CHANGES OF PLASMIN AND PLASMINOGEN ACTIVATORS IN LACTATION AND OVULATION

BY

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ABSTRACT

The role of plasmin and plasminogen activators (PA) in bovine lactation and porcine ovulation has been examined. There is no difference in the activation pattern of plasminogen to plasmin throughout the whole range of somatic cell counts (SCC) and from third to ninth month in lactation. The ratio of (plasminogen + plasmin)/plasmin, which serves as an index of the activation process, was 7.27 during early (first and second month) and 4.23 during late lactation (tenth month) and both values are different ($p<0.01$) from all the other ratios throughout the whole range of SCC and from third to ninth month in lactation suggesting limited and increased activation of plasminogen to plasmin during early and late lactation, respectively. Macrophages produce but they do not secrete urokinase-PA, suggesting a minor role in influencing milk plasmin. Somatotropin administration resulted in a suppression of milk plasmin in vivo. Insulin-like growth factor-1 (IGF-1), the most likely mediator of the effects of somatotropin on the bovine mammary gland, inhibited the induction of tissue-PA (t-PA) production which is observed when mammary epithelial cells are cultured in the absence of IGF-1. Plasmin

and t-PA increased while PA inhibitor-1 decreased in porcine granulosa, theca interna cells and follicular fluid just prior to the time of expected ovulation suggesting a role for plasmin in follicle rupture.

LES CHANGEMENTS DE LA PLASMINE ET DE L`ACTIVATEUR DU PLASMINOGENE AU COURS DE LA LACTATION ET DE L 'OVULATION.

Ioannis Politis **Ph. D.** **Animal Science**

RESUME

Les rôles de la plasmine et des activateurs du plasminogène dans le processus de lactation chez la vache et d`ovulation chez la truie ont été examiné. Il n'y a pas de différence dans le processus d'activation du plasminogène à la plasmine chez les cellules somatiques du troisième au neuvième mois de lactation. Le ratio plasminogène + plasmine / plasmine, qui sert d'indexe dans le precessus d'activation, était de 7.27 au début de la lactation (premier et deuxième mois) et de 4.23 vers la fin de la lactation (dixième mois). Ces deux valeurs étaient différentes ($P<.01$) de toutes les autres obtenues sur tout l'échantillonnage des cellules somatiques du troisième au neuvième mois de lactation. Ceci a suggéré que l'activation du plasminogène à la plasmine est limitée et augmentée durant le début et la fin de la période de lactation respectivement. Les macrophages synthétisent mais ne sécrètent pas l`urokinase, un activateur du plasminogène, ce qui suggère un rôle mineur dans l`action de la plasmine du lait. L`administration de la somatomédine chez la vache laitière a résulté en la suppression de la plasmine du lait in vivo. La somatomédine c, la plus importante médiatrice de l`effet lactogénique de la somatotropine, a supprimé la production de l`activateur tissulaire du plaminogène par les cellules

epithéliales de la glande mammaire observée lorsque les cellules mammaires épithéliales sont cultivées sans la somatomédine-c . La plasmine et l'activateur tissulaire du plasminogène ont augmenté tandis que l'inhibiteur de l'activateur du plasminogène a diminué dans les cellules de la granulosa, de la thèque interne et dans le fluide folliculaire porcin, juste avant la période d'ovulation, suggérant un rôle de la plasmine dans la rupture du follicule.

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1.GENERAL INTRODUCTION

Tissue remodelling is the hallmark of many physiological processes and involves the localized breakdown of extracellular matrix and a topographical rearrangement of cells. A positive relationship exists between tissue remodelling and protease production and it has been hypothesized that proteolytic activity is required for tissue remodelling to occur.

Plasminogen activators (PA) are serine proteases which convert the proenzyme plasminogen to active plasmin. Plasmin, in turn, can convert latent collagenase to the active enzyme. Recent evidence suggests that PA and collagenase are key enzymes in the cascade of reactions leading to tissue remodelling.

Involution of mammary gland caused by abrupt termination of lactation is the most dramatic example of tissue remodelling in the post-natal life. In mice, it has been estimated that 90% of the glandular weight is lost, within 2-3 days. Another example of tissue degradation is the dissolution of the several layers of the follicle wall during ovulation in order to expel the ovum. Even though several studies have proposed a role for PA during involution and ovulation in rodents, the importance of PA, its cellular source and hormonal regulation in bringing about these processes in farm animals like dairy cow or pigs is uncertain

and remains to be elucidated. The complexity of these processes makes them difficult to study. Success in dissecting the various participating factors is relevant to an understanding of the biochemical events involved and how the organism maintains an effective control of this proteolytic activity.

This research was undertaken:

1. to assess the importance of plasmin in terms of casein proteolysis in bovine milk during cold storage
2. identify factors affecting plasmin and plasminogen levels in bovine milk
3. determine cells within the bovine mammary gland which produce plasminogen, PA and PA inhibitors (PAI) and study some aspects of the hormonal regulation of plasmin production
4. determine the cellular source of porcine follicular PA and PAI and examine their role during the process of ovulation

2. REVIEW OF LITERATURE

2.1. Biochemistry of Plasmin/Plasminogen System

2.1.1. Plasminogen

Plasminogen (Pg), a protein ubiquitous in body fluids, is the precursor of the serine protease, plasmin (Pm). It occurs in plasma in a concentration of 2 μM and is primarily involved in the maintenance of vascular homeostasis (Dano et al., 1985). Approximately 40% of the plasminogen appears to be extravascular (Collen et al., 1972) and its presence has been demonstrated in a wide variety of locations such as milk (Kaminogawa et al., 1972), follicular fluid, yolk of hen eggs, human and porcine uterine fluids (Dano et al., 1985). The native form of human plasminogen consists of a single polypeptide chain with a molecular weight (MW) of 90,000 (Saksela, 1985). The N-terminal amino acid of this form of Pg is glutamic acid (Glu) and for this reason is termed Glu-Pg. Glu-Pg is converted to Lysine-Pg by proteolytic removal of the first 76 amino acids (Castellino and Powell, 1981). The two different pathways of plasminogen activation by the action of plasminogen activators (PA) are presented in Fig. 1. Removal of the peptide (Glu-X) by the action of plasmin greatly enhances the conversion of Lys-Pg to Lys-Pm. This activation occurs through the cleavage of an Arginine-Valine (560-561)

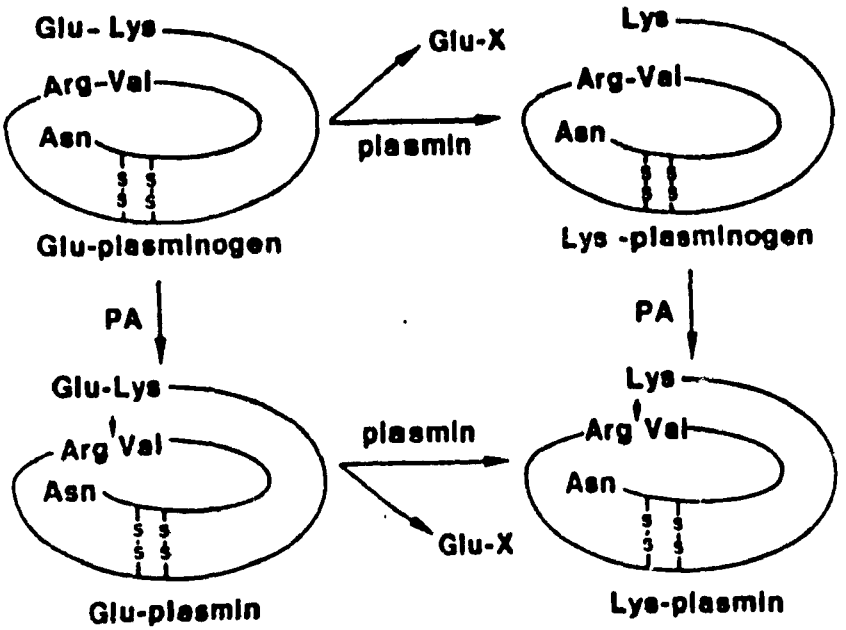


Figure 1 . Activation of plasminogen to plasmin. Proteolytic removal of the peptide (Glu-X) enhances the rate of plasminogen activation. (From Saksela, 1985)

peptide bond. Thus, the cleavage of two protease sensitive bonds are the principal events in the conversion of Pg to Pm. The activation of other mammalian plasminogens (rabbit, dog, bovine) takes place with a similar mechanism (Robbins et al., 1973).

The active protease plasmin consists of two polypeptide chains held together by disulfide bonds. The light chain with a MW of 27,500 contains the active site which consists of Aspartic acid, Histidine and Serine. These three amino acids constitute a "charge relay network" which functions as a proton shuttle during catalysis by plasmin (Murray et al., 1988). The mechanism of action of other serine proteases such as trypsin, chymotrypsin and thrombin is very similar. Plasmin has a trypsin-like specificity and hydrolyses proteins adjacent to Lysine- or Arginine-. Plasmin in milk hydrolyses α_s- and β- caseins while k-casein is probably resistant to proteolysis (Fox, 1981). Plasmin does not hydrolyse any of the whey proteins in milk while, in fact, β-lactoglobulin behaves as an inhibitor (Kaminogawa et al., 1972). No explanation was provided by these authors for this substrate specificity of plasmin.

2.1.2. Plasminogen Activators (PA)

The use of plasminogen activators for the dissolution of fibrin clots in myocardial infarction or deep vein thrombosis was the driving force for investigating their structural and

enzymatic properties. The presence of two forms of PA, termed tissue-PA (t-PA) and urokinase-PA (u-PA) has been demonstrated based on differences in primary structure (MW, isoelectric point) and antigenic nature.

Tissue-PA is a single polypeptide chain (527 amino acids) with a MW of 70,000 (Harris, 1987). In humans and rodents, t-PA has been identified in a wide variety of tissues including oviduct, pituitary, heart and ovary (Rickles et al., 1988) while in pigs t-PA is present in heart, kidney, and ovary (Dano et al., 1985). A cleavage site Arginine-Isoleucine (275-276) recognized by plasmin allows the formation of the two two-chain t-PA form (Fig. 2). Tissue-PA has a strong affinity for fibrin. This has been attributed to the presence of the two "kringles" and the "finger" regions of the molecule (Fig.2). Activation of plasminogen by t-PA in the presence of fibrin occurs through binding of Pg and PA to fibrin to form a ternary complex. Kinetic studies show that the catalytic efficiency of t-PA is enhanced in the presence of fibrin and this is mediated by a conformation change which decreases the k_m of t-PA for plasminogen (Zamarron et al., 1984).

Tissue-PA mRNA has been identified and characterized in humans (Pennica et al., 1983) and mouse (Rickles et al., 1988). Ny et al., (1984) and Degen et al., (1986) using a t-PA cDNA probe isolated fragment from human genome bearing the entire single gene for t-PA which contains 14 exons.

Urokinase-PA occurs in one polypeptide chain form, consisting of 411 amino acids. Larsson et al., (1984) detected

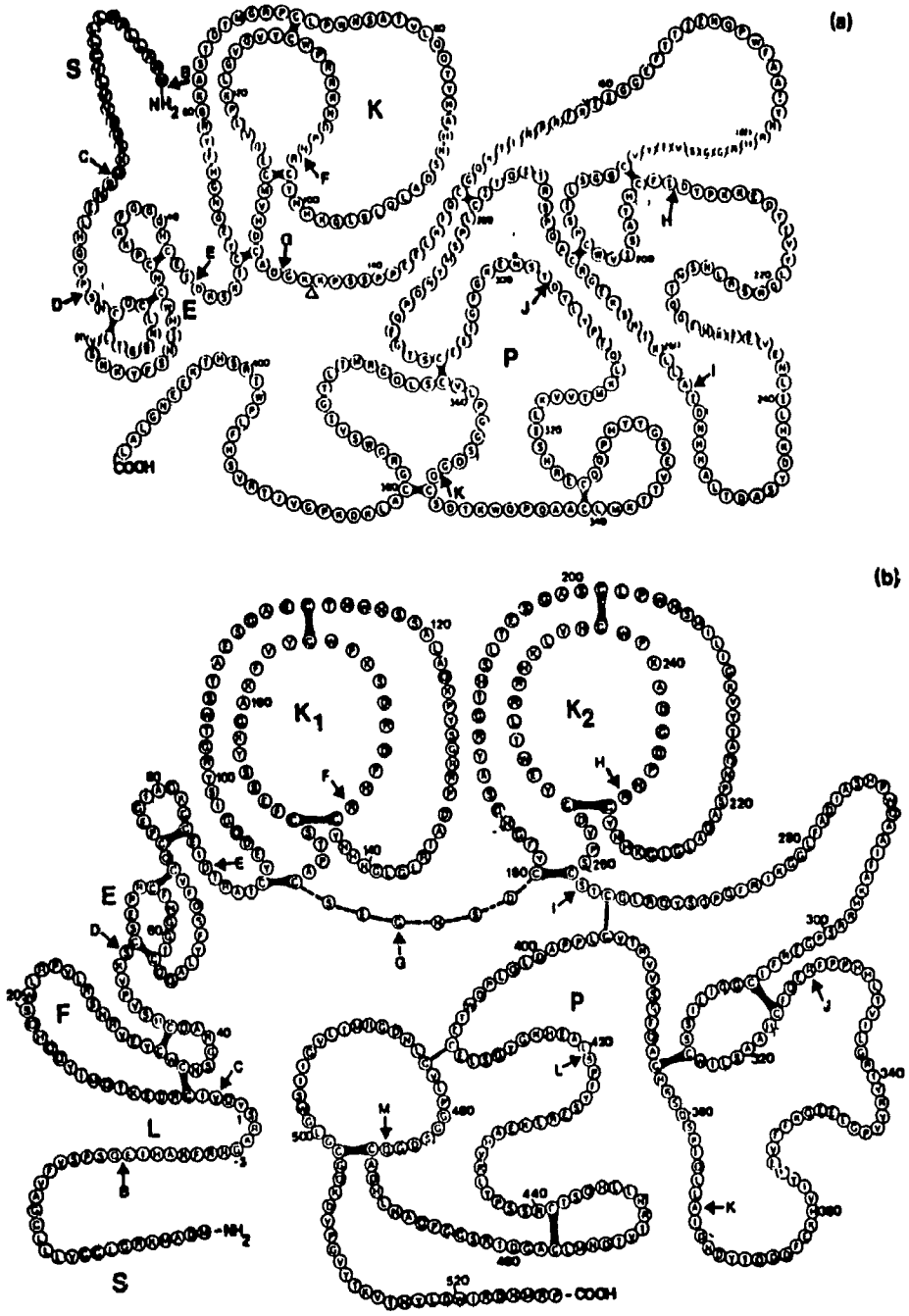


Figure 2. Structure of (a) urokinase-plasminogen activator and (b) tissue-plasminogen activator. The letters in the circle represent the single letter aminoacid code. S, signal peptide; L, leader segment; F, fibronectin finger domain; E, epidermal growth factor domain; K, kringle structure; P, serine protease domain. The arrows in (a) A-K and (b) A-M indicate intron-exon junctions. (From Harris, 1987).

u-PA in a wide variety of murine cells including epithelial cells of involuting mammary gland, fibroblasts, epithelial cells of kidney tubules, and in cells of placenta decidua in late pregnancy. Dano et al., (1985) suggested that u-PA is generally released from cells as an inactive proenzyme, converted to the active two-chain form by the action of plasmin. There are two forms of this two chain molecule. The high MW (MW=54,000) is formed by the cleavage at the Lysine-Isoleucine (158-159) while the low MW (MW=32,000) form by two additional cleavages after Lysine[135] and Arginine[156] (Fig. 2) The presence of only one "kringle" structure (Fig. 2) is the main reason of the relative inefficiency of u-PA to activate Pg in the presence of fibrin.

The nucleotide sequence of the entire u-PA gene has been established in porcine (Nagamine et al., 1984), humans (Riccio et al., 1985) and mouse (Degen et al., 1987). All the genes are organized in 11 exons. Comparison between these u-PA genes revealed extensive homology not only in terms of exon size and sequence but also between the introns and the flanking sequences as well.

A relationship may exist between the expression of u-PA gene and the growth state of cells. Pollanen et al., (1987) speculated that the role of u-PA in cell proliferation may involve breaking cell-matrix interactions during cell division or cell movement. The recently discovered u-PA receptors present in many cell surfaces such as monocytes, fibroblasts, and sperm cells (Saksela and Rifkin, 1988) have been

implicated in stimulating cell division (Kirchheimer et al., 1987).

2.1.3. Plasminogen Activator Inhibitors (PAI)

Regulation of PA activity can be exerted by specific and fast-acting inhibitors. By immunochemical criteria three types of PA inhibitors can be distinguished: PAI-1, PAI-2 and protease nexin I. All of these inhibit both u-PA and t-PA (Sprengers and Kluft, 1987).

PAI-1 is mainly present in plasma and is synthesized by a variety of cells including endothelial, hepatocytes, granulosa and smooth muscle cells (Erikson et al., 1985; Knudsen et al., 1987; Sprengers and Kluft, 1987). Molecular cloning of PAI-1 cDNA has been reported (Ny et al., 1986; Pannekoek et al., 1986; Ginsburg et al., 1986; Wun and Kretzmer, 1987). Using a PAI-1 cDNA, Loskutoff et al., (1987) and Bosma et al., (1988) isolated a genomic fragment bearing the entire PAI-1 gene which consists of 9 exons. It should be noted that there are two mRNA species (3.4 and 2.4 Kb) which hybridize with human PAI-1 cDNA (Andreasen et al., 1987). Sequence determination showed that these two mRNA's differ in the 3' noncoding region due to different polyadenylation sites (Ginsburg et al., 1986; Ny et al., 1986).

PAI-2 is a protein present in plasma and placenta extracts (Vassali et al., 1984) and is synthesized by macrophages (Schleuning et al., 1987) endothelial cells and fibroblasts

(Kruithof and Cousin, 1988). It plays an important role in inflammatory processes by providing the means to block local fibrinolysis (Kruithof and Cousin, 1988). PAI-2 occurs intracellularly and this raises the possibility that PAI-2 is stored in the cell and channelled into secretory pathways in response to as yet undefined signals (Genton et al., 1987). Molecular cloning of PAI-2 cDNA has been reported (Schleuning et al., 1987; Ye et al., 1987) but the detailed gene structure is not available.

Protease nexin I, a protein with a MW of 43,000, has been identified in different tissues such as heart and kidney and is synthesized by anchorage-dependent cells in vitro including fibroblasts, heart muscle cells and kidney epithelial cells (Sprengers and Kluft, 1987). Even though, protease nexin I reacts with u-PA and t-PA is considered a less efficient inhibitor when compared to PAI-1 and PAI-2. The biological role and significance is currently under investigation.

2.1.4. Plasmin Inhibitors

There are three plasmin inhibitors in plasma: α_2-macroglobulin, α-protease inhibitor and α_2-antiplasmin which constitute approximately 10% of the plasma protein (Sprengers and Kluft, 1987). Quantitatively, α_2-macroglobulin is the most prominent plasmin inhibitor. However, α_2-antiplasmin is a very fast-acting inhibitor based on the second order rate constant with plasmin and this renders it

the most important plasmin inhibitor in blood. α_2-antiplasmin deficiency results in severe lifelong bleeding tendencies (Wiman, 1981). Elimination of α_2-antiplasmin leads to utilization of α_2-macroglobulin which is not an active site directed enzyme. It reacts with all known proteases, justifying its nomination as a "wandering scavenger of endoproteinases" (Saksela, 1985).

2.2. Physiology of Plasmin

The occurrence of PA in a wide variety of cells suggests that PA and plasmin play a role in many physiological processes. Our survey is not meant to be complete but I have chosen examples related to this research project.

2.2.1. Involution

Three tupes of mammary gland involution have been described (Hurley, 1989). Initiated involution is defined as the regression of the lactation function resulting from suddenly stopping milking. Gradual involution is the regression of the lactation function during the course of the normal lactation generally encompassing the declining phase of lactation (3rd to 10th month). Senile involution occurs at the end of the reproductive life and is not of any interest for this research effort.

A non lactating period or a stage of mammary gland

involution between successive lactations is necessary during the lactation cycle of the dairy cow to optimize milk production in the subsequent lactation (Nickerson, 1989). It is common management practice to "dry off" dairy cows by suddenly stopping milk removal. The non-lactating period is generally 45-60 days with periods less than 40 days resulting in lower milk production in the subsequent lactation (Hurley, 1989). Non-lactating periods greater than 60 days will increase feeding and maintenance costs with no associated return and may have a negative impact in cow's lifetime performance with respect to milk production.

The non-lactating period of the dairy cow has been divided in three stages (Smith and Todhunter, 1982). Active involution initiates just 12 h after the final milking and is the transition period from a lactating to non-lactating state. This is followed by a non-lactating steady state and finally by a new transition period prior to parturition which is characterized by colostrum formation.

Marked changes in mammary secretion composition, mammary gland size, structure and function occur during active involution. Lactose, fat, total and individual caseins, α_s-lactalbumin, β-lactoglobulin contents of mammary secretion decrease during involution and this reflects the declined synthesis and secretion of milk components by mammary epithelial cells (Hurley, 1989). Despite the decreasing concentrations of caseins, total protein content of the mammary secretion increases due to substantial increases in

immunoglobulins, serum albumin and lactoferrin concentrations (Wheelock et al., 1967; Whelty et al., 1976; Watson et al., 1972; Ziv and Gordin, 1973; Hurley, 1989). These changes reflect the increased transport of blood-originated proteins to milk. Hurley (1989) suggested that the permeability of the mammary epithelium is compromised during active involution. This results in greater concentrations of blood-originated components entering the milk through paracellular diffusion via loosened junctional complexes between epithelial cells and/or in association with invading leucocytes.

In addition to changes in the mammary gland secretion, the mammary gland undergoes some striking morphological and structural changes. Holst et al., (1987) suggested that the most important change is the appearance of large vacuoles in epithelial cells. These vacuoles are thought to be formed by fusion of secretory vesicles and lipid droplets. Epithelial cells become inactive within 48 h from the last milking (Holst et al., 1987). Hurley et al., (1989) observed two types of autophagocytic structures in the mammary gland during early involution. The first appears 24 h after cessation of milk removal. It consists of densely packed membranes associated with lysosomes and it is not membrane bound. The second structure appears 36 h after stopping milking. It is a membrane bound structure found in the cytoplasm of mammary epithelial cells. Both autophagocytic structures remained until 60 h in involution. Their presence coincides with the greatest decline in rough endoplasmic reticulum and Golgi

apparatus. This led to the suggestion that they may be responsible for the degradation of rough endoplasmic reticulum and Golgi apparatus in mammary epithelial cells.

The key feature of the mammary gland involution in mice is the extensive loss of epithelial cells (Lascelles and Lee, 1978). Ossowski et al., (1979) estimated that 90% of the glandular weight is lost within the first few days of the involution process. They also reported that an induction of PA production by mammary epithelial cells may be responsible for precipitating the events leading to the "initiated involution". This was observed irrespectively of the timing during lactation at which involution was initiated. Administration of lactogenic hormones (prolactin, hydrocortisone) counteracted the "initiated involution", lactation was resumed and repressed PA production. On the other hand, insulin which promoted involution induced PA production. At this point it is not certain if it is the induction of PA which initiates involution or vice versa. Ossowski et al., (1979) suggested that PA converts plasminogen to plasmin which in turn degrades or initiates degradation of basement membranes, epithelial cells and some residual milk proteins.

The declining phase of lactation (gradual involution) is not well understood. Studies in goats showed that it is the net decrease in the number of mammary secretory epithelial cells rather than a decrease in milk production per cell that is responsible for the decrease in milk yield during gradual

involution (Wilde and Knight, 1989). Wilde and Knight (1989) suggested that a feedback inhibitor may act primarily on the secretory process rather than through inhibition of synthesis of individual milk components.

To the best of my knowledge there is no information on factors determining cell longevity in the bovine mammary gland. Despite the importance of PA in rodent mammary gland involution, little or nothing is known about a possible role of this enzyme in bovine mammary gland involution. There is only an abstract by Hurley (1986) suggesting an increase of PA activity during bovine mammary gland involution. Extrapolation of the data obtained in mice to the dairy cow may not be justified because bovine mammary gland involution is very different from that in mice. First, involution of mammary gland in dairy cows is a less extensive phenomenon with respect to cell loss. Second, dairy cows are pregnant at the time of milking cessation, which is not the case in mice. Third, dairy cows are producing significant amounts of milk at the time of milk removal which is again in contrast to other species (Oliver and Sordillo, 1989). The possibility that PA could be involved directly or indirectly in bovine mammary gland will be investigated in this research effort.

2.2.2. Inflammation-Mastitis

Macrophages and polymorphonuclear leucocytes (PMNL) play an essential role in the body's defense against pathogens. They

secrete a number of proteins that subserve the defense against bacterial invasion, thus initiating a process referred to as "inflammation". In addition to releasing an large number of soluble factors, macrophages carry out the fundamental function of ingesting and killing invading pathogens (Johnston, 1988).

Macrophages and their precursors in circulation monocytes, make up the monocyte/macrophage system. In response to bacterial or viral infection, macrophages are converted from normal, resting or resident to "activated". This is accomplished through the release of macrophage-activating lymphokines from T lymphocytes specifically responding to antigens from the infecting organism (Johnston, 1988). Critical to monocyte's protective function is their ability to emerge from blood vessels and carve paths to the sites of microbial invasion, a process called diapedesis. Vassali et al., (1976) suggested that PA through the generation of plasmin may be essential for diapedesis. Indirect evidence has been provided by the fact that administration of anti-inflammatory steroids at physiological levels such as dexamethasone or hydrocortisone suppresses PA production by macrophages and their recruitment to defend infection (Saksela et al., 1985).

