane ECM components including collagen IV, proteoglycans, laminin and fibronectin (Mignatti *et al.*, 1986). Plasmin may assist in cumulus expansion by terminating oocyte-cumulus cell communication (Liu *et al.*, 1986). Plasmin is the most likely physiological activator of uPA (Binnema *et al.*, 1991). Furthermore, plasmin can activate downstream proteases such as proMMP-3, proMMP-9, leading to the ECM remodelling indirectly by both MMP-3 and MMP-9 as well as by itself directly (Hahn-Dantona *et al.*, 1999). A cascade involving uPA, uPAR and plasminogen is proposed to activate MMPs at the cell surface (Murphy *et al.*, 1999a). In addition, plasmin is able to process or release ECM bound growth factors such as TGF-β (Pedrozo *et al.*, 1999), VEGF

(Park *et al.*, 1993), and FGF-2 (Whitelock *et al.*, 1996), which can contribute to ovarian function.

Tissue type plasminogen activator

Both tPA and uPA are released from cells as single-chain forms with no (uPA) or low (tPA) activity, with cleavage of a polypeptide bond leading to fully active forms. Tissue type PA is synthesized in endothelial cells (Binder, 1995) and many other cell types. Mature tPA is a single-chain glycoprotein of 530 amino acids (70 kDa in MW). Human tPA gene is localized to chromosome 8 (Degen *et al.*, 1986).

The rat tPA promoter contains a cAMP-responsive element (CRE) (TGACGTCA) at position -178 to -185, nuclear factor 1 (NF 1) and SP1 binding sites, and a GC box binding factor. All the factors play a role in constitutive expression as well as cAMP activation of the tPA gene. The first 621 nucleotides of the 5'-flanking region of the rat tPA gene is sufficient to confer both the basal and the FSH-induced promoter activity to a reporter gene construct when transfected into primary GC cultures (Feng *et al.*, 1990; Ohlsson *et al.*, 1993; Leonardsson & Ny, 1997).

Urokinase plasminogen activator

Urokinase PA is synthesized and secreted from cells as a proenzyme with little or no activity (pro-uPA). The human uPA gene, 6.4 kb in length, is located on chromosome 10. It contains 11 exons and gives rise to a 2.5-kb-long mRNA, which encodes a single-chain glycosylated polypeptide of 50 kDa. Single-chain pro-uPA (scuPA) is converted by the limited proteolysis into an active 50 kDa enzyme uPA consisting of two polypeptide

chains held together by one disulfide bond. The 33 kDa form of uPA is a partial degradation product, and does not interact with the receptor (Blasi *et al.*, 1987).

uPA receptor

uPAR is a 45-65 kDa glycoprotein (31.5 kDa of non-glycosylated uPAR) that is localized to the outer layer of the plasma membrane of cells, via a glycosyl phosphatidylinositol linkage (GPI anchor) and does not possess a transmembrane domain. uPAR is comprised of three domains with structural homology based on the spacing of the disulfide bonds (Ploug *et al.*, 1991). Domain 1 contains most of the determinants required for binding to uPA, and additional determinants in either domain 2 or domain 3 are required to realize the full affinity of the binding (Behrendt *et al.*, 1991). Despite the lack of a transmembrane domain in uPAR, adaptor molecules must exist and be capable of coupling uPA-uPAR binding to signal transduction. Various candidate integrins are proposed to act as this adaptor, and uPAR appears to play a central role in integrin-mediated signal transduction and cell-cell adhesion (Tarui *et al.*, 2001; Mazar, 2001). For example, co-localization studies demonstrate an association of uPAR with $\alpha 5\beta 1$ integrin for human tumor cell survival (Aguirre Ghiso *et al.*, 1999). Most recently, Jo et al. suggest that uPAR functions in concert with co-receptors, including integrins and epidermal growth factor receptor (EGFR), to initiate cell signalling, and the EGFR selectively cooperates with uPAR to mediate mitogenesis in MCF-7 cells (a breast cancer cell line) (Jo *et al.*, 2005). However, the interaction of uPAR, integrins and EGFR in ECM remodelling of ovarian follicle has not been studied yet. In addition, low-density lipoprotein (LDL) receptors are important for internalization of proteases and protease-inhibitor complexes

(Strickland *et al.*, 2002). For example, uPAR recycling is dependent on the LDL receptor-related protein (LRP) or the very-low-density lipoprotein (VLDL) receptor. PAI-1 can bind to a uPAR-uPA complex, which in turn binds tightly to LRP which leads to LRP-dependent endocytosis of the uPAR-uPA complex, followed by recycling of uPAR back to the cell surface (Nykjaer *et al.*, 1997).

Plasminogen activator inhibitor-1

PAI-1 is a single-chain glycoprotein of approximate 50 kDa molecular weight consisting of 379 amino acids. The target proteases of PAI-1 include uPA, tPA, plasmin, and thrombin (Silverman *et al.*, 2001). The reactive centre of the inhibitor (Arg346/Met347) is contained within the exposed 'strained loop' region at the carboxy-terminus of the molecule, and serves as a pseudo-substrate for the target serine proteases (Loskutoff, 1993). The lack of cysteine residues (and hence disulfide bonds) may in turn account for its biological instability in solution. It appears to be synthesized in the active form by cultured cells but is unstable in solution and rapidly decays into the inactive form upon secretion into serum-free cell culture media (Loskutoff, 1993). The binding of PAI-1 to the adhesive glycoprotein vitronectin stabilizes the inhibitor in its active conformation, thus increasing its biological half-life. Vitronectin is the primary PAI-1 binding protein in the ECM (Seiffert *et al.*, 1990). The human PAI-1 gene is approximately 12.2 kilobase pairs in length, contains 9 exons and 8 introns and is located on the long arm of chromosome 7. It encodes two distinct transcripts approximately 2.3 and 3.2 kb in length, differing only in their 3' untranslated regions (Andreasen *et al.*, 1990). The 5'–flanking region of the human PAI-1 gene was shown to contain the transcription initiation site and a

TATA box, suggesting that this region contains the PAI-1 promoter and is important for PAI-1 gene expression (Lewin, 1990). Most interestingly, one upstream region of the human PAI-1 gene (-1520 to -1008) has high homology with human tPA gene, raising the possibility that two genes may be co-ordinately regulated under some circumstances (Bosma *et al.*, 1988).

Plasminogen activator inhibitor-2

PAI-2 is also a secreted protein of approximately 60 kDa, containing 415 amino acids. PAI-2 is a major product of macrophages and monocytes in response to inflammatory conditions (Schwartz *et al.*, 1988; Gyetko *et al.*, 1992). The two different forms (extracellular and intracellular) of PAI-2 in monocytes probably have distinct functions. The extracellular form inhibits uPA activity, whereas the predominant intracellular form inhibits tumor necrosis factor α (TNF-α)-directed apoptosis (Dickinson *et al.*, 1995). The target protease of PAI-2 includes uPA (Silverman *et al.*, 2001). PAI-2 is a relatively poor tPA inhibitor compared to PAI-1. Among the serpins, PAI-2 shares only 26% homology with PAI-1. Like PAI-1, PAI-2 also forms equimolar complexes with uPA and tPA, and is present in biological samples in such complexes. The complexes are at least partially SDS-resistant. In plasminogen activator inhibitor assays, PAI-1 and PAI-2 activity can be distingushed by the fact that only PAI-1 is detectable by reverse zymography. Unlike PAI-1, PAI-2 does not appear to be able to resume an active confirmation after SDS denaturation (Andreasen *et al.*, 1990). The secretion mechanisms of PAI-1 and PAI-2 are different. PAI-1 has an amino-terminal 21-23 signal peptide that is cleaved in the mature protein, which is common to most secreted proteins (Ny *et al.*, 1986),

while the secretion of PAI-2 relies on an internal signal sequence, which is not cleaved during translocation to the endoplasmic reticulum (ER) (Ye *et al.*, 1988). The human PAI-2 gene, present on chromsome 18, is transcribed into a 2 kb mRNA (Andreasen *et al.*, 1990).

α_2-antiplasmin

α_2-AP is a 70 kDa single chain Serpin with a peptide bond Arg-Met as reactive site. It is a plasmin-specific inhibitor, which forms an inactive 1:1 stoichiometric complex. The high reaction rate requires the presence of a free active site and free lysine-binding site(s) in plasmin. Homozygous α_2-AP deficient mice display normal fertility, viability and development (Lijnen, 2001), suggesting its role in follicle development is not essential.

A schematic proteolytic cascade is illustrated in Fig 3.

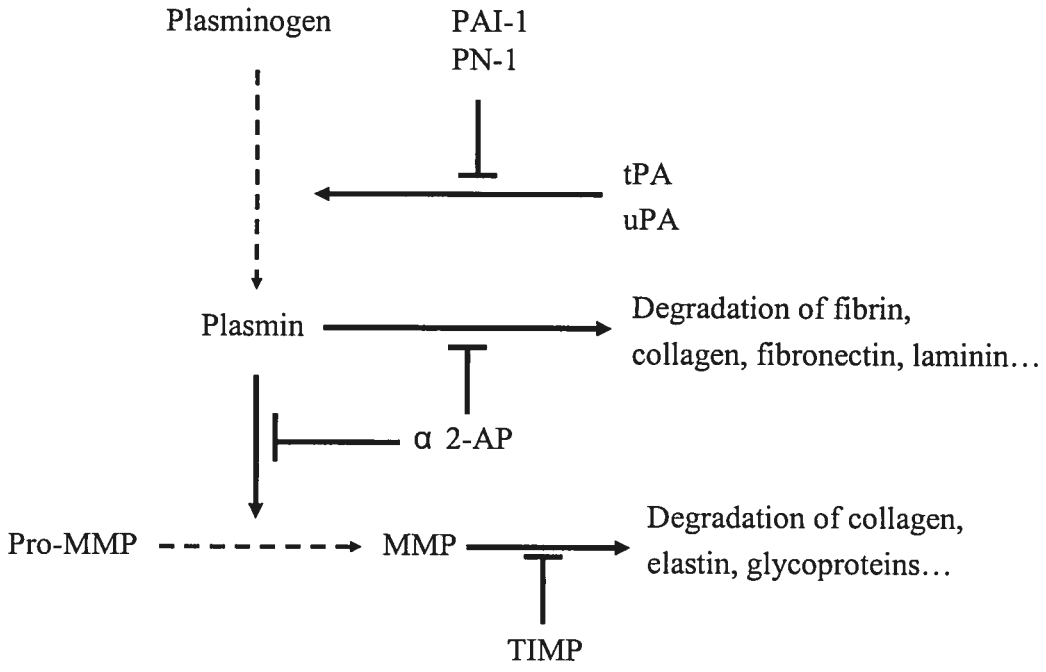

Fig 3. A schematic Proteolytic Cascade

PROTEASE NEXIN-1: MOLECULAR AND BIOCHEMICAL CHARACTERISTICS

PN-1, also referred to serine protease inhibitor-E2 (SERPIN-E2), and glia-derived nexin (GDN), is a secreted 43 kDa glycoprotein, and is a broad spectrum, trypsin-like serine protease inhibitor that rapidly inhibits a number of target proteases including trypsin, thrombin, uPA, tPA, and plasmin (Silverman *et al.*, 2001). PN-1 slowly inhibits Factor Xa and the γ subunit of nerve growth factor but does not inhibit chymotrypsin-like proteases or leukocyte elastase (Scott *et al.*, 1985). Apart from its role as a soluble protease inhibitor, PN-1 is also a component of the ECM and might control its proteolysis (Farrell *et al.*, 1988). PN-1 inhibits plasminogen activation-induced apoptosis of adherent cells via the formation of inhibitory complexes with plasmin and tPA, indicating PN-1 may be an important anti-apoptotic factor for adherent cells (Rossignol *et al.*, 2004). PN-1 is expressed and secreted by several cell types or organs, including neurons (Mansuy *et al.*, 1993; Citron *et al.*, 1996; Kury *et al.*, 1997), rat aortic smooth muscle cells (Richard *et al.*, 2004), endothelial cells and fibroblasts (Baker *et al.*, 1980), seminal vesicle in mice (Vassalli *et al.*, 1993), and human placenta (White *et al.*, 1993), suggesting PN-1 has a role in these cells or organs. PN-1 expression and secretion as well as its regulation in the ovary will be described in the next section.

PN-1 acts as a pseudo-substrate to form 1:1 stable complexes with its target proteases, and once formed, the complex binds to the cell surface where it is quickly internalized and degraded. Further studies show that the PN-1-uPA complex is internalized and degraded through a mechanism that requires both uPAR and α2-macroglobulin receptor (α2- MR) in two human and murine cell lines (Conese *et al.*, 1994).

Previous studies show that PN-1 can be regulated in terms of activity, specificity, and localization by glycoprotein or ECM co-factors. PN-1 binds tightly to and is regulated by the ECM. Once secreted, it binds tightly to collagen Type IV, regulating both its activity and its target protease specificity. Donovan et al. report that PN-1 inhibits thrombin even when bound to collagen IV, but has less inhibitory effect on uPA and plasmin (Donovan *et al.*, 1994). However, Crisp et al. find that PN-1 remains a potent uPA inhibitor in the presence of collagen IV (Crisp *et al.*, 2002), and suggests that the disagreement between studies results from the PN-1 purification protocol (Donovan *et al.*, 1994), which affects PN-1 allosteric interaction with collagen IV, leading to altered protease specificity. Moreover, PN-1 has a heparin-binding site, heparin greatly accelerates the rate of linkage between soluble PN-1 and its substrate thrombin (Baker *et al.*, 1980).

Gaining insight into how the PN-1 gene is regulated at the molecular level should lead to a better understanding of the physiological control of PN-1. The PN-1 gene has been cloned and studied in several species. The rat PN-1 promoter is highly GC rich, and methylation of these sequences is thought to play a role in suppressing PN-1 transcription (Erno & Monard, 1993).

The human PN-1 gene, localized on chromosome 2, contains nine exons spanning more than 40 kb. The 5' end of the human PN-1 gene closely resembles the genomic structure of its rat counterpart (Carter *et al.*, 1995). The human PN-1 promoter contains an activation domain at position -199 to -45, which contains multiple putative Sp1 binding sites. Multiple Sp1 proteins bind the PN-1 promoter and they act synergistically to stimulate PN-1 transcription most likely through the TATA-binding protein-associated factors that are part of the TFIID complex (Courey *et al.*, 1989; Pugh & Tjian, 1990; Pascal

& Tjian, 1991). A silencer element upstream of position -480 may exert negative regulation of PN-1 gene expression (Guttridge & Cunningham, 1996). Transcriptional regulation by Sp1, which is a ubiquitously expressed transcription factor, is often associated with housekeeping genes. PN-1 gene may not be a traditional housekeeping gene (Erno & Monard, 1993), therefore Sp-1 functions to ensure a steady-state level of PN-1 gene expression. Furthermore, Sp-1 is suggested to play a significant role in regulation of PN-1 in the nervous system (Mansuy *et al.*, 1993). Therefore, we speculate that a spatially and temporally regulatory mechanism for PN-1 gene expression and activity might exist during other events such as follicle development.

The bovine PN-1 mRNA is 2096 bp in length and contains 174 bp of 5'-untranslated region (UTR), 1191 bp of open reading frame (ORF), and a 731 bp 3'-UTR that include two signals of mRNA instability (ATTTA) and two poly-adenylation signals followed by a poly(A)$^+$ tail (Bédard *et al.*, 2003). This gene encodes a 397 amino acid (AA) protein of 43.8 kDa with an isoelectric point (IP) of 9.9. The bovine PN-1 shares 91.4%, 83.9%, and 83.1% identity of human, rat, and mouse PN-1 ortholog, respectively. Bovine PN-1 is 42% identical to bovine PAI-1 paralog.

Restricted information is available about PN-1 function in male and female reproductive systems. Mouse PN-1 is expressed in a wide variety of tissues (Mansuy *et al.*, 1993), but in the adult the highest levels are under androgen control in the seminal vesicle (Vassalli *et al.*, 1993). Furthermore, PN-1 exhibits male-specific expression prior to overt gonad differentiation, suggesting a possible role in mammalian sexual development (Grimmond *et al.*, 2000). In females, high levels of PN-1 mRNA and antigen were expressed by mice GC in periovulatory follicles (Hägglund *et al.*, 1996). As PN-1 can

neutralize several proteases, including PAs and plasmin (Scott *et al.*, 1985), it could play a role in regulating PA activity. Although the level of PN-1 mRNA is high, it is not regulated by gonadotropins (Hägglund *et al.*, 1996). Functional studies using a PN-1 knock-out (KO) approach show that PN-1deficient male mice are infertile, but female mice are fertile (Murer *et al.*, 2001). In homozygous male mice, absence of PN-1 results in altered semen protein composition, and leads to inadequate semen coagulation and deficient vaginal plug formation upon copulation, and integrity of the seminal gland is not maintained duo to the increased and uncontrolled proteolytic activity (Murer *et al.*, 2001). The data demonstrate that the level of extracellular proteolytic activity regulated by PN-1 is a critical element in controlling male fertility in mice. PN-1 KO female mice are fertile, indicating that PN-1 is not essential for female reproduction, and other protease inhibitors likely play a compensatory role in regulating proteolysis in PN-1 deficient animals.

EXPRESSION AND REGULATION OF THE PA SYSTEM IN OVARIAN FOLLICLE

Biosynthesis and secretion of PA system in ovarian follicle

Plasminogen activators are synthesized in ovarian follicles of several mammalian species. However, species differences exist in the expression and secretion of the PAs. Rat GC secrete predominantly tPA in response to gonadotropins (Canipari & Strickland, 1985; Canipari & Strickland, 1986), whereas the same hormonal stimulation induces uPA expression or secretion in mouse GC (Canipari *et al.*, 1987; Hägglund *et al.*, 1996). uPA contributes 70% of the total ovarian PA activity in PMSG/hCG treated mouse model (Liu *et al.*, 1989). In the chicken, uPA is the predominant PA type present in GC during early

follicular development (Lafrance *et al.*, 1993a), whereas tPA is the principle PA in preovulatory follicles (Politis *et al.*, 1990a). These latter data suggest the synthesis and secretion of tPA and uPA also differs in different follicle stages in the same species. Little is known about the synthesis and secretion of the PA system in humans. Human GC collected from preovulatory follicles contain little or no tPA or uPA mRNA (Jones *et al.*, 1988), nevertheless, low level tPA activity is detected in the FF of human preovulatory follicles (Jones *et al.*, 1989). In the monkey, Liu and colleagues have systematically investigated the roles of the PA system in ovulation. They find that GC conditioned media contains predominantly tPA and some uPA activity (Liu *et al.*, 2004). The ovaries of rabbits, cats, hamsters, and giant pandas also contain mainly tPA activity, which is regulated by gonadotropins (reviewed by Liu, 2004). Pig GC also secrete predominantly tPA in response to gonadotropins (Politis *et al.*, 1990b). Bovine follicular cumulus layer produces tPA in cultured oocyte-cumulus complexes (Yamada *et al.*, 1996). Furthermore, tPA (mostly expressed in granulosa layer) and uPA (both granulosa and theca layer) mRNA are localized in bovine preovulatory follicles, and both activities are detected in bovine follicular homogenates after GnRH injection (Dow *et al.*, 2002a).

One mechanism for regulation of plasminogen activation, and hence plasmin activity, is through production of specific plasminogen activator inhibitors. In the rat, PAI-1 mRNA and activity are synthesized predominantly by theca-interstitial cells (Tls) (Liu *et al.*, 1987b; Liu & Feng, 1992) and PAI-1 is secreted into follicular fluid (Peng *et al.*, 1993). A similar expression pattern occurs in mice (Leonardsson *et al.*, 1995), monkey (Liu *et al.*, 2004) and cattle (Dow *et al.*, 2002b). Stimulation with hCG upregulates PAI-1 expression in theca cells, and induces PAI-1 mRNA expression in GC in rats (Chun *et al.*, 1992). In

mice the expression of PAI-1 mRNA is also localized to theca layer (Leonardsson *et al.*, 1995; Hägglund *et al.*, 1996). PAI-2 mRNA expression in mice is localized primarily to a few individual cells that may be macrophages in the theca layer (Hägglund *et al.*, 1996), in contrast, PAI-2 mRNA is localized specifically to the granulosa cell layer in cattle (Dow *et al.*, 2002b).

The gene expression and regulation of PN-1 has been examined in ovarian follicles. In contrast to PAI-1, PN-1 is exclusively expressed in GC in mice (Hägglund *et al.*, 1996), rats (Hasan *et al.*, 2002) and cattle (Bédard *et al.*, 2003). Furthermore, studies show that the level of PN-1 mRNA is high in GC throughout the periovulatory period, and decrease in ovulated follicles of mice (Hägglund *et al.*, 1996) and rats (Hasan *et al.*, 2002). In cattle, PN-1 mRNA is expressed in GC of small follicles and reaches the highest levels in growing dominant follicles (Bédard *et al.*, 2003; Fayad *et al.*, 2004b). Moreover, PN-1 mRNA expression in granulosa cells is not regulated by gonadotropins in mice (Hägglund *et al.*, 1996), but may be regulated by anticoagulant heparan sulfate proteoglycans (aHSPGs) at the protein level, as aHSPGs are colocalized with PN-1 in rat follicles (Hasan *et al.*, 2002). Taken together, biosynthesis and secretion of PA system in ovarian follicle may be regulated in a species-specific, cell-specific, and stage-dependent manner.

Ovulation requires coordinated expression of PAs and PAIs in ovary

Ovulation, triggered by a LH surge, is an essential prerequisite for fertilization and subsequent embryonic development. A mature follicle destined to ovulate usually protrudes markedly from the surface of the ovary. For the egg to escape from a follicle, extensive breakdown and remodelling of basement membranes and connective tissus that constitute

the follicular wall is required (Richards *et al.*, 2002; Curry *et al.*, 2001). Ovulation is a very complex event that involves localized digestion of ECM components, including laminin, proteoglycans, and type IV collagen in the basement membrane and different types of collagen in the connective tissue. There are growing indications that the PA system cooperates with the MMP system to activate a proteolytic cascade leading to the follicle wall rupture (Palotie *et al.*, 1987; Woessner *et al.*, 1989). As the different ECM components of basement membrane need to be sequentially degraded, a network between the two proteases systems is suggested. This section will focus on the PA system.

Early studies using in vitro approach suggest that the production of plasminogen activators by Graafian follicles may be essential for gonadotropin-induced ovulation. For example, a pioneer study demonstrates that rat GC from preovulatory follicles contain plasminogen activator activity that reaches a maximum level prior to ovulation (Beers *et al.*, 1975). Ten years later, secreted tPA, uPA amd PAI-1 are identified by immunoprecipitation in cultured rat GC (Ny *et al.*, 1985). Furthermore, inhibitors of serine proteases block gonadotropin-induced ovulation (Reich *et al.*, 1985). Canipari and Strickland (1985) find that cultured rat GC produce exclusively tPA, and theca cells secreted uPA, suggesting that ovulation requires both types of PA and the neighbouring GC and theca cells cooperate to stimulate follicle wall rupture (Canipari & Strickland, 1985). However, separated cell culture experiments reveal that both compartments produce both types of PA, even though GC contribute most of the follicular PA (Reich *et al.*, 1986). To analyze the interaction between tPA produced by GC and PAI-1 synthesized by theca cell in rats, the two cell types are obtained from ovaries at various time points after PMSG/hCG treatment, and are incubated together. Net tPA activity in the conditioned media is

remarkably inhibited 8 h after hCG, but increases at 12 h after hCG, despite the presence of high level of PAI-1 in the conditioned media (Liu, 1988).

In vivo studies in rats show that ovulation is preceded by a transient and cell-specific increase in both tPA and PAI-1 expression (Liu *et al.*, 1991; Peng *et al.*, 1993). The mRNA level and activity of GC-derived tPA and theca PAI-1 mRNA increase with time following PMSG/hCG injection, however PAI-1 dramatically decline at about 4 h prior to ovulation, which allows an increase in net tPA activity immediately prior to ovulation (Liu *et al.*, 1991; Peng *et al.*, 1993; Shen *et al.*, 1997). Furthermore, anti-tPA and anti-uPA administration immediately before hCG treatment significantly block ovulation rate in rats (Macchione *et al.*, 2000). A similar event occurs in mice, although the main PA is uPA and the major inhibitor appears to be GC-derived PN-1 (Hägglund *et al.*, 1996). Furthermore, Hagglund and colleagues (1996) find a complementary gene expression between uPA and PN-1 in different subpopulations of mouse GC in periovulatory follicles. Mural GC localized next to the basement membrane express high levels of uPA mRNA and low levels of PN-1 mRNA. In contrast, cumulus cells localized close to the oocyte express high levels of PN-1 mRNA and low levels of uPA mRNA (Hägglund *et al.*, 1996). The author suggests that such an expression pattern provides a shift toward inhibition of proteolytic activity around the COC.

Using non-rodent models, messenger RNAs for tPA, uPA, and uPAR (Dow *et al.*, 2002a), as well as PAI-1 and PAI-2 (Dow *et al.*, 2002b) increase in bovine preovulatory follicles within 24 h following GnRH induction of LH surges. Activities for tPA and plasmin, but not PAI-1 and -2 increase in follicular fluid or follicle homogenates within 12 h after gonadotropin surge. Interestingly, the increase in tPA activity in the follicle base

(non-ovulatory region of follicle) is transient, whereas the increased activity in the follicle apex (the site of ovulation) is maintained through the 24 h time point, indicating tPA activity is differentially regulated in the follicle apex versus the base (Dow *et al.*, 2002a). uPA activity is increased in follicle homogenates of the basal and apical regions within 12 h after gonadotropin surge and remains elevated through the 24 h time point. However, uPA activity in FF is not detectable (Dow *et al.*, 2002a). Thus, the authors concluded that increased tPA, uPA, and plasmin activities may contribute to follicle rupture in cattle. However, the differential up-regulation of tPA activity in the follicular apex versus base seems unlikely to be a result of regional differences in up-regulation of PAI activity in response to the gonadotropin surge. The contribution of both tPA and uPA to follicle wall rupture, and PAI-1 and PAI-2 mRNA regulation by gonadotropin during ovulation are different from previous reports in rodents. In addition, in cattle the expression of PN-1 mRNA in GC decreases in dominant follicles following hCG injection (Bédard *et al.*, 2003).

Functional studies using knock-out (KO) mice provide further evidence for the role of PA during ovulation. Mice lacking tPA, uPA or PAI-1 expression have normal reproduction, but mice with combined deficiencies of tPA and uPA are significantly less fertile (Carmeliet *et al.*, 1994). Ovulation efficiency is normal in mice with a single deficiency of tPA or uPA but reduced by 26% in mice lacking both physiological PAs (Leonardsson *et al.*, 1995). In mice lacking either tPA or PAI-1, plasmin activity in the ovary prior to ovulation is similar to that of wild-type mice, whereas the ovarian extract prepared from uPA-deficient mice contain only 10% or less plasmin activity of the normal wild-types, indicating that most of the plasmin activity in the mouse ovary is generated by

uPA (Ny *et al.*, 1997). Using casein in situ zymography, Hagglund et al. (1996) report that a plasminogen-dependent proteolytic activity appears mainly at the surface of the ovary and around large preovulatory follicles. In mice that lack the uPA gene, tPA produces a lytic activity (Hägglund *et al.*, 1996). These data indicate that the amount of plasmin generated by PAs before ovulation in wild-type mice greatly exceeds the amount required for efficient ovulation, thereby providing functionally redundant mechanisms for plasmin formation during ovulation. Surprisingly, ovulation is not delayed in plasminogen-deficient mice during gonadotropin-induced ovulation, which suggests that plasmin is not required for efficient follicle wall rupture or for activation of other proteases involved in this process, although there is a trend toward slightly reduced (13%) ovulation efficiency in plasminogen-deficient mice (Bugge *et al.*, 1995; Ny *et al.*, 1999). However, whether the phenomena obtained from mice is the case for other species is unknown. Nevertheless, these data imply that (1) a potential compensatory mechanism among PAs may be involved, by which the loss of an individual PA appears to be functionally compensated for by the remaining PA; (2) a functionally redundant mechanism for plasmin formation may be present during gonadotropin-induced ovulation; (3) the PA system together with other proteases such as MMPs play a role in ovulation (Smith *et al.*, 1999); and (4) a potential compensatory mechanism between the PAs and the MMPs can be speculated, by which MMPs and/or other proteases efficiently degrade follicular wall during ovulation if plasmin is absent. Moreover, the PA contribution to ovulation occurs at the initial steps, since plasmin can activate pro-MMP-2 and pro-MMP-9 to their active forms MMP-2 and MMP-9, subsequently contributes ovultion via MMPs activation (Mazzieri *et al.*, 1997; Ramos-DeSimone *et al.*, 1999; Murphy *et al.*, 1999a). Further investigations are required to

elucidate the precise physiological roles of the interactions between the PAs and MMPs during ovulation.

Expression and regulation of PA and PAIs in the small growing follicles

It is likely that tissue remodelling in follicular growth is distinct from that occurs during follicle wall rupture in ovulation. Ovulation involves an extensive breakdown and degradation of basement membrane and connective tissue, and is a transient but vigorous process. Follicular growth, however, involves a chronic but comparatively mild tissue remodelling of ECM and cell migration. During follicular growth, the follicular cavity becomes progressively large, which requires the follicle wall to expand but not break. Thus, follicular growth seems to need a good balance between proteases and their inhibitors, to ensure limited and localized proteolysis. Therefore, a coordinated expression and regulation of PAs and PAIs are also suggested in follicular growth as shown in ovulation. However, little is known about the PA system and its contribution to ECM remodelling in the earlier stages of follicular development.

uPA mRNA and protein levels are highest at the earliest stage of follicular growth and they decrease dramatically before the expected time of ovulation in rats (Li *et al.*, 1997b). Evidence from other studies indicates that uPA may be important in ECM remodelling during early follicular growth (Tilly & Johnson, 1987; Karakji & Tsang, 1995a; Shen *et al.*, 1997). PAI-1 and PAI-2 are low in the developing follicles, whereas PN-1 is highly expressed in small growing follicles in the rodent, where it accumulates until the onset of ovulation (Hägglund *et al.*, 1996; Hasan *et al.*, 2002). The expression of PN-1 mRNA in bovine GC increases as follicles grow from small antral to dominant

follicles (Bédard *et al.*, 2003; Fayad *et al.*, 2004b). Combined with the previous finding that uPA is co-localized with PN-1 in GC in growing follicles of mice (Hägglund *et al.*, 1996), it is reasonable to propose that PN-1 may be a major inhibitor of uPA and both are coordinately expressed to regulate tissue remodelling during follicular growth and/or follicle wall rupture.