Mastitis is a serious wide-spread inflammation of the bovine mammary gland. Bacteria that enter the mammary gland through the streak canal find the proper environment to grow and multiply (Guirdy, 1985). In milk, macrophages and PMNL are

the principal phagocytes and constitute the first line of defense against bacterial infection. However, both types of cells have reduced phagocytic abilities once present in milk when compared to those in blood (Guidry, 1985). This is consisted with observations that a high number (500,000/ml) of macrophages and PMNL is required to protect the mammary gland, once mastitis occurs. This level will be reached approximately 24 h post-infection, eventhough E. Coli will cause an earlier response (Guidry, 1985). This time lapse, however, allows the establishment of the bacteria.

Macrophages are more predominant than PMNL in uninflamed mammary gland and for this reason are important in the very early stages of the infection. Macrophages become again the predominant cell type several days post-infection after the influx of PMNL occurs (Guidry, 1985). Macrophages present in mastitic milk are in the activated state. Activated macrophages have an enhanced capacity to produce and release a variety of hydrolytic enzymes including PA (Johnston, 1988). In the absence of definitive evidence concerning the ability of bovine milk macrophages to produce and secrete PA and in view of the importance of a controlled proteolysis during mastitis, the role of PA and PAI production by macrophages has been investigated in this research project.

For both the above processes, namely involution and inflammation, the link between PA and the respective phenomenon is still circumstantial with problems of interpretation emphasizing the fact that more rigorous studies

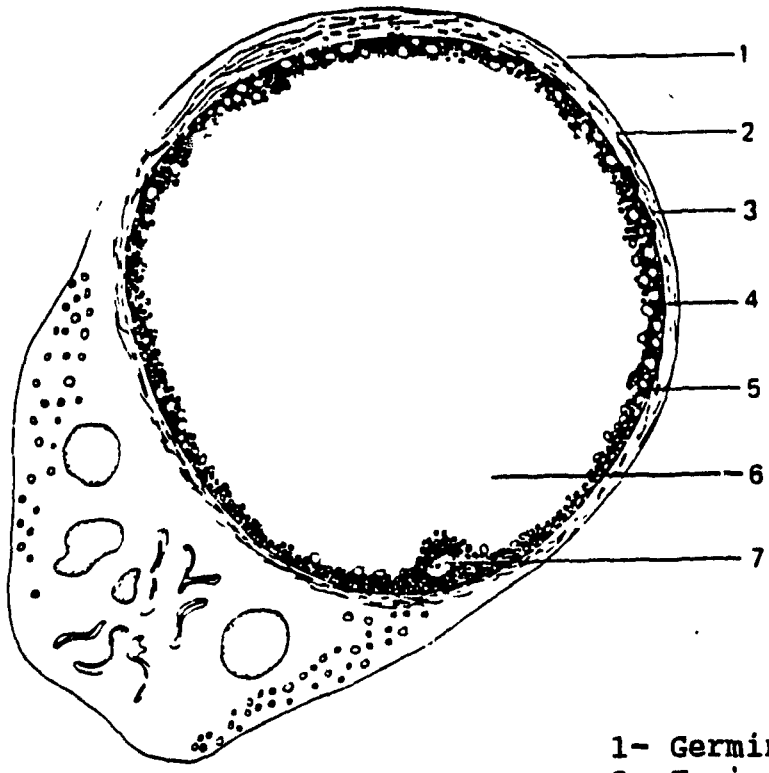
are required to establish more firmly whether PA is involved in both processes. Ultimately, selective inhibition of u-PA and t-PA, in vivo, would be the proper approach in order to clarify the long sought biological role of PA.

2.2.3. Ovulation

Ovulation is defined as the series of events initiated by the luteinizing hormone (LH) surge and characterized by resumption of meiosis and germinal vesicle breakdown, luteinization of the granulosa cells, follicular rupture and release of the mature fertilizable ovum (Lipner, 1988).

The wall of the preovulatory follicle is made up from six layers (Fig. 3). The outside layer on the surface of the follicle, is called germinal epithelium. It rests on a basal lamina which in turn merges with the tunica albuginea. The next layer moving towards the middle is theca externa, and is composed by fibroblasts and fusiforms, cells very similar to smooth muscle. Theca interna constitutes the next layer which is separated from the innermost layer, the granulosa cells by the lamina granulosa (Lipner, 1988).

At the initiation of ovulation, preovulatory follicles become edematous which led to the hypothesis that ovulation is a type of inflammatory process (Espey, 1980). The growing follicle undergoes a transformation from a solid mass of cells enclosing the ovum to a fluid-filled follicle containing a centrally located antrum into which projects a column of



1- Germinal epithelium
2- Tunica albuginea
3- Theca externa
4- Theca interna
5- Granulosa
6- Antrum
7- Ovum

Figure 3. Follicle with ovum mass (Sorensen,1979).

cells, the cumulus oophorus continuous with the membrana granulosa (Lipner, 1988). As the time of ovulation approaches thecal cells at the base of the follicle break through lamina propria and intermingle with granulosa cells that are being transformed to lutein cells. At the apex the germinal cells flatten as the follicle protrudes and give rise to the stigma and then disappear as rupture begins. Following the rupture of the mature follicle and the liberation of the ovum, the follicle fills up with blood forming the corpus hemorrhagicum. The granulosa and thecal cells increase in number and absorb clotted blood. Vascular elements from the theca cells penetrate and convert the avascular granulosa cells to a highly vascular structure. The granulosa cells start accumulating large quantities of cholesterol. This process leads to the formation of the corpus luteum (Lipner, 1988).

In addition to the morphological changes, there are considerable chemical changes. During the follicular phase of the estrus cycle, estradiol is the major hormone produced in the ovary (Lipner, 1988). Controversy surrounds the source of estrogen in the ovary. The best explanation is provided by the two-cell two-hormone hypothesis (Erickson et al., 1985). This hypothesis states that estrogen production is monitored by LH and follicle stimulating hormone (FSH); FSH induces the LH receptors and LH shifts theca cells from a progesterone to an androgen secreting cell. In granulosa cells, the androgen is converted to estrogen and follicular development starts. The preovulatory LH surge terminates estrogen production and

theca shift once again to a progesterone secreting cell. Lipner (1988) reported that in cases where the ovulation is blocked, steroid production is reduced but not completely blocked, which raises questions as to what levels of steroids, if any, are needed for ovulation to occur (Lipner, 1988).

The PMSG/hCG treated prepubertal gilt model has been used to elucidate the sequence of events leading to follicle rupture and ovulation. This model exploits the ability of pregnant mare's serum gonadotropin (PMSG) to stimulate ovulation and human chorionic gonadotropin (hCG) to control precisely the time of ovulation (Webel and Day, 1982). Injection of 750 IU PMSG to four month old gilts, followed by 500 IU hCG 72 h later, induces the production of a population of uniformly developing follicles which will ovulate 42 h following hCG injection (Ainsworth et al., 1982). Tsang et al., (1985) suggested that the development of these follicles, and the number of ovulations is similar to that observed in spontaneously ovulating animals. Using this model, Tsang et al., (1985) reported a rapid increase (400 x) in cAMP concentration 4 h following hCG and then a decrease to the pre-hCG levels. A second rise (2.5 to 4 x) occurred 30 h post-hCG and was sustained up to the ovulation time. Concentrations of PGE and PGF in follicular fluid increased markedly just prior to ovulation. A single injection of indomethacin, a prostaglandin synthesis inhibitor, suppressed the preovulatory rise in PGE and PGF and blocked ovulation. In contrast, other ovarian responces occuring during the same time like oocyte

maturation and luteinization are not prevented by indomethacin administration. Administration of $PGF_{2\alpha}$, but not PGE_2, shortly before the time of ovulation can reverse the indomethacin-blocked ovulation. This supports the suggestion that PGs of the F series may be important in the sequence of events leading to follicle rupture and ovulation in the pig (Tsang et al., 1985).

Enzymatic degradation of the follicle wall is the best hypothesis explaining follicle rupture (Lipner, 1988). A temporal correlation between the disruption of the follicle wall and PA production by granulosa cells in rodents provided circumstantial evidence that PA activity may be required for lysis of the follicle wall (Beers et al., 1975). Following gonadotropin administration, granulosa cells from preovulatory mice follicles exhibited high PA activity while those from non preovulatory follicles in the same ovary showed low PA activity (Strickland and Beers, 1976). Espey (1980) suggested the following sequence of chemical events concerning the degradation of the follicle wall: the preovulatory follicle stimulated by gonadotropin and $PGF_{2\alpha}$ in the pig and PGE_2 in other species produce and secrete PA; PA converts plasminogen to plasmin; plasmin activates latent collagenase; collagenase breaks down collagen giving rise to telopeptide-free collagen which is degraded by non-specific proteases. The observation that ovulation can be blocked by indomethacin, a prostaglandin synthesis inhibitor, administration without concomitant

suppression of PA suggested that PA may not be necessary for follicle rupture (Shimada et al., 1983; Espey et al., 1985). However, Tsafriri et al., (1989) demonstrated that antibodies against t-PA and α_2-antiplasmin suppress ovulation in rats when administered locally in the ovarian bursa. In addition Ichikawa et al., (1983) reported that talopeptin, inhibits collagenase and blocks ovulation in explanted hamster ovary. These results, collectively provide strong support for the inference that PA and collagenase play a crucial role in the proteolytic processes leading to follicle rupture.

2.3. Regulation of Plasminogen Activator Production

The hypothesis that PA play a causal role in tissue remodelling, combined with the fact that most cells in the intact animal produce high levels of PA in specific cases and for a limited time imply the concept of hormonal regulation of PA production. The regulation of u-PA and t-PA production is of interest because of the diversity of biological context in which they are involved and because PA production is influenced by many agents including hormones, growth factors, modulators of cAMP and tumour promoters. The following review was not meant to be a detailed historical or chronological review, but a brief summary of what is presently known about PA regulation by steroid and peptide hormones.

2.3.1. Effect of Steroids Hormones on Plasminogen Activator Production

Synthesis of u-PA in various cell types is particularly sensitive to glucocorticoids and a suppression of PA synthesis has been observed in macrophages (Vassali et al., 1976; Werb, 1978; Neuman and Sorg, 1980), human PMNL (Granelli-Piperno et al., 1977), bovine endothelial cells (Levin and Loskutoff, 1982; Sherpo et al., 1980; Laug, 1983), bovine Sertoli cells (Jenkins and Ellison, 1986) primary cultures of involuting murine mammary glands (Ossowski et al., 1979) and some human cell lines (Roblin and Young, 1980; Dano et al., 1982; Laug, 1983; Medcalf et al., 1988). A major point that should be addressed concerning the suppression of PA production by these cells is that this effect can be the outcome of a general suppression of cellular metabolism or even a toxic effect of glucocorticoids rather than a specific effect on PA. Even though most of these studies made no mention of this point, it appears that glucocorticoids did not suppress total protein synthesis but, in fact, PA was one of the few cellular products whose levels were decreased rather than increased by this drug (Rousseau, 1984; Medcalf et al., 1986).

The response of t-PA production to glucocorticoids appears to be less consistent. In a number of human melanoma cell lines (Roblin and Young, 1980) and bovine endothelial cell lines (Levin and Loskutoff, 1982; Laug, 1983) there was no effect while a suppression of t-PA was reported in cultured

pituitary cells grown in the presence of glucocorticoids (Granelli-Piperno and Reich, 1983). Gelehrter et al., (1987) also reported an inhibition of PA activity by glucocorticoids in rat hepatoma cells, which was the outcome of a paradoxical simultaneous induction of PAI-1 and t-PA, the first one being more pronounced. Wang and Leung (1989) reported an induction of t-PA by glucocorticoids in rat granulosa cells.

Estrogens do not appear to have an effect on PA of the same magnitude with glucocorticoids. An increase of u-PA activity by estrogens has been reported in rat uterus (Katz et al., 1976; Kneifel et al., 1982; Peltz et al., 1983). Estrogens and progesterone enhance the follicle-stimulating hormone (FSH) stimulated PA production by rat granulosa cells (Wang and Leung, 1987). Progesterone blocked the prolactin-mediated stimulation of PA in explants of rat mammary tumours (Mira-Y-Lopez et al., 1983).

Medcalf et al., (1988) using a human fibrosarcoma cell line (HT-1080) reported a significant decrease of u-PA protein content while t-PA and PAI-1 were increased, following dexamethasone administration. These effects are occuring at the level of gene transcription rather than translation. The opposing effects of dexamethasone on u-PA and t-PA are quite remarkable. Two possibilities exist: either the transduction of the signal from the glucocorticoid receptor to the nucleus is transmitted by multiple mechanisms or that regulatory sequences in these genes (u-PA and t-PA) respond in a different way to the same signal. The fact that the effect of

dexamethasone is inhibited by RU 38486, a glucocorticoid antagonist, suggests that this effect is mediated through the glucocorticoid receptor. It is also intriguing that dexamethasone modulates in a similar fashion t-PA and PAI-1 which appears biologically futile. The authors gave no explanation for these results. They only suggested the existence of a positive regulatory element in t-PA and PAI-1 genes, presumably similar to that described by Yamamoto (1985).

2.3.2. Effect of Polypeptide Hormones on Plasminogen Activator Production

Gonadotropin administration results in a dose dependent increase in PA production by granulosa cells (Strickland and Beers, 1976; Liu et al., 1981; Wang and Leung, 1983; Ny et al., 1985; Reich et al., 1985; Knecht, 1986). Granulosa cells prepared from rats primed with PMSG respond to FSH or LH *in vivo* and *in vitro* and produce high amounts of PA. While FSH administration results in a linear increase in PA production starting 2 h after FSH administration, LH shows a longer lag phase. However, both FSH and LH reach the same peak stimulation value (Canipari and Strickland, 1986). Granulosa cells are directly stimulated by FSH but only a small number of these cells are stimulated by LH and start producing prostaglandins (PGs). It is the PGs produced by these cells that stimulate the entire population. O' Connell et al.,

(1987) found that t-PA mRNA levels mirror those obtained at the protein level suggesting that the effect of gonadotropins occurs at the steady-state level of mRNA.

Activity of PA was suppressed by prolactin administration in vivo in the mouse (Ossowski et al., 1979). Contradictory results were obtained in culture as prolactin had no effect. PA activity was decreased in cultured bovine endothelial cells (Sherpo et al., 1980) and in macrophage cell lines (Dano et al., 1985) when compounds like phosphodiesterase inhibitors and cholera toxin which increase cAMP were administered. Certain caution should be exercised in interpreting some of the above studies, particularly those using as a basis for their conclusions PA activity measurements. The sole determination of PA activity makes difficult to discriminate between true changes in PA production and changes in net PA activity due to inhibitors.

2.3.3. Effect of Prostaglandins on Plasminogen Activator Production

During the past two decades, researchers have investigated the effect of prostaglandins on PA production, but the overall picture is still confusing. Prostaglandins have been reported to enhance PA production in rat granulosa cells (Strickland and Beers, 1976; Canipari and Strickland, 1986). However, inhibition of prostaglandins synthesis by indomethacin had no effect on PA production by rat ovaries (Shimada et al., 1983;

Reich et al., 1985). Espey et al., (1985) also reported that low levels of indomethacin (0.1-1 mg/rat) inhibited ovulation but did not inhibit the increase in PA activity. However, when high doses (10 mg/rat) of indomethacin were administered, inhibition of both ovulation and PA production was observed. Interpretation of these results is very difficult. Espey et al., (1985) postulated that an excessive dose of indomethacin (10 mg/rat) could produce a cytotoxic effect on whole cellular metabolism rather than a specific effect on PA.

Most of the above studies have inherent problems, the most significant of which was that they did not discriminate between u-PA and t-PA. This does not allow us to draw any conclusion on possible differential inhibition of these two enzymes. The work of Shimada et al., (1983) and Espey et al., (1985) could additionally be criticized because they measured PA activity in extracts of whole ovaries and they did not discriminate between granulosa and theca cells.

The above mentioned studies on the hormonal regulation of PA synthesis have contributed to a significant understanding of the PA system. However, closer scrutiny reveals that unambiguous results are not easily obtained. It therefore seem pertinent to discuss in a few lines some very basic problems. All the studies that report negative results concerning the presence of u-PA activity should be questioned. This is due to the fact that u-PA is released from cells in an inactive proenzyme form which is converted to the active enzyme by the action of plasmin. Thus, in the absence of plasmin, u-PA will

remain in the proenzyme form and will not be detected in activity assays. Furthermore the release of u-PA in the proenzyme form suggests the existence of an additional activation step in the cascade of reactions leading to plasmin formation, a step presumably regulated by an unknown mechanism.

Most of the above studies have been performed using different cell lines which may not be representative of the same cells in the intact organism. Also, the possible presence of different regulatory factors between the microenvironment of cell in culture and that in the intact organism where very delicate secretory and regulatory mechanisms exist, may significantly compromise the interpretation of the results. Furthermore, selection of certain cells when these cells are established may favour certain cell types, thus, resulting in non representative patterns of the in vivo situation. This is not to imply that cell cultures do not provide us with useful information. To the contrary, they may play an important role not only in the identification of underlying mechanisms but also in suggesting new directions that the in vivo studies should follow.

2.4. Plasmin-Plasminogen and Dairy Technology

The presence of proteolytic enzymes in milk has a major impact on the technological characteristics of milk and the quality of dairy products (Politis and Ng Kwai Hang,1988a;

1988b). The first report on the presence of proteolytic enzymes in milk was published in 1897 (Fox, 1981). The original suspicion was that they might be of bacteria origin. Kaminogawa et al., (1972) reported that plasmin is, in fact, the main proteolytic enzyme in milk and milk plasmin has similar MW and enzymatic properties with blood plasmin. This led to the speculation that blood is the origin of milk plasmin. Presumably, any condition such as mastitis that leads to increased permeability of the mammary epithelium would result in higher levels of plasmin in milk (Politis and Ng Kwai Hang, 1989). Adding to the confusion, Dulley (1972) suggested that alkaline milk protease, a term synonymous to plasmin, was an enzyme native in the mammary gland. However, the evidence presented was rather weak. This research effort will be directed towards identifying cells within the mammary gland producing plasminogen and PA.

2.4.1. Variation of Plasmin Levels in Bovine Milk

Plasmin concentration in milk is generally low (0.1-0.3 mg/l) (Korycka-Dahl et al., 1983) and is dependent upon the health status of the udder and the stage of lactation. Politis and Ng Kwai Hang (1989) reported a two-fold increase in plasmin activity in milk containing more than one million somatic cell counts (SCC)/ml when compared to that containing less than 100,000 SCC/ml. Higher levels of plasmin have been observed at the end of lactation period (Korycka-Dahl et al.,

1983; Politis and Ng Kwai Hang, 1989). This is particularly important in countries like Australia or New Zealand where milk production is highly seasonal and late lactation milk yields cheese of poor quality (Fox, 1981). The significance of the above studies is limited from an understanding point of view because they simply report correlations and no explanation is provided as to why mastitis or late lactation are associated with higher levels of plasmin in milk.

2.4.2. Significance of Plasmin in Dairy Technology

The consequence of the proteolytic breakdown of caseins due to the presence of proteases in milk has extensively been investigated. Gelation during storage of ultra high temperature (UHT) milk, textural defects in milk powders and cheese have been observed in milk with high contents of proteolytic enzymes (Reimerdes, 1982). There are no studies, however, discriminating between proteolysis caused by plasmin and the other somatic cell proteases present in milk. Pearse et al., (1986) reported that the addition of exogenous plasmin had no effect on the coagulating properties of milk. On the other hand, Politis and Ng Kwai Hang (1988) reported a 10% decrease in cheese yield and inferior coagulating properties associated with elevated levels of SCC in milk and attributed this to the presence of high levels of plasmin in that kind of milk. It is important to note that plasmin has high heat stability (Korycka-Dahl et al., 1983) and this renders it very

important in terms of potential for proteolysis in heat-treated dairy products. To the best of my knowledge, there are no means of preventing the detrimental effects of plasmin once present in milk.

2.5. Conclusions and Perspectives

As described in the previous pages, intensive research has led to a better understanding of the role of plasmin and PA in various biological processes. A number of recent findings have been discussed and now will be summarized.

There are two forms of PA, u-PA and t-PA immunologically distinct and products of different genes. Regulation of PA activity can be exerted by specific PA inhibitors: PAI-1, PAI-2, and protease nexin I. A variety of peptide and steroid hormones has been shown to regulate PA and PAI synthesis. In many cases, this regulation is exerted at the level of mRNA.

Production of PA accompanies the degradative processes of a large diversity of cell types during tissue remodelling. The evidence although consistent, remains rather circumstantial, in the sense that most of these studies have not demonstrated a "cause:effect" relationship between plasmin and tissue remodelling.

Research on PA system has great prospects. An area of research certain to receive attention is the differential role of u-PA and t-PA. Although t-PA function primarily in fibrinolysis and fibrinogenolysis, t-PA has been shown to be

present in a number of other systems, i.e. in endocrine organs which indicates some yet unknown function for this enzyme. A second promising area is the understanding of how matrix degradation is controlled. The mechanism which directs the u-PA receptors to those areas of the cell where proteolysis is needed is currently unknown.

A wide variety of cells synthesize and secrete both PA and PAI. Precise regulation of both proteins is of critical importance. I foresee further research on the identification of factors that modulate PA and PAI production. A detailed analysis of the mechanism of action of these factors should aid in understanding conditions underlying tissue remodelling.

3. FACTORS AFFECTING PLASMIN AND PLASMINOGEN CONCENTRATIONS IN BOVINE MILK.

Abstract

Differences in plasmin, plasminogen and serum albumin levels were examined in milk samples of varying somatic cell counts (SCC), obtained from cows at different stages of lactation and during exogenous somatotropin administration. An increase of SCC from less than 250,000 to more than 1,000,000/ml resulted in significant ($p < 0.05$) increases of plasmin, plasminogen and serum albumin by 105, 74 and 140% respectively. During this increase of SCC there was a highly significant ($p < 0.001$) correlation between milk plasminogen and milk serum albumin ($r=0.74$) This supports the suggestion that plasminogen and serum albumin, two blood components, gain access to the alveolar lumen through ruptured epithelium caused by mastitis. Concerning the effect of stage of lactation, plasmin and plasminogen concentrations increase during lactation and reach peak values immediately before the dry period. There are, however, no significant ($p < 0.05$) differences in the ratio (plasminogen + plasmin)/plasmin, throughout the whole range of SCC and from third to ninth month in lactation. The ratio was 7.27 during early lactation and 4.29 during late lactation and both values are statistically ($p < 0.01$) different from all the other ratios. This indicates limited and increased activation of plasminogen to plasmin during early and late

lactation, respectively. In somatotropin injected cows, milk plasmin was constant and milk production was enhanced. Cessation of somatotropin injections at drying off led to a rapid elevation of milk plasmin to reach control levels.

Introduction

Bovine milk contains several serine proteases. Plasmin which occurs in milk together with its inactive zymogen, plasminogen is the most significant protease in terms of total proteolytic activity (Eigel, 1977; Fox, 1981). Plasmin hydrolyses α_s-casein and β-casein while k-casein is probably resistant to degradation (Fox, 1981). The extent of proteolysis will affect the coagulating properties of milk (Politis and Ng Kwai Hang, 1988) and also its ability to withstand processing during cheese making. As plasmin has high heat stability (Driessen and Van der Waals, 1978), increased concentrations can potentially represent a problem to the dairy industry.

Mastitic infection of the mammary gland results in increased proteolytic activity that is related to a higher concentration of plasmin (Barry and Donelly, 1981; Andrews, 1983; Donelly and Barry, 1983; Schaar and Funke, 1986; Politis and Ng kwai Hang, 1989). Stage of lactation also affects plasmin with late lactation associated with higher concentrations of plasmin (Richardson and Pearce, 1981; Korycka-Dahl et al., 1983; Politis and Ng Kwai Hang, 1989). This increase in plasmin

coincides with the process of gradual involution which is characterized by a loss of milk synthetic capacity by bovine mammary epithelial cells at late lactation. Plasmin has been implicated as the key enzyme in mammary gland involution in rodents (Ossowski et al., 1979) but this issue remains unresolved in the case of bovine mammary gland involution.

Bauman et al., (1985) reported that administration of recombinantly derived somatotropin (STH) to dairy cows caused a 15-40% increase in total milk production, that predominates in the latter stages of lactation. Milk production is influenced by many factors such as the nutritional and the hormonal status of the animal. Ultimately, however, it is the number and the activity of the mammary secretory epithelial cells that determine milk yield. It is obvious that during gradual involution the number and/or the activity of these cells is reduced to account for the reduction in the milk production. Thus, any factor that could prevent this decrease is likely to have long-term effects on the rate of milk production. This led us to suggest that the effect of STH may be exercized through a retardation of involution which allows a persistence of milk production. This hypothesis taken together with the fact that gradual involution coincides with increased milk plasmin concentrations suggest that the effect of STH on milk plasmin and plasminogen is of considerable interest.

This study examined relationships between several factors (stage of lactation, somatic cell counts, lactation number and

season) and concentrations of plasmin and plasminogen in bovine milk. In addition, the relationship between exogenous administration of rSTH and milk plasmin , plasminogen, and milk yield within the context of bovine lactation was investigated.

Materials and Methods

Effect of Environmental Factors on Milk Plasmin and Plasminogen Concentrations

Origin of Milk Samples and Determination of Somatic Cell Count Content

Individual milk samples were collected biweekly during the morning milking by farm workers from 66 Holstein cows at the Macdonald College farm. The samples were transported to our laboratory by E. Lachance, Dept. of Animal Science. Data for stage of lactation and lactation number were obtained from the Dairy Herd Analysis System (DHAS) records. A Fossomatic cell counter (Foss Electric, Hillerod, Denmark) was used to determine SCC in milk. All the SCC determinations in milk were processed by DHAS staff due to the limited accessibility of this instrument.

Treatment of Milk Samples

Milk samples were defatted by centrifugation at 2,000 X g for 10 min. The resulting lipid layer on the surface was discarded. The skim milk was incubated with 50 mM ϵ-amino caproic acid (ϵ-ACA, Sigma Chemical Co., St. Louis, MO) for 2 h at room temperature to dissociate plasmin and plasminogen from the casein micelles (see below). Treated skim milk samples were then centrifuged at 100,000 X g for 1 h at 4°C to obtain a supernatant denoted "milk serum fraction" and a pellet containing the casein micelles denoted "casein fraction". The casein micelle was reconstituted in the original volume in a 50 mM Tris buffer (pH 8.0) containing 110 mM NaCl.

Determination of Milk Plasmin and Plasminogen Contents

Plasmin plus plasminogen-derived activity was determined in milk serum and casein fractions. Plasminogen-derived activity is defined as the plasmin activity after addition of 150 Plough Units of urokinase. For the combined determination of plasmin plus plasminogen-derived activity 50 μl of milk serum or casein fraction were mixed with 700 μl of 50 mM Tris buffer (pH 7.4) containing 110 mM NaCl, 0.6 mM H-D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride (S-2251, Sigma), 3.3 mM ϵ-ACA, and 150 Plough Units of urokinase (Sigma). The reaction mixture was incubated for 60 min at 37°C. This time

allows the conversion of plasminogen to plasmin (see below). The absorbance at 405 nm was then measured at 30 min intervals. The increase in absorbance was linear for up to 3 h (Fig. 5). The rate of p-nitroanilide formation was computed from the linear parts of the absorbance versus time curves. A similar reaction mixture without milk serum served as a control for spontaneous hydrolysis of S-2251 in our reaction buffer and in all cases was negligible.

Plasmin activity was measured in the same reaction mixture without added urokinase. Plasminogen-derived activity was computed by the difference of plasmin plus plasminogen activity and plasmin activity. Plasmin and plasminogen activities were expressed in units, with one unit being defined as the amount of the enzyme that produces a A_{405nm} of 0.001 in 1 min when p-nitroanilide is measured in our reaction mixture. Recoveries of exogenous plasmin (Sigma) when added to the reaction mixture at concentrations normally encountered in milk were in the range of 87 to 96%. Reproduciblity of this assay system was tested in 20 samples and was found to be + 10% of the mean and was similar to that suggested by Korycka-Dahl et al., (1983).

To convert plasmin activity to plasmin concentration a standard curve was prepared (Fig. 4). For this purpose, replicate tubes containing different concentrations of plasmin (Sigma) (0.02-0.1 μg) were incubated in the previously described reaction mixture without milk serum. Plasmin activities were recorded. A standard curve was prepared to

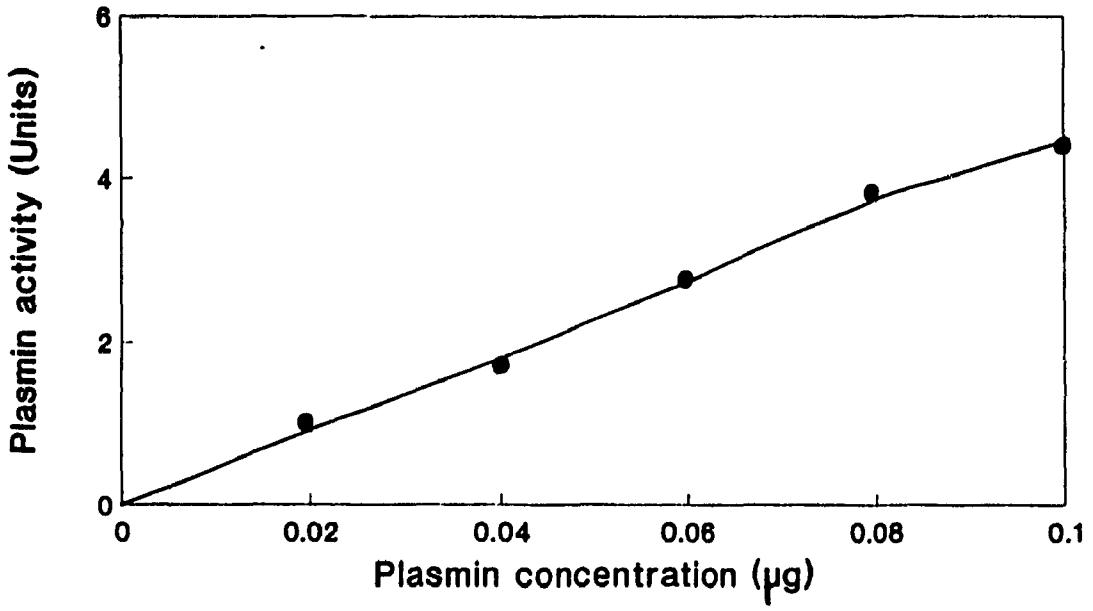


Figure 4 . Standard curve converting plasmin activity (units) to plasmin concentration (ug). Plasmin activity was determined in three independent samples for each plasmin concentration. Definition of units is in material and methods.

convert plasmin activity to plasmin concentration by plotting differences in absorbance versus plasmin concentrations. This conversion was made to overcome the difficulty in comparing plasmin activity units, which are not uniform in the literature. Such a transformation is inappropriate if significant quantities of plasmin inhibitors are present in milk samples. Korycka et al., (1983) found very low levels of plasmin inhibitors present in milk.

Validation of Plasmin and Plasminogen Assay System

In a series of preliminary experiments the validity of using the above assay system for measuring plasmin and plasminogen in milk was confirmed. In the first experiment time-related changes in the rate of the reaction were examined by incubating replicate tubes containing 50 µl of milk serum for different times: 30, 60, 90, 120, 150, 180 min. The second experiment was conducted to examine the effect of sample volume on plasmin plus plasminogen determinations by assaying different milk serum volumes: 25, 50, 75, or 100 µl. The final volume assayed in all cases was adjusted to 100 µl by addition of H_2O.

The third experiment examined the optimum concentration of chromogenic substrate, S-2251 which was used in our assay system. Replicate tubes containing 50 µl of milk serum were assayed for plasmin plus plasminogen activities in the presence of various concentrations of S-2251 in the range of

0.1-1.0 mM.

The fourth experiment examined the effect of ϵ-ACA on the plasmin plus plasminogen determination. The purpose of this experiment was to confirm that ϵ-ACA at the concentration being used in our assay system (3.3 mM) does not inhibit the rate of hydrolysis of S-2251 by plasmin. Replicate tubes containing 50 µl of milk serum were incubated with different concentrations of ϵ-ACA in the range of 2.5-120 mM.

To study the effect of preincubating the milk samples with 50 mM of ϵ-ACA on the distribution of plasmin and plasminogen between casein and milk serum fractions of bovine milk, skim milk prior to and after treatment with 50 mM ϵ-ACA was ultracentrifuged at 100,000 X g for 1 h at 4°C. The supernatant (milk serum) and the casein micelle pellet were recovered. The casein micelle was reconstituted in the original volume in a 50 mM Tris buffer (pH 8.0) containing 110 mM NaCl. Plasmin and plasminogen were determined in casein and milk serum fractions of milk as described above.

The efficiency of plasmin and thrombin in hydrolyzing the chromogenic substrate S-2251 was compared by adding pure thrombin (Sigma) or plasmin (Sigma) (1 µg/ml) in our reaction mixture without serum. This was done to assess the ability of thrombin, which is the main serine protease except plasmin in milk, (Reimerdes, 1978) to hydrolyze the chromogenic substrate S-2251. The possibility that non-serine proteases present in milk could hydrolyze the chromogenic substrate S-2251 was evaluated in the case of normal and mastitic milk.

Ten milk samples of low SCC (< 250,000/ml) and ten of high SCC (>1,000,000/ml) were processed as above to obtain milk serum and the reactions were run in the presence and in the absence of added 0.6 IU of aprotonin (Sigma). At this concentration, aprotonin is a potent inhibitor of plasmin (Rollema et al., 1983). The remaining hydrolysis of S-2251 is therefore attributed to the presence of non-serine proteases.

Determination of Bovine Serum Albumin Content of Milk

Bovine serum albumin content in milk was determined as described by Ng Kwai Hang and Kroeker (1984) with the following modifications. Milk samples were centrifuged at 2,000 X g for 10 min to produce skim milk. Casein was removed by isoelectric precipitation at pH 4.6 by mixing 1 ml of milk sample with 30 µl of 33% (v/v) acetic acid. After 5 min, 30 µl of 33% (w/v) sodium acetate were added to the reaction mixture and then centrifuged at 2,000 g for 10 min. The supernatant which contained the whey proteins was recovered and stored at -20°C. Individual whey or serum proteins were separated by polyacrylamide gel electrophoresis. The gel was made from a mixture of 6 g of acrylamide (Bio-Rad, Mississauga, ON) and 150 mg of N, N'-methylenebisacrylamide (BIS, Bio-Rad) dissolved in 100 ml 0.375 M Tris buffer (pH 8.9) containing 50 µl of N,N,N',N' tetramethylenediamine (TEMED, Bio-Rad) and 130 mg ammonium persulfate (Bio-Rad). Running buffer was made by dissolving 12 g of Tris-base and

57.6 g of glycine into 1 lt of H_2O (pH 8.3). Samples of 20 µl 5% (w/v) sucrose solution containing 1 µl of supernatant (milk serum fraction) were then loaded and run for 1.5 h at 200 V. Gels were stained for 1 h in 0.25% (w/v) Coomassie Blue (Bio-Rad), 10% (w/v) trichloroacetic acid (TCA) in methanol:H_2O (1:1, v/v). Excess stain was removed by washing the gels for 3 h in 2% (w/v) TCA in methanol:H_2O (1:4, v/v) and then overnight in 5% (v/v) acetic acid. Peak areas corresponding to serum albumin were quantified by densitometry by reference to peak areas of standards containing 0.5 and 1.5 mg/ml of bovine serum albumin (Sigma).

Statistical Analysis

An analysis of variance was performed to examine the effect of the environmental factors on plasmin, plasminogen and serum albumin concentrations in milk. Model fitted to the data included stage of lactation, SCC, season, lactation number and cow effect as fixed classification effects and a random residual term. Dependent variables in this model were: plasmin, plasminogen and serum albumin concentrations in milk and the ratio of (plasmin + plasminogen)/plasmin. There were 6 subclasses for stage of lactation with subclasses 1, 2, 3 and 4 consisting of cows at 1st and 2nd, 3rd and 4th, 5th and 6th, and 7th and 8th month of lactation, respectively. Subclass 5 included cows in lactation for 9 months and subclass 6 included cows at more than 9 months in lactation.

The five subclasses for SCC were < 250,000, 250001-500,000, 500,001- 1,000,000 and >1,000,000 SCC/ml of milk. Three subclasses for seasons: Fall (October, November), Winter (December, January, February), Spring (March, April) were chosen. There were five subclasses for lactation number:1, 2, 3, 4, >4. All the analyses were conducted using the General Linear Model procedure of the Statistical Analysis System (SAS, 1982). Differences between the means were evaluated with Duncan's Multiple Range Test.

Assessment of the Ability of Milk Somatic Cells to Affect Plasmin and Plasminogen in vitro

The ability of somatic cells to synthesize and secrete plasmin and plasminogen activators was assessed in vitro. Whole milk was centrifugated at 8000 X g for 10 min and the supernatant was saved. Pelleted somatic cells were washed in Dulbecco's phosphate buffered saline (DPBS, Gibco, Grand Island, NY) and were resuspended at the desired cell density of 1,000,000/ml in the milk supernatant. Cell density was subsequently counted with a Fossomatic cell counter. Milk samples were incubated at 37°C for 24 h and plasmin and plasminogen concentrations were determined. The same experiment was repeated with the only difference that the somatic cell used for resuspension were passed through 6 cycles of freezing and thawing to disrupt their membranes and release intracellular components. Results are expressed as

means + SD. Differences between the means were evaluated with Duncan's Multiple Range Test (SAS, 1982).

Effect of Exogenous Somatotropin Administration on Milk Plasmin and Plasminogen Concentrations

In a second independent experiment the effect of exogenous administration of rSTH on milk plasmin and plasminogen was investigated. Seventy-seven Holstein cows housed at the Macdonald College farm were used in this experiment and were part of a safety-efficacy trial for rSTH which was conducted by Dr. E. Block. Injections of the animals with rSTH was done by farm staff. Forty-two cows were assigned to groups of 14 animals each. The experimental grouping of the animals was designed by Dr. E. Block. Group 1 was injected with 12.5 mg rSTH animal^{-1} day^{-1} subcutaneously. Groups 2 and 3 received 175 or 350 mg rSTH animal^{-1} 14 days^{-1}, respectively in a sustained release preparation injected subcutaneously. The remaining 35 cows, serving as controls, received placebo injections. Cows were fed total mixed rations formulated for NRC (1978) requirements for production. Ration ingredients were alfa alfa haylage, dry ground corn, high moisture ear corn, roasted soybeans and a vitamin-mineral premix.

One thousand two hundred milk samples were collected at morning milking by farm workers and were transported to our laboratory by E. Lachance. Milk yield of individual cows, their stage of lactation and lactation number data were

obtained from the DHAS records. Plasmin, plasminogen and SCC were determined as described above.

Statistical Analysis

Least squares analysis of variance was performed to examine the effect of exogenous rSTH administration on milk plasmin and plasminogen and milk yield. Mathematical model included cow effect, lactation stage and number, treatment as fixed classification effects, the interaction between stage of lactation and treatment, and log somatic cell counts (SCC) and milk yield as covariates plus a random residual term. Dependent variables in this model were plasmin and plasminogen concentrations in milk, the ratio of (plasminogen + plasmin)/plasmin and the total plasmin and plasminogen production per day (milk yield X plasmin or plasminogen concentration) and milk yield. The effect of milk yield was removed from the model when testing the significance of the other factors on milk yield. For data analysis there were five subclasses for lactation number (1,2,3,4,>4). Stage of lactation was classified into 10 subclasses 1 to 9 consisting of cows in their first to ninth month of lactation, respectively. The last subclass contained samples from cows with more than 9 months in lactation. Treatment consisted of two subclasses; control and treated. All rSTH treated animals were grouped in one category, treated. This was possible as there was no statistically significant difference among the

three different treatments: animals injected with 12.5 mg rSTH animal^{-1} day^{-1}, or 175 mg or 350 mg rSTH animal^{-1} 14 days^{-1}. Differences between the treatments were evaluated with Duncan's Multiple Range Test (SAS, 1982). All the analyses were conducted using the General Linear Model procedure of the Statistical Analysis System (SAS, 1982).

Short-Term Effects of Exogenous Somatotropin on Milk Plasmin and Plasminogen

To determine the time course and the reversibility of rSTH effects on milk plasminogen and plasmin and milk yield, four cows were selected as they concluded the safety efficacy trial. The cows belonged to the group receiving 12.5 mg of rSTH animal^{-1} day^{-1} subcutaneously. rSTH injections were ceased for 4 days and then were resumed for another period of 3 days. Plasmin and plasminogen were determined as described earlier and milk yield was recorded.

An analysis of variance was performed to examine significant effects of time (day) on milk plasmin and plasminogen concentrations and total plasmin production per day (milk yield X plasmin concentration). Differences between the treatments were evaluated with Duncan's Multiple Range Test (SAS, 1982).

Results and Discussion

Determinations of Plasmin and Plasminogen Contents of Milk

The validity of the method of Korycka et al., (1983) for measuring plasmin and plasminogen in milk was confirmed prior to the experimental measurements. The linear ranges of this assay system were established in the first two experiments with respect to milk serum volume, reaction incubation time and total plasmin activity. Fig. 5A shows that the reaction rate was linear up to 3h incubation time while p-nitroanilide formation was proportional to milk serum volume in the range of 25 to 100 μl while (Fig. 5B). In our assay system we used 50 μl of milk serum volume and the reaction was terminated at 1 h, both well within the linear ranges.

Fig. 6A shows the dependency of p-nitroanilide formation on the concentration of the chromogenic substrate, S-2251. Increasing concentrations of S-2251 resulted in increases in the rate of p-nitroanilide formation with the maximum formation achieved at 0.4 mM. We choose the concentration of 0.6 mM (3 X k_m) for S-2251 in our reaction mixture because at this concentration the chromogenic substrate is in excess. Fig. 6B shows the effect of exogenous ϵ-ACA on the p-nitroanilide formation. This experiment was performed to confirm that the quantity (3.3 mM) of ϵ-ACA used in our reaction mixture would not inhibit the hydrolysis of S-2251 by plasmin. Within the range of 2.5-10 mM there was no

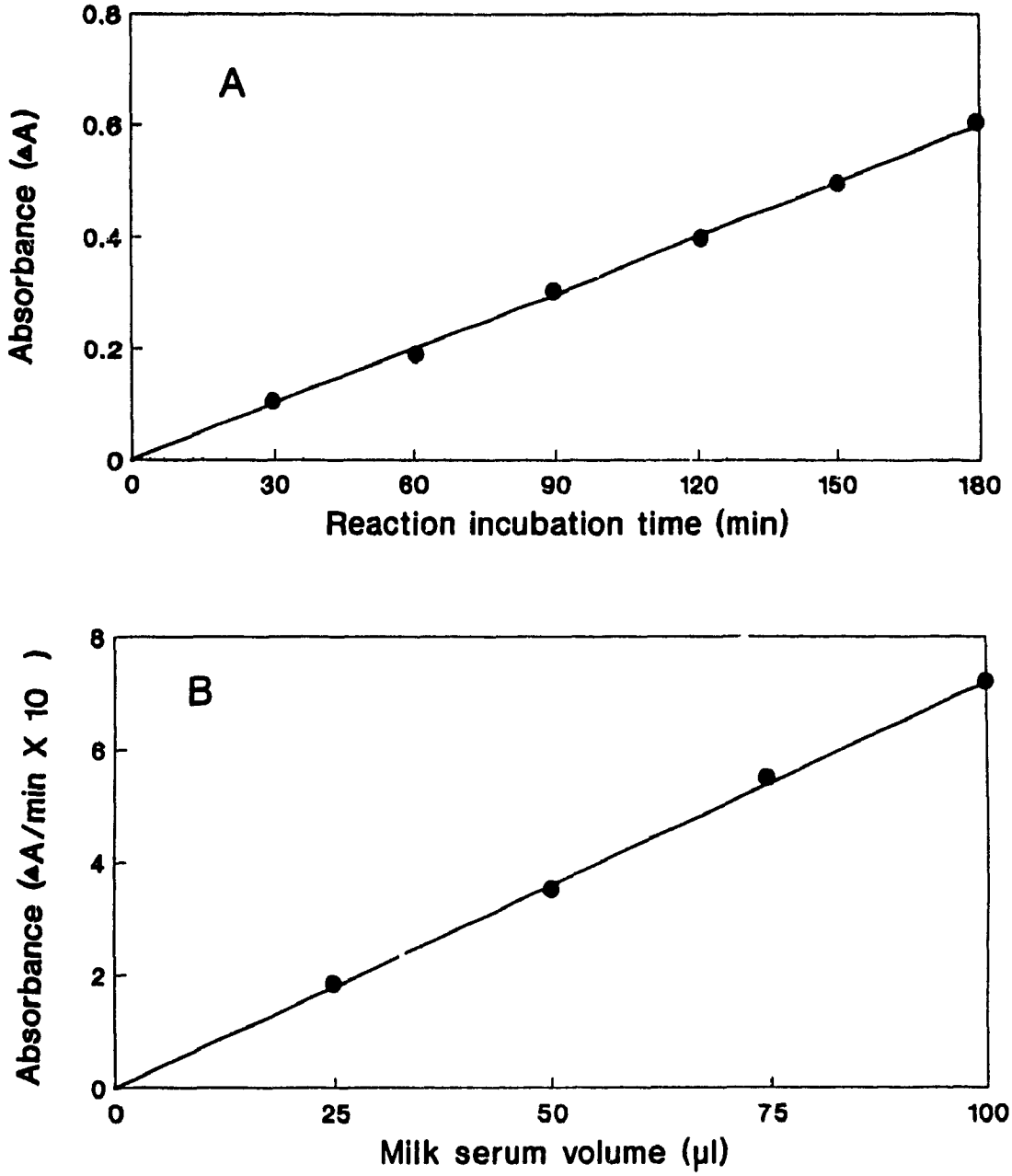


Figure 5. p-nitroanilide formation (A) as a function of the reaction incubation time and (B) as a function of the milk serum volume. Each point represents the average of three independent samples.

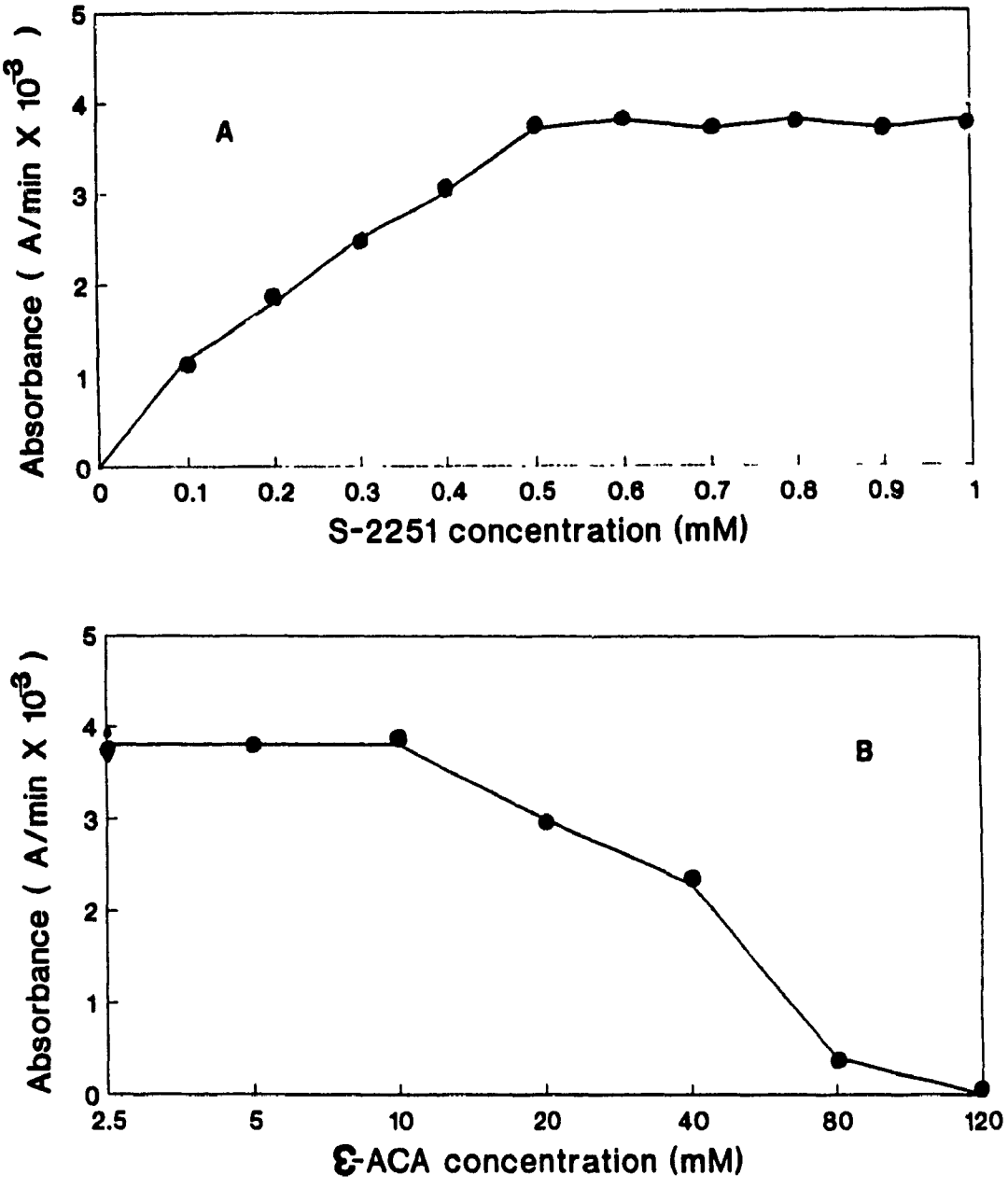


Figure 6 . p-nitroanilide formation (A) as a function of the chromogenic substrate H-D-Val-Leu-Lys-p-nitroanilide (S-2251) concentration and (B) as a function of ε-amino-caproic acid (ε-ACA) concentration. Absorbance was determined in three independent samples for each S-2251 and ε-ACA concentration.

inhibition (Fig. 6B). The hydrolysis of S-2251 by plasmin was partially inhibited between concentrations 20 and 80mM and completely inhibited at 120mM of ϵ-ACA (Fig. 6B). Plasmin determinations in this study were conducted with 3.3 mM ϵ-ACA. Searls (1980) reported that the use of ϵ-ACA can be advantageous as ϵ-ACA strongly inhibits, the binding of plasmin inhibitor, antiplasmin to plasmin by blocking lysine-binding sites in plasmin.

Reimerdes (1978) reported the presence of two serine proteases in milk, the first being plasmin and the second a thrombin-like enzyme. To evaluate the relative contribution of these enzymes, pure bovine thrombin or plasmin (1 μg/ml) was added to the reaction mixture devoid of serum. The thrombin hydrolysed the S-2251 substrate at a rate approximately 3%, compared to the 100% of plasmin. These results corroborate earlier findings by Lottenberg et al., (1981) who reported that thrombin hydrolyses S-2251 at a negligible rate. Therefore, the hydrolysis of S-2251 in our reaction mixture can be attributed to the action of plasmin rather than thrombin. It should be noted that plasmin occurs in milk in higher concentrations than thrombin (Reimerdes, 1978).

The presence of non-serine proteases in milk could confound the interpretation of plasmin assay results through the hydrolysis of the chromogenic substrate S-2251. To quantitate the extent of hydrolysis, plasmin activity was inhibited with 0.6 IU/ml aprotonin. At this concentration aprotonin is a

powerful inhibitor of plasmin (Rollema, 1982). Addition of aprotonin reduced S-2251 hydrolysis by 95 and 92% for normal (<250,000 SCC/ml) or mastitic (>1,000,000 SCC/ml) milk, respectively (Table 1). The residual hydrolysis could be attributed to incomplete inhibition of the enzyme or to the presence of non-serine proteases. In the second case, the residual hyrdolysis would cause a slight overestimation of plasminogen and plasmin concentration.