ROLES OF PA SYSTEM IN CORPUS LUTEUM AND OVARIAN ANGIOGENSIS

The corpus luteum (CL), a temporary endocrine organ, is transformed from the residual GC and theca cells of the ovulated follicle. CL formation involves dramatic morphological and biochemical changes involving invasion of the capillary network from the theca tissue into the granulosa layers, and the transformation of the granulosa and theca cells into large or small luteal cells, respectively. The functional CL secretes progesterone for maintenance of pregnancy, which primes the uterus for implantation and early fetal development. However, if fertilization does not occur, or if implantation is unsuccessful, the functional phase of the CL is terminated and luteolysis is initiated (Murphy, 2004). This involves a rapid loss of progesterone production (functional luteolysis) followed by degradation of the luteal tissue into small fibrous remnants (structural luteolysis) in days (Auletta & Flint, 1988). Therefore, matrix-degrading proteases including PAs and MMPs are thought to play important roles in CL formation and regression.

The regulatory and functional roles of the PA system in the CL have been widely studied for the last decade (reviewed by Smith *et al.*, 1999; Ny *et al.*, 2002; Liu, 2004). In the rat, proteolytic activities mediated by tPA and regulated by its inhibitor PAI-1 are

important for CL formation and regression (Liu *et al.*, 1995; Liu *et al.*, 1996b). In rhesus monkeys high levels of uPA and PAI-1 mRNA are simultaneously expressed in functional CL, indicating that the interplay of uPA and PAI-1 may be necessary for CL formation and functional maintenance (Liu *et al.*, 1997a). Furthermore, uPA, but not tPA, is the only active PA identified in the early CL (Liu *et al.*, 2003b), suggesting a role for uPA in tissue remodelling and angiogenesis during CL formation. However, a substantial elevation in tPA mRNA and protein is observed in monkey CL during luteolysis, when serum progesterone levels and StAR (a key regulator of CL function and a marker of steroidogenesis in many species) mRNA expression in the CL decline dramatically (Liu *et al.*, 2003a). Moreover, tPA and PAI-1 mRNAs and proteins show a coordinated expression in the regressing monkey CL, suggesting a role for tPA and PAI-1 in tissue degradation during CL regression, and PAI-1 regulated tPA activity might be important during the initiation of luteolysis (Feng *et al.*, 1993). If we compare the roles of the PA system of follicles with CL, we discover that uPA mainly involves tissue remodelling (a chronic and mild process) such as follicular growth or CL formation, whereas tPA mainly involves tissue degradation (a transient but vigorous process) such as ovulation or CL regression.

The ovary is a highly vascularized organ in which the formation and regression of blood vessels are required during growth and development of follicles and CL. In the developing CL, both uPA and PAI-1 are expressed in the ovary during neo-vascularization of growing follicles, and in the early stages of CL development (Bacharach *et al.*, 1992). After ovulation, uPA is dramatically expressed in the developing CL mainly in the capillary sprouts of vessels (Liu *et al.*, 2003b). PAI-1 is also expressed during early CL development stage, mainly in the vicinity of uPA expressing capillary-like structures

(Bacharach *et al.*, 1992; Gospodarowicz & Thakral, 1978). This observation suggests that there is a functional interplay between uPA and PAI-1 in CL vascularization, the presence of PAI-1 in the capillary-like structures may protect neovascularized tissues from excessive proteolysis during angiogenesis in developing CL.

The expression and regulation of the different PA system members during CL formation and regression and during angiogenesis remain to be investigated in non-rodent models.

HORMONAL REGULATION ON THE PA SYSTEM IN GRANULOSA CELLS

Most of the available information concerning the regulation of PAs in GC is obtained using in vitro approaches. Cultured GC are an excellent in vitro model for studies on the hormonal regulation of cell differentiation (Hsueh *et al.*, 1984). Using cell culture models, a number of hormones and growth factors have been shown to be involved in regulating PA and PAI gene expression and activity in rodents and other species.

Gonadotropins

To test the effects of gonadotropins on the expression and/or secretion of PAs and inhibitors in GC, many experiments have been done use cultured rat GC. FSH or the combination of FSH and dihydrotestosterone (DHT) increase tPA activity in a dose-dependent manner, while LH has no effect on the activators in undifferentiated GC (Ny *et al.*, 1985). When GCs are primed with low concentration of FSH (20 ng/ml) for two days to induce functional LH receptors, both FSH and LH increase tPA activity. Interestingly, uPA

is more prominent in undifferentiated cells and decreases upon prolonged culture. Furthermore, both FSH and LH suppress the synthesis and /or activity of PAI-1 (Ny *et al.*, 1985). Other studies show that both FSH and LH induce tPA mRNA and protein secretion in cultured rat GC, but the time courses of their effects are different and the effect of LH on tPA production is later and longer than FSH (Canipari & Strickland, 1986; O'Connell *et al.*, 1987). An FSH agonist stimulates (Inaba *et al.*, 1998), but FSH antagonist inhibits (Timossi *et al.*, 1998) tPA activity by cultured rat GC in a dose-dependent manner. Furthermore, Forskolin or cAMP (an activator of PKA) induces ovarian tPA activity in vitro (Liu *et al.*, 1986). Both FSH and cAMP have divergent effects on secreted tPA (increased) and uPA (decreased) in cultures of rat GC (Macchione *et al.*, 2000).

The action of FSH on tPA expression is rapid and coincides with increased levels of intracellular cAMP (Kolena & Channing, 1972). tPA mRNA levels are enhanced in response to the phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine (MIX), suggesting that FSH activates the tPA gene through the cAMP-dependent protein kinase A pathway, leading to phosphorylation of the cAMP-responsive element binding protein (CREB) and activation of the promoter containing a cAMP-responsive element (CRE) (Comb *et al.*, 1986). tPA gene regulation by cAMP differs between rats, mice and humans, and the difference is related to a one-nucleotide substitution in the CRE of the promoters. At the position where the rat tPA promoter contains a consensus CRE, the mouse and human counterparts contains a CRE variant, which drastically reduces the binding affinity for CREB (Holmberg *et al.*, 1995).

In other species, FSH has no stimulatory effect on plasminogen activator production by cultured porcine GCs, while hCG stimulates enzyme activity (Shaw *et al.*, 1985). This is

likely due to the absence of FSH receptors in granulosa cells, but the presence of LH receptors as porcine GC luteinize in culture (Pescador *et al.*, 1999). In the avian ovary, the granulosa layer is the site of mRNA and protein regulation of PA production by LH. PA mRNA, protein and activity are high before the LH surge and low after the LH surge (Tischkau *et al.*, 1996).

GnRH

GnRH and its agonist (GnRHa) are known to stimulate tPA expression in cultured rat GCs (Ny *et al.*, 1987). GnRHa stimulates the induction of tPA (but not uPA) activity in GC in a time-dependent manner as hCG does in vivo, reaching a maximum before ovulation. However, administration of GnRH antagonist blocks GnRHa, but not hCG-induced ovulation in vivo, indicating GnRH and hCG induce ovulation through different receptor pathways (Hsueh *et al.*, 1988b). Further studies indicate that GnRH induces tPA mRNA and activity in GC through the protein kinase-C (PKC) pathway, whereas FSH and LH induce tPA mRNA and activity through protein kinase-A (PKA) pathway (Hsueh *et al.*, 1988a), and PMA (phorbol myristate acetate, an activator of PKC) is able to induce tPA mRNA in rat GC in vitro (Liu *et al.*, 1986; Ohlsson *et al.*, 1988). The combined effect of FSH and GnRH on the stimulation of tPA gene expression and secretion is additive (Ny *et al.*, 1987). In contrast to FSH, GnRH-induced tPA mRNA is blocked by cycloheximide, indicating that the synthesis of an intermediate protein is required for the effect of GnRH (Ohlsson *et al.*, 1988).

Recently, two forms of GnRH and two types of GnRH receptors have been reported in mammalian reproductive tissues (reviewed by Kang *et al.*, 2003; Pawson *et al.*, 2003). It

is proposed that GnRH-II is a potent regulator of ovarian function in baboons (Siler-Khodr *et al.*, 2003). GnRH-I and GnRH-II increase uPA mRNA and protein expression in human decidual stromal cells in vitro in a dose- and time-dependent manner (Chou *et al.*, 2003). In contrast, GnRH-I increases, whereas GnRH-II decreases PAI-1 mRNA and protein expression in these cells (Chou *et al.*, 2003). These data suggest GnRH-I and GnRH-II differentially regulate the balance between uPA and PAI-1 expression levels, possibly via distinct receptor-mediated signal pathways. Messenger RNA of type-I and type-II GnRH receptors but not their respective proteins are detected in bovine GC (Ramakrishnappa *et al.*, 2003). Although up-regulation of tPA, uPA mRNA and activity by GnRH (-I) injection in bovine GC is reported in vivo (Dow *et al.*, 2002a), the actions of GnRH in vivo seems to be through stimulating preovulatory surges of FSH and LH.

Growth factors

Transforming growth factor-alpha (TGFα), and the closely related epidermal growth factor (EGF) bind to EGF receptors and stimulate the secretion of tPA activity in cultured rat GC in dose-and time-dependent manners, through receptor tyrosine kinase intracellular pathways (Galway *et al.*, 1989). TGFα increases basal tPA activity in both undifferentiated and differentiated rat GC, but inhibits uPA activity in undifferentiated GC (uPA activity is undetectable in differentiated cells), irrespective of the presence of FSH (Karakji & Tsang, 1995a). In cultured avian GC, TGFα stimulates uPA activity, TGFβ enhances TGFα-induced PA activity in GC from F3-6 but not F1 follicles, whereas LH attenuates TGFα-induced PA activity in GC from F1-3 but not F5-6 follicles (Lafrance *et*

al., 1993a; Lafrance *et al.*, 1993b; Li *et al.*, 1997a). EGF enhances uPA secretion from bovine cumulus cells in vitro (Park *et al.*, 1999).

TGFβ is produced in granulosa and theca cells and oocytes. In other cell types, such as WI-38 human lung fibroblasts, TGFβ decreases extracellular proteteolytic activity through stimulating PAI-1 gene transcription and protein synthesis and decreasing uPA and tPA activities (Lund *et al.*, 1987). In cultured avian GC, other growth factors including IGF-I, EGF, and platelet-derived growth factor (PDGF) stimulate secreted and cell-associated PA activity (Lafrance *et al.*, 1993a).

Basic fibroblast growth factor (FGF-2) stimulates tPA mRNA and enzyme activity in cultured rat GC, in a time- and dose-dependent manner, suggesting that FGF-2 acts as an intraovarian inducer of tPA gene expression in GC (LaPolt *et al.*, 1990). In cultured bovine GC, FGF-2 and aFGF show a potent stimulation of cell proliferation and TIMP-1 mRNA expression and protein production, while insulin stimulates cell growth but inhibits TIMP-1 mRNA levels (Hoshi *et al.*, 1995).

Other factors

A number of other factors also affect PA secretion, but their physiological relevance is unclear. Glucocorticoid agonist dexamethasone (DEX) or androgen agonist R1881 increases tPA secretion and mRNA levels in rat GC, but addition of diethylstilbestrol (DES, an estrogen agonist) has no effect on tPA levels in rat GC (Jia *et al.*, 1990). Estradiol has a modest stimulatory effect on tPA mRNA expression and activity in a human breast adenocarcinoma cell line (MCF-7 cells) while concomitant treatment with laminin increases tPA mRNA but decreases PAI mRNA levels (Sonohara *et al.*, 1998).

Vasoactive intestinal peptide (VIP), originally considered to be a hormone in the digestive system, increases tPA activity in GC and COC of rats (Liu *et al.*, 1987a), and domestic chickens (Johnson & Tilly, 1988). This may be related to the ability of VIP to induce ovulation (Schmidt *et al.*, 1990). Pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the VIP /secretin /glucagon /growth hormone-releasing hormone family, stimulates steroidogenesis and increases cAMP levels in cultured rat GC (Zhong & Kasson, 1994). PACAP acts synergistically with relaxin to stimulate secretion of 63 kDa gelatinase (MMP-1 or active MMP-2) in rat GC and 71 kDa gelatinase (MMP-2) in theca-interstitial cells, respectively. PACAP alone has no effect on MMP-2 secretion in theca cells (Teng *et al.*, 2000). In addition, cAMP signalling mediators (cholera toxin, activator of stimulatory G protein; forskolin; 8-Br-cAMP, cAMP analog) act similarly to PACAP on gelatinase secretion in rat ovarian cells, suggesting PACAP acts through the cAMP signalling pathway, whereas relaxin does not (Teng *et al.*, 2000). Relaxin increases secreted PA and MMP-1 activity in rat GC (Too *et al.*, 1984), and different major gelatinases including MMP-2 from rat GC and thecal-interstitial cells (Hwang *et al.*, 1996).

The cytokine interleukin-1 beta (IL-1β) is involved in modulating GC progression from a proliferative to a differentiated state. In vitro treatment of both undifferentiated and differentiated rat GC with FSH elicited a significant increase in secreted and cell-associated PA activities, which is inhibited by IL-1β (Karakji & Tsang, 1995b). The inhibitory effect of IL-1β is accompanied by an increase in PAI activity, irrespective of the stage of follicular development. Basal PAs activities are stimulated in cultures of undifferentiated GC by IL-1 β but attenuated in differentiated ones (Karakji & Tsang, 1995b).

Prolactin (PRL) is a pituitary hormone mainly involved in stimulating milk production. In vitro studies demonstrate that both tPA and uPA activities in mouse GC are down-regulated by PRL in a dose-dependent fashion (Hu & Liu, 1993). PRL inhibits LH- and FSH-induced tPA mRNA and activity in rat GC but stimulates PAI-1 gene expression, and decreases secreted tPA activity and increases secreted PA-PAI complexes (Liu *et al.*, 1998). PRL decreases PA activity of the mature rabbit follicles (Yoshimura *et al.*, 1990) and inhibits plasmin generation in preovulatory follicles (Yoshimura *et al.*, 1992). Injection of PRL in the rat inhibits hCG-induced ovulation in a dose-dependent manner by disturbing the normal coordinated expression of tPA and PAI-1 leading to ovulation (Liu *et al.*, 1997b). Similarly, the N-terminal fragment of PRL (16K PRL), an antiangiogenic factor, inhibits FGF-2-induced uPA activity by activation of PAI-1 gene expression and subsequent increase in PAI-1 protein in bovine capillary endothelial cells (BCEC) (Lee *et al.*, 1998).

OVERALL HYPOTHESIS & SPECIFIC OBJECTIVES

As PN-1 would appear to be the only potent PA inhibitor expressed in GC, we hypothesize that the expression of PN-1 in bovine GC is developmentally and hormonally regulated during follicular growth and ovulation.

The objectives of this study were:

1). To measure PN-1, tPA and uPA expression and secretion from cultured bovine GC at different stages of follicle development;

2). To examine the regulation of PN-1, tPA and uPA expression and secretion from bovine cultured GC by FSH and growth factors including IGF-I, BMP-7, FGF-2 and EGF;

3). To determine PN-1 mRNA expression in GC and examine secreted PN-1 protein and PA activity in FF collected from follicles at defined stages of development.

ARTICLES

Article 1

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Plasminogen Activator and Serine Protease Inhibitor-E2 (Protease Nexin-1) Expression by Bovine Granulosa Cells In Vitro[1]

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ABSTRACT

Remodeling of the extracellular matrix (ECM) occurs during antral follicle growth, and the plasminogen activators (PA) have been implicated in this process in rodents. In the present study, we measured the expression and secretion of PA and the PA inhibitor protease nexin-1 (SerpinE2) in antral and basal bovine granulosa cells from small (<6 mm), medium (6–8 mm), and large follicles (>8 mm) during 6 days of culture in serum-free medium. Casein zymography revealed that the cells secreted predominantly tissue-type PA (tPA) with urokinase (uPA) being associated mainly with cell lysates, and Western blot demonstrated that the cells secreted SerpinE2. Overall, secreted tPA activity was higher in cultures of cells from small follicles compared with large follicles, and secreted SerpinE2 levels were higher in cultures of cells from large follicles. In cultures of cells from small follicles, secreted tPA levels increased with time of culture for antral but not basal cells, and SerpinE2 levels increased with time for basal but not antral cells. In cultures of granulosa cells from large follicles, tPA activity increased significantly with time of culture, whereas SerpinE2 levels decreased. Cell-associated uPA activity decreased with time in cells from medium and large follicles. Reverse-transcription polymerase chain reaction and Northern blot analysis showed that SerpinE2 secretion was regulated largely at the transcriptional level, whereas tPA secretion was not. The data suggest stage-dependent regulation of granulosa cell PA and SerpinE2 production, consistent with a role in ECM remodeling during follicle growth.

follicle, granulosa cells, ovary

INTRODUCTION

Ovarian follicular growth and development involve extensive tissue remodeling, cell proliferation, and differentiation [1]. Tissue remodeling involves a number of protease enzyme cascades, including the matrix metalloproteinases (MMP) and plasminogen activators (PA). Plasminogen activators are serine proteases that convert the abundant extracellular zymogen plasminogen into plasmin, an active protease that degrades components of the extracellular matrix (ECM) [2]. Two forms of PA, tissue type (tPA) and urokinase (uPA), have been described in mammals [3] and are products of the *Plat* and *Plau* genes, respectively. The type of PA secreted is species- and cell-specific. Although rat [4–6] and pig [7] granulosa cells secrete predominantly tPA, uPA secretion is predominant in mice and chicken granulosa cells [8–10]. In bovine preovulatory follicles, *Plat* mRNA was localized primarily in granulosa cells, whereas *Plau* mRNA was detected in granulosa and in theca cells [11, 12].

One mechanism for the regulation of plasminogen activation is through the production of PA inhibitors (PAI). The three major inhibitors are PAI-1, PAI-2, and protease nexin-1 (PN-1) [13, 14], encoded by the serine protease inhibitor (Serpin) family of genes [15]. In rodents and cattle, *Serpine1* (encoding PAI-1) is expressed predominantly by theca-interstitial cells [16–18]. *Serpinb2* encodes the weak inhibitor, PAI-2, and is expressed at low levels in the theca layer of hCG-treated rats and in cumulus and granulosa-lutein cells of hCG-stimulated human ovaries [19, 20]. In contrast to *Serpine1* and *Serpinb2*, the gene encoding PN-1, *Serpine2*, is strongly expressed in granulosa cells of rats and cattle [9, 21, 22].

The PAs and Serpins are widely considered to be important during the process of ovulation when proteolytic degradation of the follicle wall occurs. During final preovulatory growth in rats and monkeys, there are concomitant increases in granulosa cell *Plat* expression/tPA secretion and thecal *Serpine1* expression/secretion. However, a few hours prior to ovulation there is a significant decrease in *Serpine1* mRNA and protein levels, presumably resulting in an increase in net tPA activity that initiates the proteolytic cascade necessary for the degradation of the follicle wall [23, 24]. Periovulatory increases in PA activity/mRNA have also been described for pigs, sheep, and cattle [12, 25, 26].

Tissue remodeling is also important for the growth and development of small follicles, as bovine follicles typically increase in size several hundred-fold between preantral and preovulatory stages. Studies in rats have shown that uPA is the predominant PA in small growing follicles, whereas tPA is predominant in preovulatory follicles [27, 28], suggesting a role for uPA in early follicle growth. It is not clear if or how PA activity is regulated by inhibitors at this stage of follicle development, although available evidence suggests that SerpinE1 and SerpinB2 are not involved. In rats, *Serpine1* expression was low in small growing follicles and increased as follicles differentiated [28], consistent with the role in ovulation described above. *Serpinb2* expression was not readily detected in bovine preovulatory follicles before the induction of ovulation by GnRH [18]. Interestingly, *Serpine2* is highly expressed in small growing follicles in rats [9, 21] and in preantral and growing antral follicles in cattle [22].

As SerpinE2 would appear to be the major PA inhibitor expressed in granulosa cells and in small growing follicles, we hypothesize that this member of the Serpin family plays a role in the remodeling of the membrana granulosa during

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Key words: SerpinE2, tPA, granulosa cell, ovarian follicle, extracellular matrix

Running title: SerpinE2 secretion from bovine granulosa cells

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INTRODUCTION

Ovarian follicular growth and development involve extensive tissue remodeling, cell proliferation and differentiation (Smith *et al.*, 1999). Tissue remodeling involves a number of protease enzyme cascades, including the matrix metalloproteinases (MMP) and plasminogen activators (PA). Plasminogen activators are serine proteases that convert the abundant extracellular zymogen plasminogen into plasmin, an active protease that degrades components of the extracellular matrix (ECM) (Blasi *et al.*, 1987) . Two forms of PA, tissue type (tPA) and urokinase (uPA), have been described in mammals (Macchione *et al.*, 2000), and are products of the *Plat* and *Plau* genes, respectively. The type of PA secreted is species- and cell-specific. While rat (Canipari & Strickland, 1985), (Canipari & Strickland, 1986), (Galway *et al.*, 1989) and pig (Politis *et al.*, 1990b) granulosa cells secrete predominantly tPA, uPA secretion is predominant in mice and chicken granulosa cells (Canipari *et al.*, 1987), (Hagglund *et al.*, 1996), (Lafrance *et al.*, 1993a). In bovine preovulatory follicles, *Plat* mRNA was localized primarily in granulosa cells whereas *Plau* mRNA was detected in granulosa and in theca cells (Yamada *et al.*, 1996), (Dow *et al.*, 2002a).

One mechanism for the regulation of plasminogen activation is through the production of PA inhibitors (PAI). The three major inhibitors are PAI-1, PAI-2 and protease nexin-1 (PN-1) (Kruithof, 1988), (Roberts *et al.*, 1995), encoded by the serine protease inhibitor (Serpin) family of genes (Silverman *et al.*, 2001). In rodents and cattle, *Serpine1* (encoding PAI-1) is expressed predominantly by theca-interstitial cells (Liu *et al.*, 1987b), (Chun *et al.*, 1992), (Dow *et al.*, 2002b). *Serpinb2* encodes the weak inhibitor,

PAI-2, and is expressed at low levels in the theca layer of hCG-treated rats and in cumulus and granulosa-lutein cells of hCG-stimulated human ovaries (Piquette *et al.*, 1993), (Leonardsson *et al.*, 1995). In contrast to *Serpine1* and *Serpinb2*, the gene encoding PN-1, *Serpine2*, is strongly expressed in granulosa cells of rats and cattle (Hagglund *et al.*, 1996), (Hasan *et al.*, 2002), (Bedard *et al.*, 2003).

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above. *Serpinb2* expression was not readily detected in bovine preovulatory follicles before the induction of ovulation by GnRH (Dow *et al.*, 2002b). Interestingly, *Serpine2* is highly expressed in small growing follicles in rats (Hagglund *et al.*, 1996), (Hasan *et al.*, 2002) and in preantral and growing antral follicles in cattle (Bedard *et al.*, 2003).

As SerpinE2 would appear to be the major PA inhibitor expressed in granulosa cells and in small growing follicles, we hypothesize that this member of the Serpin family plays a role in the remodeling of the membrana granulosa during follicle growth by regulating PA activity, mainly uPA. The objective of the present study was to measure *Serpine2*, *Plau* and *Plat* expression and protein secretion from bovine granulosa cells at different stages of development. To do so, we employed an established cell culture system that permits long-term estradiol secretion in vitro and maintains the follicular phenotype of the cells (Gutierrez *et al.*, 1997), (Manuel Silva & Price, 2000).

MATERIALS AND METHODS

Cell culture

The cell culture system was based on that described by Gutiérrez et al. (Gutierrez *et al.*, 1997), with slight modifications (Manuel Silva & Price, 2000). All materials were obtained from Invitrogen Life Technologies (Burlington, ON, Canada) except where otherwise stated. Briefly, bovine ovaries were collected from adult cows, irrespective of stage of the estrous cycle, at a local abattoir and were transported to the laboratory in PBS at 35°C containing penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (1

μg/ml). Follicles were dissected from the ovaries, and those with obvious signs of atresia (avascular theca, debris in antrum) were discarded. Lightly adherent 'antral' granulosa cells from small (2-5 mm in diameter), medium (6-8 mm) and large (>8 mm) follicles were released by dissection or aspiration; and the adherent 'basal' granulosa cells were subsequently collected by repeatedly passing the follicle wall through a pipette. Cells were washed twice by centrifugation at 980 × g for 20 min each and suspended in α-MEM, containing Hepes (20 mM), sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), BSA (0.1%; Sigma-Aldrich Canada, Oakville, ON, Canada), penicillin (100 IU/ml), streptomycin (100 μg/ml), transferrin (2.5 μg/ml), non-essential amino acid mix (1.1 mM), androstenedione (10^{-7} M), insulin (10 ng/ml), 1 ng/ml FSH (AFP-5332B, NIDDK), and human recombinant insulin-like growth factor-1 (IGF-1, 10 ng/ml). Cell viability was estimated with 0.4% Trypan Blue Stain. Cells were seeded into 24-well tissue culture plates (Corning Glass Works, Corning, NY) at a density of 10^6/well in 1 ml medium. Cultures were maintained at 37°C in 5% CO_2 in air, with 700 μl medium being replaced every 2 days. Medium and cells were recovered on day 2, day 4 or day 6 of culture. Medium samples were stored at -20°C until assay, while cells were collected in Trizol and stored at -70°C until RNA, DNA, and protein extraction.

RNA, DNA and protein extraction and quantification

Total RNA, DNA and protein were extracted using Trizol according to the manufacturer`s instructions. Total RNA was quantified by absorbance at 260 nm. Total DNA was quantified in duplicate by measuring fluorescence in the presence of

bisbenzimide (Hoechst 33258) and compared with a calf thymus DNA standard (Sigma-Aldrich) curve. Total protein was measured by the Lowry assay (Lowry, 1951), using BSA (Sigma-Aldrich) as standard.

Casein zymography

Casein zymography was used to measure tPA and uPA activity in culture medium and granulosa cell extracts (Dow *et al.*, 2002a). Briefly, samples were subjected to electrophoresis at 120 V for 90 min in 10% non-denaturing polyacrylamide gels containing 0.2% casein (Sigma-Aldrich), 0.1% SDS and 3.75 mU/ml bovine plasminogen (Sigma-Aldrich). After electrophoresis, gels were washed once in 2.5% Triton X-100 for 45 min to remove SDS, and placed in incubation buffer (50 mM Tris, 0.1 M NaCl , pH 7.6) at 37°C for 16 h with gentle shaking. The gels were then stained using 0.05% Coomassie blue in 10% acetic acid, 40% methanol for 2 h, destained in 10% acetic acid, 40% methanol, and then fixed in 10% glycerol. The identity of the enzymatic activities was investigated by comparing molecular size with human tPA (Calbiochem, Darmstadt, Germany) and uPA (NIBSC, Hertfordshire, UK) standards. Amiloride (1mM), a specific inhibitor of uPA, was included in some gels. Plasminogen-free gels were used to confirm that the activity detected was plasminogen dependent. Bands of activity were visualized as clear zones where casein degradation occurred, against a dark (blue) background. The volume of medium analyzed was corrected for cell number (total DNA). To correct for gel-to-gel variation, all samples were expressed relative to a control sample (spent medium from a culture of cells from small follicles) that was included in every gel.

Western blot

SerpinE2 protein abundance was analyzed by Western blot. Media samples were concentrated by lyophilization (Dura-Dry ™ MP Corrosion Resistant Freeze-Dryer, Stone Ridge, NY) and the volume analyzed was adjusted to correct for cell number. Samples were subjected to electrophoresis at 120 V for 90 min in 10% denaturing polyacrylamide gels. Proteins were then electrotransferred onto nitrocellulose membrane (0.45 μm) (Bio-Rad, Hercules, CA) at 22 V overnight at 4°C in transfer buffer (39 mM glycine, 48 mM Tris-base, 0.037% SDS and 20% methanol, pH 8.3). After blocking for 1 h in TTBS (0.2% Tween 20, 10 mM Tris-HCl, 150 mM NaCl), blots were incubated with 1:5000 rabbit anti-bovine SerpinE2 (Bedard *et al.*, 2003) for 4 h with agitation, followed by three washes (10 min each) with 0.2% TTBS. The blots were then incubated with 1:2500 alkaline phosphatase-linked anti-rabbit IgG (Sigma-Aldrich) for 1.5 h with agitation, following by three washes (10 min each) with 0.2% TTBS. Finally, the blots were incubated with NBT/BCIP solution (Roche Diagnostics, Indianapolis, IN). Rainbow™ Coloured Protein Molecular Weight Marker (Pharmacia, Piscataway, NJ) was used to estimate molecular size of the target protein, and bovine follicular fluid (2 μl) was used as positive control.

Semi-quantitative RT-PCR

Plau, *Plat* and *Serpine2* mRNA were assayed by reverse-transcription polymerase chain reaction (RT-PCR). Total RNA (1 μg) was reverse transcribed in the presence of 0.2 mM oligo(dT) primer and 200 U SuperScript II (Invitrogen Life Technologies), 2.5 mM

$MgCl_2$, 0.5 mM dideoxy-nucleotide triphosphate (dNTPs) mix, 10 mM dithiothreitol (DTT) in a volume of 50 µl. The RNA samples were heated to 70°C for 10 min and added to the prewarmed (42°C) reaction mixture. The reaction was incubated for 50 min at 50°C, then for 15 min at 70°C. Residual RNA was removed by incubating 20 min at 37°C with 1 µl of RNase H.

A duplex reaction was performed for *Serpine2* in which both *Serpine2* primers and primers for glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were amplified together for each sample, whereas *Plau* and *Plat* primers and *Gapdh* primers were amplified separately for each sample. The primers used were: *Plau* sense: 5'-GTCTGGTGAATCGAACTGTGGC-3', antisense: 5'-GGCTGCAAACCAAGGCTG-3' (Balcerzak *et al.*, 2001); *Plat* sense: 5`-AAGGTTGCAGAAGAAGATGG-3`, antisense: 5`-GTGAGGCGGGTACCTCTCCTGGAA-3 (Macchione *et al.*, 2000); *Serpine2* sense: 5`-TCCGTGACGTTGCCCTCTGTG-3`, antisense: 5`-CCGTGATCTCCACAAACCCTT-3` (Bedard *et al.*, 2003); *Gapdh* sense: 5`-TGTTCCAGTATGATTCCACC-3`, antisense: 5`-TCCACCACCCTGTTGCTG-3` (Tsai *et al.*, 1996).