The overall mean plasmin and plasminogen concentrations and their distribution between the casein and the serum fraction in untreated and treated with 50 mM ϵ-ACA bovine milk samples appear in Table 2. In untreated samples, 98% of the plasmin and plasminogen were present in the casein fraction. Addition of ϵ-ACA led to a transfer of plasminogen from the casein to the milk serum fraction since most of the plasminogen (90%) and 94% of plasmin was present in the milk serum fraction. We decided to measure plasmin and plasminogen in the milk serum fraction after addition of ϵ-ACA because the accuracy of plasmin and plasminogen determinations in the casein fraction is very doubtful. This is because plasmin in the casein fraction has the option of attacking its natural substrate (casein) or the chromogenic substrate. Bastian et al., (1988) reported that casein inhibited the breakdown of S-2251 by plasmin. Similar problems were reported by Richardson and Pearce (1981) who found that casein inhibited the breakdown of their coumarin chromogenic substrate by plasmin.

The average value for plasminogen in milk serum (0.94 mg/l)

Table 1. Impact of adding aprotonin on the rate of hydrolysis of S-2251[1] ($\bar{X}$ + SD), n=10) after addition of 150 Plough units of urokinase.

Source	Normal[2]	Mastitic[3]
	Units[4]	
Milk serum	2.8 ± 0.32	4.23 ± 0.58
Milk serum + aprotonin (0.6 IU)	0.14 ± 0.01	0.29 ± 0.04

1 S-2251: Val-Leu-Lys-p-nitroanilide

2 Normal: < 250,000 SCC/ml

3 Mastitic: > 1,000,000 SCC/ml

4 One plasmin unit is defined as the amount of the enzyme that produces a A_{405nm} of 0.001 in 1 min when p-nitroanilide is measured in our reaction mixture.

Table 2. Effect of ε-ACA on the distribution of plasmin and plasminogen concentrations ($\bar{x}$ + SD) between the casein and the serum fraction of bovine milk[1]

Milk fraction[2]	Plasmin	Plasminogen
	mg/l	
Untreated		
Serum (n=24)	0.003±0.001	0.021±0.002
Casein (n=24)	0.202±0.006	0.922±0.028
Treated with ε-ACA		
Serum (n=96)	0.212±0.052	0.942±0.300
Casein (n=774)	0.013±0.032	0.104±0.022

1 Values are presented as means ± standard deviations

2 Casein fraction is the pellet that precipitates after centrifugation of milk at 100,000 X g for 1 h. The supernatant is referred to as milk serum fraction.

is within the range reported in the literature. Richardson and Pearce (1981) and Richardson (1983) reported values ranging from 0.55-2.75 mg/l while Korycka-Dahl et al., (1983) found an average plasminogen concentration of 1.02 mg/l. The average plasmin concentration of 0.21 mg/l found in this study is higher to that of Korycka-Dahl et al., (1983) 0.1 mg/l, similar to those of Richardson (1983) (0.15-0.37 mg/l), but lower than those of Rollema et al., (1983) (0.7-2.4 mg/l). Discrepancies could be attributed to the widely different methods of measuring plasmin and plasminogen used in these studies. Also, the nature of milk being tested (origin, previous treatment) has not always been sufficiently documented to allow a direct comparison of the results.

Factors Affecting Plasmin and Plasminogen Concentrations in Milk

Results of the analysis of variance that was conducted to investigate variation in milk plasmin and plasminogen concentration due to environmental factors appear in Table 3. From the factors included in the model individual cow, stage of lactation, SCC, season and lactation number had a significant effect on plasmin and plasminogen concentration in milk ($p < 0.01$). Sum of the squares (SS) in Table 3 are the marginal sum of the squares (type III SS in SAS). In this way the effect of any factor on plasmin and plasminogen concentration in milk can be calculated with adjustments being

Table 3. Analysis of variance for factors affecting the plasmin-plasminogen system in milk

Sources of variation	df	Sum of squares		Relative importance[1]	
		Plasmin	Plasminogen	Plasmin	Plasminogen
Individual cow	65	1.40*	15.68*	16.7	5.89
Lactation stage	5	0.40*	3.80*	4.77	1.43
SCC	4	0.40*	32.19*	4.77	12.09
Season	2	0.11*	6.15*	1.31	2.31
Lactation no.	4	0.01	0.15	0.00	0.00
Residual	694	3.81	75.93	-	-

* $p < 0.01$

[1] Relative importance was calculated by dividing the partial sum of the squares by the corrected total sum of the squares

made for the effects of all the other factors included in the model. Thus, the described associations are direct. Table 3 also allows a comparison of the significance of the effects of all the environmental factors on plasmin and plasminogen in milk. Relative importance is not a measurement of the contribution of a particular effect to the total variation but one that enables the comparison of the effects relative to each other. Stage of lactation and SCC were of greater importance (4.77%) than season (1.31%) and lactation number in explaining variation of plasmin in milk. Somatic cell counts were of greater importance (12.09%) than either season (2.31%) or stage of lactation (1.43%) in determining plasminogen variation in milk.

Influence of Somatic Cell Count on Milk Plasmin and Plasminogen Concentrations

Milk plasmin and plasminogen concentrations were increased with increasing SCC (Fig. 7). An increase of SCC from 250,000 to 750,000/ml resulted in a significant ($p < 0.05$) increase of plasmin concentration in milk from 0.18 to 0.28 mg/l. The increase in this range is linear, where every 250,000 increase in SCC/ml led to an increase in plasmin concentration of 0.03 mg/l. Milk containing more than 1,000,000 SCC/ml was associated with a further significant ($p < 0.05$) increase in concentration of plasmin to 0.37 mg/l. These findings are in agreement with those of Barry and Donelly (1981), Donelly

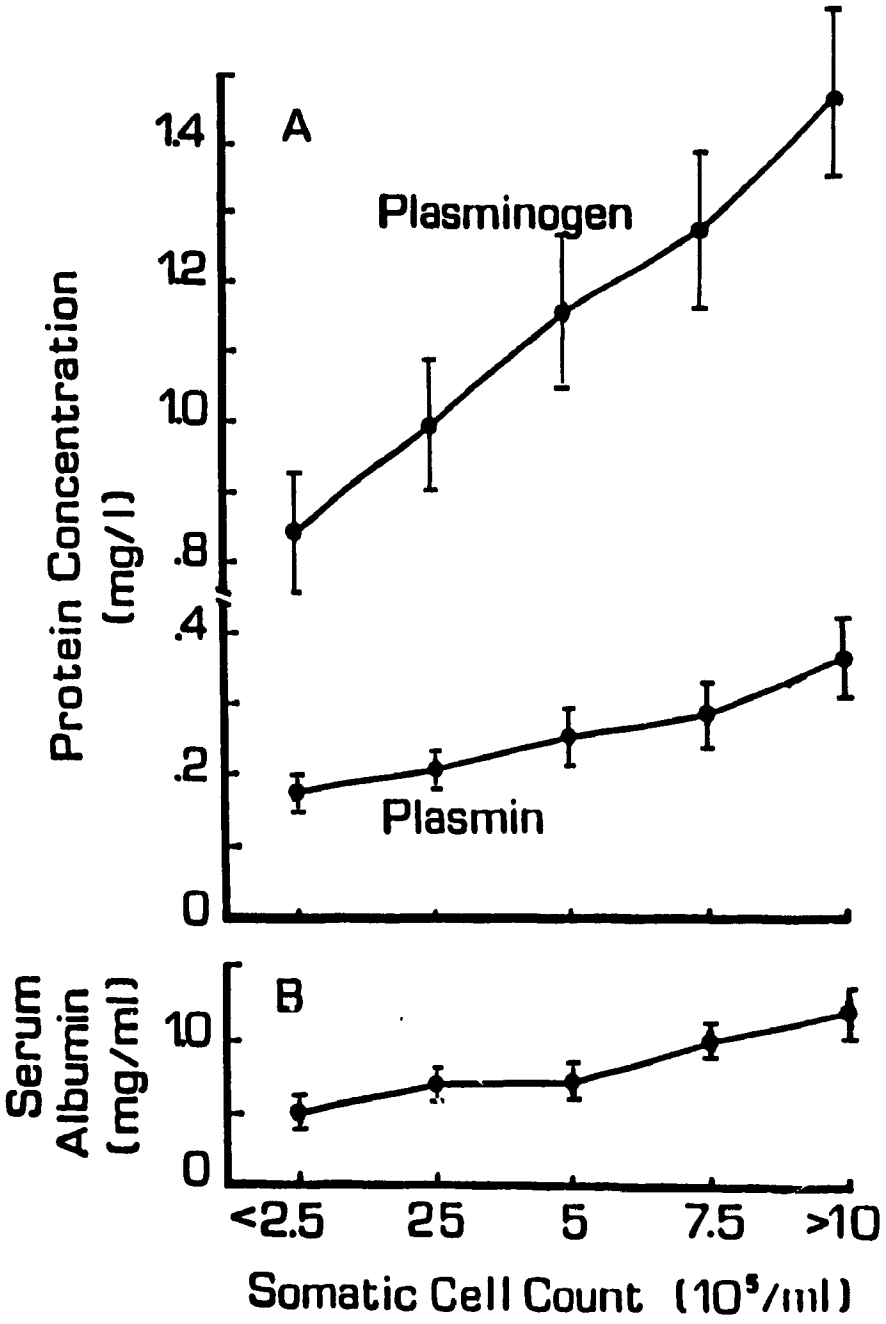


Figure 7 . Effect of somatic cell count on (A) milk plasminogen (o); and plasmin (●). (B) Effect of somatic cell count on milk serum albumin concentration. All values represent means ± SD.

and Barry, (1983), and Politis and Ng Kwai Hang (1989). The trend for plasminogen was similar to that of plasmin in that the increase of SCC from 250,000/ml to more than 1,000,000/ml led to a significant ($p < 0.01$) increase of plasminogen concentration from 0.85 to 1.48 mg/l. The ratio of (plasminogen + plasmin)/plasmin can be used as an index of the activation of plasminogen to plasmin and is a measurement independent of milk volume. This ratio was 5.72 at 250,000 SCC/ml and decreased non-significantly ($p < 0.05$) to 5.0 when SCC exceeded 1,000,000/ml (Fig. 8A). This suggested that increased plasminogen rather than increased activation of plasminogen is the major difference between milk taken from mastitic and normal quarters regarding the plasmin/plasminogen system.

An obvious question then arises: from where is the plasminogen coming? There are two possibilities: transfer of plasminogen from blood to milk or local production in the mammary gland. Even though the actual mode of transport of plasminogen to plasmin from blood to milk is unknown, it seems reasonable to assume that in mastitic quarters where the permeability of the mammary epithelium is compromised, more plasmin and plasminogen will be transferred to the milk. Other plasma proteins with slightly lower molecular weights than plasminogen (82,000) such as serum albumin and antitrypsin with a molecular weight of 70,000 and 68,000 respectively pass through mammary epithelium (Schaar and Funke, 1986). Philippy and McCarthy (1979) estimated that at least 80% of the albumin

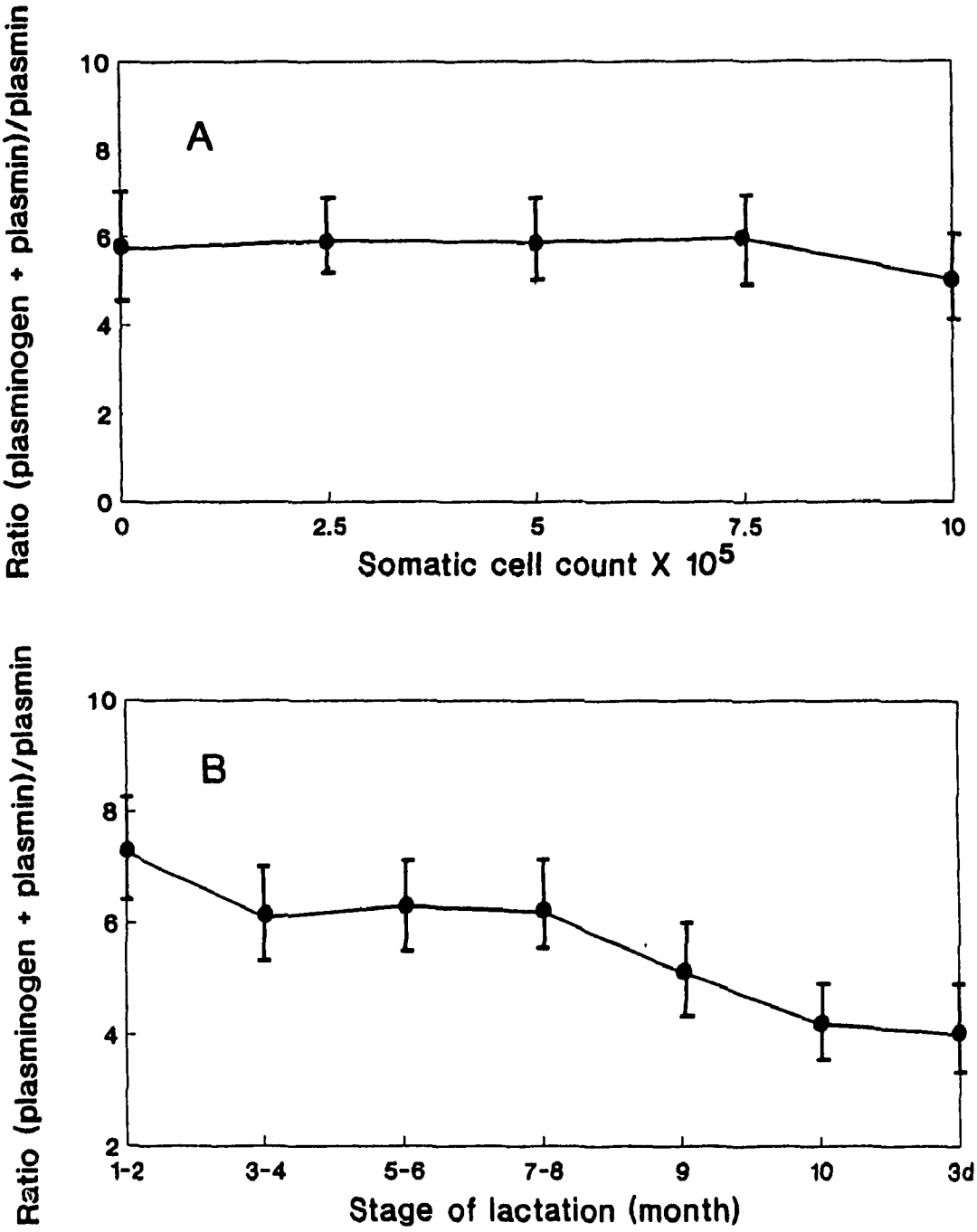


Figure 8 . Effect of somatic cellc counts content of milk (A) and stage of lactation in months and 3 days after drying off (B) on the calculated ratio of (plasminogen + plasmin)/plasmin.

All values represent means ± SD.

found in milk was of blood origin.

To provide some inference on the origin of plasminogen, we decided to analyze the relationship between SCC and serum albumin and the relationship between plasminogen and serum albumin in milk. Figure 7B shows the relationship between SCC and serum albumin in milk. With an increase of SCC from 250,000 to >1,000,000/ml there was a concurrent rise in milk serum albumin level from 0.5 to 1.2 mg/ml. Milk serum albumin and plasminogen followed similar trends with increasing SCC. This conclusion is further strengthen by a highly significant ($p < 0.001$) correlation between serum albumin and plasminogen ($r=0.74$). This supports the hypothesis that these two blood components gain access to the alveolar lumen through ruptured epithelium caused by mastitis.

Somatic cells in bovine milk are mostly neutrophils and macrophages. They are present in high numbers in milk (10-200,000/ml in normal quarters and >1,000,000/ml in mastitic quarters). Both cell types produce plasminogen activators in blood once being activated (PA) (Dano et al., 1985). If this occurs when these cell are present in milk, PA could potentially activate plasminogen to plasmin and therefore decrease the ratio of plasminogen to plasmin. This was not the case as the (plasminogen + plasmin)/plasmin ratio was relatively constant at 5.7-5.0 over the range of SCC from <250,000 to >1,000,000/ml. The potential effect of somatic cells on milk plasmin and plasminogen was further investigated by <u>in vitro</u> mixing experiments. Addition of intact somatic

cell or lysed somatic cells did not significantly ($p < 0.05$) affect plasmin and plasminogen concentrations in milk (Table 4). The results of this in vitro experiment taken together with the observation that the ratio of (plasminogen + plasmin)/plasmin remains unaltered through the whole range of SCC in milk provided strong support for the argument that somatic cells do not affect directly the plasmin and plasminogen system in milk. Even though somatic cells do not have a direct effect on plasmin/plasminogen they may contribute to elevated proteolytic activity through higher levels of non-serine proteases as proposed by Seaman et al., (1988) and Verdi and Barbano (1988).

Influence of Stage of Lactation on Milk Plasmin and Plasminogen

The association of stage of lactation and the plasmin/plasminogen concentrations in milk is shown in Fig. 9. Plasmin concentration was increased from 0.15 mg/l in early lactation to 0.22 mg/l during middle lactation to reach the maximum value of 0.38 mg/l during the latter part of the lactation. All these changes are statistically significant at the level of 0.05. Plasminogen was also increased during lactation from 0.94 mg/l during early lactation to 1.25 mg/l during late lactation (Fig. 9). There was a decrease in the ratio of (plasminogen + plasmin)/plasmin from 7.27 at early lactation to 6.20 during middle lactation (3-4 mo) (Fig.8 B). The ratio

Table 4. Impact of adding isolated somatic cells (1,000,000) on the plasmin and plasminogen concentrations ($\bar{x}$ + SD, n=5) in milk.

Source	Plasmin	Plasminogen
	(mg/L)	
Control	0.20 ± 0.04	1.02 ± 0.24
Control + SC[1]	0.21 ± 0.05	1.04 ± 0.28
Control + SC[2]	0.25 ± 0.06	0.96 ± 0.26

1 SC =somatic cells

2 Somatic cells lysed by passing them through 6 cycles of freezing and thawing

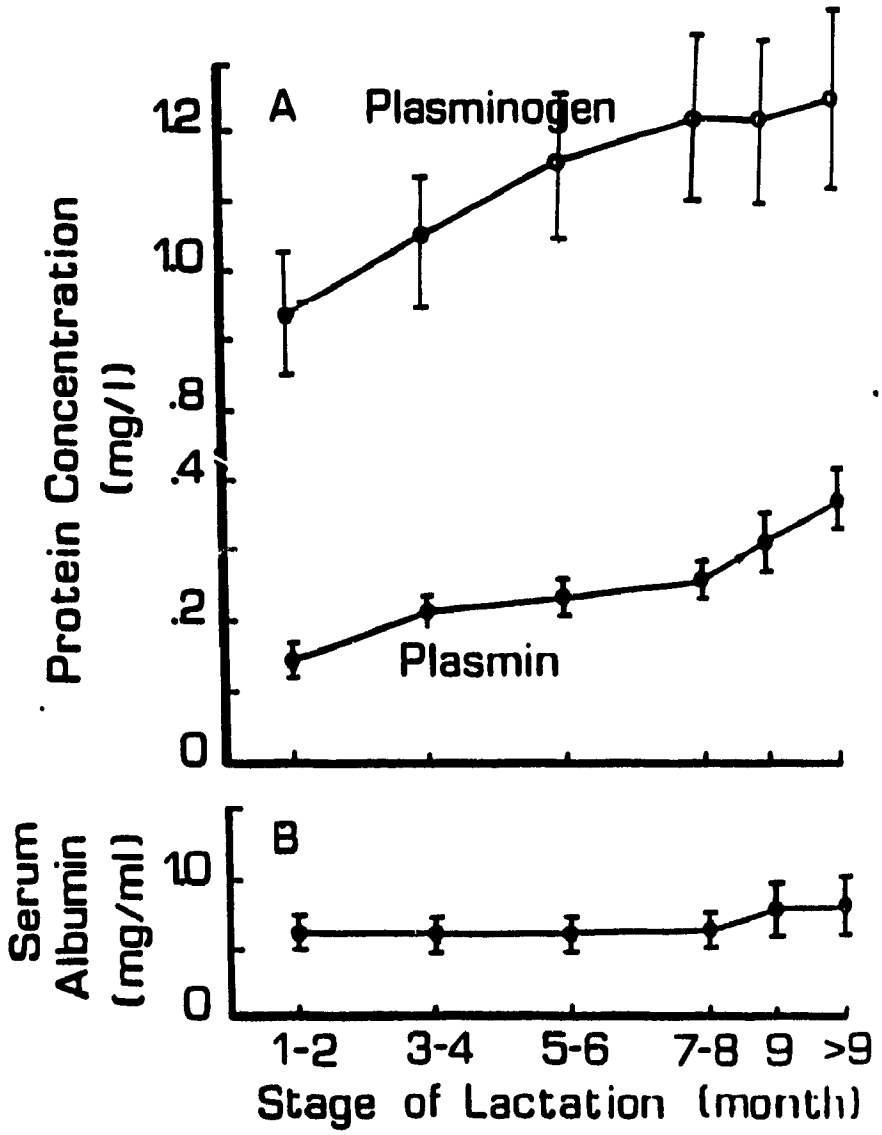


Figure 9 . Effect of stage of lactation on (A) milk plasminogen (○); and plasmin (●) concentrations (B) Effect of stage of lactation on milk serum albumin concentration.

All values represent means ± SD.

remained constant 6.2-6.1 during the next four months in lactation followed by a significant ($p < 0.01$) decrease from 6.1 to 4.3 during late lactation (Fig.8 B).

To evaluate whether this trend persists into the dry period, milk samples were collected from the cows 72 h after the cessation of milk collection. The 72 h interval was chosen because it was assumed that this would be enough time for the involution process to be sufficiently advanced (Hurley, 1989). Plasmin concentration was increased to 0.60 mg/l while plasminogen concentration was increased to 1.80 mg/l resulting in a non-significant ($p < 0.05$) drop of the ratio of (plasminogen + plasmin)/plasmin from 4.3 that was at the end of lactation to 4.0.

To avoid any misleading conclusions with respect to differences in the patterns of activation of plasminogen to plasmin and throughout the lactation period, we analyzed for statistically significant differences in the ratio of (plasminogen + plasmin)/plasmin between different levels of SCC and different stages of lactation. This was possible by fitting the interaction of stage of lactation and SCC in our statistical model. There were no significant ($p < 0.05$) differences in the above ratio throughout the whole range of SCC and for most of the lactation period (3rd to 9th mo). Thus, there is no difference in the activation pattern of plasminogen to plasmin from 3rd to 9th month in lactation when compared to the activation pattern throughout the whole range of SCC. The ratio was 7.27 during early lactation (1-2 mo) and

4.3 during late lactation (10th mo) and both values are different ($p < 0.05$) from all the ratios from the whole SCC range or the other part of the lactation. The higher ratio in early lactation suggests limited activation of plasminogen to plasmin. In contrast, the lower ratio at the end of lactation suggests increased plasminogen activation.

The kinetics of this activation process can be influenced by many factors such as: availability of plasminogen, presence of PA and PAI and milk volume. Enhancement of PA production by mammary epithelial cells which results in local plasmin production is known to be involved in the "initiated involution" in rodents (Ossowski et al., 1979).The most reasonable then explanation of our findings is that plasminogen activator secretion increases within the bovine mammary gland as lactation goes to an end and results in local plasmin production. Increases in plasmin, could initiate surface proteolysis within the alveolus which may affect negatively the ability of mammary epithelial cells to produce and secrete milk.

Influence of Season and Lactation Number on Milk Plasmin and Plasminogen Concentrations

Differences in plasmin and plasminogen concentrations were found between samples from different seasons (Fig.10 A). There was a significant ($p < 0.05$) increase in plasminogen concentration from 0.89 during the fall months to 1.34 during

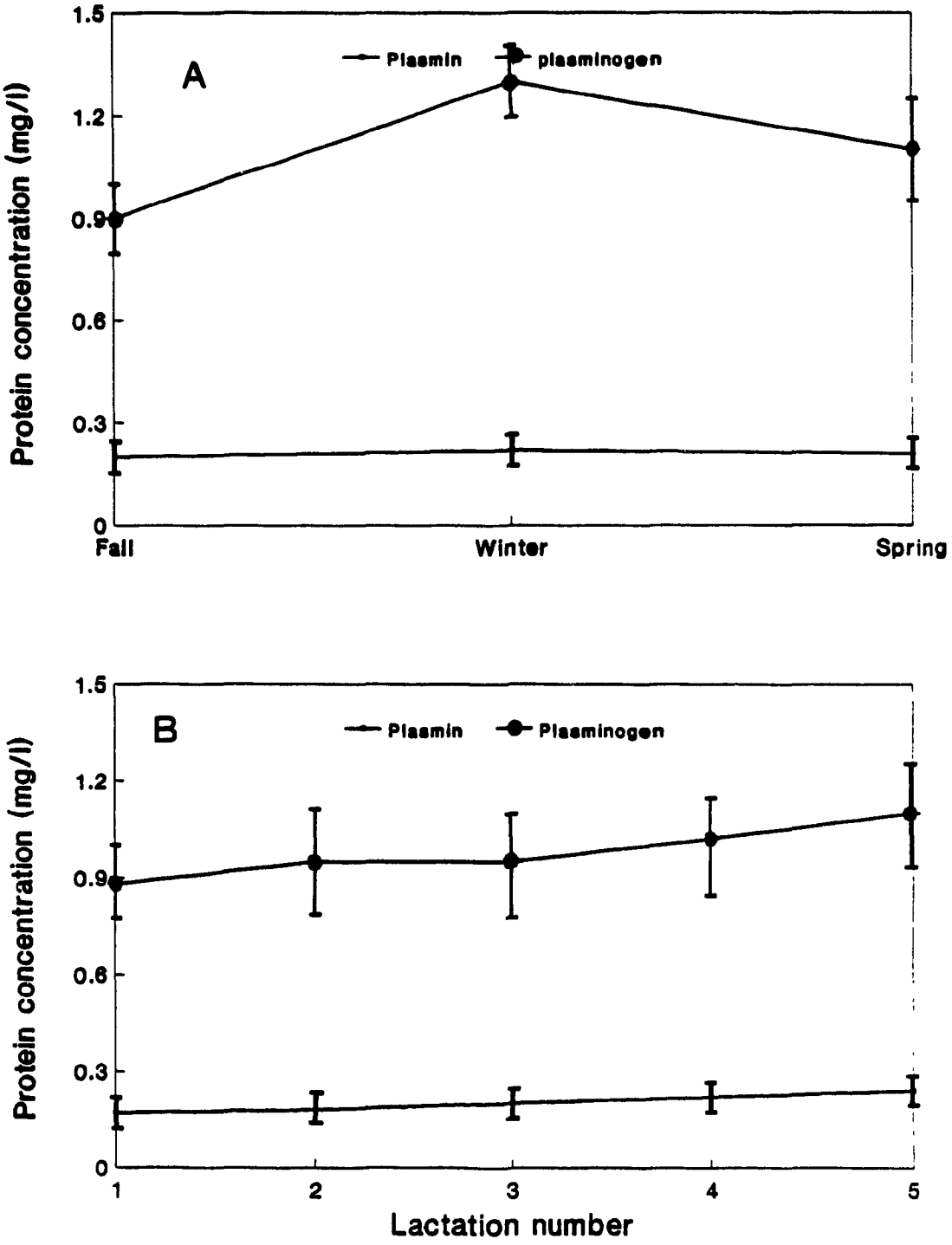


Figure 10. Relationships between (A) season of the year and milk plasmin and plasminogen and (B) lactation number and plasmin and plasminogen.

All values represent means ± SD.

the winter months. Plasminogen concentration decreased during the spring months. There were non-significant ($p < 0.05$) changes in plasmin concentrations during different seasons of the year. A similar trend for plasmin has been observed by Politis and Ng Kwai Hang (1989).

There was a non significant ($p < 0.05$) variation in plasmin and plasminogen concentrations between milk from cows of different lactation numbers (Fig.10 B). However, there was a trend for higher plasmin and plasminogen concentrations with increasing lactation number. Our findings are not in agreement with those of Schaar and Funke (1986) who reported that plasminogen tended to decrease with increasing lactation number.

Results obtained up to this point suggest that there is increased activation of plasminogen to plasmin during late lactation. We have postulated that this increase may be related to the gradual involution process of bovine mammary gland. In the light of the suggestion that exogenous somatotropin increases milk production by retarding involution, we decided to investigate the relationship between exogenous somatotropin administration and plasmin and plasminogen concentrations of milk.

Effect of Exogenous Somatotropin Administration on Milk Plasmin and Plasminogen Concentrations

In Fig. 11 the relationship between milk plasmin concentration and milk yield is presented for control cows and for those treated with rSTH. The three different rSTH treatments have been pooled into one group because there were no significant differences between them. Plasmin concentration remain depressed throughout lactation in cows injected with rSTH (Fig. 11). Similarly, total plasmin production per day (milk volume X plasmin concentration) is maintained at low levels in milk from cows injected with rSTH (Fig. 12). The milk yield data have been superimposed in Fig. 11 to illustrate the inverse relationship between milk plasmin concentration and milk yield. The rSTH-injected cows have persistently higher milk yields and lower milk plasmin concentration and total plasmin production per day.

Milk plasminogen levels expressed as concentration in milk (mg/l) (Fig. 13) or total plasminogen production per day (Fig. 12) are not significantly different ($p < 0.05$) between rSTH-injected and control cows. Milk plasmin is influenced by the availability of precursor plasminogen and the presence of plasminogen activators and inhibitors (Korycka-Dahl et al., 1983). Plasminogen is not the limiting factor for plasmin production in animals injected with rSTH. This suggests that the process by which STH suppresses milk plasmin production in not plasminogen dependent.

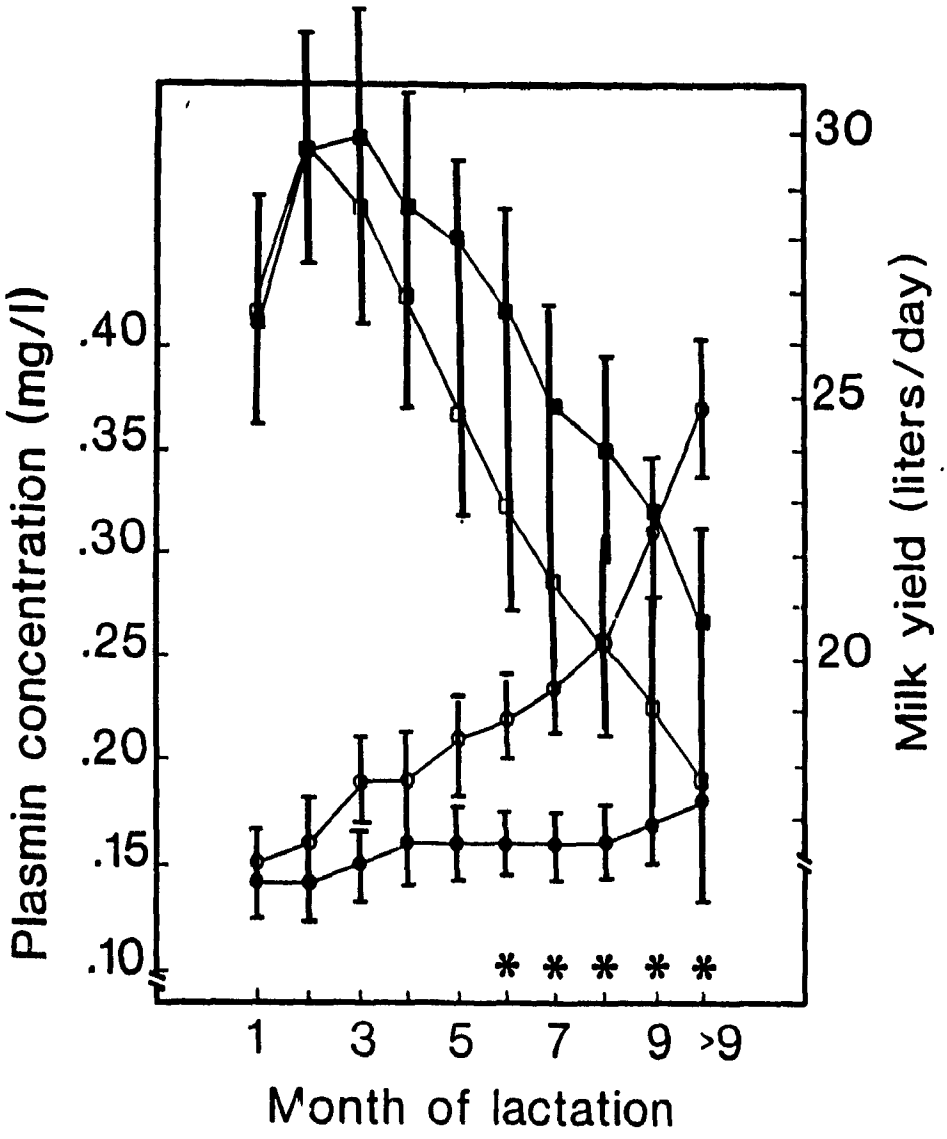


Figure 11. Relationship between milk plasmin concentration and milk yield in control and rSTH-injected cows. Plasmin concentration shown in circles for milk from control (o) or rSTH-injected cows (●). Daily milk yield shown in squares for milk from control (□) or rSTH-injected cows (■).

All values represent least squares means±SD.

* Milk plasmin differences between control and treated samples are significant ($p < 0.05$)

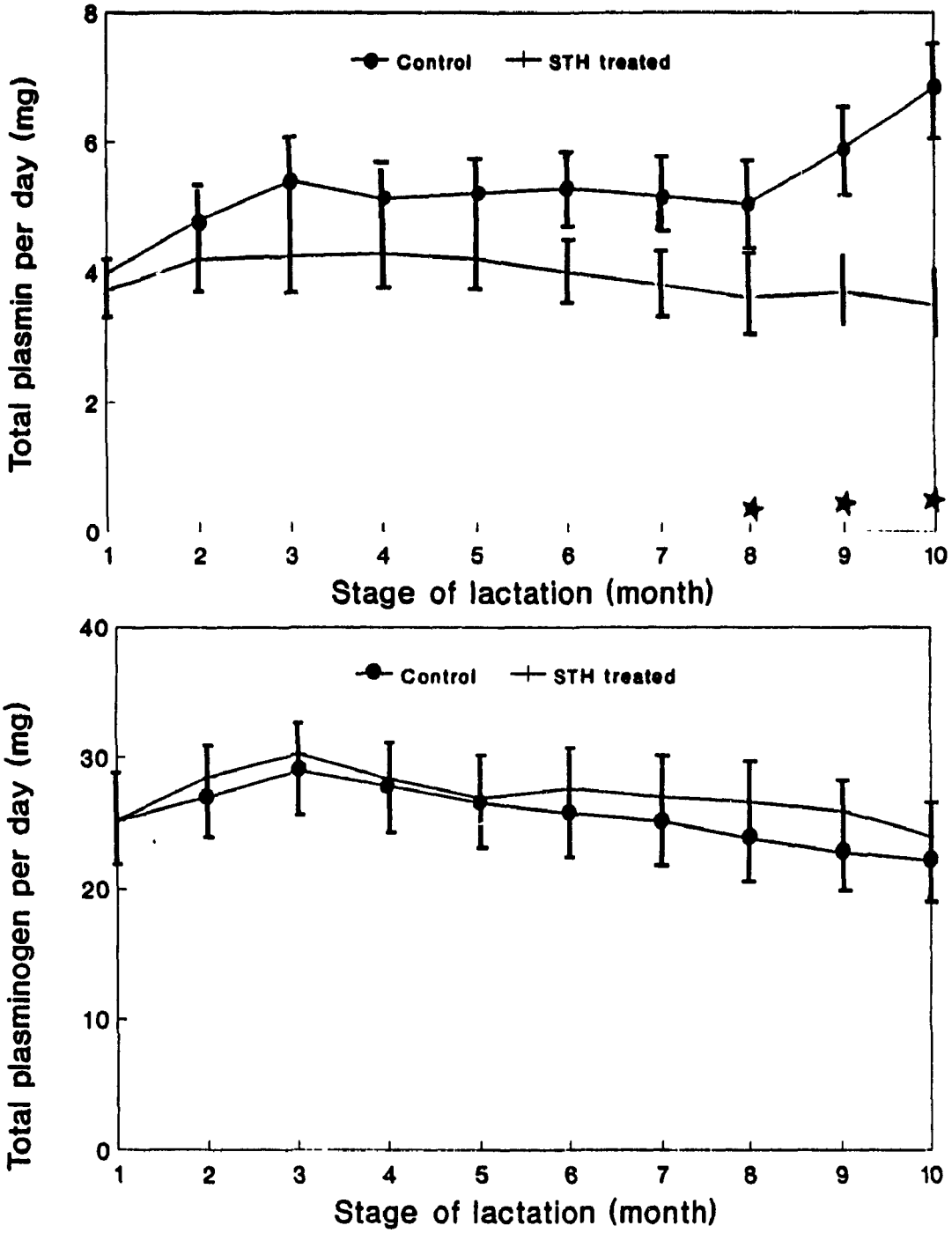


Figure 12. Relationships between rSTH injections and total plasmin (A) and plasminogen (B) production per day throughout the lactation period.

All values represent means ± SD.

★Differences in total plasmin are significant ($p < 0.05$).

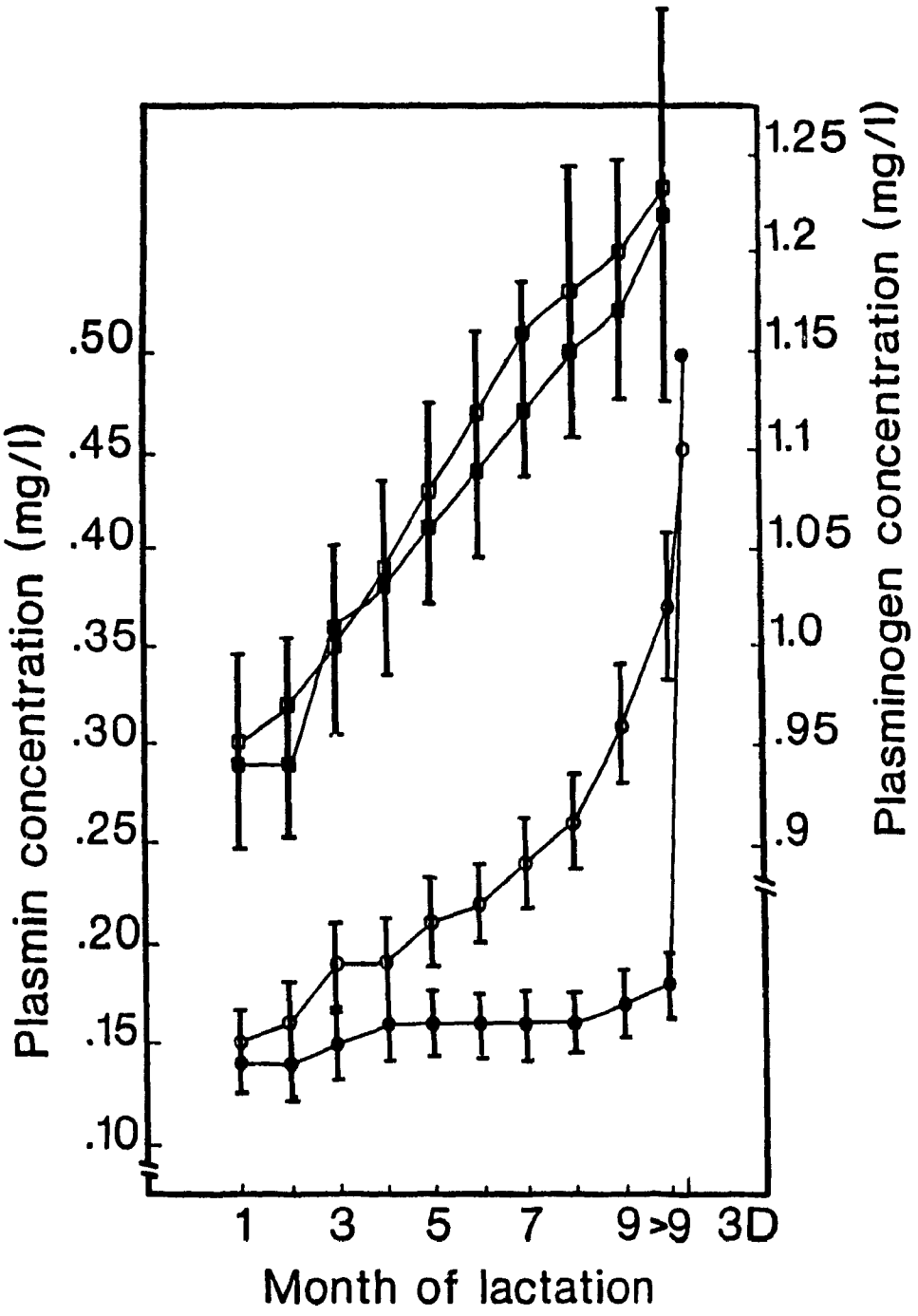


Figure **13** . Effect of rSTH injections on milk plasminogen and plasmin concentration through lactation and 3 days after drying off. Plasmin concentrations are represented for control cows (*o*) and rSTH injected cows (●). Milk plasminogen concentrations are shown for control cows (□) and rSTH-injected cows (■). All values represent means ± SD.

The ratio of (plasminogen + plasmin)/plasmin in milk from rSTH-injected cows, remains remarkably constant throughout lactation (Fig. 14). There are significant differences between treated and non-treated animals during late lactation (6th to 10th month) (Fig. 14). This suggests that rSTH administration is correlated with a suppression in the overall pattern of plasminogen activation during late lactation. It is remarkable that the ratio which was constant throughout the lactation period for the rSTH injected animals dramatically fell from 8.18 during late lactation to 3.35 three days after drying off the animals indicating massive activation of plasminogen to plasmin (Fig. 14). This is not an effect of rSTH since the cows are not injected with the hormone during the three days in the dry period. It illustrates, however, that when the injections were ceased the ratio decreased very quickly to reach the level of the control animals.

Short-Terms Effects of Somatotropin on Milk Plasmin and Plasminogen Concentrations

Administration of rSTH has an immediate effect on milk yield (24-48 h) (McBride et al., 1988). Thus, the time course and the reversibility of the effects of rSTH on milk plasmin is of considerable importance. To study this effect, four cows were selected when they concluded the safety-efficacy trial. The last three days of this trial in which cows received rSTH injections constitute period 1. Injections of rSTH were ceased

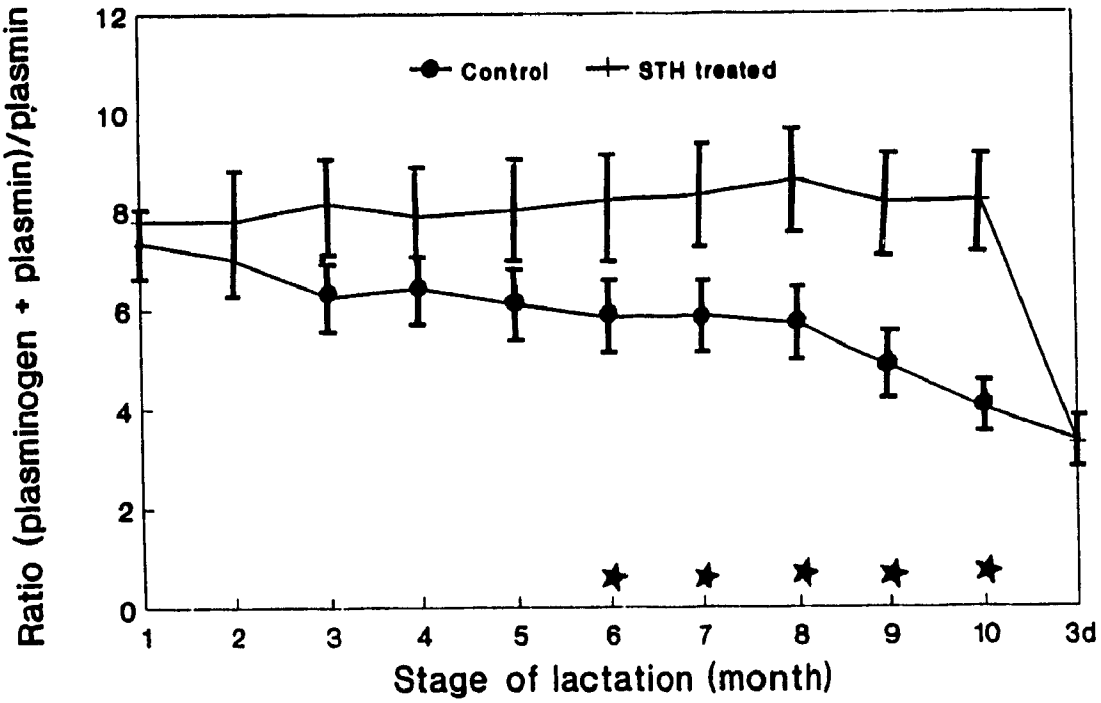


Figure 14. Effect of rSTH injections on the calculated ratio of (plasminogen + plasmin)/plasmin throughout the lactation period and 3d in the dry period for control cows (●) and rSTH injected cows (—+—).

All values represent means +SD.

★Differences in the ratio are significant ($p < 0.05$)

for the next 4 days (period 2) and resumed during the next 2 days (period 3). In this experiment each cow serves as its own control. Plasmin concentration (mg/l) and total plasmin production per day (mg) were not statistically different ($p < 0.05$) during the first three days of this experiment (period 1) when cows received rSTH (Fig. 15A and 15C). On the second and third day following rSTH cessation (period 2) milk plasmin concentration (Fig. 15A) or total plasmin (Fig. 15C) increased ($p < 0.05$). Plasmin decreased slightly during the last day of the rSTH cessation followed by further significant ($p < 0.05$) decreases in plasmin concentration (Fig. 15A) or total plasmin (Fig. 15C) when rSTH injections were resumed (period 3). Plasminogen levels were unaffected by the cessation and resumption of the rSTH treatment (Fig. 15B). These data suggest that cessation of rSTH administration resulted in significant increases in milk plasmin within 48 h, remained high during 72 h and decreased slightly at 96 h after the cessation of rSTH treatment. Resumption of rSTH injection led to a further decrease of milk plasmin levels.

These data suggest that the effect of rSTH on milk plasmin occurs as quickly as the effect on milk yield. To the best of my knowledge, milk plasmin is the only known milk component that changes significantly during STH administration. This led us to suggest that the decrease in milk plasmin which coincide with the increase in the milk production may be closely related phenomena.

The above short-term experiment, allowed a very short

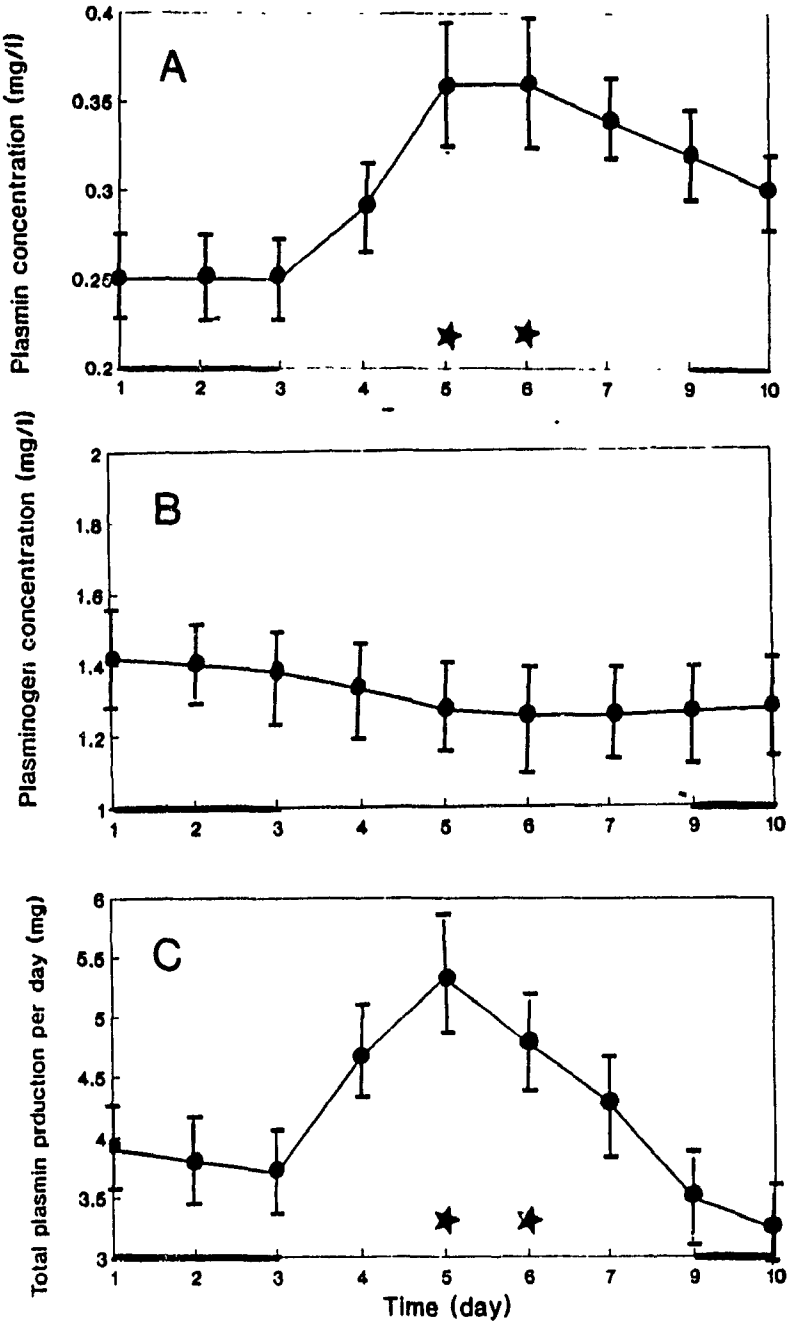


Figure 15. Concentrations of milk plasmin (A), plasminogen (B) and total plasmin production per day (C) (n=4) during cessation and resumption of rSTH treatment in late lactation. Dark areas on the X axis indicate daily rSTH injections. Light areas on the Y axis indicate cessation of rSTH injections.

All values represent means +SD.

★ Differences are significant ($p < 0.05$)

window of rSTH cessation (4 days). Further experiments using larger windows will establish if the increased plasmin levels following rSTH cessation would have been sustained for a longer period of time.

The fact that rSTH administration is correlated with decreased plasmin levels in milk taken together with our suggestion that plasmin may be related to the gradual involution led us to speculate that the lactogenic effect of exogenous rSTH may be exerted through reducing milk plasmin levels, thereby retarding involution (hypothesis) and therefore creating conditions permissive for lactation. Clarification of this possibility must await future experiments. Bovine involution does not involve large-scale epithelial cell sloughing but rather regression (Holst et al., 1987). The role of plasmin may involves surface proteolysis of alveolar epithelial cells leading to the sudden regression of these cells during initiated involution. The extensive breakdown of proteins in the apical region of the mammary epithelial cells is probably non-plasmin mediated. The opposite would require an intracellular role for plasmin which is highly unlikely. Dano et al., (1985) reported that all cell types studied produce inactive plasminogen which is converted to plasmin extracellularly. An alternative possibility is that PA, that are produced by mammary epithelial cells (see next chapter) may be related to this process. Clarification of this possibility is relevant to the question of whether PA exert any function while associated with producer cells.