An aliquot (0.4 µl) of the reverse transcription reaction was amplified by PCR using 0.2 µl (2.5 U) Taq Polymerase (Amersham Pharmacia Biotech Inc., Oakville, ON, Canada) in a 20-µl PCR buffer (Amersham Pharmacia Biotech Inc.) containing 0.1 mM dNTP mix, and 0.2 µM specific primers. Target cDNA was amplified under the following conditions: 1) an initial denaturation step for 3 min (*Plat*) and 5 min (*Serpine2*, *Plau*) at 94°C; 2) amplification cycles with denaturation at 94°C for 30 sec, annealing for 45 sec at 65°C (*Plau*), 55°C (*Plat*) and 62°C (*Serpine2*), and elongation at 72°C for 1 min; and 3) final elongation at 72°C for 5 min.

Semiquantitative RT-PCR was validated for each gene product. Preliminary experiments verified that PCR product increased with amount of RNA in the RT reaction. Reactions were performed for 30 cycles for *Plau*, 26 cycles for *Plat* and *Gapdh*, and for 24 cycles for *Serpine2*. The PCR products were separated on 1% agarose gels with 0.001% ethidium bromide, and visualized under UV. Quantification of band intensity was performed with NIH Image software. Target gene mRNA abundance was expressed relative to *Gapdh* mRNA abundance.

Northern blot

To verify the RT-PCR results, we performed Northern hybridizations on a subset of samples, where the amount of RNA available permitted. The complete *Serpine2* cDNA (Bedard *et al.*, 2003) was subcloned into pBK-CMV phagemid and digested by *Eco*RI and *Xho*I restriction endonuclease to generate radioactive probes. To prepare *Plat* and *Gapdh* cDNA probes, PCR products (see above) were cloned into pGEM-T Easy Vector (Promega, Madison, WI) and digested by *Eco*RI enzyme for *Plat* and by *Pvu*II enzyme for *Gapdh* respectively. The cDNA probes were labeled with [^{32}P]dCTP (DuPont NEN Research Products, Boston Massachusetts, USA) using the Random Primed DNA Labeling Kit (Roche Diagnostics, Indianapolis, IN, USA), and purified by centrifugation through a Microspan S-200 HR Column (Pharmacia, Piscataway, NJ).

Electrophoresis of 15 μg total RNA, performed through a 1% denaturing formaldehyde-agarose gel, was followed by overnight capillary transfer onto a nylon membrane (Hybond-N; Amersham Pharmacia Biotech Inc.). Membranes were UV cross-linked in a commercial UV chamber (Bio-Rad, Mississauga, ON, Canada) and incubated

for 2 h at 65°C in prehybridization solution containing 5X saline-sodium phosphate-EDTA buffer (SSPE), 5X Denhardt's solution, 0.5% SDS, 10% dextran sulfate, and 1% herring sperm DNA (10 mg/ml). Upon adding the purified probe, hybridization was carried out in hybridization buffer at 65°C overnight. After hybridization, membranes were washed in 2X SSPE-0.1% SDS twice at room temperature and twice at 65°C (15 min each). The labeled membranes were exposed to Kodak X-Omat film at -70°C in the presence of an intensifying screen.

Statistics

Each experiment was carried out at least three times. Data are presented as means ± SEM. The data were analyzed by ANOVA with follicle size, day of culture, cell subpopulation and culture replicate as main effects. Where main effects and/or interactions involving follicle size were found, effects of cell subpopulation and/or day of culture on antral and basal cells were analyzed separately. Data were transformed to logarithms when not normally distributed. Means comparisons were performed with the Tukey-Kramer HSD test. All analyses were performed with JMP software (SAS Institute, Cary, NC).

RESULTS

To confirm the identity of PA activities observed, activities were examined in bovine granulosa cell lysates by zymography. Bovine tPA migrated slightly less (approx 70 kDa) than the human standard (66 kDa) and was not inhibited by amiloride (Fig 1).

Bovine uPA activity migrated at approx 45 kDa (compared to 53 kDa for the human standard) and was attenuated by amiloride. No proteolytic activity was observed in plasminogen-free gels (Fig 1). Granulosa cells secreted predominantly tPA, with low and variable amounts of uPA being detected (Fig 2). Although it appeared that cells from small follicles secreted low amounts of uPA throughout the culture period and that cells from medium and large follicles secreted uPA mainly during the first two days of culture (see zymograph in Fig 2C), the proteolytic bands were too weak or too often absent for accurate quantification and analysis.

There were significant effects ($P<0.001$) of follicle size and day of culture on secreted tPA activity. Overall, secreted tPA activity was higher from cells of small follicles compared to cells of medium and large follicles, and was higher on Day 6 of culture compared to Day 2. When tPA secretion from cells of small, medium and large follicles were analyzed separately, there was a significant effect of cell subpopulation and an interaction between cell subpopulation and day of culture ($P<0.05$) for cells from small follicles. Secreted tPA activity increased with time for antral cells, but there was no significant increase with time for basal cells (Fig 2A). On day 2 of culture, basal cells secreted significantly more tPA than did antral cells, whereas on day 6, antral cells secreted slightly ($P=0.06$) more tPA compared to basal cells. There were significant effects of time in culture ($P<0.05$) but not of cell subpopulation in cultures of cells from medium and large follicles (Fig 2B, C).

There were no main effects of culture or follicle size on cellular tPA activity, but significant effects of time in culture and of follicle size for cellular uPA activity (Fig 3).

Cellular uPA was significantly lower on day 6 of culture than on day 2 for cells from medium and large follicles ($P<0.01$), but not from small follicles. There was no effect of cell subpopulation.

Abundance of *Plat* expression was measured by semiquantitative RT-PCR. There were no significant effects of follicle size, cell population or time in culture (data not shown). RT-PCR results were verified by performing Northern analysis on two replicates of cultures from medium follicles (as there was sufficient RNA); there was a significant correlation between Northern and PCR data ($r= 0.6$, $P<0.05$, $n=12$). We could not detect *Plau* mRNA in samples from cultured granulosa cells after 30 cycles of PCR, although the positive control (uterus) provided a strong band at the expected size.

Granulosa cells from all follicle size groups secreted SerpinE2 as detected by Western blotting. There were significant main effects of cell population and follicle size, and an interaction between day of culture and follicle size ($P<0.01$). Overall, cells from large follicles secreted more SerpinE2 compared to cells from small and medium follicles. SerpinE2 secretion from basal but not antral cells of small and of medium follicles increased with time in culture (Fig 4 A, B; $P<0.05$), whereas SerpinE2 secretion from antral (and not basal) cells of large follicles decreased with time in culture (Fig 4C; $P<0.01$).

There were significant effects of time in culture and follicle size on *Serpine2* mRNA levels, and an interaction between time and follicle size ($P<0.001$). When data from different follicle sizes were analyzed separately, there were no main effects of time or cell population on *Serpine2* mRNA levels in cells from small or medium follicles, but there was

a significant decrease ($P<0.01$) in *Serpine2* mRNA with time in culture in cells of large follicles (Fig 5). RT-PCR results were verified by performing Northern analysis on two replicates of cultures from medium follicles (as there was sufficient RNA); there was a significant correlation between Northern and PCR data ($r= 0.65$, $P<0.05$, $n=12$). Overall, there was a significant correlation between SerpinE2 protein and mRNA levels ($r= 0.5$; $P<0.01$); when the data from different follicle sizes were analyzed separately there was a correlation between protein and mRNA for large ($r=0.6$, $P<0.01$) but not small or medium follicles.

Estradiol secretion was significantly affected by follicle size, with interactions between follicle size and time, and between follicle size and cell population. For cells of small follicles, estradiol secretion increased with time in culture for antral but not basal cells, whereas for medium follicles estradiol secretion increased with time for basal and not for antral cells (Fig 6). Estradiol secretion from cells of large follicles decreased significantly with time in culture (Fig 6; $P<0.01$). There was a main effect of time in culture on progesterone secretion, and no main effects of follicle size or cell population; progesterone concentrations were consistently higher on day 4 and 6 of culture compared to day 2 (Fig 6; $P<0.05$).

DISCUSSION

This is, to our knowledge, the first study to describe the secretion of members of the PA system from granulosa cells at different stages of development in a non-rodent mammal. Bovine granulosa cells secreted predominantly tPA in culture, with very low amounts of

uPA secreted. This is consistent with measurements of PA activity in bovine follicular fluid (Dow *et al.*, 2002a). In rats, uPA has been described as a major secreted PA in some studies (Macchione *et al.*, 2000), whereas it is low or absent in other studies (Galway *et al.*, 1989), (Liu *et al.*, 1991). The present data show that, overall, secreted tPA activity was higher from cells of small and less differentiated follicles compared with that of the more differentiated medium and large follicles. This is in contrast to the lower level of tPA activity in undifferentiated versus differentiated rat follicles (Karakji & Tsang, 1995a), and presents an important species difference in PA secretion at these stages of follicle growth. A species difference in tissue uPA and tPA activity between rodents and cattle has been described for the periovulatory period (Dow *et al.*, 2002a).

Also novel is the measurement of secreted SerpinE2 from granulosa cells. *Serpine2* mRNA and protein has previously been localized in rat (Hagglund *et al.*, 1996), (Hasan *et al.*, 2002) and bovine (Bedard *et al.*, 2003) granulosa cells by in-situ techniques, and has been detected by Western blotting in bovine follicular fluid (Bedard *et al.*, 2003). During the first 2 days of culture particularly, granulosa cells of large follicles secreted more SerpinE2 than did cells from small follicles, in agreement with the relative intensity of immunostaining described in rat and cow follicles (Hasan *et al.*, 2002), (Bedard *et al.*, 2003). As *Plat* or *Plau* expression would not be expected in bovine granulosa cells until after the LH surge (Dow *et al.*, 2002b), SerpinE2 is a candidate for the regulation of PA activity within the membrana granulosa of growing follicles.

The pattern of changes of secreted tPA and SerpinE2 differed. Secretion of tPA from antral cells of small follicles increased with time of culture, whereas SerpinE2 secretion did not change. Conversely, tPA secretion from basal cells did not change but

SerpinE2 secretion increased. This difference may reflect the needs of the sublayers of granulosa cells. Although the separation of 'antral' and 'basal' may be simplistic, the basal cells are nearer the basal lamina and more firmly attached to ECM components than are antral cells. In sheep, there are immunocytochemical differences in the intensity of staining for fibronectin and collagen between antral and basal granulosa cells (Huet *et al.*, 1997). A difference between cell subpopulations was not seen in cells of medium and large follicles in the present study, potentially reflecting the changes in the basal lamina ECM that occur with follicle growth (reviewed in (Rodgers *et al.*, 2003)).

The most striking divergence between tPA and SerpinE2 secretion occurred with cells of large follicles, for which tPA secretion increased with time of culture and SerpinE2 secretion decreased. This would result in a net increase in extracellular PA activity. This is consistent with increases in *Plat* expression and protein secretion observed with rat granulosa cells (Galway *et al.*, 1989). The relationship between tPA and SerpinE2 is related to stage of follicle development, as tPA secretion from cells of medium follicles increased with time of culture in a manner very similar to that of large follicles, but SerpinE2 secretion did not decrease from cell of medium follicles. The most likely explanation is that the cells of large follicles undergo at least partial luteinization in culture, whereas those of medium follicles do not. This is indicated by the steroid data, which show that estradiol secretion decreases with time of culture from cells of large but not medium follicles, as previously observed with this cell model (Gutierrez *et al.*, 1997). This is supported in part by data from studies of rats, which show increased *Plat* expression and enzyme activity during early development of the corpus luteum, and in granulosa cells following the ovulatory LH surge (Macchione *et al.*, 2000), (Liu *et al.*, 1996a); it is possible that some of

the large follicles may have been periovulatory follicles, and thus exposed to elevated concentrations of LH in vivo. Further, there was increased expression of genes encoding collagen and the MMP inhibitor TIMP-1 during in vitro luteinization of bovine granulosa cells (Zhao & Luck, 1996), demonstrating that changes to the ECM occur during luteinization.

Another explanation is a change in the ECM that occurs during follicle growth, that may in consequence alter the amount and/or type of protease activity secreted and granulosa steroidogenesis. It has been shown that collagen type 1 levels within the granulosa cell layer increase significantly during follicle development in sheep (Huet *et al.*, 1997), and that estradiol secretion from ovine granulosa cells of large follicles is inhibited when cultured in the absence of collagen (Huet *et al.*, 2001). Thus, the cells of large follicles in the present study may have an increased requirement for collagen type 1 which was not met by the culture conditions, resulting in reduced estradiol (but not progesterone) secretion ((Huet *et al.*, 2001), present study). The cellular response to this collagen deprivation may therefore be a reduction in the normally high secretion of SerpinE2, in order to increase extracellular protease activity and alter the local ECM structure.

Steady-state levels of *Serpine2* mRNA largely reflected secreted protein levels. Over the first 2 days of culture, *Serpine2* mRNA levels were higher in cells of large follicles compared to those of small and medium follicles, consistent with data from Northern analysis in cattle (Bedard *et al.*, 2003; Fayad *et al.*, 2004a). Similarly, the decrease in SerpinE2 secretion observed during culture of cells from large follicles was tightly coordinated with a decline in *Serpine2* mRNA levels. Thus we conclude that SerpinE2 secretion is controlled at the transcriptional level, at least in vitro. This does not

appear to be the case for PA however, as consistent increases in secreted tPA activity were not correlated with *Plat* mRNA levels. Although the measurement of tPA activity may be confounded by inhibitor activity, we do not believe this to be the case in the present study for the following reasons. First, when SerpinE1 inhibits tPA activity, a reversible protein-protein complex is formed which is visible by zymography as an additional high molecular mass lytic zone [34]. Second, potential complexes between SerpinE2 and PA should also be visible on Western blots as an additional high molecular mass band. As we did not observe lytic or protein bands corresponding to a PA-inhibitor complex, we conclude that the majority of the PA activity and SerpinE2 protein measured in culture medium occurs in a 'free' non-complexed state. Collectively, this data suggest that secreted tPA concentrations are controled at the post-translational level, possibly involving regulation of secretory mechanisms.

We were unable to detect *Plau* gene expression in bovine granulosa cells in vitro, and uPA was a minor secretory product. Most of the uPA activity was detected in cell lysates, most likely bound to the cell surface [3]. This is agreement with the readily detectable uPA activity in cell lysates of bovine follicle wall [12]. The decrease in secreted and cell-associated uPA in cells of medium and large bovine follicles in vitro is consistent with the developmental decrease seen in rats (Li *et al.*, 1997b). In cultures of cells from medium and large follicles, cell-associated uPA activity decreased with time of culture whereas secreted tPA activity increased. It is not clear what effect this change would have on net PA activity in the ECM immediately surrounding the cell, nor the impact of altered SerpinE2 concentrations on local tPA and uPA activity.

In conclusion, we have demonstrated that secretion of tPA and SerpinE2 from granulosa cells and cell-associated uPA activity are regulated in a follicular stage dependent manner in cattle. There appear to be several differences between rodents and cattle in terms of PA secretion, which makes generalization difficult, but a common theme is a decrease in granulosa cell uPA content as follicles develop. During the first 2 days of culture, this decrease in cell-associated uPA activity occurred as SerpinE2 secretion increased, suggesting a functional link between these two proteins during follicle development. As SerpinE2 is the only known PA inhibitor secreted by the granulosa cell layer, it may play an important role in tissue remodeling of this follicular compartment during early follicle growth.

Acknowledgements

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FIG 1. Zymographic demonstration of PA activity in bovine granulosa cells cultured for 6 days in serum-free medium. PA activity in granulosa cell lysate (bGC) was compared with human (hum) tPA and uPA standards. These samples were run in a 10% non-denaturing PAGE gel. Specificity of PA activity was tested by running a cell lysate sample in a 10% gel in the absence (-AM) or presence of 1 mM amiloride (+AM) and in a plasminogen-free gel (-PL). Molecular weight markers (MW x 10^{-3}) are shown.

Fig 1

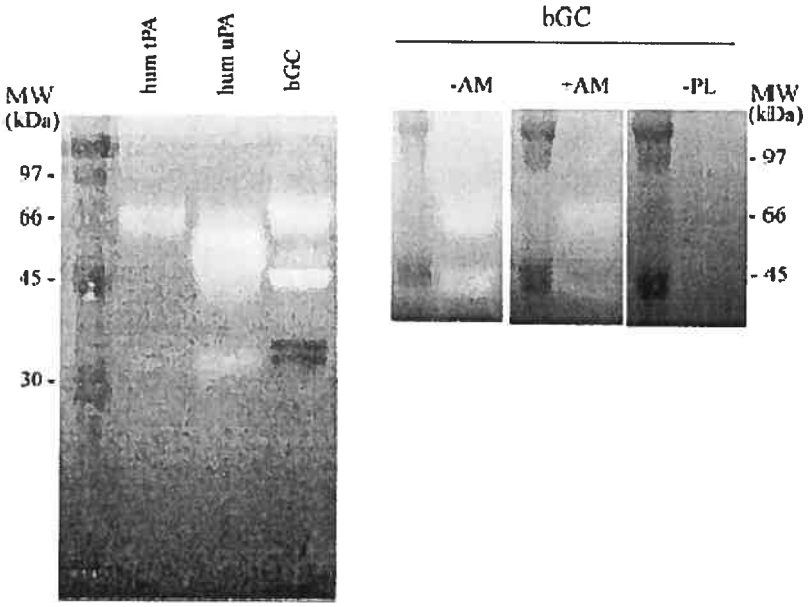


FIG 2. Secreted PA activity from cultured bovine granulosa cells from (A) small (2 – 5 mm diameter), (B) medium (6-8 mm) and (C) large (>8mm) follicles. Antral (open bars) and basal (solid bars) granulosa cells were cultured with 1 ng/ml bFSH, 10 ng/ml insulin and 10 ng/ml IGF-1 for 2, 4 or 6 days. A control medium sample was used to normalize the variation between gels. The loading volume was adjusted to correct for cell number. Inserts show representative zymographs. Data are least-squares means (relative units) ± SEM. Bars with different letters within follicle size group are significantly different. M, molecular weight markers.

Fig 2

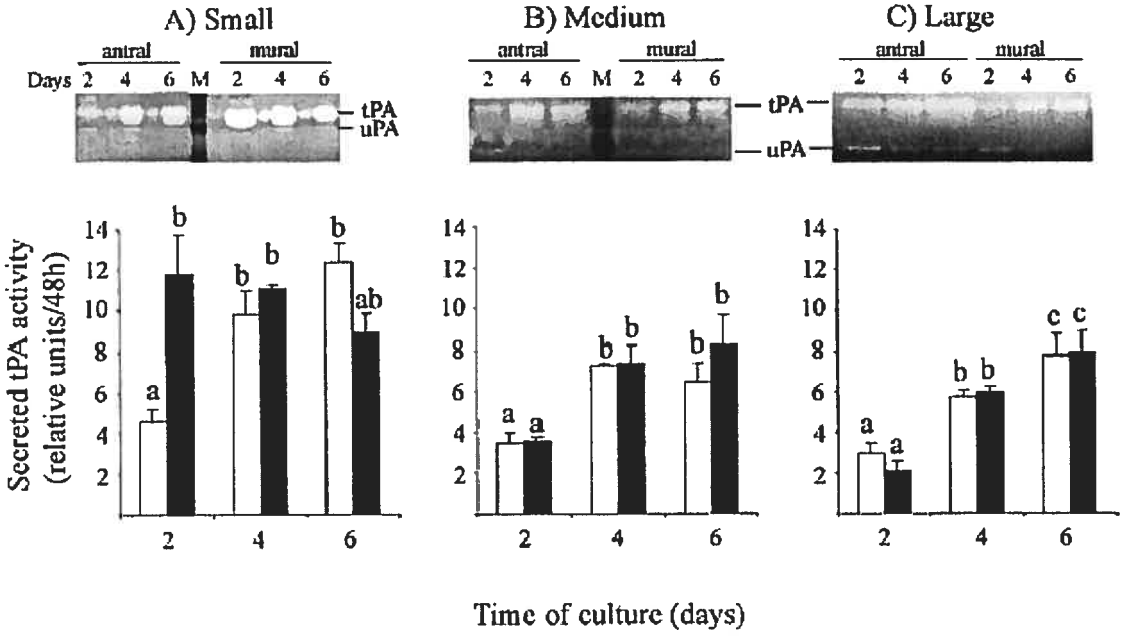

A) Small
B) Medium
C) Large
antral
mural
Days 2 4 6 M 2 4 6
tPA
uPA
Secreted tPA activity (relative units/48h)
Time of culture (days)

FIG 3. Cellular PA activity from cultured bovine granulosa cells from (A) small (2 – 5 mm diameter), (B) medium (6-8 mm) and (C) large (>8mm) follicles. Antral (open bars) and basal (solid bars) granulosa cells were cultured with 1 ng/ml bFSH, 10 ng/ml insulin and 10 ng/ml IGF-1 for 2, 4 or 6 days. A control medium sample was used to normalize the variation between gels. Inserts show representative zymographs. Data are least-squares means (relative units) ± SEM. Asterisks indicate means significantly different from day 2.

Fig 3

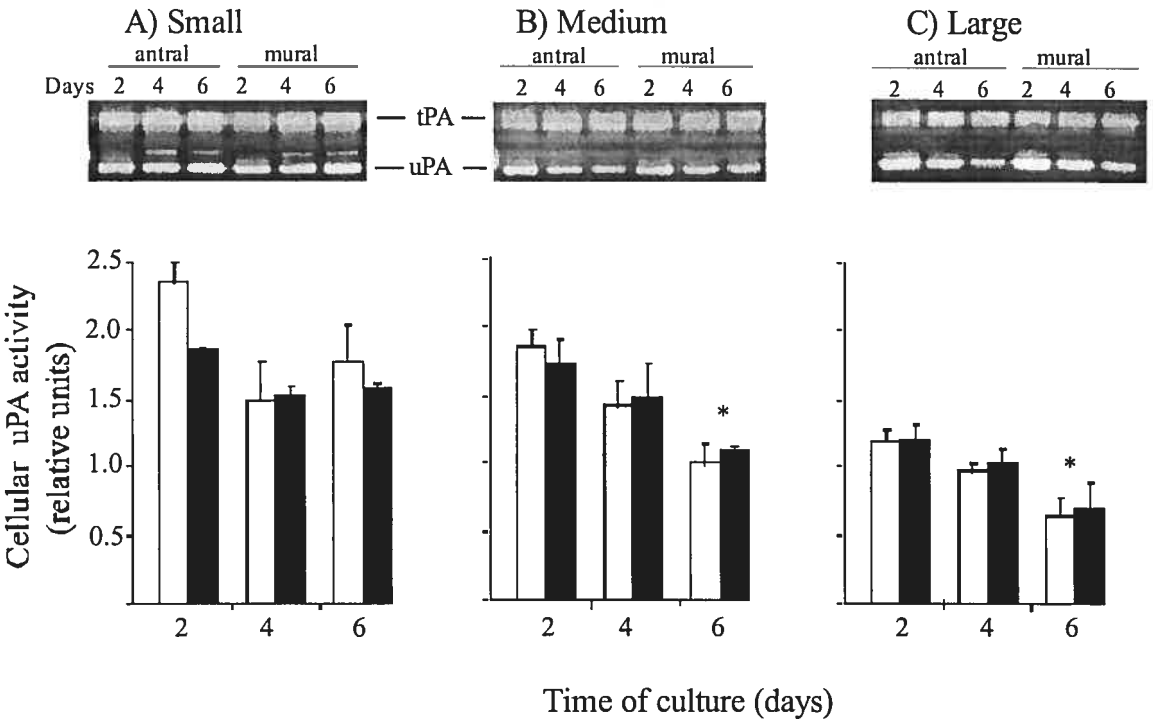

A) Small
B) Medium
C) Large
antral
mural
Days 2 4 6 2 4 6
tPA
uPA
Cellular uPA activity (relative units)
2.5
2.0
1.5
1.0
0.5
*
Time of culture (days)

FIG 4 . Western analysis of secreted protease nexin-1 (SerpinE2) from cultured bovine antral (open bars) and basal (solid bars) granulosa cells from (A) small (2 – 5 mm diameter), (B) medium (6-8 mm) and (C) large (>8mm) follicles. Granulosa cells were cultured with 1 ng/ml bFSH, 10 ng/ml insulin and 10 ng/ml IGF-1 for 2, 4 or 6 days. A control sample (follicular fluid) was used to normalize the variation between blots. The loading volume was adjusted to correct for cell number. Inserts show representative blots. Data are least-squares means (relative units) ± SEM. Asterisks indicate differences between means ($P<0.05$).

Fig 4

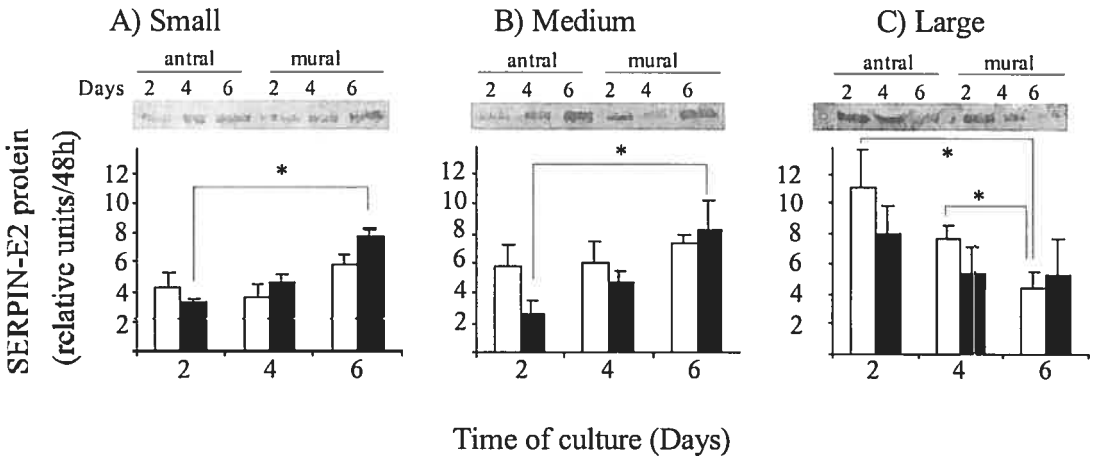

A) Small
B) Medium
C) Large
antral
mural
Days 2 4 6 2 4 6
SERPIN-E2 protein
(relative units/48h)
12
10
8
6
4
2
*
2 4 6
Time of culture (Days)

FIG 5. *Serpine2* mRNA levels in cultured bovine antral (open bars) and basal (solid bars) granulosa cells from (A) small (2 – 5 mm diameter), (B) medium (6-8 mm) and (C) large (>8mm) follicles. Granulosa cells were cultured with 1 ng/ml bFSH, 10 ng/ml insulin and 10 ng/ml IGF-1 for 2, 4 or 6 days. *Serpine2* mRNA was expressed relative to *Gapdh*. Inserts show representative agarose gels. Data are least-squares means ± SEM. Bars with different letters are significantly different ($P<0.05$). Note the difference in scale of the y-axis for large follicles.

Fig 5

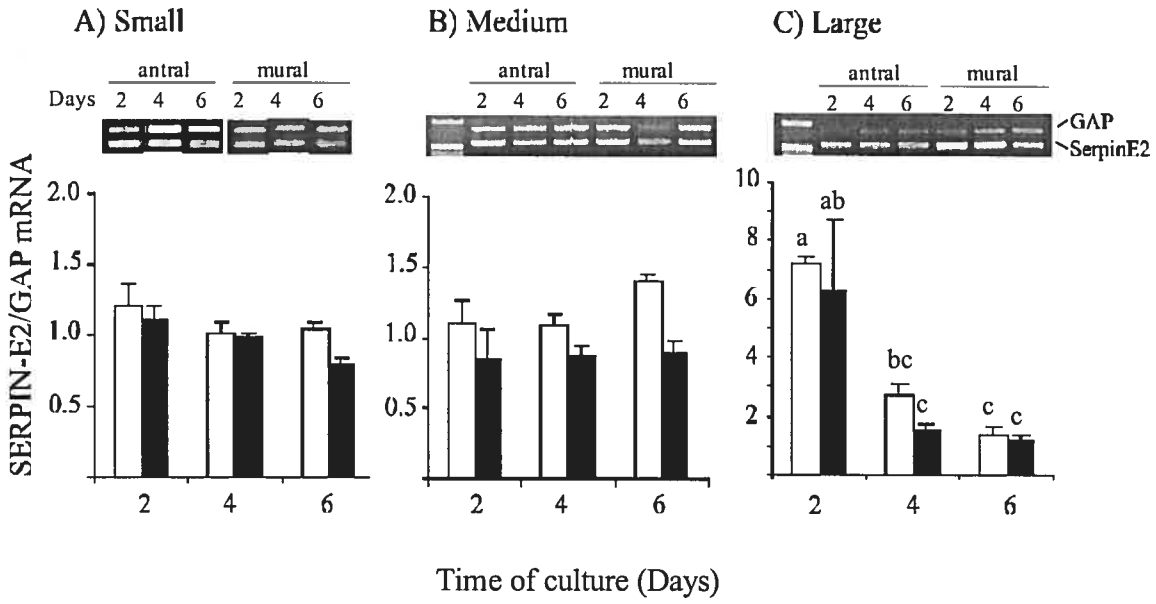
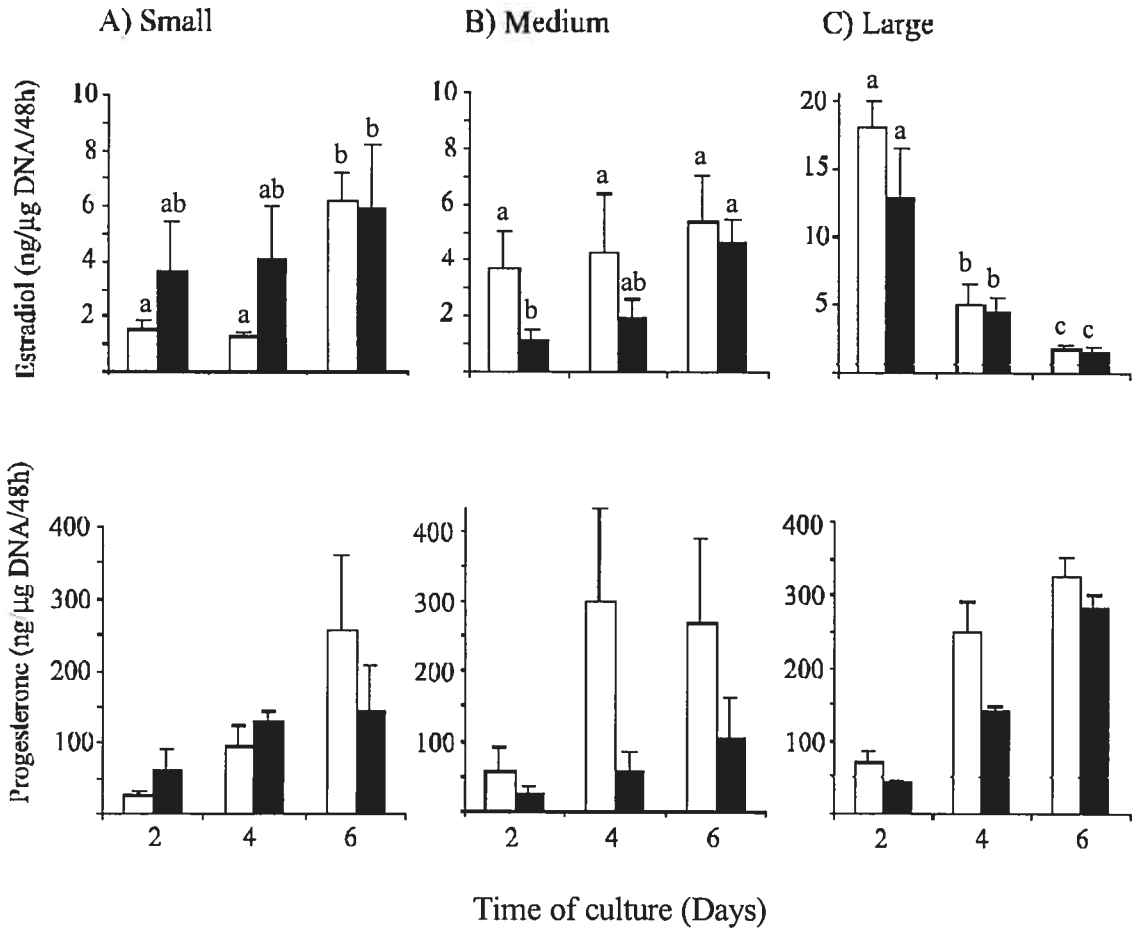

A) Small
B) Medium
C) Large
antral
mural
Days 2 4 6 2 4 6
GAP
SerpinE2
SERPIN-E2/GAP mRNA
2.0
1.5
1.0
0.5
10
8
6
4
2
a
ab
bc
c
c c
2 4 6
Time of culture (Days)

FIG 6. Culture medium estradiol and progesterone concentrations after culture of bovine antral (open bars) and basal (solid bars) granulosa cells from (A) small (2 – 5 mm diameter), (B) medium (6-8 mm) and (C) large (>8mm) follicles. Granulosa cells were cultured with 1 ng/ml bFSH, 10 ng/ml insulin and 10 ng/ml IGF-1 for 2, 4 or 6 days. Data are least-squares means ± SEM. Within follicle size, bars with different letters are significantly different ($P<0.05$).

Fig 6


A) Small
B) Medium
C) Large
Estradiol (ng/µg DNA/48h)
Progesterone (ng/µg DNA/48h)
Time of culture (Days)

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Article 2

Regulation of Serpin-E2 and Plasminogen Activator Expression and Secretion by Growth Factors in Non-luteinizing Bovine Granulosa Cells in Vitro

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Abstract

During antral follicle growth, there is expansion of the basal lamina and changes in composition of the follicular extracellular matrix (ECM). These changes likely occur through proteolytic enzyme cascades, such as the plasminogen activators (PA) and their inhibitors, including serpin-E2. In this study, we hypothesized that PA and serpin-E2 expression/secretion by non-luteinizing bovine granulosa cells are regulated by FSH and growth factors (IGF-I, BMP-7, FGF-2 and EGF). Serpin-E2 mRNA and protein levels, tPA gene expression and uPA secretion were stimulated by FSH, but FSH had no effect on secreted tPA activity or uPA gene expression. IGF-I stimulated serpin-E2 secretion and uPA activity, and decreased secreted tPA activity and tPA gene expression. BMP-7 had a stimulatory effect overall, increasing cell proliferation, estradiol production & serpin-E2 secretion in FSH- and IGF-I stimulated cells, and increasing secreted tPA activity in IGF-I- but not FSH-stimulated cells. In contrast, FGF-2 was generally inhibitory, decreasing estradiol and tPA secretion in FSH- and IGF-I stimulated cells, and decreasing serpin-E2 secretion in IGF-I stimulated but not FSH stimulated cells. EGF inhibited cell proliferation, and estradiol and serpin-E2 secretion, but increased secreted tPA activity. In conclusion, the coordinated regulation of uPA and serpin-E2 expression/secretion by FSH and IGF-I supports a role for these proteins in follicle development.

Introduction

Understanding follicle growth leads to improved control of reproduction in monovular species such as humans and cattle. During folliculogenesis follicles increase in diameter, necessitating the expansion of the basal lamina and changes in the follicular extracellular matrix (ECM) [1]. These changes likely occur through proteolytic enzyme cascades involving plasmin and matrix metalloproteinases (MMP). These cascades start with the conversion of the abundant extracellular zymogen plasminogen into plasmin by plasminogen activators (PA). Plasmin is an active protease that promotes degradation of components of the ECM as well as activating MMPs [2, 3].

In cattle, tissue-type plasminogen activator (tPA) synthesis occurs mainly in the granulosa layer, whereas urokinase plasminogen activator (uPA) is expressed in granulosa and theca layers [4]. Granulosa cells but not theca cells express the PA inhibitor serpin-E2 (also known as protease nexin-1) [5], but not other inhibitors [6, 7]. Serpin-E2 is a potent inhibitor of tPA, uPA and plasmin activity [8]. We have suggested that the interplay between PA and serpin-E2 may be important for follicle growth in cattle, as expression and secretion of these proteins in granulosa cells is dependent on stage of follicle development [9].

The regulation of the PAs and their inhibitors is poorly understood. FSH is an essential factor in the regulation of follicle development from primary follicles through to dominant preovulatory follicles. In rodents, tPA secretion from granulosa cells was upregulated by FSH [10-12], whereas in cultured pig granulosa cells, FSH did not alter tPA production [13]. Both FSH and cAMP decreased uPA secretion from cultured rat granulosa cells [12].

A number of growth factors are also involved in follicle development, including IGF-I, bone morphogenetic protein-7 (BMP-7), basic fibroblast growth factor (FGF-2) and epidermal growth factor (EGF). Bovine granulosa cells express type-I IGF receptors [14], and IGF-I is known to stimulate cell proliferation and steroidogenesis in cattle [15, 16], rats [17-19] and pigs [20-22].

BMP-7, a member of the transforming growth factor-β (TGF-β) superfamily, is expressed in theca cells [23-25], and its receptors are expressed in granulosa cells in rats [26], chickens [24], sheep [27] and cattle [25]. In vitro studies demonstrated that BMP-7 enhanced granulosa estradiol production and cell proliferation, but reduced progesterone production [23, 25, 28]. FGF-2 belongs to a family of heparin-binding growth factors and is produced by many tissues including granulosa cells [29]. FGF-2 and its receptors have been identified and localized in the follicle [30, 31]. FGF-2 stimulated granulosa cell proliferation in several mammals including cattle [32, 33], and inhibited FSH-induced estrogen production in rat [34-36] and bovine granulosa cells [37]. EGF promoted cell proliferation [38] and was associated with a loss of differentiated function such as estradiol production in vitro [39-41] and in vivo [42].

However, little is known about the regulation of PAs by these growth factors. FGF-2 stimulated tPA mRNA and enzyme activity in cultured rat granulosa cells [43]. EGF stimulated the secretion of tPA in cultured rat granulosa cells [44] and uPA from bovine cumulus cells [45]. Despite the different effects of various growth factors on granulosa cell proliferation, nothing is known about their regulation of serpin-E2 expression or secretion. The objective of the present study was to investigate the

regulation of PA and serpin-E2 secretion and expression by FSH, IGF-I, BMP-7, FGF-2, and EGF in non-luteinizing bovine granulosa cell in vitro.

Materials and Methods

Cell culture

The cell culture system was based on that described by [46], with slight modifications [47]. All materials were obtained from Invitrogen Life Technologies (Burlington, ON, Canada) except where otherwise stated. Briefly, bovine ovaries were collected from adult cows, irrespective of stage of the estrous cycle, at a local abattoir, and were transported to the laboratory in PBS at 35°C containing penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (1 μg/ml). Follicles (2 – 5 mm diameter) were dissected from the ovaries, and those with obvious signs of atresia were discarded. Cells were collected by repeatedly passing bisected follicle walls through a pipette, were washed twice by centrifugation at 219 × g for 20 min each, and suspended in α-MEM, containing Hepes (20 mM), sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), BSA (0.1%; Sigma-Aldrich Canada, Oakville, ON, Canada), penicillin (100 IU/ml), streptomycin (100 μg/ml), transferrin (2.5 μg/ml), non-essential amino acid mix (1.1 mM), androstenedione (10^{-7} M at start of culture, and 10^{-6} M at each medium change) and insulin (10 ng/ml). Cell viability was estimated with 0.4% Trypan Blue Stain.

Treatments and sample collection

In the first series of cultures, cells were cultured in the presence of FSH (AFP-5332B, NIDDK) or IGF-1 analog (LR3; Sigma-Aldrich Canada) at the doses given in Results. Cells were seeded into 24-well tissue culture plates (Sarstedt, Montreal, QC) at a density of 1 x 10^6 viable cells per well in 1 ml medium. Cultures were maintained at 37°C in 5% CO_2 in air for up to 6 days, with 700 µl medium being replaced every 2 days. Medium samples were collected on day 6, and stored at -20°C until assay, and cells were collected in Trizol and stored at -70°C until RNA extraction. Total RNA, DNA and protein were extracted using Trizol according to the manufacturer's instructions. Total RNA was quantified by absorbance at 260 nm. Total DNA was determined by measuring fluorescence in the presence of bisbenzimide (Hoechst 33258, Sigma) [48] and compared with a calf thymus DNA (Sigma-Aldrich) standard curve. Total protein was measured by using BSA (Sigma-Aldrich) as standard [49]. FSH and IGF-I treatments were performed as separate experiments on different pools of cells, and all cultures were performed three times.

In the second series of cultures, the effects of growth factors on FSH- & IGF-I-treated cells were determined. Cells were seeded into 48-well tissue culture plates (Falcon, Lincoln Park, NJ) at a density of 2.5x10^5 cells/well in 500 µl medium. Cultures were maintained at 37°C in 5% CO_2 in air for 4 days, with 350 µl medium being replaced on day 2. In the presence of FSH (1 ng/ml) or IGF-I analog (10 ng/ml), cells were cultured with graded doses (indicated in Results) of recombinant human BMP-7 (R&D Systems, Minneapolis, MN), FGF-2 (Sigma-Aldrich) or recombinant human EGF (R&D Systems). Vehicles for the reconstitution of each growth factor (4 mM HCl with 0.1% BSA for BMP-7; buffered saline with 0.5% BSA for FGF-2; 10mM acetic acid with 0.1%

BSA for EGF) were used as controls. Medium samples were collected and stored at -20°C until assay. Cells were lysed with 100 µl of 1 N NaOH for 2 h and neutralized with 100 µl of 1 N HCl, and total cell protein was measured by the Bradford protein assay (Bio-Rad, Mississauga, ON, Canada). The effect of each growth factor (on both FSH- and IGF-I-stimulated cells) was tested in separate cultures, and all experiments were performed on three independent cultures.

Cell proliferation assay

To measure cell proliferation, cells were seeded into 48-well tissue culture plates (Falcon, Lincoln Park, NJ), at a density of $2.5x10^5$ cells/well in 500 µl medium. At the medium change on day 2, 1µCi of [^{3}H]-thymidine (Amersham Biosciences, Baie d'Urfé, QC) was added to each well, and cells were incubated for a further 23 hr. Cells were then scraped from the wells, pelleted by centrifugation and washed twice with 0.5ml of ice-cold PBS. Centrifugation was performed at 100 g for 5 min at 4°C for cell pellet and wash. The cells were fixed by adding 0.5 ml ice-cold methanol/acetic acid (3:1) for 15 min, followed by two washes with 0.5ml of methanol/acetic acid. Centrifugation was performed at 3000 g for 5 min. Cell pellets were stored at -20°C before assay. The cell pellets were lysed in 0.25ml of 0.1 N NaOH for 2 hr at room temperature, neutralized with 0.25ml of 0.1 N HCl, and transferred to scintillation vials. The incorporated [^{3}H]-thymidine was measured by liquid scintillation counting. Cell proliferation was expressed as DPM.

Casein zymography

Casein zymography was used to measure tPA and uPA activity in culture medium [4], and validated for bovine granulosa cell conditioned medium [9]. Briefly, samples were subjected to electrophoresis at 120 V for 90 min in 10% non-denaturing polyacrylamide gels containing 0.2% casein (Sigma-Aldrich), 0.1% SDS and 3.75 mU/ml bovine plasminogen (Sigma-Aldrich). After electrophoresis, gels were washed once in 2.5% Triton X-100 for 45 min to remove SDS, and placed in incubation buffer (50 mM Tris, 0.1 M NaCl , pH 7.6) at 37°C for 16 h with gentle shaking. The gels were then stained using 0.05% Coomassie blue in 10% acetic acid, 40% methanol for 2 h, destained in 10% acetic acid, 40% methanol, and then fixed in 10% glycerol. Bands of activity were visualized as clear zones where casein degradation occurred, against a dark (blue) background. The volume of medium analyzed was corrected for cell number (total DNA). To correct for gel-to-gel variation, all samples were expressed relative to a control sample (spent granulosa cell medium) that was included in every gel.

Western blot

Serpin-E2 protein abundance was analyzed by Western blot [9]. Medium samples were concentrated by lyophilization and the volume analyzed was adjusted to correct for cell number. Samples were subjected to electrophoresis at 120 V for 90 min in 10% denaturing polyacrylamide gels. Proteins were then electrotransferred onto nitrocellulose membrane (0.45 μm) (Bio-Rad, Hercules, CA) at 22 V overnight at 4°C in transfer buffer (39 mM glycine, 48 mM Tris-base, 0.037% SDS and 20% methanol, pH 8.3). After

blocking for 1 h in TTBS (0.2% Tween 20, 10 mM Tris-HCl, 150 mM NaCl), blots were incubated with 1:5000 rabbit anti-bovine serpin-E2 [5] for 4 h with agitation, followed by three washes (10 min each) with 0.2% TTBS. The blots were then incubated with 1:2500 alkaline phosphatase-linked anti-rabbit IgG (Sigma-Aldrich) for 1.5 h with agitation, followed by three washes (10 min each) with 0.2% TTBS. Finally, the blots were incubated with NBT/BCIP solution (Roche Diagnostics, Indianapolis, IN). Rainbow colored protein molecular weight markers (Amersham) was used to estimate molecular size of the target protein, and bovine follicular fluid (2 μl) was used as positive control.

Nucleic acid extraction & semi-quantitative RT-PCR

Gene expression was assayed by RT-PCR essentially as described [9]. Total RNA (1 μg) was first treated with 1 U DNase (Promega, Madison, WI) at 37°C for 30 min to digest any contaminating DNA, followed by adding 1 μl of EDTA stop buffer at 65°C for 10 min. The RNA was reverse transcribed in the presence of 1 mM oligo(dT) primer and 4 U Omniscript RTase (Omniscript RT Kit, Qiagen, Mississauga, ON), 0.25 mM dideoxy-nucleotide triphosphate (dNTP) mix, and 19.33 U RNase Inhibitor (Amersham Biosciences, Baie D'Urfé, QC) in a volume of 20 μl at 50°C for 2 h. The reaction was terminated by incubation at 93°C for 5 min.

Bovine-specific primers for uPA (sense: 5'-GTCTGGTGAATCGAACTGTGGC-3', antisense: 5'-GGCTGCAAACCAAGGCTG-3' [50], tPA (sense: 5`-AAGGTTGCAGAAGAAGATGG-3', antisense: 5'-GTGAGGCGGGTACCTCTCCTGGAA-3' [12], and serpin-E2 (sense: 5'-TCCGTGACGTTGCCCTCTGTG-3', antisense: 5'-CCGTGATCTCCACAAACCCTT-

3' [5] were used as described [9]. Variability in mRNA amounts was assessed by amplifying the housekeeping gene histone H2a (H2a) with published primers (sense: 5'-GTCGTGGCAAGCAAGGAG -3', antisense: 5'-GATCTCGGCCGTTAGGTACTC -3' [51]. An aliquot (0.4 µl) of the cDNA template was amplified by PCR using 0.2 µl (2.5 U) Taq Polymerase (Amersham Pharmacia Biotech Inc., Oakville, ON, Canada) in a 20-µl PCR buffer (Amersham Pharmacia Biotech Inc.) containing 0.1 mM dNTP mix, and 0.2 µM specific primers. After an initial denaturation step for 3 min at 94°C, target cDNA was amplified with a denaturation step at 94°C for 15 sec (serpin-E2), 30 sec (tPA & uPA), or 45 sec (H2a), annealing for 45 sec at 65°C (uPA), 55°C (tPA) and 62°C (serpin-E2), or at 55°C for 30 sec (H2a), and elongation at 72°C for 1 min. All reactions were terminated with a final elongation at 72°C for 5 min.

Semiquantitative RT-PCR was validated for each gene product [9]. Preliminary experiments verified that PCR product increased with amount of RNA in the RT reaction. Reactions were performed for 30 cycles for uPA and H2a, 26 cycles for tPA, and for 24 cycles for serpin-E2. The PCR products were separated on 1% agarose gels with 0.001% ethidium bromide, and visualized under UV light. Quantification of band intensity was performed with NIH Image software. Target gene mRNA abundance was expressed relative to H2a mRNA abundance.

Steroid Assay

Estradiol was measured in conditioned medium in duplicate with the RIA described by Bélanger et al. (1990), without solvent extraction. Intra- and inter-assay

coefficients of variation were 8.5% and 6.3%, respectively. Progesterone was measured in duplicate as described [52] with mean intra- and inter-assay coefficients of variation were 7.2% and 18%, respectively. The sensitivity of these assays were 10 pg and 4 pg per tube for estradiol and progesterone, equivalent to 0.3 and 20 ng/μg DNA or μg protein, respectively.

Statistical analysis

Data are presented as means ± SEM. The data were analyzed by ANOVA with dose of hormones and growth factors as main effects, and culture replicate was included in the model as a random effect. Data were transformed to logarithms when not normally distributed (Shapiro-Wilk test). Means comparisons were performed with the Tukey-Kramer HSD test. All analyses were performed with JMP software (SAS Institute, Cary, NC).

Results

Effect of FSH on granulosa cell steroidogenesis, proliferation and on PA and serpin-E2 secretion and expression

FSH stimulated both estradiol (Fig. 1A; $P<0.01$) and progesterone (Fig 1B; $P<0.05$) secretion by granulosa cells in a dose-dependent manner. FSH enhanced granulosa cell proliferation, but only at a dose of 10ng (Fig 1C; $P<0.05$). Zymography demonstrated that both tPA and uPA are secreted by cultured cells in the absence of FSH (Fig 2 A&B), and that FSH increased uPA ($P<0.05$) but not tPA activity (Fig 2A&B). In

contrast, tPA but not uPA mRNA levels were up-regulated by FSH (Fig 2A, $P<0.01$). Both serpin-E2 protein secretion measured by Western blot and serpin-E2 mRNA detected by RT-PCR (Fig 2C) were stimulated by FSH in a biphasic manner ($P<0.05$), with maximal responses observed with 1ng of FSH.

Effect of IGF-I on granulosa cell steroidogenesis, proliferation and on PA and serpin-E2 secretion and expression

IGF-I stimulated estradiol secretion (Fig 3A; $P<0.05$) by granulosa cells in a dose-dependent manner, but had no effect on progesterone secretion (Fig 3B). IGF-I had a dose-dependent effect on cell proliferation (Fig 3C; $P<0.01$). IGF-I exerted a divergent effect on tPA and uPA secretion. As shown in Fig 4A, tPA activity and gene expression were inhibited by IGF-I, whereas uPA activity but not mRNA levels were stimulated by IGF-I (Fig 4B). IGF-I stimulated serpin-E2 protein secretion but not mRNA levels (Fig 4C).

Effect of BMP-7 on granulosa cell steroidogenesis, proliferation and on PA and serpin-E2 secretion

Based on the regulation of PA and serpin-E2 secretion by IGF-I, we examined also the effects of other growth factors known to regulate granulosa cells. Cells were cultured with FSH or IGF-I, in combination with graded doses of growth factors. BMP-7 stimulated estradiol secretion in IGF-I-stimulated cells in vitro (Fig 5A, $P<0.05$), and had

a modest effect on FSH-stimulated cells (P=0.08 at 50ng/ml). BMP-7 stimulated cell proliferation in FSH- but not IGF-I-stimulated cells (Fig 5B, $P<0.05$). BMP-7 stimulated tPA secretion from IGF-I-stimulated cells (Fig 5C; $P<0.01$) but not from FSH-stimulated cells. uPA secretion was not changed by BMP-7 (Fig 5C, insert). BMP-7 increased serpin-E2 secretion in FSH-stimulated cells, and had a biphasic effect on IGF-I-stimulated cells, inhibiting at lower concentrations and stimulating at higher concentrations (Fig 5D; $P<0.05$).

In the absence of BMP-7, cell proliferation and serpin-E2 were higher in IGF-I-stimulated compared to FSH-stimulated cells ($P<0.05$), whereas tPA was lower (Fig 5).

Effect of FGF-2 on granulosa cell steroidogenesis, proliferation and on PA and serpin-E2 secretion

FGF-2 inhibited estradiol secretion from both FSH- and IGF-I-stimulated cells in a dose-dependent manner (Fig 6A, $P<0.01$), but had no effect on cell proliferation either in FSH- or in IGF-I-stimulated cells (Fig 6B). FGF-2 had no effect on progesterone production by granulosa cells (data not shown). FGF-2 decreased tPA secretion from FSH- and IGF-I-stimulated cells at the highest dose used (Fig 6C, $P<0.01$), and inhibited serpin-E2 secretion by cells cultured in the presence of IGF-I (Fig 6D; $P<0.05$) but not in the presence of FSH.

In the absence of FGF-2, cell proliferation and serpin-E2 were higher in IGF-I-stimulated compared to FSH-stimulated cells ($P<0.05$; Fig 6).

Effect of EGF on granulosa cell steroidogenesis, proliferation and on PA and serpin-E2 secretion

EGF inhibited estradiol secretion in both FSH- and IGF-I-stimulated cells (Fig 7A), but had no effect on progesterone production (data not shown). EGF inhibited cell proliferation in FSH- and IGF-I-stimulated cells (Fig 7B, $P<0.01$). Secreted tPA activity increased with EGF treatment in IGF-I-stimulated cells (Fig 7C, $P<0.01$) but not in FSH-stimulated cells. In FSH-stimulated cells, lower doses of EGF stimulated serpin-E2 secretion ($P<0.001$), but a high dose (10ng) was ineffective (Fig 7D). In IGF-I-stimulated cells, serpin-E2 secretion was inhibited by EGF in a dose-dependent manner ($P<0.05$; Fig 7D).

In the absence of EGF, cell proliferation and serpin-E2 were higher in IGF-I-stimulated compared to FSH-stimulated cells ($P<0.05$), whereas tPA secretion was lower (Fig 7).

Discussion

Plasminogen activators initiate a wide range of proteolytic cascades, therefore PA inhibitors play potentially important roles in tissue remodeling. Within the ovarian follicle, the predominant inhibitor expressed in granulosa cells is serpin-E2 [5, 6], which is not expressed in theca cells. This is, to our knowledge, the first study to describe growth factor regulation of serpin-E2 expression and/or secretion in granulosa cells. We have previously shown that serpin-E2 and PA production by granulosa cell is dependent

on stage of follicle development [9], consistent with localized remodeling of the ECM as follicles grow and cells multiply. Here we report that serpin-E2 and PA production are regulated by FSH, IGF-I, BMP-7, FGF-2 and EGF, providing novel insights into the regulatory function of FSH and growth factors for ECM remodeling during follicular growth.

Two major stimulators of granulosa cell function are FSH and IGF-I. Both increased estradiol production and cell proliferation in non-luteinizing bovine granulosa cells as previously reported [16, 46, 47]. Both hormones also stimulated uPA and serpin-E2 secretion. Although tPA is the major PA in follicular fluid and secreted from granulosa cells in cattle [4, 9], uPA activity is higher in small compared with large follicles in rats and cattle [9, 10, 53] suggesting that extracellular uPA activity may be important for earlier stages of antral follicle growth. Consistent with this hypothesis, uPA but not tPA activity was upregulated by FSH in cells from small antral follicles in the present study. Further, IGF-I not only stimulated secreted uPA activity but also inhibited tPA activity. As FSH and IGF-I are important for early antral follicle growth (reviewed in [54]), the present data suggest that uPA and serpin-E2 are also involved in this process at this stage of development.

These data differ from those obtained with rodent granulosa cells, as FSH upregulated tPA mRNA and tPA secretion [10-12] and decreased uPA secretion [12]. In pigs, FSH did not alter tPA production in cultured granulosa cells (Shaw et al., 1985), likely due to the absence of FSH receptors on luteinized porcine granulosa cell [55]. In rodents, serpin-E2 protein levels or gene expression do not change during the estrous cycle and are not regulated by gonadotropins [6, 56], whereas in the present study FSH

clearly stimulated serpin-E2 protein secretion and gene expression. IGF-1 also stimulated protein secretion, but did not alter gene expression. There is therefore a major difference between rats and cattle in the regulation of granulosa cell PAs and serpin-E2 expression.

Other growth factors also regulate granulosa cell proliferation and differentiation. EGF, FGF-2 and BMP-7 have all been reported to stimulate granulosa cell proliferation, but to have different effects on steroidogenesis. In the present study, BMP-7 stimulated estradiol production and cell proliferation in cultured cells, as has been shown in rats [23, 28] and in cattle [25]. BMP-7 did not affect progesterone secretion, whereas others have reported a decrease [23, 25, 28]. This discrepancy maybe related to the presence or absence of FSH, as BMP-7 did not affect granulosa cell steroidogenesis in the absence of FSH in the rat [23]. FGF-2 and EGF decreased estradiol secretion, as previously reported in rodent and bovine granulosa cells [16, 36, 37]. However, FGF-2 did not affect cell proliferation in the present study, and EGF inhibited proliferation. Previous studies have shown that FGF-2 was ineffective in modulating cell proliferation in cultured rat [33] or porcine granulosa cells [57], but stimulated proliferation in bovine cells [33]. However, the effect of FGF-2 on cell proliferation in cattle may only become evident after periods of culture longer than used here [58]. These earlier studies were also performed with serum-containing culture medium, which alters cell responsiveness to hormones (discussed by [46]). EGF moderately increased cell number in serum-free bovine granulosa cell culture [16], whereas it inhibited cell proliferation in the present study. The reason for this discrepancy is unclear, as cells were cultured under similar conditions in the two studies.

EGF and FGF2 have been reported to stimulate tPA secretion from cultured rat granulosa cell [43, 44], whereas in the present study FGF2 markedly inhibited secreted tPA activity in FSH- and IGF-1-treated bovine cells and EGF stimulated tPA activity in IGF-I treated cells. Again, this is likely to reflect the difference between cattle and rodents in PA secretion (see above). The effects of BMP-7 on tPA activity were very similar to those of EGF (stimulation in IGF-I and not FSH-treated cells), despite the opposing effects of these two ligands on estradiol secretion and cell proliferation. This suggests that changes in extracellular tPA activity are not simple sequelae of cell proliferation, but are specifically regulated by hormones.

These growth factors also regulated serpin-E2 secretion from bovine granulosa cells. The effects of BMP-7 and FGF-2 on serpin-E2 secretion largely mirrored the effects of these ligands on tPA secretion. BMP-7 increased both tPA and serpin-E2 in IGF-1 stimulated cells, and FGF-2 decreased tPA and serpin-E2 in IGF-I stimulated cells. It is therefore possible that serpin-E2 secretion changes to counter a corresponding change in net extracellular PA activity, or vice versa. However, there were discordant changes in tPA and serpin-E2 secretion in the present study, as FGF-2 inhibited extracellular tPA activity in FSH-stimulated cells but did not affect serpin-E2 secretion. Further, EGF increased extracellular tPA activity and decreased serpin-E2 secretion in IGF-I treated cells. In the latter case, it is possible that EGF increased net tPA activity by decreasing the amount of inhibitor present, rather than altering expression/secretion of tPA protein. Most interestingly, EGF had a biphasic effect on serpin-E2 secretion from FSH-stimulated cells, which did not impact extracellular tPA activity. To our knowledge, there are no other reports of growth factor regulation of serpin-E2 secretion.

A discrepancy between enzyme activities and corresponding mRNA levels was observed in the present study. These data indicated that secreted tPA and uPA activity are regulated at a post-transcriptional level, possibly involving regulation of secretory mechanisms, in agreement with our previous report [9]. Redistribution of uPA from secreted to the cell-bound fraction in cells of rat preovulatory follicles has been suggested [12], and this redistribution may be modified by FSH or growth factors.

It is of interest to compare the effect of FSH and IGF-I on granulosa cell function in present study. Cell proliferation and serpin-E2 secretion were consistently lower in FSH-treated cells than in IGF-I-treated cells, whereas tPA secretion was higher in FSH-treated cells than in IGF-I-treated cells. Importantly, extracellular tPA activity was altered in IGF-1 stimulated cells by BMP-7 and EGF, whereas these two ligands did not affect extracellular tPA activity in FSH-stimulated cells. As future dominant follicles grow, they become critically dependent on IGF-I stimulation [54], thus we propose that growth factors play an important role in modulating the PA system at this stage of development.

In summary, the present study provides comprehensive evidence for the regulation by FSH and a number of growth factors of gene expression and secretion of members of the plasminogen activator system by bovine granulosa cells in vitro. Overall, FSH, IGF-I and BMP-7 had a stimulatory effect on cell proliferation, estradiol production, and PA and serpin-E2 secretion. In contrast, FGF-2 had an inhibitory effect on estradiol production, and on tPA and serpin-E2 secretion. EGF inhibited estradiol production, cell proliferation and serpin-E2 secretion but enhanced tPA secretion. These

data support a role for these proteins in follicle development, especially during the IGF-I dependent process of follicle selection and establishment of dominance.

Acknowledgements

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FIG. 1. Dose-dependent stimulation of A) estradiol and B) progesterone production, and C) cell proliferation by FSH in bovine granulosa cells cultured in serum-free medium. Granulosa cells were cultured in the absence or presence of FSH for 6 days (for steroid measurement) or 4 days (for cell proliferation assay). Steroid concentrations were corrected for cell number (total DNA). Results shown represent the mean ± SEM of at least three separate experiments. Bars with different letters are significantly different ($P<0.05$).