4. PRODUCTION OF PLASMINOGEN ACTIVATORS AND INHIBITORS BY CLONED BOVINE MAMMARY EPITHELIAL CELLS AND FIBROBLASTS.

Abstract

Cloned bovine mammary epithelial cells and fibroblasts produce tissue-plasminogen activator (t-PA) consisting of two molecular weight (MW) forms (70,000 and 74,000). Two forms of PA inhibitor (PAI-1) with apparent MWs of 120,000 and 126,000 which constitute enzyme-inhibitor complexes were produced by epithelial cells but not fibroblasts. Treatment of epithelial cell extracts with 0.5% SDS and 1.5 NH_4OH resulted in a partial dissociation of both complexes and the formation of a common 50,000 PAI-1 protein. Insulin-like growth factor-I (IGF-I) causes an inhibition of the induction (2.5X) of t-PA protein content which is observed when mammary epithelial cells are cultured in the absence of IGF-1. The levels of PAI-1 protein content were unaffected by the IGF-1 treatment. The effect is specific to t-PA protein and is not the outcome of a general suppression of the cellular metabolism.

Introduction

Previous work from our laboratory (Chapter 3) has demonstrated variations in milk plasmin and plasminogen levels depending upon the stage of lactation and health status of the udder. We have also demonstrated that somatotropin (STH)

administration to dairy cattle maintained milk plasmin levels at the early lactation levels in contrast with untreated animals where plasmin was increased throughout the lactation period. An important question remains unanswered: How somatotropin modulates plasmin levels in milk? Somatotropin cannot exert its effect directly on mammary epithelial cells because these cells do not possess high affinity STH receptors (Akers, 1985; Kazmer et al., 1986). Exogenous STH, does, however increase circulating levels of the peptide hormone, insulin-like growth factor-1 (IGF-1) (McBride et al., 1988). These findings collectively led us to speculate that the effects of STH on milk plasmin are mediated through elevated levels of insulin-like growth factor-1 (IGF-1).

A greater understanding of the relationship between STH or IGF-1 and milk plasmin /plasminogen system in milk is hampered by the absence of very basic information concerning this system. The origin of these proteins be they blood or mammary gland remains unknown. If, indeed, these proteins are native to the mammary gland, the cellular source of plasminogen, plasminogen activators (PA) and PA inhibitors (PAI) must be determined.

Two types of plasminogen activators both serine proteases, have been identified in mammals: urokinase-PA (u-PA, 50 Kd) and tissue-PA (t-PA, 70 Kd) (Dano et al., 1985; Saksela, 1985). Three types of specific PAI have been characterized by immunochemical criteria and have been designated: PAI-1, PAI-2 and protease nexin I (Saksela and Rifkin, 1988). All these

inhibitors are proteins with MWs of approximately 50,000 and all react with both t-PA and u-PA (Sprengers and Kluft, 1987).

This study describes the identification and primary characterization of PA and PAI proteins produced by bovine mammary epithelial cells and fibroblasts in vitro. In addition, the effect of IGF-I on t-PA production by mammary epithelial cells in culture was examined.

Materials and Methods

Origin of Cell Lines

Cloned bovine mammary epithelial cells and fibroblasts were used in these experiments. Each clone was developed from a single cell that grew out of a primary cell preparation of mammary epithelial cells or fibroblasts. These clones were developed, characterized and kindly provided by H.T. Hung from our laboratory.

The mammary epithelial cells are immortalized and not transformed and they have the following characteristics: 1) they grow in monolayers only; they do not overgrow or form loci 2) they have an obligatory requirement for 5% fetal calf serum for long term maintainence 3) are not tumorigenic in immunodeficient mice 4, are anchorage dependent and do not grow on soft agar. The line used resulted from a spontaneous event and is denoted LMH17.

The epithelial nature of the LMH17 cell line was verified

by the characteristic array of cytoskeletal proteins, namely intermediate filaments which were recognized by monoclonal antibodies which react with cytokeratin (type II, subfamily No 1-8). Schmid et al., (1983) used the same criterion for verifying the epithelial nature of cells obtained from bovine mammary gland. In addition, these cells are hormone responsive and produce caseins and lactose. These two milk components are specific to mammary secretory epithelial cells (Fox, 1981). Administration of prolactin (5 μg/ml) led to lactose secretion (2-8 nmol/μg DNA/24h). Western blot analysis showed that extracts of these cells contained α_s-casein and β-casein. Detailed information about these cells was provided by Turner and Hung (1989).

Fibroblasts cell lines were produced in the same manner as the LMH17 line, however they are less well characterized. Criteria for indentification included: 1) morphology under phase contrast 2) positive immunoreactive staining with vimentin and negative with cytokeratins.

Cell Cultures and Preparation of Cell Extracts

Both cell types were grown in 70 cm^2 tissue culture flasks (NUNC, Gibco) in Dublecco's modified Eagle's minimum essential medium (Gibco), supplemented with 10% fetal calf serum, 100 U/ml streptomycin, 100 IU/ml penicillin, insulin (5 μg/ml) and hydrocortisone (1 μg/ml). Cells were passed with calcium free Dublecco's phosphate buffered saline and 10 mM EDTA, 0.5%

(w/v) trypsin. Cells were then placed at high density 3 X 10^7 cells/ 100 mm plate on type I calf tail collagen gels prepared as described by Pitelka et al., (1973). Stock solutions of collagen were prepared by dissolving 1 g of calf tail tendons in 250 ml of 0.1% (v/v) acetic acid. Neutralizing solution was 2 parts 10 X concentrated Waymouth's medium (Sigma) and 1 part 0.34 N NaOH. 1.5 parts of cold neutralizing solution was mixed with 7 parts of cold collagen solution and the mixture was dispensed quickly, 1.25 ml/ 35 mm petri dish. The gel was sterilized by UV exposure for a minimum of 60 min.

The effect of IGF-1 on PA production by mammary epithelial cells was investigated. In the following experiment, H. T. Hung from our laboratory prepared the cells, collagen and added IGF-1. I was responsible for preparing cell extracts, measurements of PA activity and content and characterization of PA and PAI produced by these cells. At the onset of the experiment the medium was replaced with 10 ml of fresh media supplemented with IGF-I (Chemicon, La Jolla, CA) at a final concentration of 50 ng/ml. This is the typical circulating concentration of IGF-1 <u>in vivo</u> following rSTH administration (McBride et al., 1988). Cells without added IGF-1 served as controls. At the beginning of the treatment, collagen gels were released from attachment to the plastic dish by loosening the edges with a glass rod and shaking gently. The floating gel was contracted to about 1/3 of the original diameter. After incubations for 0, 12, 24, 48 or 72 h treated and controls gels washed twice with 10 ml PBS and sonicated for

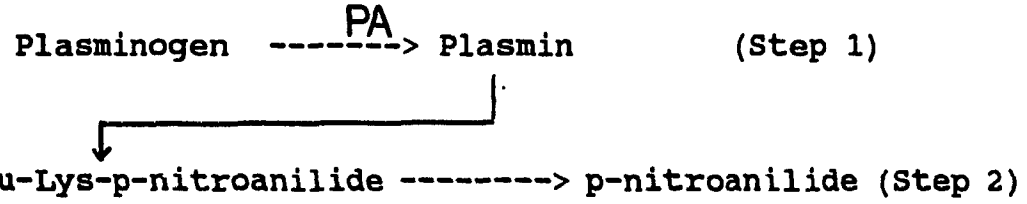
15 sec in 3 ml distilled water as suggested by Searls (1980). The sonicate was centrifuged at 2,500 X g for 10 min to remove cellular debris and collagen and was stored at -20°C until used. The conditioned media was stored in aliquots at -20°C.

Fibroblasts were not subjected to IGF-1 treatment. Fibroblast cell extracts were prepared using the same methodology except that they were grown on plastic substrata and were removed by using a rubber policeman. These cells extracts were used to characterize PA and PAI produced by fibroblasts.

All mammary epithelial and fibroblast samples were analyzed for the presence of PA and PAI after having been frozen and thawed only once. Repeated freezings and thawings resulted in loss of PA and PAI. We estimated that after two cycles of freezing and thawing, there was a 30% and 10% decrease in PA and PAI activity, respectively.

Determination of Plasminogen Activator Activity in Cell Extracts and Conditioned Medium

Plasminogen activator activity was assayed by the spectrophotometric method of Searls (1980). This is a two step assay system:



In the first step, PA present in the sample to be assayed will convert plasminogen to plasmin. In the second step, plasmin so produced will attack the chromogenic substrate adjacent to lysine and will liberate the chromophore p-nitroanilide. The method in details has as follows: Ten μl of epithelial or fibroblast cell extracts were mixed with 140 μl 50mM Tris buffer (pH 7.8) containing 3 mg/ml bovine plasminogen and 50 mM ε-amino-caproic acid (ε-ACA; Sigma). ε-ACA at this concentration enhances the conversion of plasminogen to plasmin (Searls, 1980). The reaction mixture was incubated at 37°C for 1h; 750 μl of 120 mM L-lysine containing 0.9 mM of the chromogenic substrate Val-Leu-Lys-p-nitroanilide dihydrochloride (S-2251; Sigma) were added. L-lysine at this concentration will inhibit further conversion of plasminogen to plasmin without affecting the rate of hydrolysis of S-2251 by plasmin. The reaction mixture was incubated for 30 min and the reaction terminated by the addition of 300 μl 40% trichloroacetic acid (TCA). The liberated p-nitroanilide was diazotized and coupled to N-(1-naphthyl) ethylenediamine dihydrochloride (NED; Sigma) by the addition of 100 μl of 0.1% (w/v) sodium nitrite, then 100 μl of 0.5% (w/v) ammoniun sulfamate and finally 100 μl of 0.1% (w/v) NED. This step results in a four-fold increase in the molar absorbancy index of the final product (Searls, 1980). In all assays, a sample without added plasminogen served as a control. No plasmin activity was detected in the absence of a source of

plasminogen which suggests that these cells do not produce plasminogen. Plasminogen activator activity was expressed as change in absorbance (545 nm) per hour per μg of protein. Protein content was determined by the method of Bradford (1976) using bovine serum albumin (Sigma) as a standard.

Validation of the Plasminogen Activator Activity Assay

A series of preliminary experiments to validate the above assay system for measuring PA activity in mammary epithelial cell extracts was performed prior to experimental measurements. The first two experiments were performed to establish the linear range of the reaction with respect to cell extract volume and the reaction incubation time. Effect of cell extract volume was evaluated in the first experiment by assaying different cell extract volumes: 10, 20, 30, 40 or 50 μl with the final volume assayed in each case being adjusted to 50 μl by addition of H_2O. In the second experiment, time-related changes in the determination of PA activity were examined by incubating the reaction mixture for 15, 30, 45 and 60 min in order to determine the appropriate reaction incubation time to measure PA.

The third experiment established the optimum concentration of chromogenic substrate (S-2251). Replicate tubes containing 10 μl of cell extracts were assayed for PA activity in the presence of various concentrations of S-2251 in the range of 0.1-1 mM. The fourth experiment established the optimum

concentration of exogenous plasminogen added to the reaction mixture by assaying replicate tubes containing 10 μl of cell extracts in the presence of different plasminogen concentrations in the range of 0.5-4 mg/ml. Plasminogen should be added in high enough concentration to ensure that the reaction rate will not be limited by plasminogen availability.

Characterization of the PA and PAI Activity

Our methodlogy in order to characterize PA and PAI proteins produced by mammary epithelial cells and fibroblasts included:

1) Western blot analysis using specific antibodies against PA or PAI.

2) Localization of PA and PAI within the electrophoretic lane following SDS-PAGE, extraction of the protein and determination of its activity, molecular weight and isoelectric point.

The first step in the characterization of PA activity and PAI activity in mammary epithelial and fibroblast cell extracts prepared as described above, involves SDS-PAGE (Laemmli, 1970) using 10% acrylamide resolving and 4% acrylamide stacking gels. The resolving gel was made from a mixture of 10 g of acrylamide- N',N'-methylenebisacrylamide (Bio-Rad) (29:1) and 0.1 g of sodium dodecyl sulfate (SDS) dissolved in 100 ml of 0.375 M Tris buffer (pH 8.8) containing 60 μl of N, N, N', N' tetramethylenediamine (TEMED) (Bio-Rad)

and 0.5 ml of 10% (w/v) ammonium persulfate (Bio-Rad). The stacking gel was made from a mixture of 4 g of acrylamide-N,N' methylenebisacrylamide (29:1) and 0.1 g of SDS dissolved in 100 ml of 0.125 M Tris (pH 6.8) containing 60 µl of TEMED and 0.5 ml of 10% (w/v) ammonium persulfate. Twenty-five µl of mammary epithelial or fibroblast cell extracts (3 X 10^7 cells sonicated in 3 ml of H_2O) were mixed with 25 µl of 1 M Tris sample buffer containing 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue and 5% (w/v) SDS. Samples were not reduced or boiled. This was necessary to preserve the integrity of the PA molecule which contains disulfide bridges (Dano et al., 1985). Samples were then applied to the gels in the Bio-Rad mini-gel apparatus. Two known molecular weight (MW) standards were used (Pharmacia, Piscataway, NJ): a low MW including phosphorylase (94,000), serum albumin (67,000), ovalbumin (43,000), carboxylase anhydrase (30,000) and trypsin (20,000) and a high MW consisting of ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000) and bovine serum albumin (67,000). We used two molecular weight markers because the range of the molecular weight for PA and PAI in the literature is 25,000 to 140,000. Electrophoresis were conducted in a 0.02 M Tris-0.15 M glycine buffer (pH 8.3) at 40 V until the tracking dye reaches the bottom of the gel. When the electrophoresis was completed, the gel portions containing molecular weight standards were cut off the gel and stained for 2h in 1% (w/v) Coomassie Blue and 50% (w/v) TCA, then destained in 20% (v/v) acetic acid. The distance of

migration of each protein in the above standards from the bottom of the well was recorded. The remaining portion of the gel containing the fractionated proteins of the cell extracts was processed either for Western blots or for localization of PA activity.

Western Blot Analysis

Western blots were prepared using nitrocellulose membranes (Bio-Rad) and electrostatic transfer of the samples fractionated by SDS-PAGE (100 V, 1.5 h, 4°C, Bio-Rad Transblot apparatus) in 0.02 M Tris, 0.15 M glycine, (pH 8.8) containing 17% methanol. To facilitate transfer two filter papers soaked in 1% SDS were placed on the cathodic side of the gel. Membranes were incubated for 30 min in 50 mM Tris, 500 mM NaCL, pH 7.5 (BS) containing 3% gelatin. After 6 washes with BS, membranes were incubated for 3 h at room temperature in 10 ml of BS containing 1% gelatin and goat anti-human t-PA, goat anti-human u-PA IgG or goat anti-porcine t-PA IgG (American Diagnostica, Greenwich CT) at a final concentration of 3 µg/ml or rabbit antisera against bovine PAI-1 (Hekman and Loskutoff, 1985) at a 1:500 dilution. The antisera against bovine PAI-1 was a generous gift from Dr. D. Loskutoff, La Jolla, CA. After 6 washes with BS, membranes were incubated in 10 ml of BS containing 1% gelatin and peroxidase-conjugated anti-goat or anti-rabbit IgG at a 1:2000 dilution (Sigma). After 1 h incubation, the membranes were again washed 6 times

and peroxidase staining was performed in 100 ml of BS containing 0.003% hydrogen peroxide and 60 mg 4 chloro-1 napthol (Bio-Rad). A sister blot was developed using non-immune goat or rabbit serum (American Diagnostica) to serve as a control.

Localization of PA and PAI in Electrophoretic Gels

The position of PA or PAI within the electrophoretic lanes, was determined as described by Loskutoff et al., (1983). This procedure involves three sequential steps: 1) cutting the gel into pieces, 2) extracting the protein from each gel piece into Tris buffer after removal of the SDS and 3) assaying all the extracts for PA activity. This methodology takes advantage of the fact that PA are not irreversibly inactivated by SDS (Strickland and Beers, 1976; Levin and Loskutoff, 1982; Loskutoff et al., 1983; Dano et al., 1985; Saksela, 1985; Booyse et al., 1988). In all these cases, cell extracts were fractionated by SDS-PAGE, gels were sliced and the slices were directly assayed for PA activity after the addition of 3 volumes of Tris buffer to neutralize SDS. In our system we used Loskutoff et al., (1983) procedure who added a PA extraction step prior to PA determination.

Electrophoretic lanes were cut into approximately 5 mm slices. Each gel slice was incubated in 0.1M Tris buffer (pH 8.1) containing 0.5% Triton X-100 to remove SDS. After 1 h, the Tris buffer was discarded and the slices were allowed to

continue soaking in 0.5 ml of the same Tris buffer for further 16 h to elute the proteins. Aliquots of 100 µl of the gel extracts were tested for PA activity as described above. The gel extracts containing the inhibitor protein present in 0.6 ml of cell extracts were pooled. The inhibitory capacity of these gel extracts was determined by incubating 100, 200, 300 and 400 µl of gel extract with 70 IU/ml urokinase (American Diagnostica). Samples were subsequently tested for the remaining PA activity as described above. Extracts from a blank gel were tested for inhibitory capacity inherent in the gel itself. This accounted to 1% of the total inhibitory capacity.

Isoelectric foccusing

In order to further characterize the PA and PAI proteins present in mammary epithelial and fibroblasts cell extracts, the isoelectric points of PA and PAI were determined. The gel extract containing PA or PAI activity was electrofocussed in a 5% polyacrylamide gel containing 6M urea and 5% (w/v) ampholytes (pH 3.5-10, Bio-Rad), 0.5% (v/v) Triton X-100, and 10% (v/v) glycerol. We followed the protocol suggested by Levin and Loskutoff (1982). Electrophoresis was performed at 30 V for 3 h using 1 M NaOH and 1 M H_3PO_4 as electrode solutions. Following electrofocussing, each lane was removed from the gel, cut into 5 mm slices and each slice was incubated in 1 ml of 0.5% Triton X-100 for 30 min and the pH

was determined (Levin and Loskutoff, 1982). The slices were allowed to continue soaking for further 16 h at 4°C in 1 ml of 0.1 M Tris buffer containing 0.5% (v/v) Triton X-100 and PA or PAI activity was assayed in a 100 µl aliquot as described above.

Dissociation of the High Molecular Weight Form of PAI

In order to dissociate the high MW forms of PAI present in mammary epithelial cell extracts (see results) a 50 µl aliquot of mammary epithelial cell extracts was treated for 30 min at 37°C with 0.5% SDS and 1.5 M NH_4OH. This procedure was developed by Booyse at al., (1988) and is known to dissociate PA-PAI complexes into intact PAI and PA. Samples were then dialysed over night at room temperature against 0.1 M Tris (pH 8.1) containing 0.5% Triton X-100, and then analyzed for the presence of the low MW of PAI by Western blot analysis and localization of the protein within the electrophoretic lane.

Acid-Labile Protease Inhibitors in Fibroblasts

The methodology to test for the presence of native acid-labile protease inhibitors has been developed by Schaar and Funke (1986). One hundred µl portions of fibroblast cell extracts were treated with 200 µl 25 mM HCl to reach a final pH 2.7. After 1h, 200 µl 25 mM NaOH were added. Control samples were diluted with 400 µl of a neutral solution

containing HCl and NaOH. Assays for PA were then performed as described above. PAI activity is defined in this case as the difference between PA activity prior to and after the acid treatment.

Determination of Plasminogen Activator (PA) and PA Inhibitor Content.

Tissue-PA and PAI-1 protein content in mammary epithelial cell extracts was determined by an Enzyme Linked Immunosorbent Assay (ELISA) using goat anti human u-PA IgG or rabbit antisera against bovine PAI-1 as primary antibodies and peroxidase-conjugated anti-goat or anti rabbit IgGs (Sigma) as the secondary antibodies. Test samples were appropriately diluted (usually a three fold serial dilution) in 0.1 M bicarbonate buffer, pH 9.6 and then 100 μl aliquots were incubated overnight at 4°C in each well of the 96-well polystyrene microtiter plate (Fisher Scientific). Remaining active sites were blocked by incubation for 1 h at 37°C with 200 μl PBS (0.14 M NaCL, 0.01 M NaH_2PO_4, pH 7.2) containing 1% bovine serum albumin (BSA). Following 6 washes with PBS containing 0.05% Tween 80, 100 μl of the goat anti-human t-PA IgG at a final concentration of 0.8μg/ml or rabbit antisera against bovine PAI-1 at 1:1000 dilution in PBS were added for 1h at 37°C. After another 6 washes, 100 μl of anti-goat IgG-peroxidase conjugate in PBS were added to each well at a dilution of 1:2000. Following 1 h incubation at 37°C, the

plates were again washed 6 times and 100 µl of 0.182 mM ABTS (2', 2'- azino-diethylbenzothiazoline sulfonate, Sigma) in 0.1 M citrate, pH 5.2, containing 0.003% hydrogen peroxide were added per well and the absorbance was measured at 405 nm after 10 min. Various controls included: 1) wells coated with BSA without sample 2) wells without the first antibody layer and 3) wells with the first antibody layer substituted by non-immune serum (American Diagnostica). For the determination of t-PA content, t-PA (American Diagnostica) was used as a standard. For the PAI-1 determination, t-PA and PAI-1 were incubated for 1 h and the formatted complex was used as a standard. The detection limit for t-PA and PAI-1 in our assay system was approximately 1 ng/ml and absorbance was linearly related to t-PA and PAI-1 concentrations up to 10 and 5 ng/ml respectively.

Results

A series of preliminary experiments were initially performed to validate the method of Searls (1980) for measuring PA activity in mammary epithelial cell extracts. Formation of p-nitroanilide was directly proportional to the cell extract volume up to 40 µl (Fig. 16A) while the reaction rate was linear up to 60 min incubation time (Fig. 16B). All subsequent determinations were performed using 10 µl sample and the reaction was incubated for 15 min, well within the linear ranges of this assay system.

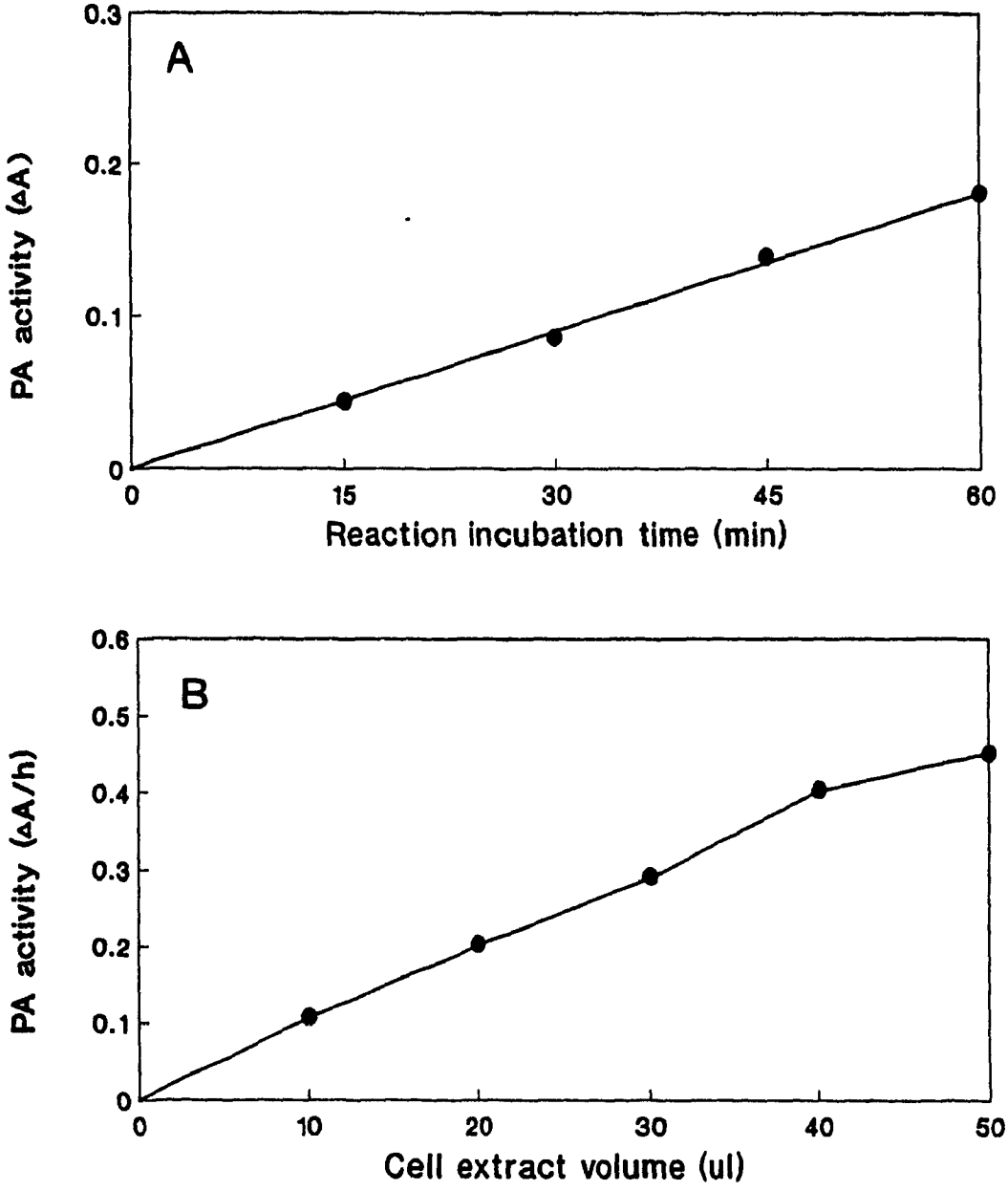


Figure 16. p-nitroanilide formation (A) as a function of the reaction incubation time and (B) as a function of the cell extract volume. Each point represents the average of three independent samples.