Fig 1

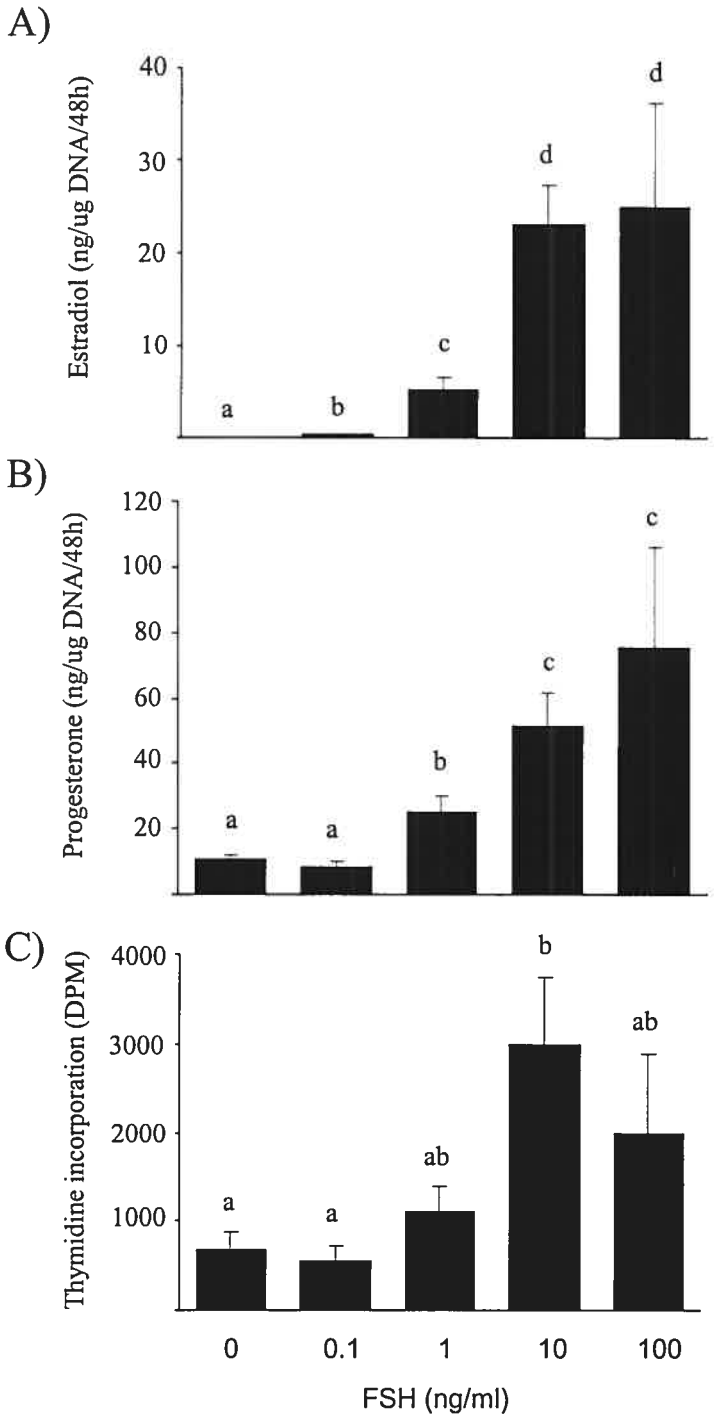

A)
Estradiol (ng/ug DNA/48h)
40
30
20
10
a
b
c
d
d
B)
Progesterone (ng/ug DNA/48h)
120
100
80
60
40
20
a
a
b
c
c
C)
Thymidine incorporation (DPM)
4000
3000
2000
1000
a
a
ab
b
ab
0
0.1
1
10
100
FSH (ng/ml)

FIG. 2. Effect of FSH on A) secreted tPA activity and gene expression, B) secreted uPA activity and gene expression, and C) serpin-E2 protein secretion and gene expression in bovine granulosa cells cultured in serum-free medium. Inserts show a representative zymograph for enzyme activity, a Western blot for serpin-E2 secretion, and agarose gels showing results of RT-PCR assays. Secreted tPA, uPA activity and serpin-E2 protein levels are expressed relative to a control sample (Ctrl; spent medium from cells cultured with FSH) included in every gel. The control (FF) in Western blots is a follicular fluid sample collected from a follicle at 8 mm in diameter. Results shown represent the mean ± SEM of at least three separate experiments. Bars with different letters are significantly different ($P<0.05$).

Fig 2.

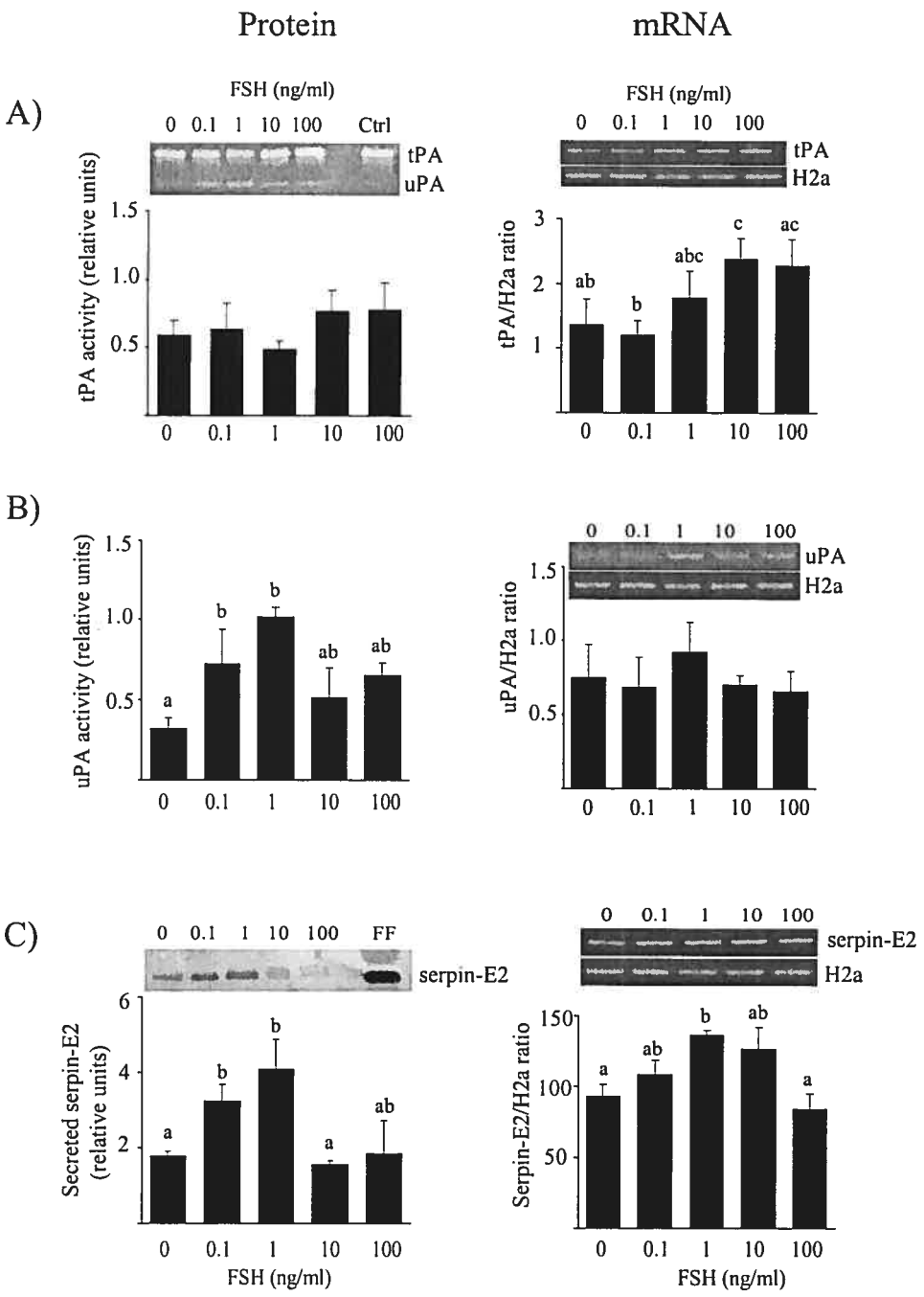
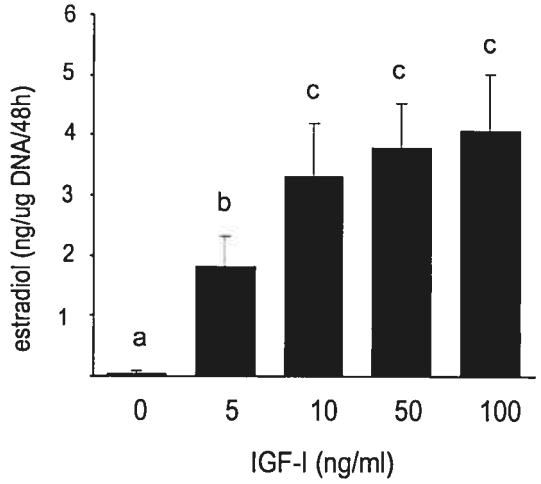

Protein
mRNA
A)
FSH (ng/ml)
0 0.1 1 10 100 Ctrl
tPA
uPA
tPA activity (relative units)
1.5
1.0
0.5
0 0.1 1 10 100
FSH (ng/ml)
0 0.1 1 10 100
tPA
H2a
tPA/H2a ratio
3
2
1
ab
b
abc
c
ac
0 0.1 1 10 100
B)
uPA activity (relative units)
1.5
1.0
0.5
a
b
b
ab
ab
0 0.1 1 10 100
0 0.1 1 10 100
uPA
H2a
uPA/H2a ratio
1.5
1.0
0.5
0 0.1 1 10 100
C)
0 0.1 1 10 100 FF
serpin-E2
Secreted serpin-E2 (relative units)
6
4
2
a
b
b
a
ab
0 0.1 1 10 100
FSH (ng/ml)
0 0.1 1 10 100
serpin-E2
H2a
Serpin-E2/H2a ratio
150
100
50
a
ab
b
ab
a
0 0.1 1 10 100
FSH (ng/ml)

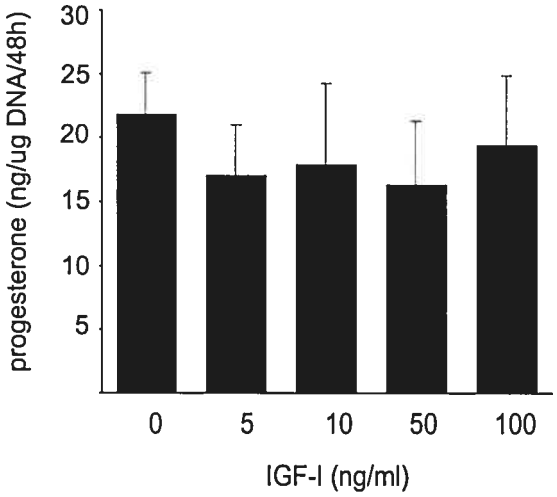
FIG. 3. Dose-dependent stimulation of A) estradiol and B) progesterone production, and C) cell proliferation by IGF-I analog (LR3) in bovine granulosa cells cultured in serum-free medium for 6 days (for steroid measurement) or 4 days (for cell proliferation assay). Steroid concentrations were corrected for cell number (total DNA). Results shown represent the mean ± SEM of at least three separate experiments. Bars with different letters are significantly different ($P<0.05$).

Fig 3

A)


estradiol (ng/ug DNA/48h)
6
5
4
3
2
1
a
b
c
c
c
0
5
10
50
100
IGF-I (ng/ml)

B)


progesterone (ng/ug DNA/48h)
30
25
20
15
10
5
0
5
10
50
100
IGF-I (ng/ml)

C)

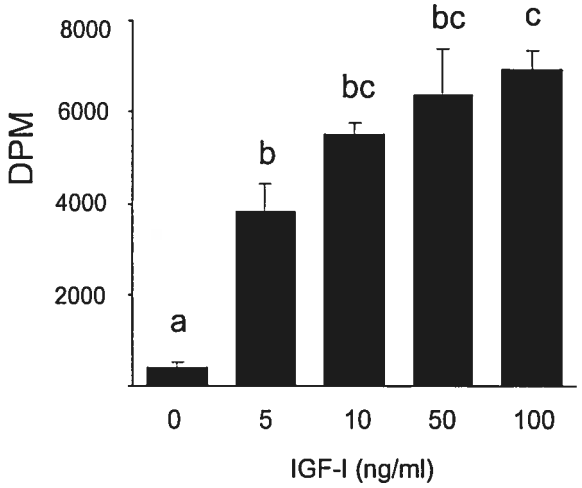

DPM
8000
6000
4000
2000
a
b
bc
bc
c
0
5
10
50
100
IGF-I (ng/ml)

FIG. 4. Effect of IGF-I analog (LR3) on A) secreted tPA activity and gene expression, B) secreted uPA activity and gene expression, and C) serpin-E2 protein secretion and gene expression. Granulosa cells were cultured in serum-free condition in the presence of the doses of IGF-I shown for 6 days. Inserts show a representative zymograph for enzyme activity, a Western blot for serpin-E2 secretion, and agarose gels showing results of RT-PCR assays. Secreted tPA, uPA activity and serpin-E2 protein levels are expressed relative to a control sample included in every gel. The control (FF) in Western blots is a follicular fluid sample collected from a follicle at 8 mm in diameter. Results shown represent the mean ± SEM of at least three separate experiments. Bars with different letters are significantly different ($P<0.05$).

Fig 4.

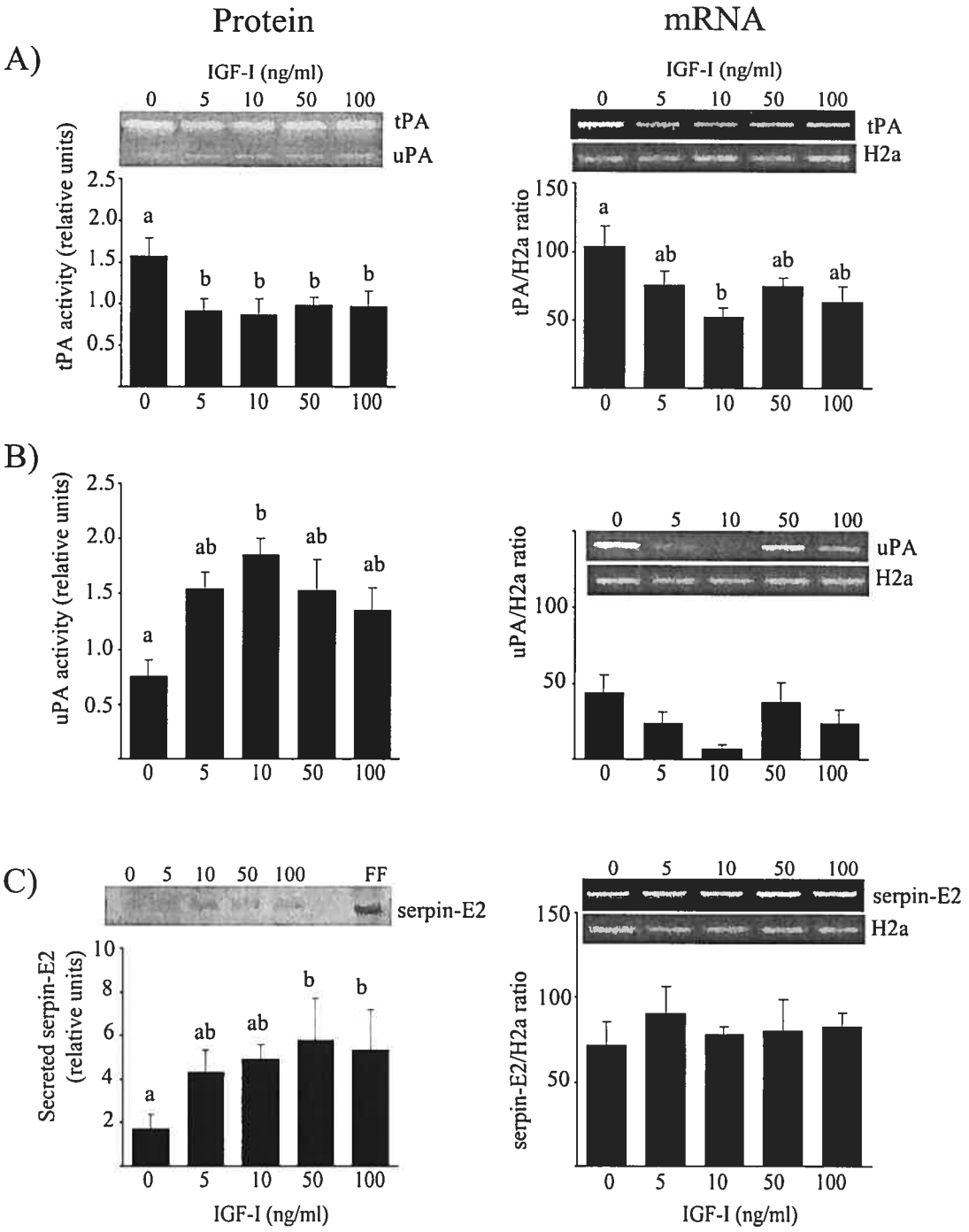

Protein
mRNA
A)
IGF-I (ng/ml)
0 5 10 50 100
tPA
uPA
tPA activity (relative units)
2.5
2.0
1.5
1.0
0.5
a
b b b b
0 5 10 50 100
IGF-I (ng/ml)
0 5 10 50 100
tPA
H2a
tPA/H2a ratio
150
100
50
a
ab b ab ab
0 5 10 50 100
B)
uPA activity (relative units)
2.5
2.0
1.5
1.0
0.5
a
ab b ab ab
0 5 10 50 100
0 5 10 50 100
uPA
H2a
uPA/H2a ratio
100
50
0 5 10 50 100
C)
0 5 10 50 100 FF
serpin-E2
Secreted serpin-E2 (relative units)
10
8
6
4
2
a
ab ab b b
0 5 10 50 100
IGF-I (ng/ml)
0 5 10 50 100
serpin-E2
H2a
serpin-E2/H2a ratio
150
100
50
0 5 10 50 100
IGF-I (ng/ml)

FIG. 5. Effect of BMP-7 on A) estradiol secretion, B) cell proliferation, C) secreted PA activity and D) serpin-E2 secretion from bovine granulosa cells cultured in serum-free medium in the presence of 1ng/ml FSH (open bars) or 10ng/ml IGF-I LR3 (solid bars). Cell proliferation was assayed by [^{3}H]-thymidine incorporation. Secreted tPA activity and serpin-E2 protein levels are expressed relative to a control sample (C) included in every gel. Inserts show a representative zymograph for enzyme activity, and a Western blot for serpin-E2 secretion. Bars with different letters within FSH- or IGF-1 treated groups are significantly different ($P<0.05$). Asterisks identify significant differences between FSH and IGF-1 treatments in the absence of BMP-7 ($P<0.01$). Results shown represent the mean ± SEM of three separate experiments.

Fig 5

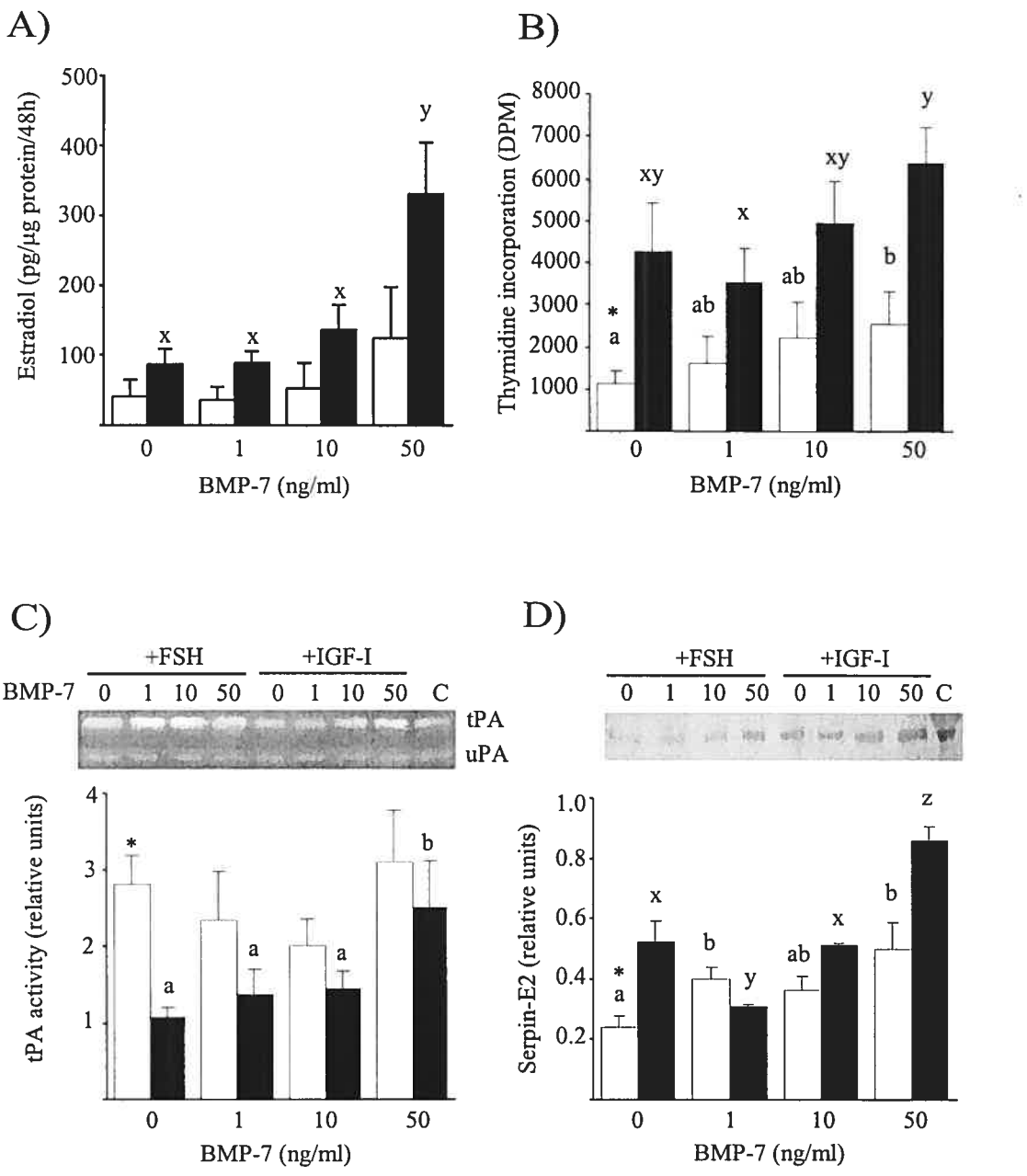

A)
Estradiol (pg/µg protein/48h)
500
400
300
200
100
x
x
x
y
0
1
10
50
BMP-7 (ng/ml)
B)
Thymidine incorporation (DPM)
8000
7000
6000
5000
4000
3000
2000
1000
*
a
ab
x
ab
xy
b
y
xy
0
1
10
50
BMP-7 (ng/ml)
C)
+FSH
+IGF-I
BMP-7 0 1 10 50 0 1 10 50 C
tPA
uPA
tPA activity (relative units)
4
3
2
1
*
a
a
a
b
0
1
10
50
BMP-7 (ng/ml)
D)
+FSH
+IGF-I
0 1 10 50 0 1 10 50 C
Serpin-E2 (relative units)
1.0
0.8
0.6
0.4
0.2
*
a
x
b
y
ab
x
b
z
0
1
10
50
BMP-7 (ng/ml)

FIG. 6. Effect of FGF-2 on A) estradiol secretion, B) cell proliferation, C) secreted PA activity and D) serpin-E2 protein secretion from granulosa cells cultured in serum-free medium in the presence of 1ng/ml FSH (open bars) or 10ng/ml IGF-I LR3 (solid bars). Cell proliferation was assayed by [^{3}H]-thymidine incorporation. Secreted tPA activity and serpin-E2 protein levels are expressed relative to a control sample (C) included in every gel. Inserts show a representative zymograph for enzyme activity, and a Western blot for serpin-E2 secretion. Bars with different letters within FSH- or IGF-1 treated groups are significantly different ($P<0.05$). Asterisks identify significant differences between FSH and IGF-1 treatments in the absence of FGF-2 ($P<0.01$). Results shown represent the mean ± SEM of three separate experiments.

Fig 6

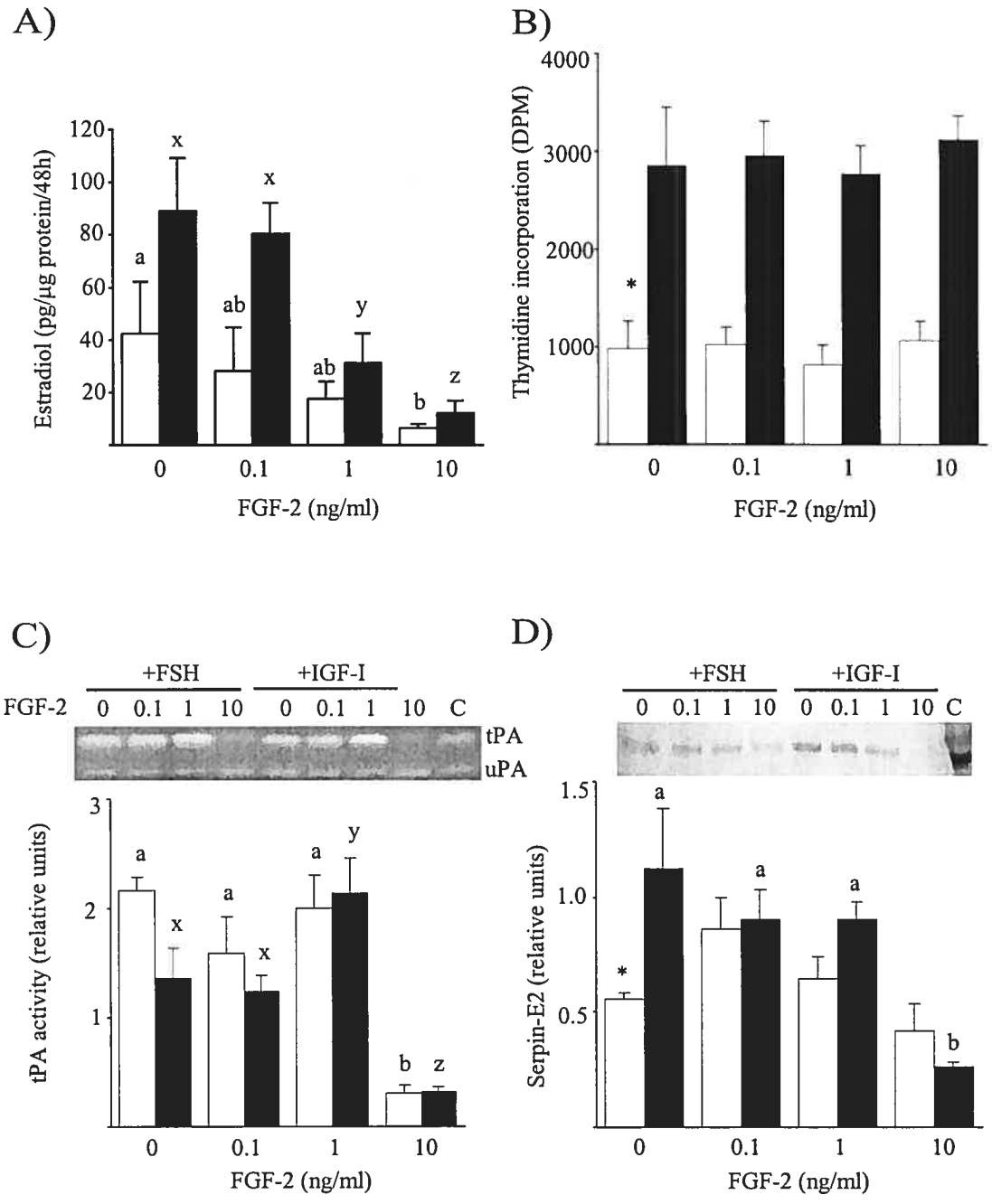
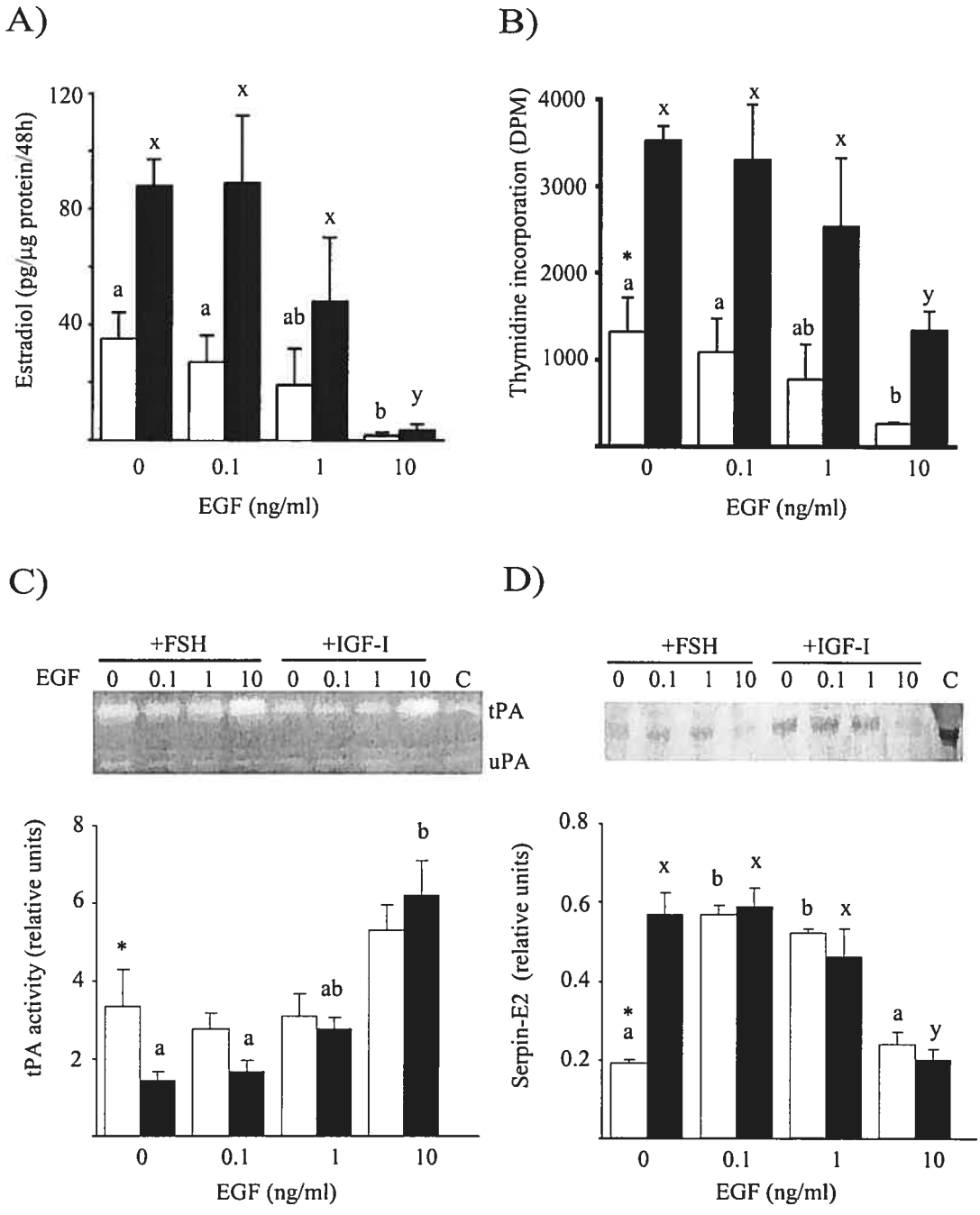

A)
Estradiol (pg/µg protein/48h)
120
100
80
60
40
20
a
x
ab
x
ab
y
b
z
0
0.1
1
10
FGF-2 (ng/ml)
B)
Thymidine incorporation (DPM)
4000
3000
2000
1000
*
0
0.1
1
10
FGF-2 (ng/ml)
C)
+FSH
+IGF-I
FGF-2
0 0.1 1 10 0 0.1 1 10 C
tPA
uPA
tPA activity (relative units)
3
2
1
a
x
a
x
a
y
b
z
0
0.1
1
10
FGF-2 (ng/ml)
D)
+FSH
+IGF-I
0 0.1 1 10 0 0.1 1 10 C
Serpin-E2 (relative units)
1.5
1.0
0.5
*
a
a
a
b
0
0.1
1
10
FGF-2 (ng/ml)

FIG. 7. Effect of EGF on A) estradiol secretion, B) cell proliferation, C) secreted PA activity and D) serpin-E2 protein secretion from granulosa cells cultured in serum-free medium in the presence of 1ng/ml FSH (open bars) or 10ng/ml IGF-I LR3 (solid bars). Cell proliferation was assayed by [^{3}H]-thymidine incorporation. Secreted tPA activity and serpin-E2 protein levels are expressed relative to a control sample (C) included in every gel. Inserts show a representative zymograph for enzyme activity, and a Western blot for serpin-E2 secretion. Bars with different letters within FSH- or IGF-1 treated groups are significantly different ($P<0.05$). Asterisks identify significant differences between FSH and IGF-1 treatments in the absence of EGF ($P<0.01$). Results shown represent the mean ± SEM of three separate experiments.

Fig 7


A)
B)
C)
D)
Estradiol (pg/µg protein/48h)
Thymidine incorporation (DPM)
EGF (ng/ml)
+FSH
+IGF-I
EGF
0 0.1 1 10 0 0.1 1 10 C
tPA
uPA
tPA activity (relative units)
Serpin-E2 (relative units)

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Article 3

Expression of protease nexin-1 and plasminogen activators during follicle growth and the periovulatory period in cattle

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Abstract

Extracellular matrix (ECM) remodeling occurs during ovarian follicular development, mediated by plasminogen activators (PA) and PA inhibitors including protease nexin-1 (PN-1). In the present study we measured expression/activity of the PA system in bovine follicles at different stages of development by timed collection of ovaries during the first follicle wave and during the periovulatory period, and in follicles collected from an abattoir. The abundance of mRNA encoding PN-1, tissue-type PA (tPA), urokinase (uPA) and PA inhibitor-1 (PAI-1) were initially up-regulated by hCG in bovine preovulatory follicle wall homogenates. PN-1, PAI-1 and tPA mRNA expression then decreased near the expected time of ovulation, whereas uPA mRNA levels remained high. PN-1 concentration in follicular fluid (FF) decreased and reached the lowest level at the time of ovulation, whereas plasmin activity in FF increased significantly after hCG. Follicles collected from the abattoir were classified as nonatretic, early-atretic or atretic based on FF oestradiol and progesterone content: PN-1 protein levels in FF were significantly higher in nonatretic than in atretic follicles, and plasmin activity was correspondingly higher in the atretic follicles. No changes in PN-1 levels in FF were observed during the growth of pre-deviation follicles early in a follicle wave. These results indicate that PN-1 may be involved in the process of atresia in nonovulatory dominant follicles and the prevention of precocious proteolysis in periovulatory follicles.

Introduction

The growth of bovine follicles from the primordial to the preovulatory stage is characterized by the proliferation of cells and considerable increase in size of the follicle (Lussier *et al.* 1987). The granulosa and theca cell layers are separated by the basal lamina, and the theca cells are enclosed in a dense extracellular matrix (ECM). Remodeling of the basal lamina and ECM occurs as follicles expand, and changes in the chemical composition of the basal lamina have been described during follicle development (Rodgers *et al.* 2003). Extensive breakdown and remodeling of the basal lamina and connective tissue of the follicular wall is required for ovulation (Curry *et al.* 2001, Richards *et al.* 2002).

The plasminogen activator (PA) system has been implicated as one of the important mediators of ECM remodeling and follicle rupture at ovulation (Ny *et al.* 2002, Liu 2004). The PA system consists of the ubiquitous proenzyme, plasminogen, that is converted to an active enzyme, plasmin, by the tissue-type (tPA) and urokinase (uPA) plasminogen activators. The activity of PA is regulated in part by inhibitors, including plasminogen activator inhibitor-1 (PAI-1) and protease nexin-1 (PN-1, also known as serine protease inhibitor E2, Serpine2). PN-1 is a secreted glycoprotein, and is a broad and rapid inhibitor of a number of serine and cysteine proteases including tPA, uPA, and plasmin (Silverman *et al.* 2001). The expression and regulation of PN-1 has been examined in ovarian follicles. In contrast to PAI-1, which is predominantly expressed in thecal-interstitial cell layers, PN-1 was exclusively expressed in granulosa cells in mice (Hägglund *et al.* 1996), rats (Hasan *et al.* 2002) and cattle (Bédard *et al.* 2003).

Previous studies indicate that ovulation requires coordinated expression of the plasminogen activators and their inhibitors. In rats and monkeys, there is an upregulation of tPA and PAI-1 expression by human chorionic gonadotrophin (hCG) during the periovulatory period, followed by a marked decrease in PAI-1 expression just before ovulation. This may allow a narrow window of increased tPA activity that results in follicle rupture and ovulation (Liu *et al.* 1987, Shen *et al.* 1997, Liu 2004). PN-1 is also expressed in preovulatory follicles, although its role is not clear. PN-1 expression decreased following an ovulatory dose of hCG in rats (Hasan *et al.* 2002) but not in mice (Hägglund *et al.* 1996). How the proteolytic cascade is controlled in cattle is not well understood, as tPA activity increases prior to ovulation whereas PAI-1 activity does not change (Dow *et al.* 2002a, 2002b). The potential role of PN-1 during the periovulatory period in cattle remains to be determined.

The PA system may also be involved in tissue remodeling at earlier stages of folliculogenesis. In rats, there is a switch from uPA to tPA activity during follicle growth (Karakji & Tsang 1995). In cattle, follicle growth occurs in waves, during which the dominant, potential ovulatory follicle undergoes rapid growth and the subordinate follicles become atretic (Fortune *et al.* 2001, Ginther *et al.* 2001b). The PA system may be involved in the growth of the dominant follicle, as cellular uPA activity was higher in granulosa cells from small antral follicles compared to those from large follicles, and PN-1 secretion and expression was lower in granulosa cells from small follicles compared to those of large follicles of rodents and cattle (Hägglund *et al.* 1996, Bédard *et al.* 2003, Cao *et al.* 2004). Follicle regression may also involve PA activity, as plasminogen activation markedly decreased attachment of Chinese hamster ovary fibroblasts to ECM

components in vitro, resulting in detachment-induced cell death (anoikis) (Rossignol *et al.* 2004). Interestingly, PN-1 inhibited PA-induced anoikis in these cells (Rossignol *et al.* 2004).

The objective of this study was to determine if cell-specific and temporal regulation of PN-1 and PA expression and secretion contribute to follicular development and ovulation in cattle. We assessed the role of PN-1 as a candidate for regulation of PA activity in bovine follicles at three stages of folliculogenesis: 1) the periovulatory period, 2) in healthy, early atretic and atretic dominant follicles classified on biochemical criteria, and 3) during the first follicle wave before and during follicle deviation.

Materials and Methods

Experiments 1 and 3 were performed with crossbred heifers aged between 1.5 and 3 years. The animals were housed indoors on the University of Montreal farm for the duration of the experiment and were fed concentrate and hay twice daily. Water was available *ad libitum*. All animal experimentation was approved by the Animal Care Committee of the Faculty of Veterinary Medicine, University of Montreal, and performed in accordance with Canadian Council of Animal Care Guidelines.

Experiment 1. hCG-induced periovulatory follicles

Experimental design

Ten heifers were induced to ovulate as described (Bédard *et al.* 2003). The animals were synchronized with one injection of prostaglandin F2α (PGF2α) (25 mg, im; Lutalyse, Upjohn, Kalamazoo, MI), and behavioural oestrus was monitored at 12 hr

intervals, from 48 to 96 hr following PGF2α injection. Ovarian follicle development was monitored by daily transrectal ultrasonography performed with a real-time linear scanning ultrasound system (LS-300; Tokyo Keiki Co, Ltd, Tokyo, Japan) equipped with a 7.5-MHz transducer (Lussier *et al.* 1994). Preovulatory follicles were obtained following a second injection of 25 mg PGF2α seven days after oestrus to induce luteolysis, thereby allowing the development of the dominant follicle of the first follicle wave into a preovulatory follicle (Sirois 1994). An ovulatory dose of hCG (3000 IU; APL, Ayerst Lab, Montreal, Quebec, Canada) was injected 36 h after the induction of luteolysis, and ovaries bearing the preovulatory follicle were collected by ovariectomy at 0, 6, 12, 18, and 24 h after hCG injection.

Follicular fluid (FF) was aspirated from the follicles with a 21G needle, centrifuged (3,000*g* for 2 min at 4°C) and stored at -20°C for PN-1 and PA assay. The follicle walls were then homogenized in lysis buffer (4 M guanidium isothiocyanate, 0.5% Na-N-laurylsarcosine, 25 mM sodium citrate, pH 7) (Chomczynski & Sacchi 1987), and total RNA was sedimented on a cesium chloride cushion by centrifugation (Ndiaye *et al.* 2005). The concentration of total RNA was evaluated by optical density at 260 nm, and quality was estimated by visualizing the 28S and 18S ribosomal bands following electrophoretic separation on a formaldehyde-agarose gel in the presence of ethidium bromide.

Granulosa cells were collected from individual follicles at 0, 12 and 24 h following hCG injection for protein extraction. Cells were homogenized in M-PER Reagent (Pierce, Rockford IL) supplemented with Complete Protease Inhibitor Cocktail (Roche Applied Science, Laval QC). Lysis of cells was achieved by repeated passage through a 25 gauge

needle attached to a 3 mL syringe. Cell lysates were centrifuged (16,000 g for 15 min at 4C) and supernatant was stored at -80C until analysis of PN-1 and PA activity.

Messenger RNA reverse transcriptase and semiquantitative RT-PCR

One microgram of total RNA was reverse transcribed and amplified for 15 cycles with the SMART PCR cDNA synthesis kit (BD Biosciences Clontech, Mississauga, ON, Canada) as described (Ndiaye *et al.* 2005). The resulting cDNA pool was diluted to 50 µl in TE buffer (10 mM Tris pH 8, 1 mM EDTA), and 1 µl of the aliquot was used in a secondary 100 µl PCR reaction for 18 cycles using the Advantage 2 DNA Polymerase Mix (BD Biosciences Clontech, Mississauga, ON, Canada) and the PCR primer AAGCAGTGGTAACAACGCAGAGT.

Complementary DNA from the secondary PCR reactions were diluted 10-fold in TE buffer, and were used as template in subsequent semi-quantitative RT-PCR for the target genes PN-1, PAI-1, tPA and uPA. Table 1 summarizes the gene-specific PCR primers used and PCR conditions. GAPDH was used as the housekeeping control (Ndiaye *et al.* 2005). Briefly, an aliquot of 2 µl of the diluted cDNA was amplified using Advantage 2 DNA polymerase (0.6 µl) in a 25-µl PCR reaction containing 0.4 mM dNTP mix, and 0.8 µM specific primers (except for GAPDH, 0.4 µM). Target cDNA was amplified in a PCR thermal cycler (Applied Biosystems, Gene AMP® PCR System 9700, Foster City, CA) under the following conditions: 1) an initial denaturation step for 1 min at 95°C; 2) amplification cycles with denaturation at 95°C for 30 sec, annealing for 45 sec at the temperatures indicated in Table 1 for each gene, and elongation at 68°C for 1.5 min. The number of PCR cycles was optimized for each gene to be analyzed (see Table

1). The amplicons were separated on 1% agarose gel containing ethidium bromide, and visualized under UV light. Quantification of band intensity was performed with NIH Image software. Target gene mRNA abundance was normalized to GAPDH mRNA abundance.

Experiment 2. Healthy & atretic dominant follicles

Tissue collection

Ovaries were obtained from an abattoir local to the São Paulo State University campus in Botucatu, and transported to the laboratory in saline on ice. Follicles ≥ 9 mm in diameter (and therefore post-deviation) were dissected from the ovaries, and follicular fluid was aspirated, centrifuged and frozen for steroid, PN-1 and PA assay. The antral cavity was flushed repeatedly with cold saline and granulosa cells recovered by centrifugation at 1200 g for 1 minute, and pooled with the follicular fluid cell pellet. The remaining granulosa cells adhering to the follicle wall were removed by gently scraping with a blunt Pasteur pipette, and pooled with the flushed cells. The theca layer was then removed with forceps and washed in saline by passing repeatedly through a 1mL syringe. Granulosa and theca cell were collected into Trizol (Invitrogen; São Paulo, Brazil) and homogenized with a Polytron. Total RNA was extracted immediately according to the Trizol protocol.

Follicles containing > 100ng oestradiol/ml and < 100ng progesterone/ml were classified as nonatretic, those containing < 40ng oestradiol/ml and < 100ng progesterone/ml were classified as early atretic, and those containing < 40ng oestradiol/ml and > 100ng progesterone/ml were classified as atretic. These represent

mature dominant growing, static and regressing follicles, respectively (Price *et al.* 1995). Cross-contamination of theca and granulosa cells was tested by detection of mRNA encoding cytochromes P450 aromatase (Cyp19) and 17α-hydroxylase (Cyp17) in each sample by PCR (Buratini *et al.* 2005). Only granulosa cell samples negative for Cyp17, and only thecal samples negative for Cyp19 amplicons were included in the analysis.

Semiquantitative RT-PCR

PN-1, tPA, and uPA mRNA expression in granulosa cells, and PAI-1, tPA, and uPA mRNA expression in theca cells were measured by semiquantitative RT-PCR. Briefly, for both theca and granulosa cells, total RNA (1μg) was incubated with DNAse I (Invitrogen) and reverse transcribed with SuperScript II (Invitrogen) and oligo-d(T) primer (Buratini *et al.* 2005). An aliquot (0.4 µl) of the cDNA template was amplified by PCR using 0.2 µl (2.5 U) Taq Polymerase (Amersham Pharmacia Biotech Inc., Oakville, ON, Canada) in a 20-µl PCR buffer (Amersham Pharmacia Biotech Inc.) containing 0.1 mM dNTP mix, and 0.2 µM specific primers (Cao *et al.* 2004). Target cDNA was amplified under the following conditions: 1) an initial denaturation step for 3 min at 94°C, except uPA which was for 5 min at 95°C; 2) amplification cycles with denaturation at 94°C for 15 sec (PN-1), 30 sec (uPA, tPA, PAI-1 and GAPDH) or 45 sec (H2a), annealing for 30 sec for H2a and 45 sec for all other genes, at the temperatures indicated in Table 1, and elongation at 72°C for 1 min; and 3) final elongation at 72°C for 5 min. The number of cycles is given in Table 1.

Semiquantitative RT-PCR was validated for each gene product (Cao *et al.* 2004). The PCR products (10 µl) were separated on 1% agarose gels containing ethidium bromide, and visualized under UV light. Quantification of band intensity was performed

with NIH Image software. Target gene mRNA abundance was expressed relative to H2a mRNA abundance in granulosa cells, and to GAPDH mRNA abundance in theca cells.

Experiment 3. before and during follicle deviation

Experimental design

Seven heifers were first synchronized with two injections of PGF2α given 11 days apart. Ovulation and follicular development was monitored daily or twice daily by transvaginal ultrasonography, and follicles were punctured as described (Ouellette *et al.* 2005) when the largest follicle of the first wave had reached 6.5, 7.5, 8.5 or 9.5 mm internal diameter (referred to as 'follicle stage'), corresponding to approximately 1, 1.5, 2 and 2.5 days after wave emergence (Ginther *et al.* 2001b). Follicle deviation is expected to occur when the largest follicle reaches 8.5 – 9 mm diameter (Beg *et al.* 2001). Follicular fluid (FF) from the largest three follicles (F1, F2 and F3) was collected separately for each follicle ('follicle rank'). Each animal was used once during a follicle wave, and 5 to 6 days after follicular puncture each animal received a single injection of PGF2α to initiate ovulation and a new first follicle wave. Each animal was in this manner sampled on four consecutive oestrous cycles. Only clear FF samples without blood contamination were used. The FF was centrifuged for 15 min at 2000 *g* and the supernatant frozen at -20°C until assayed for steroid concentrations, PN-1 content and PA activity.

Casein zymography

Casein zymography was used to measure plasmin, tPA and uPA activity in follicular fluid and cell extracts as described (Cao *et al.* 2004). Briefly, 2 μl of follicular

fluid or 30μg cell protein were subjected to electrophoresis in 10% non-denaturing polyacrylamide gels containing 0.2% casein (Sigma), 0.1% SDS and 3.75 mU/ml bovine plasminogen (Sigma). After electrophoresis, gels were washed once in 2.5% Triton X-100 for 45 min to remove SDS, and placed in incubation buffer (50 mM Tris, 0.1 M NaCl , pH 7.6) at 37°C for 16 h with gentle shaking. The gels were then stained using 0.05% Coomassie blue in 10% acetic acid, 40% methanol for 2 h, destained in 10% acetic acid, 40% methanol, and then fixed in 10% glycerol. The identity of the enzymatic activities was investigated by comparing molecular size with human tPA (Calbiochem, Darmstadt, Germany) and uPA (NIBSC, Hertfordshire, UK) standards. Plasminogen-free gels were used to confirm that the activity detected was plasminogen dependent. Bands of plasmin activity were visualized as clear zones where casein degradation occurred, against a dark (blue) background. To correct for gel-to-gel variation, all samples were expressed relative to a control sample (conditioned medium) that was included in every gel.

Western Blot

PN-1 protein abundance in follicular fluid and cell lysates was analyzed by Western blot as described (Cao *et al.* 2004). Samples were subjected to electrophoresis in 10% denaturing polyacrylamide gels. Proteins were then electrotransferred onto nitrocellulose membrane (0.45 μm) (Bio-Rad, Hercules, CA) at 22 V overnight at 4°C in transfer buffer (39 mM glycine, 48 mM Tris-base, 0.037% SDS and 20% methanol, pH 8.3). After blocking for 1 h in TTBS (0.2% Tween 20, 10 mM Tris-HCl, 150 mM NaCl), blots were incubated with 1:5000 rabbit anti-bovine PN-1 (Bédard *et al.* 2003) for 4 h

with agitation, followed by three washes (10 min each) with 0.2% TTBS. The blots were then incubated with 1:5000 alkaline phosphatase-linked anti-rabbit IgG (Sigma) for 1.5 h with agitation, followed by three washes (10 min each) with TTBS. Finally, the blots were incubated with NBT/BCIP solution (Roche Diagnostics, Indianapolis, IN). Rainbow coloured protein molecular weight markers (Pharmacia, Piscataway, NJ) were used to estimate molecular size of the target protein, and a bovine follicular fluid sample (2 μl) was used as positive control in all blots.

Steroid assays

Oestradiol and progesterone in FF from Experiment 2 were assayed by radioimmunoassay (RIA) using iodinated tracers and antibodies furnished in the 3rd Generation Estradiol RIA (DSL 39100) and the DSL-3400 Progesterone RIA kits (Diagnostic Systems Laboratories, Inc., Webster, Texas). The standard curves were prepared from crystalline steroids (Sigma Chemical Co) in PBS-gelatin (0.02 M sodium phosphate, 0.15 M sodium chloride, 0.1% gelatin, 0.01% sodium azide, pH 7.5). The assay protocols were as described in the kits, except that the oestradiol antibody and tracer were each diluted 1:1 with PBS-gelatin before use, and the progesterone antibody and tracer were diluted 3:2 and 7:3, respectively. FF samples were diluted in PBS-gelatin before assay. Intra- and inter-assay coefficients of variation were 7.4 and 13.5%, respectively for oestradiol, and 6.8 and 7% respectively for progesterone. The sensitivities of the assays were 0.05ng/ml for oestradiol (at 1:25 dilution of FF) and 0.2ng/ml for progesterone (at 1:10 dilution).

Concentrations of oestradiol in FF from Experiment 3 were analysed by RIA using double antibody precipitation and charcoal absorption methods, respectively, as validated for use with bovine FF (Carrière & Lee 1994, Price *et al.* 1995). Spiking FF samples with 2pg/tube and 5 pg/tube of oestradiol gave 81% and 107% recovery, respectively. Spiking FF samples with 100pg/tube and 500 pg/tube of progesterone gave 115% and 97% recovery, respectively. Intra- and inter-assay coefficients of variation were 5% and 10% for oestradiol, and 7% and 8% for progesterone, respectively.

Statistics

Data are presented as least-squares means ± SEM. Data were transformed to logarithms when they were not normally distributed (Shapiro-Wilk test). All analyses were performed with JMP software (SAS Institute, Cary, NC). The data from Experiments 1 and 2 were analysed by ANOVA for effect of time (Expt 1) or follicle class (Expt 2), with gel or blot included as random effect terms where samples were analysed in several gels/blots. Where main effects were found, means comparisons were performed by the Tukey-Kramer HSD test. In Experiment 3, data were analysed by 2-way ANOVA with follicle stage & rank as main effects. Owing to the lack of normal distribution of the oestradiol data, log oestradiol values were analysed for effect of follicle rank within follicle stage. The time of follicle deviation was defined as the earliest change in diameter between the largest follicle (F1) and the second largest follicle (F2). Correlations between PA activity or PN-1 secretion and FF steroid concentration or follicle diameter were assessed with Pearson's product-moment correlation coefficient (r).

Results

Experiment 1. hCG-induced periovulatory follicles

PN-1, PAI-1, tPA, and uPA mRNA expression were determined in follicle wall homogenates by RT-PCR. Abundance of mRNA encoding PN-1, PAI-1 and tPA all increased transiently after hCG injection ($P<0.05$; Fig. 1), reaching maximal values at 6h after hCG, and returning to pretreatment levels by 24 h after hCG injection. The pattern of uPA mRNA abundance differed from the other genes examined, as mRNA levels increased following hCG administration and remained elevated at 24 h after hCG injection ($P<0.05$; Fig. 1C).

To support the gene expression data, PN-1 protein and PA activities in granulosa cell lysates were measured. A major immunoreactive band corresponding to PN-1 was detected by Western blot in granulosa cell lysates, and abundance of this protein band did not differ between 0, 12 or 24 h after hCG injection (Fig. 2A). Zymography demonstrated an increase in proteolytic activity corresponding to plasmin (approximately 82 kDa) and uPA (approximately 45 kDa) in preovulatory granulosa cell lysates after hCG injection ($P<0.05$; Fig. 2B), whereas tPA activity was weak to undetectable (Fig. 2B).

Follicular fluid PN-1 protein content decreased with time after hCG injection, reaching the lowest levels at 24 h after hCG ($P<0.05$; Fig. 3A). Plasmin activity in FF increased after hCG ($P<0.05$; Fig. 3B) whereas uPA activity decreased (Fig 3B). Proteolytic activity corresponding to tPA was not detected in FF of preovulatory follicles.

Experiment 2. Healthy & atretic dominant follicles

Oestradiol and progesterone concentrations and diameters of the follicles in each class are given in Table 2. Nonatretic and early-atretic differed by oestradiol content but not by progesterone content or diameter; nonatretic and atretic follicles differed by oestradiol and progesterone content, but not by follicle diameter. Plasmin activity in FF collected from nonatretic follicles was significantly lower than that in early-atretic and atretic follicles ($P<0.05$; Fig. 4A). uPA activity in FF did not differ between groups (Fig. 4A). tPA activity was not detectable in any the follicles examined. PN-1 protein abundance in FF of nonatretic follicles was significantly higher than that of early-atretic and atretic follicles ($P<0.05$; Fig. 4B). Plasmin activity in FF was negatively correlated with PN-1 content ($r= -0.6$, $P<0.05$) but not with uPA activity ($P>0.05$).

Messenger RNA for tPA, uPA, and PN-1 were detected in granulosa cells. PN-1 mRNA levels were lower in granulosa cells of early-atretic follicles than in those of nonatretic and atretic follicles ($P<0.05$; Fig. 5A), but there were no differences in tPA or uPA mRNA levels. Messenger RNA for tPA, uPA, and PAI-1 were detected in theca cells, and message levels did not differ significantly between groups (Fig. 5B).

Overall, granulosa cell PN-1 mRNA abundance and FF PN-1 protein abundance were positively correlated with FF oestradiol concentrations ($r=0.73$ & 0.62, respectively; $P<0.01$). Plasmin activity in FF was negatively correlated with FF oestradiol concentration ($r= -0.65$, $P<0.01$). Follicle diameter was correlated with granulosa cell tPA mRNA abundance ($r= 0.57$, $P<0.05$) but with no other variable.

Experiment 3. before and during follicle deviation

Mean diameters and oestradiol and progesterone concentrations in the F1, F2 and F3 follicles are summarized in Table 3. A significant difference in diameter between the largest (F1) and second-largest (F2) follicle occurred when the F1 had reached 9.5 mm. Oestradiol concentrations did not differ between the three largest follicles of a wave when the F1 was 6.5 mm diameter, but was significantly lower in the F3 compared to F1 at all subsequent stages. Differences in oestradiol between F1 and F2 occurred only after follicle deviation. Progesterone concentrations did not differ between follicles at any stage of the wave.

A single band corresponding to PN-1 was detected in FF by immunoblotting, and PN-1 abundance did not significantly change with stage of the follicle wave or between F1, F2 or F3 follicles (Fig. 6A). Plasmin and uPA (but not tPA) activities were detected in FF, and no differences was observed between follicle stages or rank (Fig. 6B). However, uPA activity was correlated with FF oestradiol concentration and the oestradiol:progesterone ratio in subordinate (F2: $r = 0.65$, $P<0.001$; F3: $r = 0.73$, $P<0.001$, for oestradiol:progesterone ratio) but not in dominant (F1) follicles.

Discussion

The pattern of expression and potential role of PN-1 during follicle growth is not well known. In the present study, we measured PN-1 and PA activity in bovine follicles at three stages of follicle development. We demonstrate that during the periovulatory period the expression of PN-1 is initially up-regulated by hCG, and then declines in a pattern similar to the expression of PAI-1 and tPA. This profile of PN-1 expression appears to differ from that observed in rodents, where no change in expression was

observed until the onset of ovulation (Hägglund *et al.* 1996, Hasan *et al.* 2002), although these are *in situ* studies that do not lend themselves well to quantification. We also demonstrate a potential role for PN-1 in dominant follicle growth, as follicular protein levels are higher in nonatretic than in early-atretic and atretic follicles.

We first measured the pattern of expression of PA system members in the follicle wall in response to an ovulatory dose of hCG, as the ovarian PA system is best known for its role during ovulation. Follicular tPA, uPA, PAI-1 and PN-1 mRNA abundance increased sharply by 6 h after hCG, and mRNA levels declined thereafter except for uPA mRNA which remained high until the expected time of ovulation. Overall, these results support previous studies in cattle (Dow *et al.* 2002a, 2002b) in which tPA, uPA and PAI-1 mRNA levels were upregulated by an induced gonadotrophin surge. This is in contrast to that observed in other species, in which only one PA was increased in response to the gonadotrophin surge. For instance, only tPA in rats (Li *et al.* 1997) and monkeys (Liu *et al.* 2004) or uPA in mice (Macchione *et al.* 2000) and sheep (Colgin & Murdoch 1997) was upregulated during ovulation. The specific time points when mRNA abundance increased and then decreased is slightly different between the present study and the previous studies (Dow *et al.* 2002a, 2002b), possibly caused by the different methods of inducing ovulation (hCG vs GnRH).

Changes in periovulatory PN-1 expression have not previously been described in ruminants. In mice, granulosa cell PN-1 expression generally did not vary throughout the periovulatory period (Hägglund *et al.* 1996), although a decrease in immunostaining was observed 12 h after hCG injection in PMSG-stimulated rats (Hasan *et al.* 2002). The present data clearly show a transient upregulation of PN-1 expression within 6 h of hGC

treatment, indicating that PN-1 regulation in cattle is different from that of rodents. The only comparable work in ruminants is a gene-profiling study that described lower PN-1 expression in periovulatory follicles 24 h after hCG injections compared to dominant non-ovulatory follicles on day 5 of the oestrous cycle (Bédard *et al.* 2003). In rats, a model was proposed for the tight regulation of proteolytic activity in periovulatory follicles, in which both tPA and PAI-1 expression increase initially to generate high levels of inhibited enzyme within the follicle wall (Liu *et al.* 1987, Shen *et al.* 1997). According to this model, PAI-1 but not tPA expression then decreases, thus triggering activation of accumulated tPA and degradation of the follicle wall. In cattle, a modified version of this model can be proposed, in which there is an initial upregulation of tPA, uPA, PAI-1 and PN-1, followed by a decrease in tPA, PAI-1 and PN-1 expression while maintaining uPA expression. The activation of accumulated PA in theca (owing to reduced PAI-1 expression) and granulosa (owing to reduced PN-1 expression) cells would contribute to the proteolytic cascade at ovulation in this species. The cell-specific expression of PA inhibitors is physiologically relevant, as tPA expression is localized predominantly to the granulosa layer in bovine follicles (Dow *et al.* 2002a), thus regulation of PN-1 expression is likely important to prevent precocious proteolytic activity on the antral side of the basal lamina.