Chromophore (p-nitroanilide) formation as a function of the chromogenic substrate (S-2251) concentration is presented in Fig. 17A. Increasing concentrations of S-2251 led to an increase in the amount of chromophore (p-nitroanilide) released with the maximum absorbance achieved at 0.6 mM. We choose the concentration of 0.9 mM in our assay system because at this concentration the S-2251 is in excess. Formation of p-nitroanilide as a function of the exogenous plasminogen concentration is shown in Fig. 17B. Increasing concentrations of plasminogen in the range of 0 to 3 mg/ml resulted in increases in the rate of p-nitroanilide formation. A further increase in plasminogen concentration resulted in no further increase in the rate of chromophore release. Exogenous plasminogen was added in our reaction mixture at a concentration of 3 mg/ml and at this concentration was not limiting for the reaction rate. This is critical in samples containing very high PA activity, where the danger exists that PA could exhaust the exogenous plasminogen prior to the completion of the reaction (15 min).

To determine the type of PA and PAI present in mammary epithelial and fibroblast cell extracts, Western blot analysis was performed. The absence of available homologous (bovine) antibodies led us to use human and porcine antibodies that were commercially available (American Diagnostica). Antibodies against human and porcine t-PA and human u-PA gave no insight with regard to the nature of PA produced by epithelial cells and fibroblasts due to cross-reactivity between these

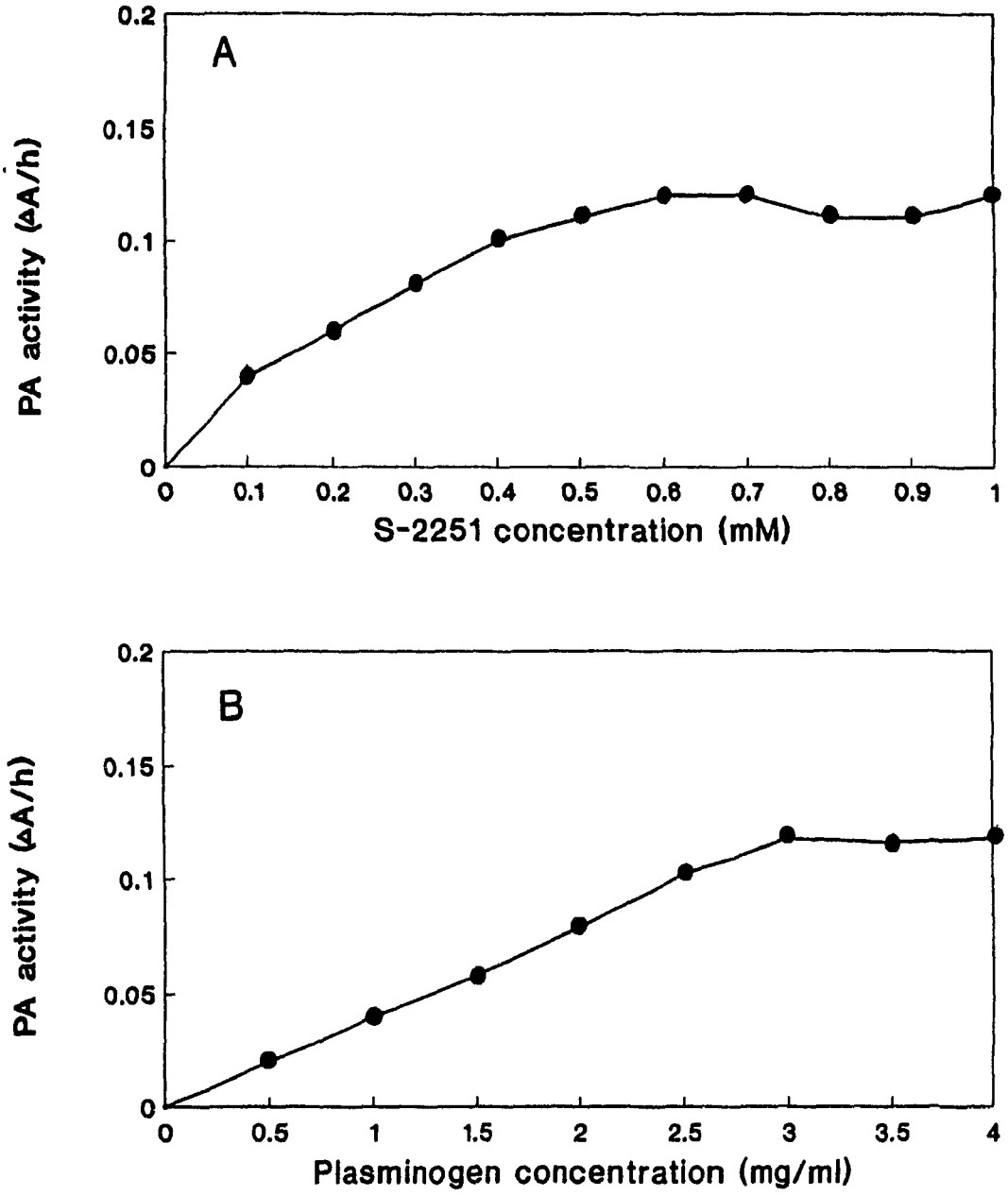


Figure 17. p-nitroanilide formation (A) as a function of the chromogenic substrate H-D-Val-Leu-Lys-p-nitroanilide (S-2251) concentration and (B) as a function of the exogenous plasminogen concentration. Each point represent the average of three independent samples.

antibodies. However, all antibodies detected two main bands of PA with apparent MWs of 70,000 and 74,000 (Fig. 18). Following SDS-PAGE, gel slices were removed from sister gel lanes, and PA activity was detected in two gel pieces corresponding to these MWs (Fig. 19A). If our recovery of PA activity after SDS-PAGE had been 100%, we should have recovered a total of PA activity 0.75 (0.05 X 15) from mammary epithelial cell extracts and 0.45 (0.03 X 15) from the fibroblasts. The above calculations are based on the fact that the activity ($\Delta A/h/\mu g$ protein) of the samples were 0.05 and 0.03 for mammary epithelial and fibroblasts, respectively (see Fig. 23) and 15 μg of protein were loaded to each gel channel. The actual recoveries were (0.38 + 0.07)/0.75 = 61.3% for mammary epithelial cells and (0.20 + 0.06)/0.75 = 57.8% for fibroblasts. The low MW form (70,000) contained 84.8% and 77% of the total activity recovered after SDS-PAGE from mammary epithelial and fibroblasts, respectively while the high MW (74,000) accounted for the remainder.

The isoelectric points (pI) of both forms of PA ranged from 7.7 - 7.9 with the peak occurring at pH 7.8 (Fig. 19B). The recovery of PA activity after isoelectric focussing was 0.24/(0.38 + 0.07) = 52% in mammary epithelial cells and 0.16/(0.20 + 0.06) = 61.5% in fibroblasts. All these recoveries are overestimated because PA activity is determined in gel extracts without the influence of PAI which is not the case for the original determination of t-PA activity in the mammary epithelial and fibroblast cell extracts.

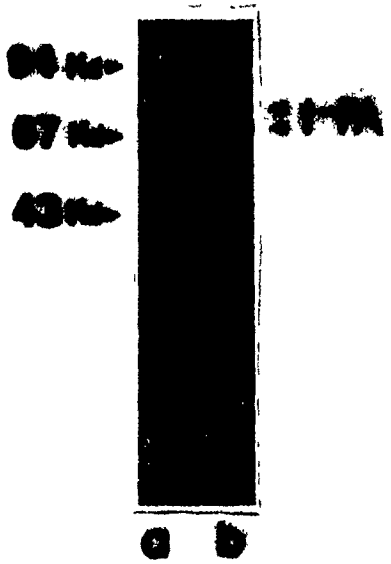


Figure 18 . Western blot analysis of bovine (a) mammary epithelial (15 μg protein) and (b) fibroblast cell extracts (25 μg protein) with purified antibodies against human tissue-plasminogen activator (t-PA).

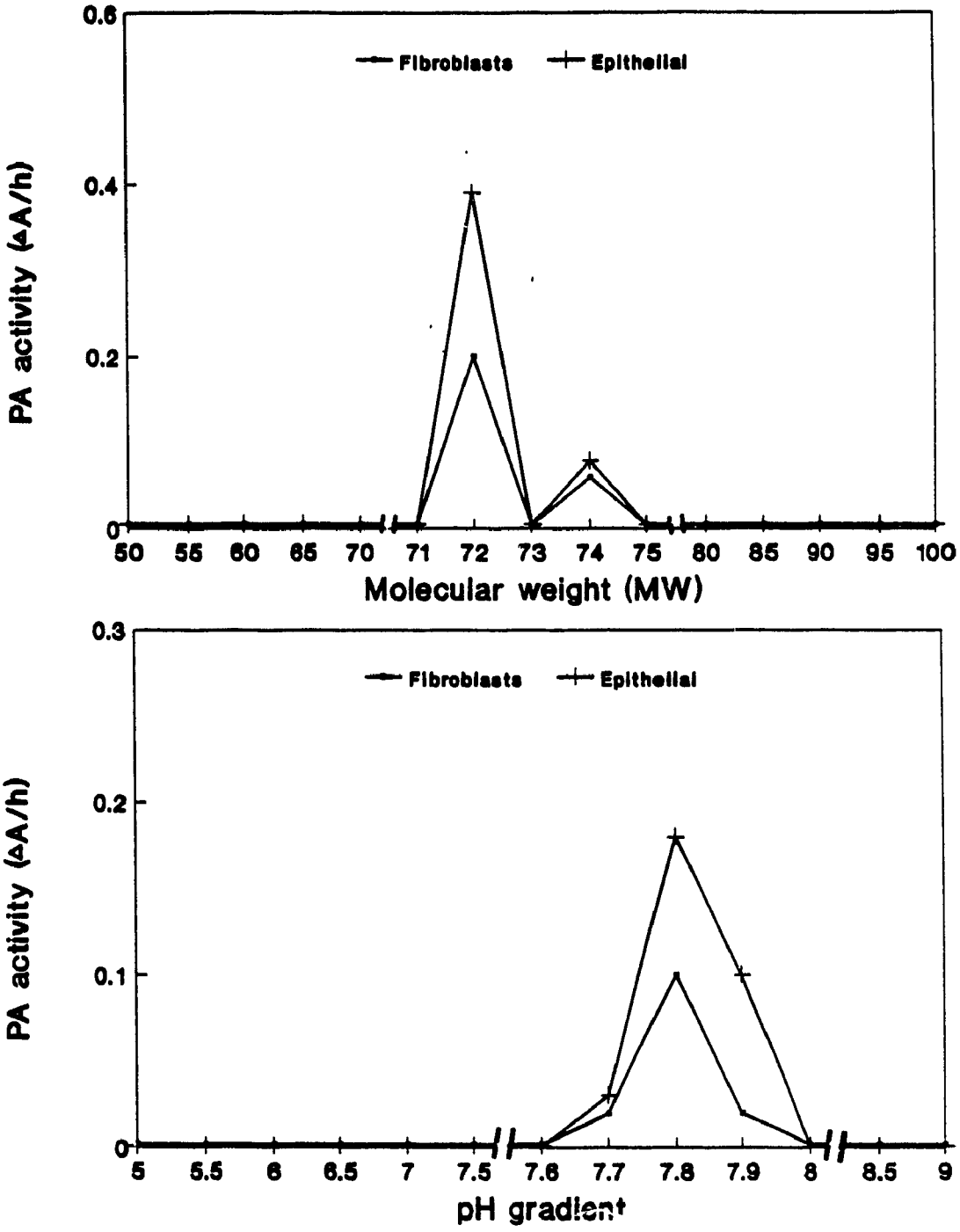


Figure 19. SDS-PAGE distribution of plasminogen activator (PA) activity of mammary epithelial and fibroblast cell extracts (A). Distribution of PA activity of mammary epithelial and fibroblast cell extracts after electrofocussing in a pH gradient (B). PA activity was assayed in 5 mm gel extracts as described in the materials and methods.

The type of PAI present in mammary epithelial extracts was determined by Western blot analysis. Antisera against bovine PAI-1 detected two high MW bands of the enzyme (120,000 and 126,000) (Fig. 20). Treatment with 0.5% SDS and 1.5 M NH_4OH led to a partial dissociation of the high MW forms and the appearance of a band at 50,000 (Fig. 20). Following SDS-PAGE the 50,000 protein was eluted from the gel slices and was shown to inhibit u-PA (Sigma) in a dose-dependent manner with 100 µl and 300 µl resulting in 50 and 100% inhibition of u-PA enzymatic activity (Fig. 21).

We were unable to detect any immunoreactive protein with antibodies against human PAI-1 and PAI-2 in fibroblasts cell extracts. However, acid treatment of fibroblasts cell extracts resulted in a 70% increase in PA activity while PA content was unaffected (Table 5) suggesting the presence of an acid-labile PAI in bovine fibroblasts.

Mammary epithelial and fibroblasts cell extracts do not contain plasminogen. In all assays for PA performed, we failed to detect plasmin activity in the absence of exogenous plasminogen. In addition, antibodies against human plasminogen failed to detect any immunoreactive protein in mammary epithelial and fibroblast cell extracts.

The time dependency of the effect of IGF-I on the t-PA content determined by an ELISA and t-PA activity of mammary epithelial cell extracts and the conditioned medium is shown in Fig. 22 and 23, respectively. PA in control samples expressed either as content (Fig. 22) or activity (Fig. 23)

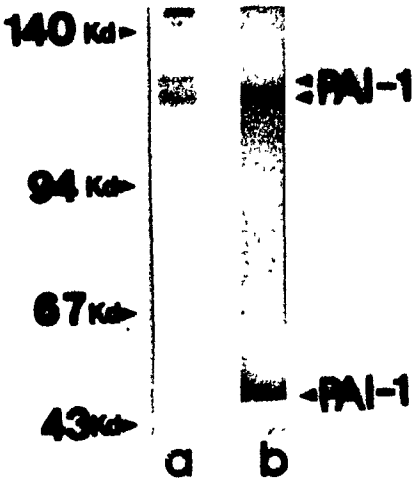


Figure 20. Western blot analysis of bovine mammary epithelial cell extracts (15 μg protein) (a) prior to and (b) after being treated for 1 h with 0.5% sodium dodecyl sulfate and 1.5 M NH_4OH with rabbit antiserum against bovine PAI-1.

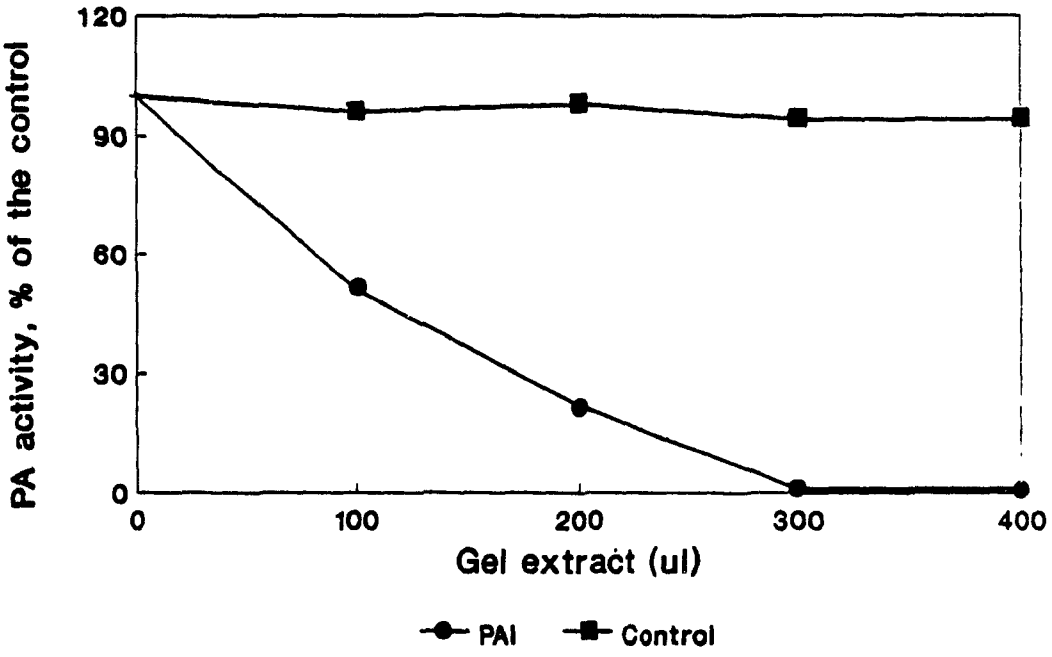


Figure 21 . Plasminogen activator (PA) inhibitory capacity of gel extracts. The active fractions were pooled from 0.6 ml of mammary epithelial cell extracts. PA activity in replicate tubes after addition of extracts from a blank gel served as a control.

Table 5. Tissue-plasminogen activator activity and content ($\bar{X}$ + SD, n=3) in fibroblast cell extracts.

Fibroblasts	t-PA content (ng/ml)	t-PA activity (ΔA/h/μg protein)
Control	82 ± 7.8	0.043 ± 0.004
Acid treated (pH 2.7) cell extracts	79 ± 7.3	0.074 ± 0.006

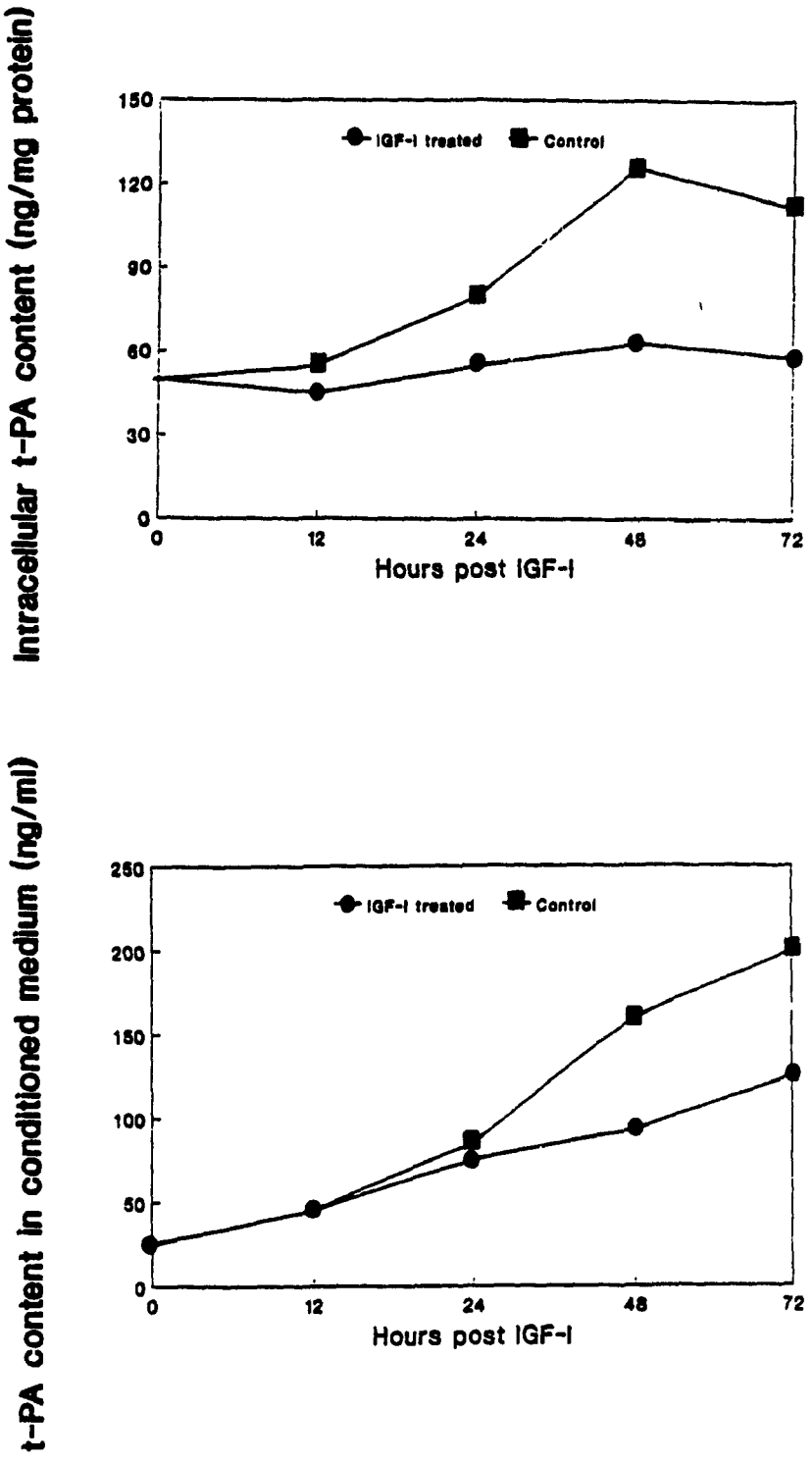


Figure 22. Effect of insulin-like growth factor-1 (IGF-1) on tissue plasminogen activator (A) intracellular protein content of mammary epithelial cells as determined by an ELISA and (B) on the protein accumulation in the conditioned medium.

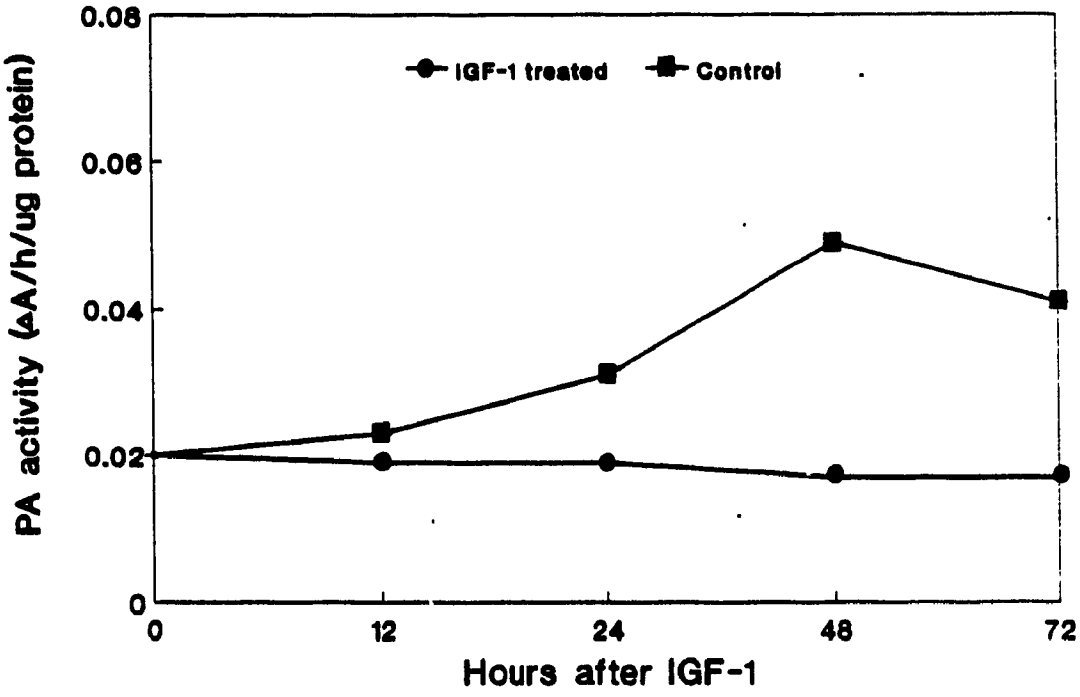


Figure 23. Effect of insulin-like growth factor 1 (IGF-1) on plasminogen activator activity (A) of mammary epithelial cell extracts

increased gradually to reach the peak value at 48 h post IGF-I and was followed by a slight decrease during a longer exposure (72 h). In marked contrast, t-PA intracellular content was maintained at low levels throughout the 72 h exposure to IGF-I. Tissue-PA content was also suppressed in the conditioned medium 48 h and 72 h following IGF-I exposure. The levels of PAI-1 were unaffected by the IGF-1 treatment and remained fairly constant at 10 ng/mg of total protein (Fig. 24).

Discussion

In the previous chapter (3) we observed variations in plasmin and plasminogen depending upon the health status of the cow, the stage of lactation and the exogenous somatotropin administration. In an attempt to understand these variations, we decided to investigate the possibility that cells within the mammary gland produce plasminogen, PA and PAI. The two cell examined here were mammary epithelial and fibroblasts.

This study showed that mammary epithelial cells and fibroblasts do not produce plasminogen. This conclusion was based on the fact that in the absence of exogenously supplied plasminogen, no plasmin activity was detected during the PA measurements in mammary epithelial and fibroblasts extracts. In addition, we failed to detect any immunoreactive proteins in cell extracts using antibodies against human plasminogen.