This model for the control of the proteolytic cascade at ovulation in cattle is supported by the increased uPA and plasmin activity in granulosa cell lysates observed 12 – 24 h after hCG, at a time when granulosa cell PN-1 protein levels were not different from pretreatment controls (see Fig. 2). The increase in plasmin activity in granulosa cells was reflected by an increase in FF plasmin activity. This is consistent with the

plasmin activity detected in sheep follicle walls (Murdoch 1998), but in contrast to a previous study in cattle (Dow *et al.* 2002a) in which FF plasmin activity increased in GnRH-treated animals, but was not detected in the lysates of whole follicle wall. The reason for this discrepancy is unknown but may be related to the methods used to prepare lysate samples and/or the use of whole follicle wall compared to granulosa cells. We also observed an increase in uPA activity in granulosa cell lysates after hCG but a decrease in activity in FF. This discrepancy may be caused by a redistribution of uPA to the cell surface or the ECM (Macchione *et al.* 2000), specific regulation of protein secretion or a consequence of theca secretion of uPA.

The expression of PN-1 has been detected in small antral follicles (Hägglund *et al.* 1996, Hasan *et al.* 2002, Bédard *et al.* 2003) suggesting that it might play a role in follicle growth prior to the preovulatory period. We explored this by measuring PN-1 and PA activity/expression in nonatretic and atretic dominant follicles, and during follicle deviation early in the follicle wave. Changes in PN-1 or PA activity in FF were not detected during the growth of the follicle cohort before deviation, even though FF oestradiol content increased in the largest follicle and decreased in smaller follicles of the cohort. Interestingly, uPA activity in FF was positively correlated with follicle oestradiol content in subordinate follicles early in a follicle wave but not in the largest, presumably dominant follicle of the cohort. This suggests that the oestrogenic subordinate follicles may be growing or have the potential to grow, and thus have different requirements for ECM remodeling and uPA activity than the less-oestrogenic subordinate follicles whose growth may have ceased. In support of this, it has been demonstrated that larger subordinate follicles have the capacity to develop into dominant follicles if the existing

dominant follicle is ablated (Ginther *et al.* 2001a). The dominant follicle is actively growing and therefore would be expected to undergo constant ECM remodeling irrespective of oestradiol content, which might explain the absence of a correlation between oestradiol and uPA in the dominant follicle. The absence of changes in PN-1 protein or expression in pre-deviation follicles suggests that PN-1 may not be tightly regulated prior to deviation, or may not play a role at this stage of development.

In dominant follicles, however, PN-1 may be regulated and/or play a more important role. Follicle fluid PN-1 content was lower in early-atretic and atretic follicles compared to nonatretic follicles, and FF plasmin activity was correspondingly higher in the early-atretic and atretic follicles. Plasmin activity in FF appears to be regulated by PN-1, as plasmin activity was correlated with PN-1 but not uPA, and uPA activity did not change significantly with follicle health. Decreased PN-1 expression and increased plasmin activity in early-atretic follicles may be related to ECM remodeling during the onset of atresia. Also, PN-1 has been suggested as an anti-apoptotic factor in adherent cells, as it inhibited plasminogen activation-induced anoikis in these cells (Rossignol *et al.* 2004). Atresia in dominant bovine follicles is characterized by apoptosis and detachment of granulosa cells near the antrum (Irving-Rodgers *et al.* 2001). Therefore it is possible that certain endocrine or paracrine signals may inhibit PN-1 synthesis and or secretion from dominant follicles at the end of their growth phase, and these lowered PN-1 levels may initiate or facilitate the onset of atresia in the membrana granulosa.

In summary, PN-1 expression/secretion changes in a manner dependent on stage of follicle development. Specifically, no changes in PN-1 or PA activity were observed during the growth of follicles early in the follicle wave before follicle deviation occurred,

suggesting a minor role if any for these proteins before follicle deviation. In dominant follicles however, PN-1 levels in FF were lower in atretic compared to nonatretic dominant follicles, and was inversely correlated with follicle plasmin activity. We suggest that PN-1 may be involved in the onset of atresia in nonovulatory dominant follicles. In periovulatory follicles, PN-1, PAI-1, tPA and uPA mRNA levels were transiently upregulated by hCG, and all decreased at the expected time of ovulation except for uPA which remains elevated. These data support a role for PN-1 in preventing precocious proteolysis in the granulosa cell layer before ovulation.

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Table I Summary on RT-PCR protocol in Expt 1 & 2.

Gene	Primers	Size	Annealing temperature		Cycles		Reference
		(bp)	Expt 1	Expt 2	Expt1	Expt2	
PN-1	S: 5'-TCCGTGACGTTGCCCTCTGTG-3'	555	64°C	62°C	17	24	(Bédard *et al.*
	AS: 5'-CCGTGATCTCCACAAACCCTT-3'						2003)
PAI-1	S: 5'-GAACAAGGATGAGATCAGCACAGC-3'	696	64°C	62°C	22	30	
	AS: 5'-GACACGTACAGAAACTCTTGATCTG-3'						NM_174137
tPA	S: 5'-AAGGTTGCAGAAGAAGATGG-3'	479	56°C	55°C	20	26	(Macchione *et al.*
	AS: 5'-GTGAGGCGGGTACCTCTCCTGGAA-3'						2000)
uPA	S: 5'-GTCTGGTGAATCGAACTGTGGC-3'	511	58°C	65°C	25	30	(Balcerzak *et al.*
	AS: 5'-GGCTGCAAACCAAGGCTG-3'						2001)
GAP	S: 5'-TGTTCCAGTATGATTCCACCCACG-3'	600	64°C		21		(Fayad *et al.*
	AS: 5'-CTGTTGAAGTCGCAGGAGACAACC-3'						2004)
H2a	S: 5'-GTCGTGGCAAGCAAGGAG -3'	182		55°C		30	(Robert *et al.*
	AS: 5'-GATCTCGGCCGTTAGGTACTC -3'						2002)
GAP	S: 5`-TGTTCCAGTATGATTCCACC-3`	860		55°C		26	(Tsai *et al.* 1996)
	AS: 5`-TCCACCACCCTGTTGCTG-3`						

Table II. Oestradiol and progesterone concentrations, and mean diameters of follicles in nonatretic, early-atretic and atretic follicles in Experiment 2.

Follicle class	**Diameter** mm (range)	*Oestradiol* ng/ml (range)	*Progesterone* ng/ml (range)
Nonatretic	11.6±0.8 [a]	1392±687 [a]	33±2 [a]
	(10 - 14)	(396 - 4084)	(28 – 38)
Early-atretic	9.7±0.6 [a]	1.3±0.6 [b]	34±6 [a]
	(9 - 13)	(0.6 – 4.6)	(18 – 63)
Atretic	10.0±0.6 [a]	13±8 [b]	207±86 [b]
	(9 - 11)	(0.4–31.0)	(106 – 465)

Table III Mean (± SEM) diameter and follicular fluid steroid content of the dominant (F1) and the two largest subordinate follicles (F2, F3) collected early in a follicle wave, when the dominant follicle reached approximately 6.5, 7.5, 8.5 or 9.5 mm diameter (Expt 3).

Follicle stage (mm)	**Follicle rank**	**Diameter** (mm)	*Oestradiol* (ng/ml)†	*Progesterone* (ng/ml)
6.5	F1	6.5±0.1	114±33	29±6
	F2	6.1±0.3	68±17	30±8
	F3	5.6±0.3	40±18	61±41
7.5	F1	7.4±0.1	226±42 [a]	37±8
	F2	6.5±0.1	127±51 [ab]	29±6
	F3	5.7±0.2	32±6 [b]	23±5
8.5	F1	8.2±0.5	423±124 [a]	30±6
	F2	6.8±0.6	168±68 [a]	26±4
	F3	6.4±0.6	14±10 [b]	60±28
9.5	F1	9.7±0.1 *	682±45 [a]	46±6
	F2	6.8±0.3	3±1 [b]	74±43
	F3	6.1±0.1	11±8 [b]	19±5

†Within follicle stage group, different superscripts denote differences between F1, F2 and F3 follicles ($P<0.05$). * F1 follicle was significantly differerent from F2 follicle.

Figure 1 Analysis of A) PN-1, B) PAI-1, C) uPA, and D) tPA mRNA expression in follicular wall lysates of preovulatory follicles by RT-PCR (experiment 1). Total RNA was extracted from bovine preovulatory follicle walls collected at 0, 6, 12, 18, and 24 hours after hCG injection, and was employed in mRNA expression analyses as described in *Materials and Methods*. GAPDH was used as a control gene, and showed no significant difference in expression levels between samples (shown in panel A). Data are least-squares means (relative units) ± SEM of two animals. Different letters denote means that are significantly different ($P<0.05$).

Fig 1

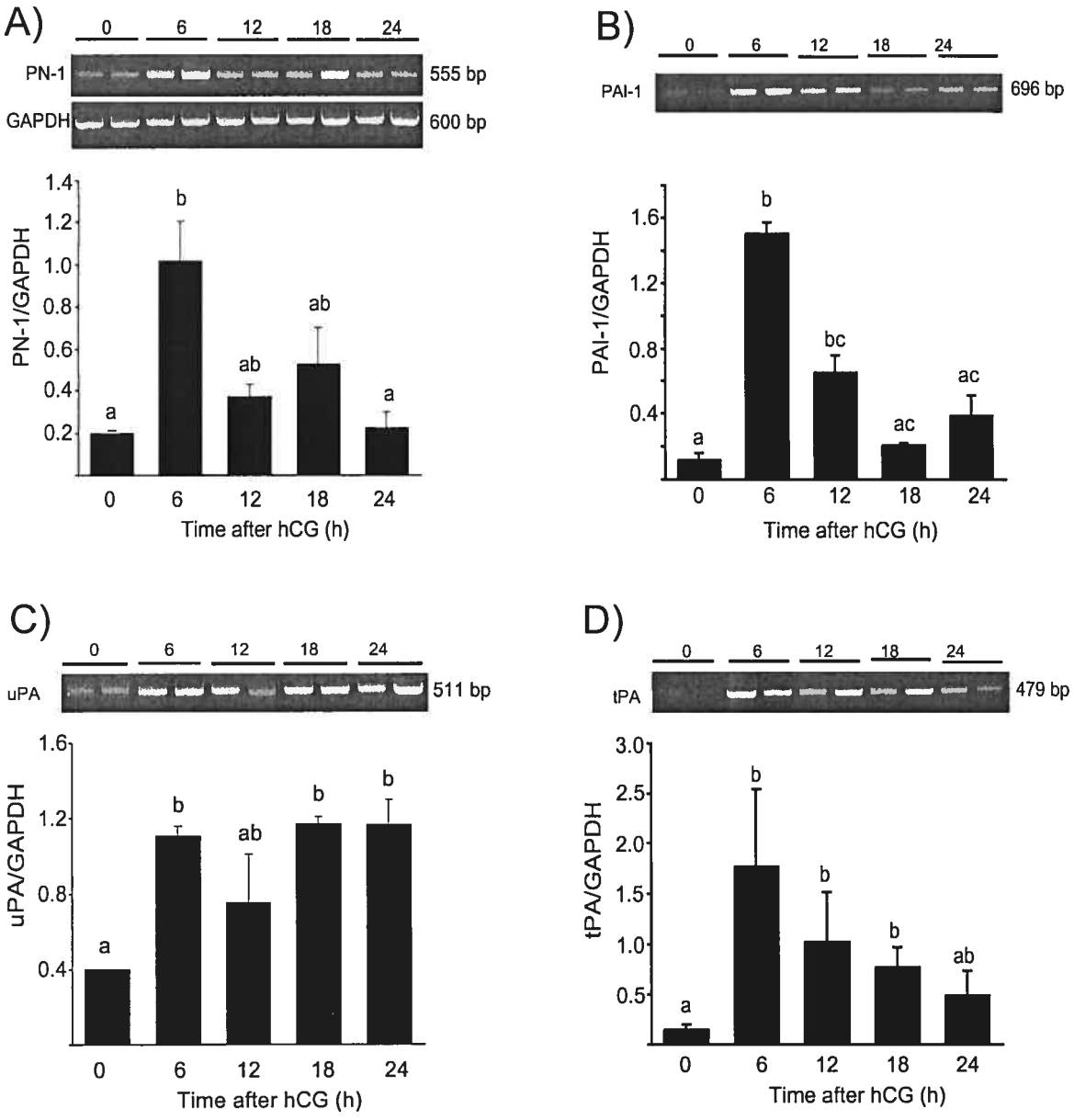

A)
0
6
12
18
24
PN-1
555 bp
GAPDH
600 bp
PN-1/GAPDH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
a
b
ab
ab
a
0
6
12
18
24
Time after hCG (h)
B)
0
6
12
18
24
PAI-1
696 bp
PAI-1/GAPDH
1.6
1.2
0.8
0.4
a
b
bc
ac
ac
0
6
12
18
24
Time after hCG (h)
C)
0
6
12
18
24
uPA
511 bp
uPA/GAPDH
1.6
1.2
0.8
0.4
a
b
ab
b
b
0
6
12
18
24
Time after hCG (h)
D)
0
6
12
18
24
tPA
479 bp
tPA/GAPDH
3.0
2.5
2.0
1.5
1.0
0.5
a
b
b
b
ab
0
6
12
18
24
Time after hCG (h)

Figure 2 Analysis of A) PN-1 protein content and B) proteolytic enzyme activities in granulosa cell lysates from periovulatory bovine follicles at 0, 12, and 24 hours after hCG (experiment 1). For PN-1, samples (50μg cell protein) were subjected to SDS-PAGE followed by blotting with an antibody raised against bovine PN-1. PA activity was measured in aliquots of 30μg cell protein by casein zymography. The zymograph presented in panel B shows lytic zones produced by human recombinant uPA standard (uPA) and samples collected at 0, 12 and 24 h after hCG injection. Location of plasmin, tPA and uPA activities are indicated to the right of the zymograph. Different contrast and brightness settings were used for uPA and plasmin image capture, and although the data are plotted on the same axis, uPA activity was significantly weaker than plasmin activity. Data are least-squares mean densitometry units ± SEM of two animals. For each enzyme, different letters denote means that are significantly different ($P<0.05$).

Fig 2

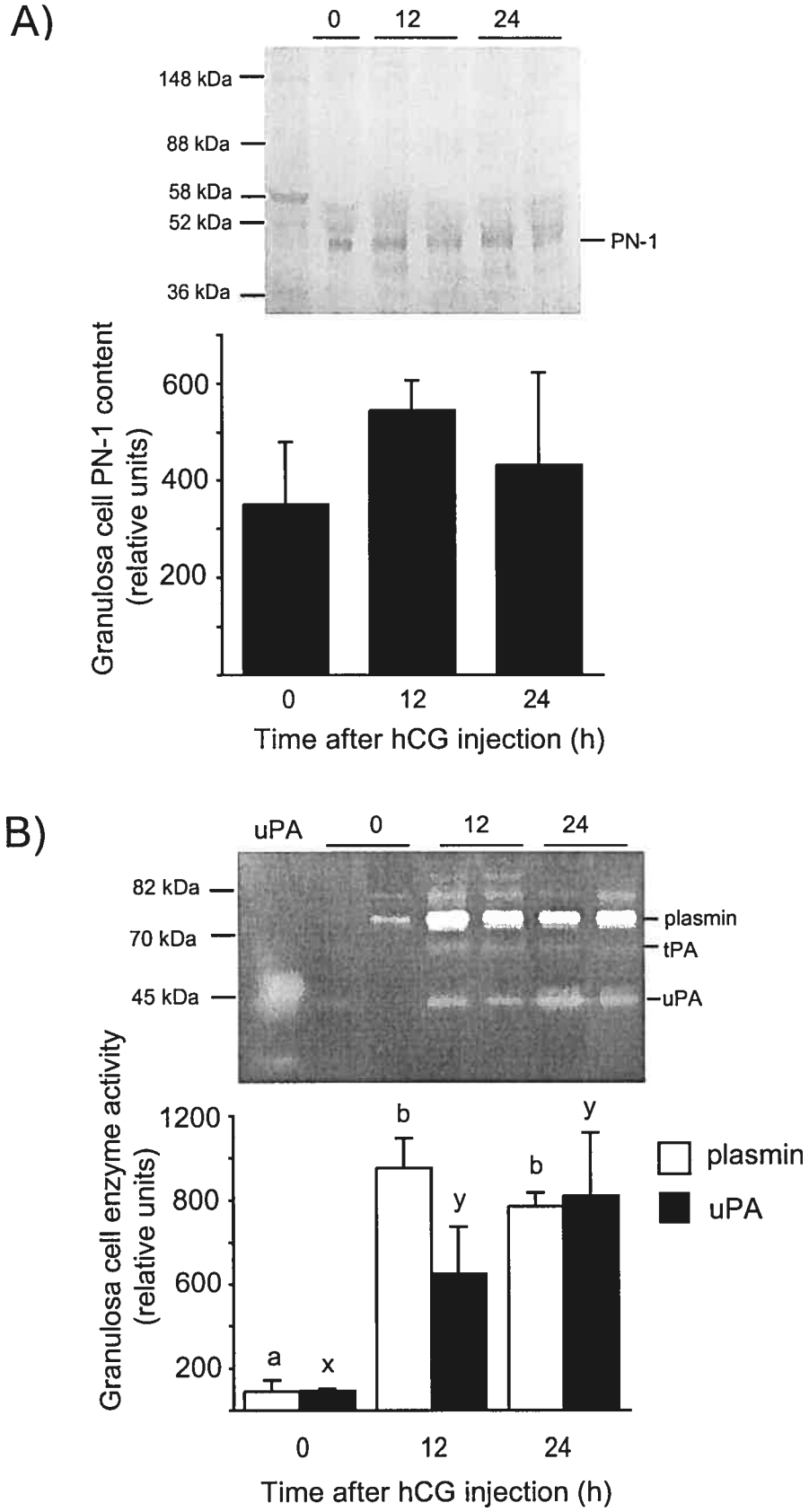

A)
0
12
24
148 kDa
88 kDa
58 kDa
52 kDa
36 kDa
PN-1
Granulosa cell PN-1 content (relative units)
600
400
200
0
12
24
Time after hCG injection (h)
B)
uPA
0
12
24
82 kDa
70 kDa
45 kDa
plasmin
tPA
uPA
Granulosa cell enzyme activity (relative units)
1200
800
600
200
a
x
b
y
b
y
plasmin
uPA
0
12
24
Time after hCG injection (h)

Figure 3 Analysis of A) PN-1 protein content and B) proteolytic enzyme activity in follicle fluid collected from periovulatory follicles at 0, 6, 12, 18, and 24 hours after hCG (experiment 1). The representative Western blot (for PN-1) and composite zymograph (for uPA and plasmin activity) presented show data for one animal at each time point, plus the control (Ctrl) sample (follicle fluid) used in Western blotting. Image capture parameters for plasmin and uPA activity were different owing to the significantly weaker uPA activity. Results shown represent least-squares means ± SEM densitometry units of two animals. For each enzyme, bars with different letters are significantly different ($P<0.05$); * mean different from time 0 ($P<0.05$).

Fig 3

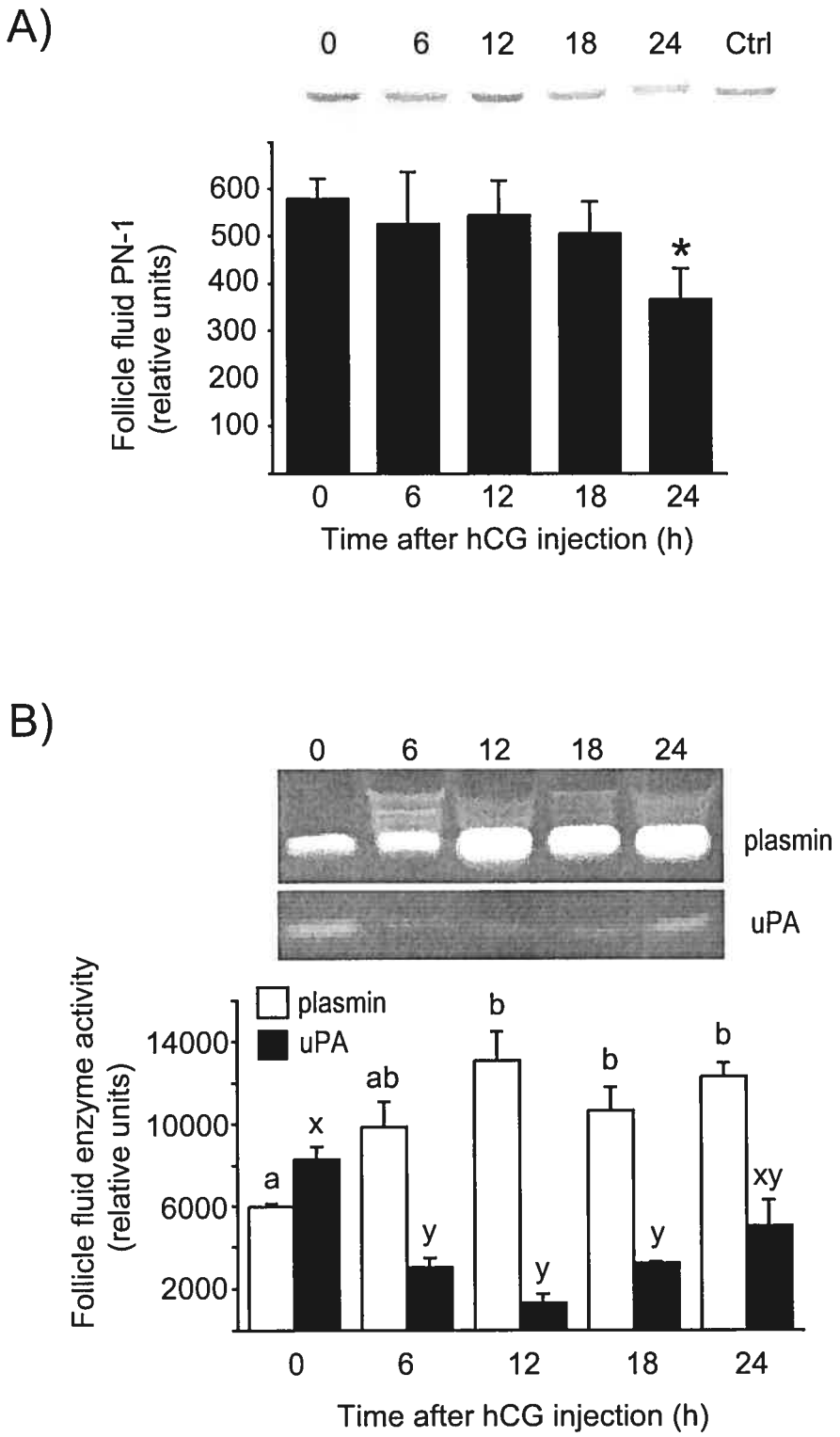

A)
0
6
12
18
24
Ctrl
Follicle fluid PN-1 (relative units)
600
500
400
300
200
100
*
0
6
12
18
24
Time after hCG injection (h)
B)
0
6
12
18
24
plasmin
uPA
plasmin
uPA
Follicle fluid enzyme activity (relative units)
14000
10000
6000
2000
a
x
ab
y
b
y
b
y
b
xy
0
6
12
18
24
Time after hCG injection (h)

Figure 4 Plasmin and uPA enzyme activities (A) and PN-1 protein (B) in follicle fluid from nonatretic (n=7), early-atretic (n=7) and atretic (n=4) follicles (experiment 2). Follicle classification is described in *Materials and Methods*. Inserts are zymographs (A) and Western blots (B) showing raw data for all follicles. Lanes marked 'C' are control samples used to correct for variation between gels. Data are least-squares means (relative units) ± SEM. Bars with different letters are significantly different ($P<0.05$).

Fig 4

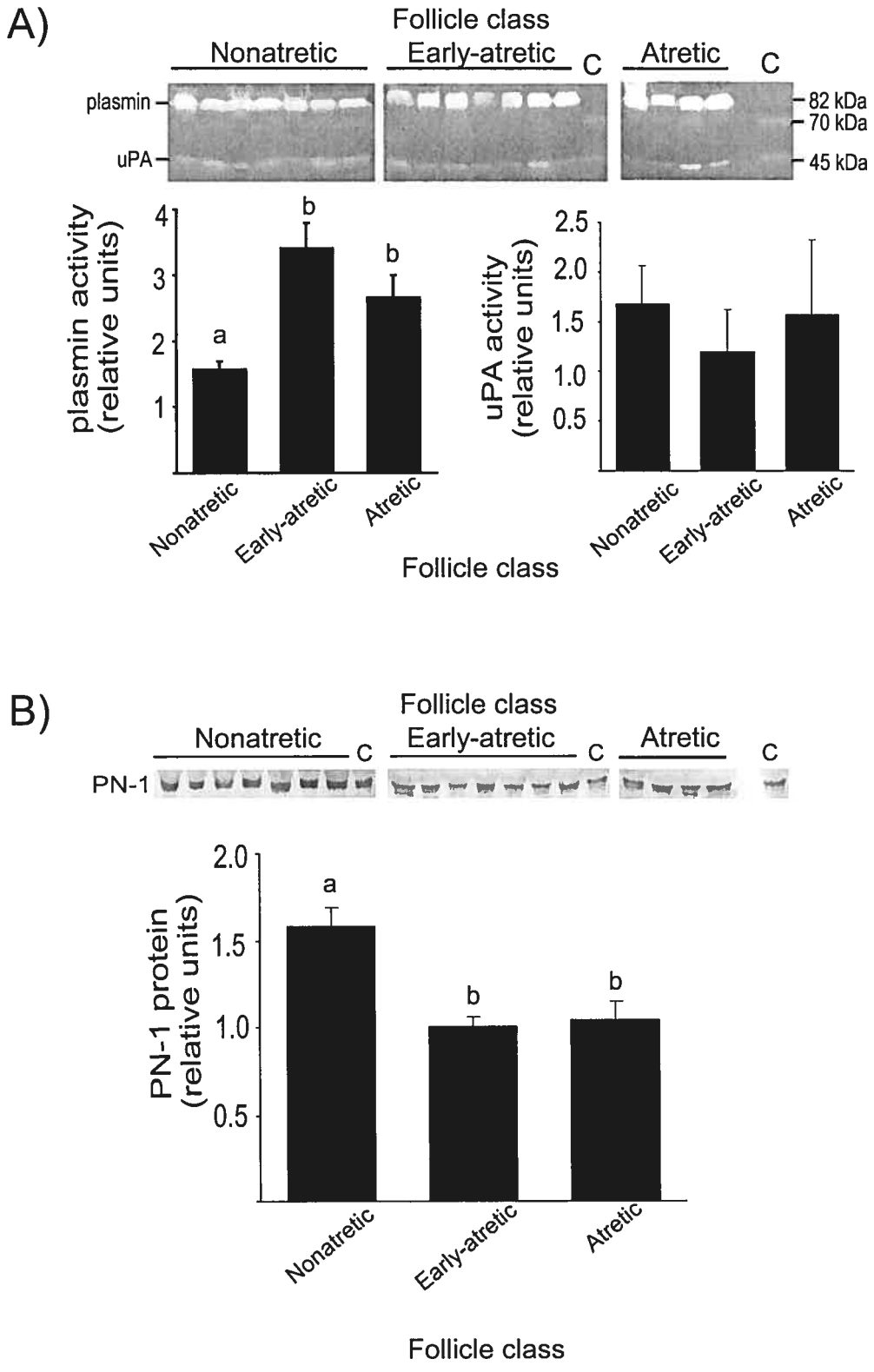
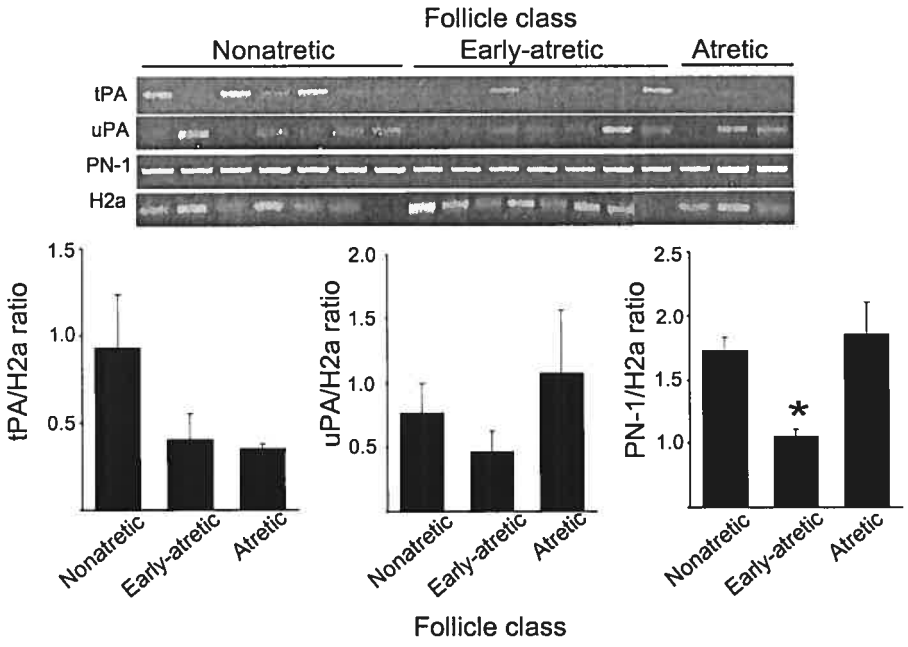

A)
Follicle class
Nonatretic
Early-atretic
C
Atretic
C
plasmin
uPA
82 kDa
70 kDa
45 kDa
plasmin activity (relative units)
uPA activity (relative units)
a
b
b
Nonatretic
Early-atretic
Atretic
Follicle class
B)
Follicle class
Nonatretic
C
Early-atretic
C
Atretic
C
PN-1
PN-1 protein (relative units)
a
b
b
Nonatretic
Early-atretic
Atretic
Follicle class

Figure 5 RT-PCR analysis of mRNA expression of PA and PA inhibitors in (A) granulosa and (B) theca cells from nonatretic (n=7), early-atretic (n=7) and atretic (n=4) follicles (experiment 2). Follicle classification is described in *Materials and Methods*. Inserts are composite images of agarose gels showing all samples. Data are least-squares means (relative units) ± SEM. Group means that differed are indicated by an asterisk ($P<0.05$).

Fig 5

A) Granulosa cells



B) Theca cells

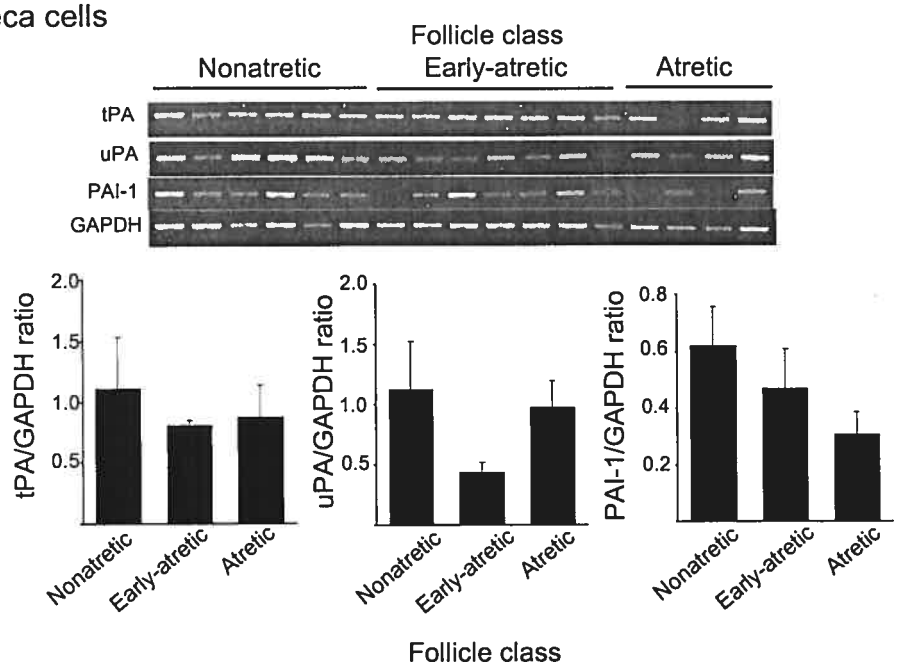
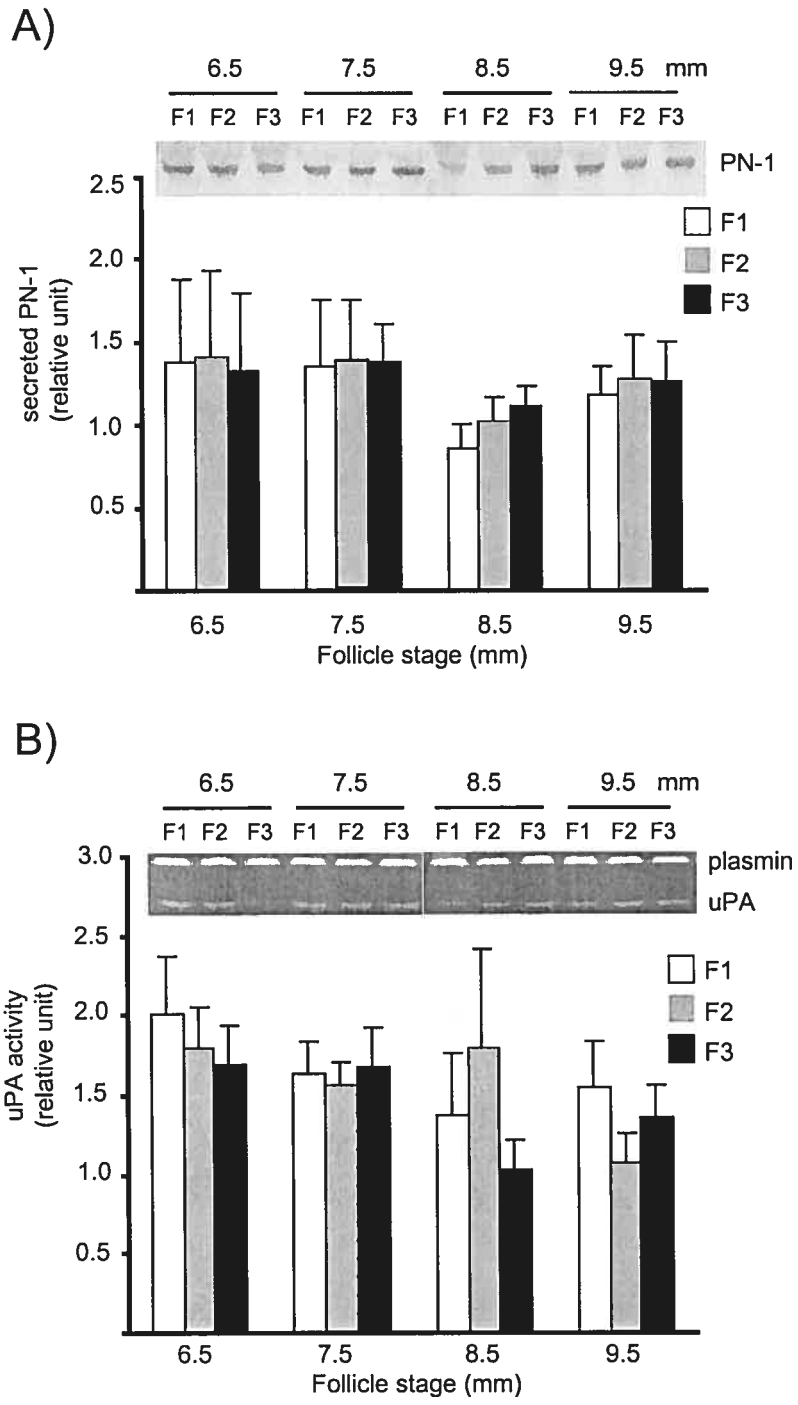


Figure 6 PN-1 protein (A) and uPA enzyme activity (B) in follicular fluid samples collected from growing dominant (F1) and subordinate (F2, F3) follicles during the first follicle wave (experiment 3). Follicle fluid was collected by ultrasound-guided aspiration when the dominant follicle reached approximately 6.5, 7.5, 8.5 and 9.5 mm diameter (follicle stage). Inserts are representative Western blots (A) and zymographs (B). Data are least-squares means (relative units) ± SEM of seven animals at each follicle stage.

Fig 6


A)
6.5
7.5
8.5
9.5 mm
F1 F2 F3 F1 F2 F3 F1 F2 F3 F1 F2 F3
PN-1
F1
F2
F3
secreted PN-1 (relative unit)
2.5
2.0
1.5
1.0
0.5
6.5
7.5
8.5
9.5
Follicle stage (mm)
B)
6.5
7.5
8.5
9.5 mm
F1 F2 F3 F1 F2 F3 F1 F2 F3 F1 F2 F3
plasmin
uPA
F1
F2
F3
uPA activity (relative unit)
3.0
2.5
2.0
1.5
1.0
0.5
6.5
7.5
8.5
9.5
Follicle stage (mm)

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GENERAL DISCUSSION

Interaction of PN-1 with ECM components: biological role of PN-1

Together with the literature, the present study suggests that PN-1 may contribute to follicle development through modulating proteolytic enzyme activity during follicle growth. Furthermore, as PN-1 is a broad-spectrum serine protease inhibitor of tPA, uPA, plasmin, trypsin and thrombin, it may be an important specific regulator of proteases activities in the peri-cellular environment, although the biological role of PN-1 has not yet been defined.

Previous studies show that PN-1 can be regulated in terms of activity, specificity and localization by ECM associated proteins. Secreted PN-1 binds tightly to proteins present in ECM such as collagen IV and heparan sulfate (Donovan *et al.*, 1994), which regulate both its activity and its target protease specificity. At the cell surface, PN-1 forms SDS-stable equimolecular complexes with its target proteases (Baker *et al.*, 1980). Once formed, the complexes are rapidly internalized and degraded (Low *et al.*, 1981). In this study, multiple bands (see Appendix) are immunostained with bovine PN-1 antibody in GC lysates from small follicles and large growing follicles by Western analysis, whereas a single band (approximately 47 kDa, secreted form) is detected in GC lysates from preovulatory follicles (Fig 2, Study 3). Multiple bands likely refer to different cell-associated forms of PN-1, corresponding to the complex of PN-1 and its substrate (148 kDa), putative degradation products of this complex (88 kDa, 58 kDa and 52 kDa), secreted PN-1 (45 kDa) and a possible degraded protein (36 kDa). We speculate that hCG may affect PN-1 redistribution within cells. The intracellular localization of PN-1 needs to be further examined.

Donovan et al. report that PN-1 inhibits thrombin even when bound to collagen IV, but has less inhibitory effect on uPA and plasmin (Donovan *et al.*, 1994). However, Crisp et al.

find that PN-1 remains a potent uPA inhibitor in the presence of collagen IV (Crisp *et al.*, 2002), and suggest that the disagreement between studies results from the PN-1 purification protocol (Donovan *et al.*, 1994), by which PN-1 is eluted from a monoclonal antibody column where exposed to acidic conditions, that affects PN-1 allosteric interaction with collagen IV, and leads to alter protease specificity.

Moreover, PN-1 has a heparin-binding site, heparin greatly accelerates the rate of linkage between soluble PN-1 and its substrate thrombin (Baker *et al.*, 1980). Binding of PN-1 to heparin-like GAG accelerates its interaction with thrombin by 1000-fold, making PN-1 a more efficient inhibitor of thrombin than uPA and plasmin (Crisp *et al.*, 2002). Recently, thrombin has been shown to down-regulate PN-1 expression in a dose- and time-dependent manner in rat aortic smooth muscle cells, and this effect is mediated via the interaction of thrombin with its receptor protease activated receptor (PAR-1) (Richard *et al.*, 2004).

In addition, bovine GC possess thrombin receptor (PAR-1) mRNA, and cells from small follicles possess more PAR-1 mRNA than cells from large follicles (Roach *et al.*, 2002). Thrombin induces cellular responses in both vascular and avascular tissues, therefore, a functional thrombin system has been suggested in bovine ovarian follicle. The high and co-localized expression of PN-1 and PAR-1 in GC of healthy growing follicles suggest that PN-1 may be a modulator of ovarian FF coagulation cascade (Gentry *et al.*, 2000) via inhibiting thrombin activity, together with PAR-1.

Cell culture model

The serum-free estrogenic GC culture model developed in cattle (Gutiérrez *et al.*, 1997) provides us a tool to study GC proliferation & differentiation, and hormonal regulation of cell

function as well. FSH and insulin are essential for inducing and maintaining cell viability and estradiol production in this culture system, and IGF-I also induces/ stimulates cell proliferation and estradiol production (Gutiérrez *et al.*, 1997). FSH in the presence of insulin stimulates estradiol production and cell proliferation in a dose-dependent manner as previously observed (Gutiérrez *et al.*, 1997; Glister *et al.*, 2001). Furthermore, the induction of cellular differentiation and re-establishment of cell-cell communication (cell 'clump' formation) in culture is obtained by increased FSHr and type I IGF receptor in the presence of FSH and IGF-I (Marsters *et al.*, 2003). Based on this understanding, we test FSH and LR3-IGF-I dose responses separately in GC from small antral follicles. However, in the present study, the effect of FSH on estradiol secretion does not follow the biphasic pattern previously described (Gutiérrez *et al.*, 1997; Silva & Price, 2000; Glister *et al.*, 2001). Gutiérrez et al. find that 1ng/ml FSH stimulates maximal estradiol production whereas 10ng/ml FSH stimulates maximal cell proliferation (Gutiérrez *et al.*, 1997). Glister et al. report that 0.33ng/ml FSH stimulates maximal estradiol production and that 3ng/ml FSH stimulates maximal cell proliferation (Glister *et al.*, 2001). In the present study, 10ng/ml stimulates cell proliferation, whereas maximal estradiol secretion occurs with a high dose of FSH. Obviously, the different culture conditions result in these differences. One possible explanation is that different cell densities are used. Cell density plating in culture is suggested to be a critical determinant for cell response (Marsters *et al.*, 2003). We plate 1 million viable cells in 1 ml media per well in 24-well plate, with a density of around 500×10^3 cells per cm^2, which is more than double of that used ($50\text{-}75 \times 10^3$ cells in 250µl media per well in 96-well plate) in the previous studies (Gutiérrez *et al.*, 1997; Glister *et al.*, 2001). Androstenedione, a precursor of estradiol, is suggested to increase estradiol accumulation in cultured bovine GC (Hamel *et al.*, 2005). Therefore, another possible reason is that we increase androstenedione in culture medium 10-

fold from Day 2 of culture compared to the previous studies (Gutiérrez *et al.*, 1997; Glister *et al.*, 2001). In terms of cell proliferation, different methodologies are used to measure cell growth (DNA synthesis in present study vs. cell numbers in previous studies). Progesterone is also stimulated by FSH in the present study and Glister's study, whereas Gutiérrez and colleagues do not show their progesterone data (Gutiérrez *et al.*, 1997).

Do GC in culture need ECM proteins?

The ECM provides a structural tissue support, and forms barriers between tissue compartments. Adhesion to ECM regulates the survival, proliferation and differentiation of numerous cell types in many tissues (Streuli & Gilmore, 1999). In the ovary, changes in ECM that occurs during follicle growth may in consequence alter the amount and/or type of protease activity secreted and granulosa steroidogenesis. In vitro studies have previously shown that ECM can modulate GC function, for example, ECM stimulates bovine GC proliferation and progesterone secretion in response to FSH (Savion *et al.*, 1981). Bovine GC in culture can also synthesize a basal lamina, which at least contains collagen IV and fibronectin (Rodgers *et al.*, 1995; Rodgers *et al.*, 1996), providing a possible model to study the origin of ECM proteins and as well as the interaction of ECM proteins and GC function.

It has been shown that collagen type I levels within the GC layer increase significantly during follicle development in sheep (Huet *et al.*, 1997) and that estradiol secretion from ovine GC of large follicles is diminished when cultured in the absence of collagen (Huet *et al.*, 2001). Thus, the cells of large follicles in the present study may have an increased requirement for collagen type I which is not met by the culture conditions, resulting in reduced estradiol (but not progesterone) secretion (present study). The cellular response to this collagen deprivation

may therefore be a reduction in the normally high secretion of PN-1, in order to increase extracellular protease activity and alter the local ECM structure.

The addition of heparin to cultured GC reduces cell proliferation and progesterone secretion but enhances estradiol secretion in sheep (Huet *et al.*, 2001). However, it remains to be determined whether the change in GC function resulting from heparin treatment is directed by the change in cell shape, or involves other mechanisms. One possible explanation is that the addition of excess heparin to the cultured cells likely disturbs the action of endogenous heparin-binding growth factors such as FGF-2, which inhibits estradiol production by regulating its bioavailability (Ruoslahti & Yamaguchi, 1991).

Although the role of growth factors in regulating the ovarian cell proliferation and differentiation has been extensively examined, the mechanisms controlling the bioavailability and activity of growth factors are less well understood. Growth factors are often secreted and sequestered in the ECM in an inactive form or in associated with their specific binding proteins, and can be subsequently released by proteolysis of ECM (Flaumenhaft & Rifkin, 1992; Logan & Hill, 1992). MMPs are shown to release bioactive FGF molecules bound to HSPGs in the ECM (Tamura *et al.*, 1996). It is of interest to test if PN-1 and PAs regulate the bioavailability and activity of growth factors, including FGF-2 and IGF-I.

Collectively, ECM proteins influence basic cellular process such as proliferation, differentiation, migration and adhesion, they are involved in the control of ovarian follicular development, and modulate interactions between growing follicles and surrounding connective tissue.

Growth factor & follicle growth: BMP-7 as an example

Antral follicle growth is under gonadotropic control (Campbell *et al.*, 2003). A number of locally produced growth factors are known to modulate follicle development. These growth factors include the IGF system, the TGFβ superfamily including BMPs, the FGF family, and EGF/TGFα. In cattle, growth of follicles from 2 mm to approximately 5 mm in diameter (recruitment) and above is characterized by induction of mRNA expression for P450scc and P450arom in GC, and around the time of selection of the dominant follicles (approximately 8-9 mm in diameter) LHr and 3β-HSD mRNA expressions can be detected in GC (Bao & Garverick, 1998). In the present study, we do not examine steroidogenic enzyme mRNA and protein expression, but measure steroid secretion and cell proliferation instead. We also report that the proteolytic enzymes, the PAs and their specific inhibitor PN-1 are differentially regulated by FSH, IGF-I, BMP-7, FGF-2 and EGF. Overall, these results indicate that intraovarian factors, acting in concert with FSH, play a role in follicular growth. However, the exact mechanisms through which these factors operate and degree of redundancy and/ or compensation need to be elucidated in the future.

The ability of growth factors to influence PA activity in ovarian cells is poorly documented. Here I present BMP-7 as an example to discuss the functional link between a growth factor and follicle development. A previous study shows that BMP-7 can promote the recruitment of primordial follicles into the growing follicle pool in rats (Lee *et al.*, 2001). BMP-7 derived from adjacent larger follicles may stimulate this transition through enhancing GC mitosis, as rat primordial follicles do not express BMP-7. Theca-interstitial cells of secondary follicles start to express BMP-7, in particular those cells near the basal lamina of the growing antral and dominant follicles (Erickson & Shimasaki, 2003; Glister *et al.*, 2004). Granulosa cells of this stage of follicle express BMP type II receptor (BMPR-II) and activin

type II receptor (ActR-II) required for BMP-7 action on GC (Shimasaki *et al.*, 1999; Souza *et al.*, 2002; Glister *et al.*, 2004). Therefore, a functional BMP system is suggested during follicle development. We report here that BMP-7 stimulated tPA and PN-1 secretion in GCs, providing a new insight on the functional role of the intrinsic ovarian BMP system. Together with others, we speculate that BMP-7 might be a stimulator of follicle growth, at least in part, through increasing tPA and PN-1 secretion by GC.

A working model: PN-1 & PA in ovulation and follicle growth

Ovulation is associated with a number of spatially and temporally expressed genes including specific proteases that degrade the follicle wall (Robker *et al.*, 2000a). Over the years, several lines of indirect evidence suggest that both MMPs and the PA system are important for generating the proteolytic activity required for ovulation (Curry & Osteen, 2003). However, functional studies using KO mice suggest the PA system is less important for ovulation (Carmeliet *et al.*, 1994; Leonardsson *et al.*, 1995; Murer *et al.*, 2001). Interestingly, the PA system is proposed to activate pro-MMPs at the cell surface (Murphy *et al.*, 1999a). The activation of MMPs can be regulated by a balance between serine proteases such as uPA and the PAIs (Nagase & Woessner, 1999; Murphy *et al.*, 1999b). The coordinated expression of tPA and PAI-1 is documented in preovulatory follicles in rats and monkey (Liu *et al.*, 1987b; Shen *et al.*, 1997; Liu *et al.*, 2004), but not in cattle (Dow *et al.*, 2002a; Dow *et al.*, 2002b). Results in the present study indicate that PAI-1 and PN-1, tPA and uPA may participate in proteolysis and subsequent follicular wall degradation during ovulation in cattle.

Ovulation, like other acute inflammatory reactions, is complicated by paradoxical metabolic process that simultaneously causes tissue damage and repair. Espey and Richards (2002) consider that protein products of genes expressed in granulosa layer might be

responsible primarily for destruction of the follicle wall, whereas the products of genes transcribed in the theca tissue and ovarian stroma might be more relevant to the healing process (Espey & Richards, 2002). Our data, together with others (Hägglund *et al.*, 1996; Hasan *et al.*, 2002; Bédard *et al.*, 2003) challenge this consideration, as granulosa PN-1 and theca PAI-1 provide a "two-layer protection system". The "protection concept" is also supported by the statement that protease inhibitor such as PAI-1, expressed in theca cells possibly act as a protective shield to ensure that toxic levels of compounds do not reach the GC or the oocyte at an inopportune time (Richards *et al.*, 2002).

Tissue remodeling is also important for the growth and development of small follicle. In nature, there must be interactions between two PAs and two PAIs to mediate proteolysis that tissue remodeling requires during follicular development. Stimulation of plaminogen activators enzymatic activity results in an increase in proteolysis and subsequent ECM degradation, whereas stimulation of plaminogen activator inhibitors result in localizing or limiting the region where proteolysis take place; as plasminogen is present in all body fluids, plasminogen activation needs to be restricted. In contrast, inhibition of PAIs facilitates proteolysis. The role of PA and PN-1 in follicle growth is unknown. In the present study, increasing tPA activity may facilitate proteolysis, whereas increasing PN-1 in non-luteinizing GC may prevent unrestrained tissue degradation and localize the proteolysis to the follicular basement membrane that are required for follicle growth.

Furthermore, there is a switch from uPA to tPA activity during follicle growth (Karakji & Tsang, 1995a), suggesting uPA and tPA may differently contribute to follicle development. If we compare the roles of the PA system in follicles and with that in CL, we find that uPA mainly involves in tissue remodelling (a chronic and mild process) such as follicular growth or CL formation, whereas tPA mainly involves in tissue degradation (a transient but vigorous

process) such as ovulation or CL regression. This concept is supported by the findings in the present study, in which GC uPA content decrease as follicles develop (study 1), and uPA is present in growing follicles and decrease after hCG injection (study 3). Although follicular development is complex, to simplify this question, we hypothesize that two PAs (tPA and uPA), two PAIs (PAI-1 and PN-1), two cell types (granulosa and theca cells), and two partnerships (uPA regulated by PN-1 and tPA regulated by PAI-1) are systemically involved in this process. The working models on the PA system during follicular growth and ovulation are illustrated in Fig 1 and Fig 2, respectively.

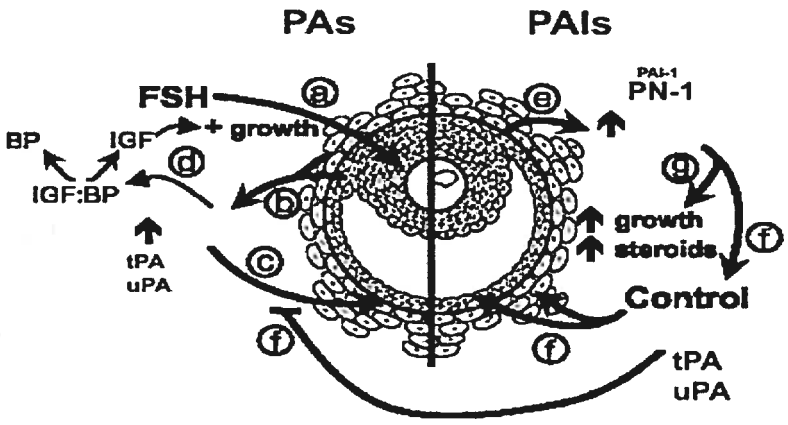

Fig 1. A working model for the PA system in follicular growth.

FSH would stimulate GC proliferation and estradiol secretion (path a), which in turn stimulate tPA & uPA secretion (path b), and regulate the expression of PN-1 in GC and PAI-1 in thecal cells (path e). The PA activities would facilate remodeling of the thecal ECM and expansion of the basement membrane as the GC proliferate (path c). In addition to directly control ECM remodeling, the PA system may act to regulate the bioavailability of growth factors such as IGFs, released from IGFBPs degradation (path d) to further stimulate follicular growth. Whereas PN-1 in GC and PAI-1 in theca cells provide control for the extent PA action (path f). Possibly, changes in PN-1 expression may be correlated with cell proliferation or steroidgenesis during follicular growth (path g). Note GC PN-1 and theca PAI-1 may provide a "two-layers" protection system in follicle growth.

(Modified from the illustration on MMP system by
Curry & Osteen, 2003. Endocrine Reviews, 24: P442)

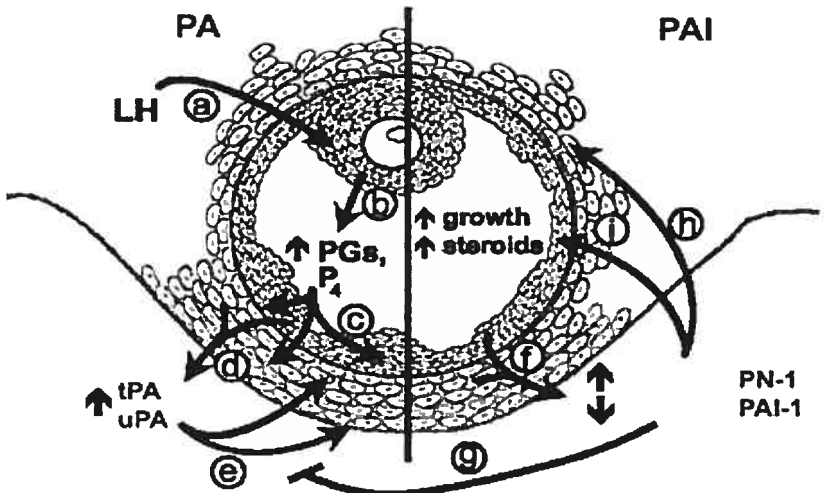

Fig 2. A working model for the PA system during ovulation.

LH initiates a series of biochemical events (path a) that stimulate progesterone (P4) and PGs (path b). These mediators induce tPA & uPA production (path d) in granulosa and theca (path c), and initially upregulate and followed an inhibition of the expression PN-1 in GC and PAI-1 in theca cells (path f). PA convert plasminogen to plasmin, its action would degrade the follicular apex (path e) allowing oocyte release. PN-1 and PAI-1 may act to control PA activity at the follicular apex (path g) while protecting the basal portion of the follicle (path h).

(Modified from the illustration on MMP system by
Curry & Osteen, 2003. Endorine Reviews, 24: P436)

FUTURE STUDY

The present study provides a number of new insights into our understanding of the physiological function of PN-1 during follicle growth and ovulation. Further studies need to be carried out in the following issues:

1) The molecular mechanism of FSH and growth factor action on PN-1 expression in GC;
2) Intracellular PN-1 localization and redistribution in GC from periovulatory follicles by immunocytochemistry (ICC) and/or immunohistochemistry (IHC); and/or PN-1 mRNA localization in these cells by in situ hybridization;
3) Improvement of cell culture model, such as adding oocytes in the current GC culture system to investigate the effect of crosstalk between oocyte and GC on PN-1 expression and secretion, as well as the effect of PN-1 on in vitro oocyte maturation. Previous studies demonstrated that oocytes secreted tPA (Liu *et al.*, 1986), and contained PN-1 in mice (Hägglund *et al.*, 1996).
4) Co-culture granulosa-theca to elucidate the interactions between them;
5) Use ECM components such as collagen I, IV, fibronectin, heparin, thrombin to study their interaction with PN-1.

GENERAL CONCLUSSION

We have demonstrated that secretion of tPA and PN-1 from GC and cell-associated uPA activity are regulated in a follicular stage dependent manner in cattle. There appear to be several differences between rodents and cattle in terms of PA secretion, but a common theme is a decrease in GC uPA content as follicles develop. During the first 2 days of culture, this decrease in cell-associated uPA activity occur as PN-1 secretion increased, suggesting a functional link between these two proteins during follicle development. As PN-1 is the only known PA inhibitor secreted by the GC layer, it may play an important role in tissue remodeling of this follicular compartment during early follicle growth.

The present study provides comprehensive evidence for the regulation by FSH and a number of growth factors on gene expression and secretion of members of the plasminogen activator system by bovine GC in vitro. Overall, FSH, IGF-I and BMP-7 had a stimulatory effect on cell proliferation, estradiol production, and PA and PN-1 secretion. In contrast, FGF-2 had an inhibitory effect on estradiol production, and on tPA and PN-1 secretion. EGF inhibited estradiol production, cell proliferation and PN-1 secretion but enhanced tPA secretion. These data support a role for these proteins in follicle development. The divergent effect of those hormones and growth factors on PA activity suggests mechanisms for precise control for ECM remodelling in proliferating and differentiating GC during follicular growth.

We report that the mRNA abundance of PN-1 was initially upregulated following an inhibition by hCG in follicular wall of bovine preovulatory follicles. Gene expression and PN-1 concentration in FF decreased in ovulatory follicles at the time of ovulation. In addition, PN-1 is expressed in GC throughout follicular development but is not a good marker for follicle deviation. Although PN-1 is not an essential factor in ovulation, as mice lacking this gene are

fertile or subfertile, GC PN-1 together with theca PAI-1 may provide two layers of protection against excessive plasmin formation during follicle growth and ovulation.

Collectively, the hormonal regulation of the PA system in GC is complex. The expression of PN-1 in bovine GC is developmentally and hormonally regulated during follicular growth and ovulation. As the estrogenic cultured GC mimics a "follicular" phenotype under the serum-free conditions, we conclude that FSH, IGF-I, and other growth factors examined in this study coordinately control net PA activity through a balance of stimulation or inhibition in GC, which subsequently modulate tissue remodeling during follicular growth and ECM degradation during ovulation.

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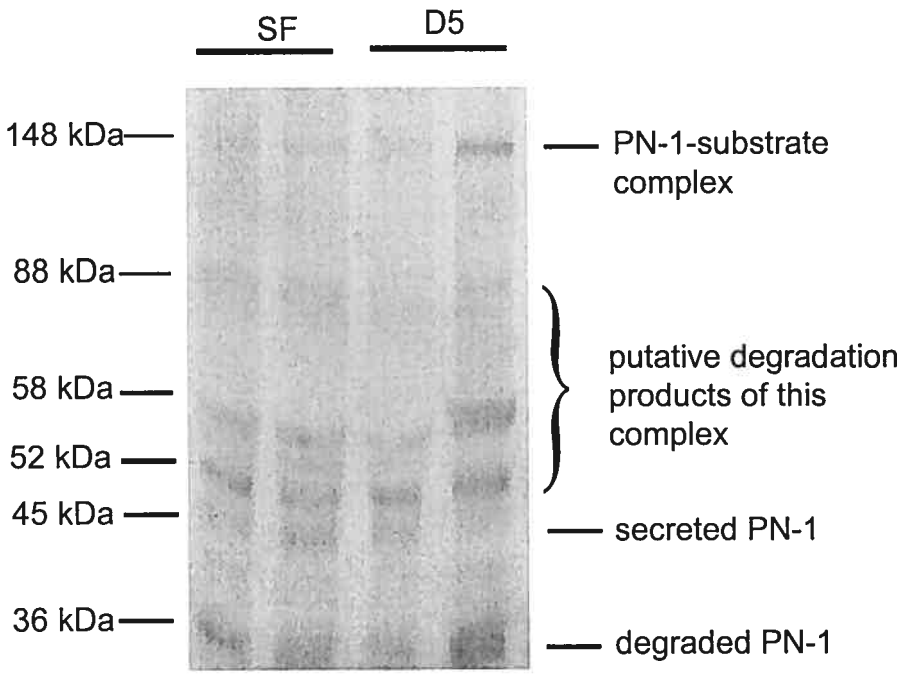
Appendix



Multiple bands are immunostainned with bovine PN-1 antibody in GC lysates from small and large growing follicles.

ACCORD ET PERMISSION DES COAUTEURS D'UN ARTICLE[1]

IDENTIFICATION DE L'ETUDIANT

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