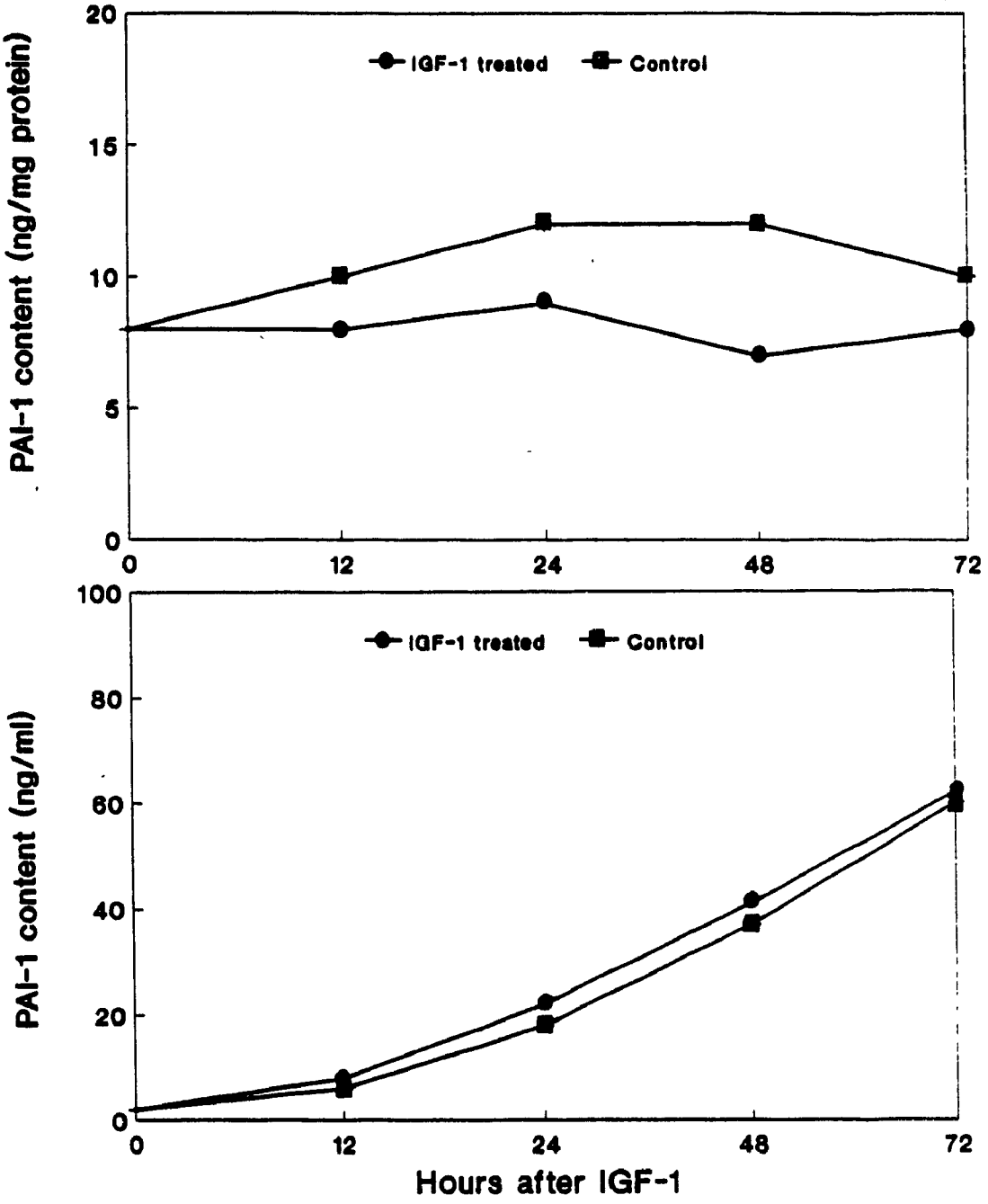


Figure 24. Effect of insulin-like growth factor-1 (IGF-1) on the plasminogen activator inhibitor-1 (PAI-1) (A) intracellular protein content of mammary epithelial cells as determined by an ELISA and (B) on the protein accumulation in the conditioned medium.

These data, collectively, suggest that mammary gland cells do not actively manipulate plasminogen levels. Plasminogen is present in high levels in all the extracellular fluids and is regarded as an extracellular "reservoir" of proteolysis. It is the presence or absence of PA which is the limiting factor for plasmin formation.

Mammary epithelial and fibroblasts cell extracts contained two forms of PA with MWs of 70,000 and 74,000 (Fig. 18). The cross-reactivity between antibodies against human u-PA and t-PA does not allow a definitive conclusion based on immunochemical criteria. To confirm that the two bands shown in the Western blot analysis are truly PA, the proteins were allowed to diffuse from the corresponding gel pieces from a sister gel and where shown to be PA (Fig. 19). In addition MW in the range of 70,000 to 75,000 and pI of 7.8 are consistent with characteristics reported for bovine t-PA isolated from bovine aortic endothelial cells (Levin and Loskutoff, 1982) and human t-PA isolated from a large variety of tissues (Dano et al., 1985; Saksela, 1985). Species differences exist since mouse mammary epithelial cells produce u-PA (Dano et al., 1985). Cultured bovine aortic endothelial cells produce u-PA and t-PA (Levin and Loskutoff, 1982).

Tissue-PA was detected in both mammary epithelial and fibroblasts in two forms: a high (74,000) and a low (70,000) MW form, the second one containing approximately 80% of the total PA activity (Fig. 19). Tissue-PA in a low and a high MW form has been detected in many species including porcine,

human, and rodents (Dano et al., 1985). It appears that the low MW represents a partially degraded product of the high MW form which retains enzymatic activity (Dano et al., 1985).

Bovine mammary epithelial cells produce PAI-1 in two high MW forms (122,000 and 126,000) (Fig. 20). The fact that all the PAI-1 forms in the literature have a MW of approximately 50,000 (Sprengers and Kluft, 1987) taken together with the existence of two t-PA of 70,000 and 74,000 suggest that the observed high MW forms may constitute enzyme-inhibitor complexes. Treatment with a nucleophilic agent (1.5 M NH_4OH) a procedure suggested by Booyse et al., (1988) led to a partial dissociation of both high MW forms and the formation of a common 50,000 PAI-1 protein (Fig. 20).

As an additional test, we showed that the gel extracts containing the 50,000 protein inhibited very efficiently u-PA enzymatic activity (Fig. 20). We are unable to provide definitive evidence that the other component of the high MW form is t-PA. This is because the antibodies against human t-PA failed to recognize the high MW molecule forms. This behaviour is consistent with information provided by the manufacturer of this antibody (American Diagnostica) that anti-human t-PA IgG has very low affinity for PA-PAI complexes. However there was an increase of 30% (0.45 to 0.59) in the PA activity recovered from the gel pieces following nucleophilic attact. We suggest that the additional PA activity is provided from the breakdown of the high MW forms.

The concept of a nucleophilic attact to break enzyme-

inhibitors complexes has been extensively used in the work of Booyse et al., (1988) who showed that NH_4OH was able to dissociate a t-PA-PAI-1 complex into active t-PA and PAI-1 in cultured human umbilical vein endothelial cells. Dissociation of protease-inhibitor complexes such as thrombin-antithrombin and plasmin-α_2-antiplasmin following nucleophilic attack into active enzymes and inhibitors has been demonstrated by Fish and Bjork (1979) and Wiman and Collen (1979).

Western blot analysis of fibroblasts cell extracts using antibodies against human PAI-1 or PAI-2 failed to detect any bands. A negative finding can be interpreted two ways: 1) true negative i.e. fibroblasts do not produce PAI or 2) PAI is not detected by our assay system because bovine PAI does not cross-react with the antibodies against human PAI-1 or PAI-2. Acid treatment of cell extracts at pH 2.7 led to a 70% increase in PA activity without concomitant increase in PA content (Table 5) which suggests that a protein with properties of PAI but not PAI-1 or PAI-2 (both of them are not acid-labile) was irreversibly inactivated. These results, collectively, suggest that fibroblasts may produce protease nexin which is the only PAI being inactivated at pH 2.7 (Van Murik et al., 1984). It should be noted that human type fibroblasts in culture are known to produce copious amounts of this protein (Sprengers and Kluft, 1987).

The immunospecific protein determination with antibodies against t-PA demonstrated that IGF-I inhibited the induction

of t-PA activity and content which is observed when mammary epithelial cells are cultured in the absence of IGF-1. Intracellular t-PA content is a useful but difficult to be interpreted measurement. This is because the intracellular t-PA protein content is influenced by the rate of protein production and the rate of secretion. To ensure that the increase in the intracellular t-PA content observed when cells are cultured in the absence of IGF-1 was not the outcome of an impairement in t-PA secretion, we examined differences in the t-PA content in the conditioned medium (Fig. 22). Here, once again the t-PA content was lower in the IGF-1 treated samples at 48 and 72 h following IGF-1 addition. Tissue-PA content was increased in the conditioned medium in control samples as the incubation time increased (Fig. 22). This argues against of an impairement in t-PA secretion as an explanation for the induction of t-PA content in the control cells.

The nature of the conditions or factors that govern the induction of t-PA in the control cells remains unclear. Whether this induction is related to the recovery of cells from seeding or to the cell cycle or even to a general disfunction remains to be established in mammary epithelial cells. Data obtained from different cell types suggest that PA production varies according to the cell cycle. Intracellular PA is high during G_1 and S phase and low during G_2 and M phases of the cycle (Carlsen, 1988). Our data argue against this possibility. IGF-1 affected neither the amount

of the PAI (Fig. 24) nor the intracellular total protein content (Fig. 25) suggesting that the decrease in t-PA is a specific effect rather than an effect on general cellular metabolism. Turner and Hung (1989) have also observed limited proliferation of mammary epithelial cells when they are placed on floating collagen gels in a variety of hormonal environments (prolactin, hydrocortisone or insulin).

Examination of the hormonal regulation of PA is difficult because the expression of PA gene varies due to culturing conditions. Perhaps, we should have added IGF-1 after 48 h in collagen because this was the time that the cells reached a plateau value for t-PA. Such an experiment is relevant to the question of how long mammary epithelial cells can remain in the collagen under optimum culturing conditions.

It is apparent that IGF-I affects PA rather than PAI. This is atypical for a hormonal effect on the PA/PAI system. Typically hormones affect different components of the PA system in a concerted manner that would promote an overall induction or suppression of the expressed PA activity (Saksela and Rifkin, 1988). If mammary epithelial cells are able to maintain t-PA production by mammary epithelial cells at their basals levels <u>in vivo</u> this would provide some support for the hypothesis that somatotropin administration through elevated levels of IGF-I suppresses PA production by mammary epithelial cells which results in reciprocal decreases in plasmin levels in milk. However, certain caution should be exercised in interpreting these results simply because it is not possible

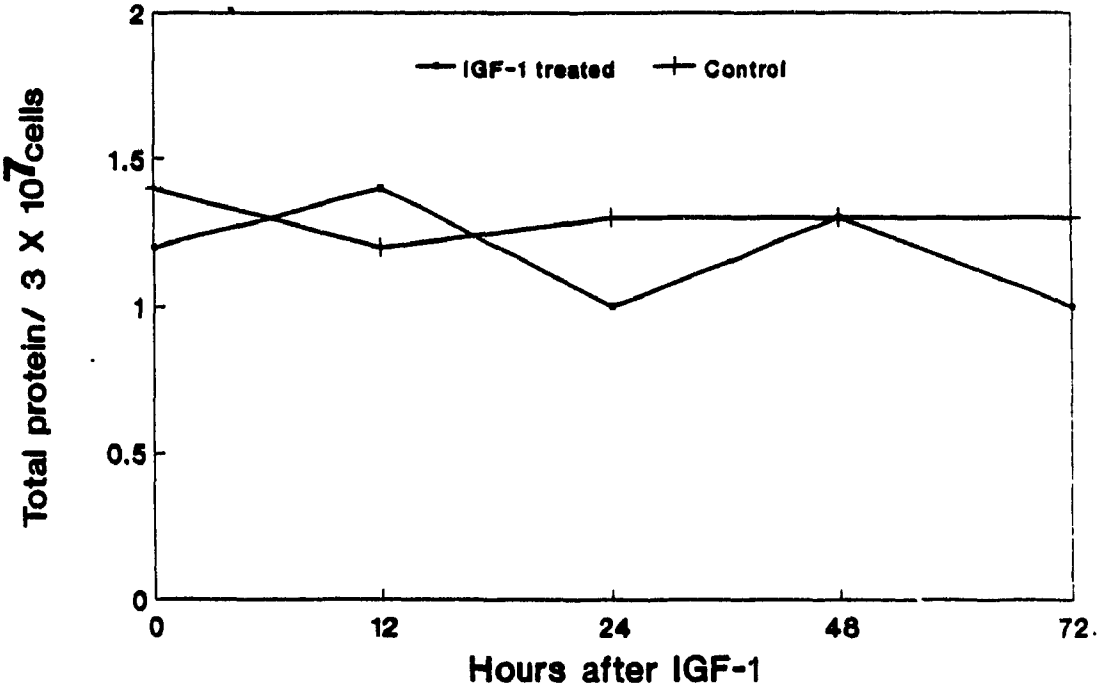


Figure 25. Effect of insulin-like growth factor-1 (IGF-1) on the total protein content of mammary epithelial cells.

to establish whether the expression of t-PA and PAI-1 is a general *in vitro* property of mammary epithelial cells only under culture conditions or whether these proteins are also expressed *in vivo*. Booyse et al., (1988) suggested that endothelial cells alter their plasminogen activator phenotypes during primary culturing due to dilution or loss of a factor(s) that originally regulated the *in vivo* expression of PA.

In summary, our results indicate that bovine mammary epithelial cells produce t-PA and PAI-1. IGF-1 resulted in an inhibition of the induction of PA activity and content which is observed when mammary epithelial cells are cultured in the absence of IGF-1. PAI-1 levels were unaffected by the IGF-1 treatment. Bovine fibroblast produce t-PA and a PAI with similar biochemical characteristics with protease nexin I.

5. PLASMINOGEN ACTIVATOR PRODUCTION BY BOVINE MACROPHAGES

Abstract

Differences in the level of urokinase-plasminogen activator (u-PA) protein production and mRNA levels were examined in macrophages isolated from milk taken from normal and mastitic cows. Macrophages isolated from milk taken from mastitic quarters had 2.4 and 2.7 fold higher levels of u-PA content and u-PA mRNA when compared to that of macrophages isolated from normal milk. Secretion of u-PA protein in the culture medium for both mastitic and normal macrophages was minimal (2%) when compared to the intracellular u-PA content. The lack of secretion of PA by milk macrophages suggests a minor role in influencing milk plasmin. Plasminogen-activator production may reflect the ability of macrophages to pass through basement membrane into milk during inflammation.

Introduction

Macrophages and polymorphonuclear leucocytes (PMNL) are the principal phagocytes present in milk and play a central role in the body's defense against mastitic infection (Guidry, 1985). They are present in high levels: 10-250,000/ml in milk isolated from normal quarters and >1,000,000/ml in milk isolated from mastitic quarters.

Macrophages carry out important protective functions in host defense such as ingesting and killing bacteria and they secrete a number of proteins (Johnston, 1988). Plasminogen activator (PA) is thought to be important for the penetration of macrophages through the capillary endothelium and secretory epithelium. If, indeed, macrophages produce PA once present in milk, their impact on the plasmin/plasminogen system in milk will be considerable. Previous work from our laboratory (Chapter, 3) has demonstrated, however, that there is no change in the activation pattern of plasminogen to plasmin in mastitic milk. This occurs despite the fact that this kind of milk contains high levels of macrophages and PMNL which are known to produce PA. In order to solve this paradox and in view of the importance of controlled proteolysis in inflammatory reactions, a detailed understanding of the role of PA produced by macrophages is of obvious interest. This study was undertaken to establish the identity of PA produced by bovine macrophages and to study its regulation during mastitis.

Materials and Methods

Milk Samples and Macrophage Isolation

Six Holstein cows in their sixth month in lactation were used. Three cows defined here as mastitic contained somatic cell counts (SCC >1,000,000/ml). Cows were not experimentally

infected. They were selected from the naturally occuring infections in the Macdonald herd. During the course of the experiment the cows were not treated with antibiotics. Cows were sampled on three occasions in a period of two weeks which covered days 4 to 20 post-infection. Day 1 of the infection is defined as the day the SCC surpass the 500,000/ml level (Guidry, 1985). Three cows serving as controls were chosen with the following criteria: no known disease or sign of mammary gland inflammation according to information provided to us by farm staff and SCC in milk less than 50,000/ml.

Milk macrophages were isolated by a procedure described by Subiza et al., (1988) with some modifications. M. Denis, Institute of Parasitology was responsible for macrophage isolation as part of a collaborative effort. Milk samples (15 l)were collected during the morning milking and then centrifuged (500 X g for 20 min). Pelleted somatic cells were washed three times with RPMI-1640 (Gibco) containing 15% fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cells were resuspended in 5 ml of 31% isotonic Ficoll-Hypaque (Pharmacia) and layered on top of a 52% Ficoll-Hypaque cushion and centrifuged at 500 x g for 45 min at 4°C. Cells at the interface were collected, assessed for viability by trypan blue exclusion (typically > 95% viable) washed twice in RPMI-1640 and plated at 5 x 10^7 cells/ml of RPMI-1640/35mm dish in 5% CO_2, 37°C overnight. Cells were then washed 5 times with warm Hank's balanced salt solution (HBSS, Gibco). Prior to PA analysis, the cell population was shown to contain more

than 90% macrophages by Giemsa staining (Denis et al., 1985).

Determination of Plasminogen Activator Activity in macrophage cell extracts

Isolated milk macrophages were removed from culture plates for PA analysis using a rubber policeman, then sonicated in 3 ml distilled water for 30 sec, centrifuged at 2000 x g for 10 min and the supernatant frozen. Plasminogen activator activity was determined by the colorimetric method of Searls (1980). Details of this method are as described in Chapter 4.

To quantitate PA secretion from macrophages into the culture medium, 1 x 10^6 macrophages were incubated in 10 ml of RPMI-1640 in 35-mm tissue culture dishes for 24 h at 37°C in a water saturated, 5% CO_2 atmosphere. The PA activity in the medium was assayed as above and is expressed in difference in absorbance per 10^6 macrophages. In another experiment including two out macrophage preparations, we studied whether the limited secretion of PA by macrophages (see results) could be increased by longer cultures times (48 h).

Characterization of Plasminogen Activator Activity

Proteins from macrophage cell extracts were resolved using 10% acrylamide resolving and 4% stacking gels (Laemmli, 1970) and run at 40V as in Chapter 4. Western blots were prepared using nitrocellulose membranes and electrostatic transfer of

the samples fractionated by SDS-PAGE. Membranes were incubated with purified goat anti-human u-PA IgG or goat anti-human t-PA IgG at a final concentration of 0.8 μg/ml. Immune complexes were detected with anti-goat IgG-peroxidase conjugate at a 1:2500 dilution. Other details of this method are as described in Chapter 4.

Isoelectric focussing in macrophage cell extracts was performed in 5% polyacrylamide gels containing 6M urea by the method of Levin and Loskutoff (1982). Other details were as in Chapter 4.

Enzyme-Linked Immunosorbent Assay (ELISA) for u-PA

Urokinase-PA was quantitated with an ELISA using goat anti human u-PA IgG as primary antibody and peroxidase-conjugated anti-goat IgG as the secondary antibody. Other details of this assay system are as described in Chapter 4.

RNA analysis

Total cellular RNA was isolated from cells by the method of (Chomczynski and Sacchi, 1987). 2 X 10^8 macrophages were lysed in the Petri dish by the addition of 2 ml of RNAzol (CINNA/BIOTECH, Frienswood, TX). The RNA was solubilized by passing the macrophage lysate 3-4 times through a 18 g needle. Two hundred μl of chloroform were added and incubated in dry ice for 15 min and then centrifuged at 10,000 g for 20 min.

The upper aqueous phase was transferred to a new tube and an equal volume of isopropanol was added. The sample was stored at -20°C for 1h and then centrifuged at 10,000 g for 20 min. The supernatant was discarded and the RNA pellet was washed twice with 95% ethanol. The pellet was dried for 15 min under vacuum.

RNA was electrophoresed in 1.2% agarose gel prior to its transfer to the nylon membrane (Zeta-probe, Bio-Rad). Four μg of RNA were dissolved in a solution containing 23.4 μl of H_2O, 3.2 μl of 0.1 M sodium phosphate buffer (pH 7.0) and 5.4 μl of deionized glyoxal that was prepared by mixing 1 ml of glyoxal (Sigma) with 1 ml of dry mixed bed resin (Bio-Rad) for 15 min. After 1 h incubation of the RNA at 55°C, 8 μl of loading buffer consisting of 10% (v/v) glycerol, 10% (v/v) 0.1 M phosphate buffer (pH 7.0) and 10% (v/v) of 1% (w/v) bromophenol blue were added. The sample was loaded to the 1.2% agarose gel prepared in running buffer (0.01 M sodium phosphate, pH 7.0) and run at 100 V until the dye was near but not off the edge of the gel. Prior to transfer, the agarose gel was soaked in 200 ml of 50 mM NaOH for 15 min to reverse the glyoxalation and then in 0.1 M Tris (pH 7.4) for another 15 min and finally in 0.1 M sodium phosphate for 15 min. To transfer RNA to Zeta-probe, the gel was then placed overnight in the appropriate transfer set-up (Davis et al., 1986). The transfer medium was 10 X SCC.

Total RNA was analyzed by hybridizing Northern blots with nick translated (Davis et al., 1986) plasmid cDNA probes for

murine u-PA (Belin et al., 1985) and bovine β-actin (Degen et al., 1983). The cDNA probe to mouse u-PA was 1 Kb insert cloned into pBR322 and has been shown to cross-hybridize with bovine u-PA mRNA (Belin, D., personal communication). The cDNA probe to bovine β-actin was 1.25 Kb insert cloned into pBR322. Prehybridizations were performed at 42°C overnight in 50% formamide, 4 x SSPE, 1% SDS, 200 ug/ml salmon sperm DNA and 5 mg/ml skim milk powder. Hybridizations were performed in the same solution containing 4 x 10^5 cpm/ml of ^{32}P-labelled probes. Filters were washed at 42°C for 20 min in 2 x SCC/0.1% SDS, then with 0.5 x SSC/0.1% SDS and then 0.1 x SCC/0.1% SDS. The filters were then exposed to Kodak XAR-S X ray film at -72°C for 24 h using two intensifying screens. Relative radioactivity was estimated from the autoradiograms by densitometry. The relative amounts of u-PA mRNA were normalized against the corresponding amounts of β-actin signal that was found not to be influenced by mastitis.

All data were expressed as means ± standard deviation. Differences between means were evaluated using student's test ($p<0.01$) (SAS, 1982).

Results and Discussion

Macrophages were isolated from milk using the methodology suggested by Subiza et al., (1987). The purity of macrophages population was greater than 90%. As assays were performed 24 h after adherence, the macrophages may have been in a quiescent state.

In order to characterize the PA forms produced by bovine milk macrophages two different methodologies were used:

1) Western blot analysis using antibodies against human u-PA and t-PA

2) Localization of PA within the electrophoretic lane following SDS-PAGE, extraction of the protein from the gel and determination of its activity, molecular weight and isoelectric point.

Antibodies against human t-PA and u-PA gave no insight as to the nature of PA since cross-reactivity between these antibodies was observed and both precipitated 100% of the PA activity present in macrophage cell extracts. However, both human antibodies against u-PA and t-PA detected a band with an apparent MW of 28,000 (Fig. 26). Mammary epithelial cells and fibroblast cell extracts produce t-PA and were included in this figure for contrast purposes. A duplicate macrophage sample was incubated with non-immune serum to serve as a control. No significant signal was observed with this treatment (data not shown).

No definitive conclusion can be drawn based on the Western blot analysis. To confirm that the 28,000 protein band is truly a PA, we examined PA activities in gel slices following SDS-PAGE. Several researchers have shown that PA is not irreversibly inactivated by SDS (Strickland and Beers, 1976; Levin and Loskutoff, 1982; Booyse et al., 1988). We found PA activity in only one gel slice which corresponded to the above

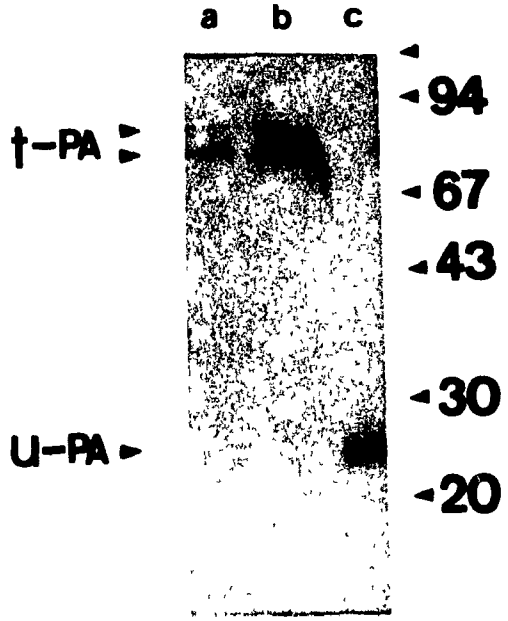


Figure 26. Western blot analysis of bovine (a) mammary epithelial (15 μg protein) (b) fibroblasts (25 μg protein) and (c) macrophages cell extracts (25 μg protein) with antibodies against human urokinase-plasminogen activator (u-PA). Mammary epithelial cells and fibroblasts produce tissue-PA (t-PA) detected due to cross-reactivity of anti human u-PA IgG with bovine t-PA antigen. Macrophages produce u-PA.

MW (28,000) (Fig. 27A). If our recovery of PA activity after SDS-PAGE had been 100%, we should have recovered a total of PA activity 0.015 (0.25 X 0.06). This calculation is based on the fact that the initial PA activity (ΔA/h/mg protein) was 0.25 and we pooled the gel extracts from 4 channels, each one being loaded with 15 μg of protein (0.015 X 4=0.06 mg). The actual recovery achieved was 0.08/0.015= 53% (Fig. 27A). The isoelectric point (pI) of bovine macrophage PA occurred at pH 8.5 (Fig. 27B). The recovery of PA activity after isoelectric focussing was (0.01 + 0.04)/0.08 = 62.5%. It is important to note that all these recoveries are overestimated because the PA activity is determined in gel extracts without the influence of PAI which is not the case for the original determination of u-PA activity in macrophage gel extracts. Molecular weight of 28,000 and pI of 8.5 are consistent with the characteristics reported for u-PA by Dano et al., (1985).

Western blot analysis using antibodies against human PAI-1 or PAI-2 failed to detect any immunoreactive bands in macrophage cell extracts. This suggests that macrophages do not produce PAI-1. We have shown in the previous chapter that antibodies against human PAI-1 or PAI-2 cross-react with bovine PAI-1 present in bovine mammary epithelial cell and bovine fibroblasts. In the case of PAI-2, we do not have a positive control. Thus, the possibility exist that our assay system could not detect the bovine PAI-2 antigen. Clarification of this possibility should await further experiments.

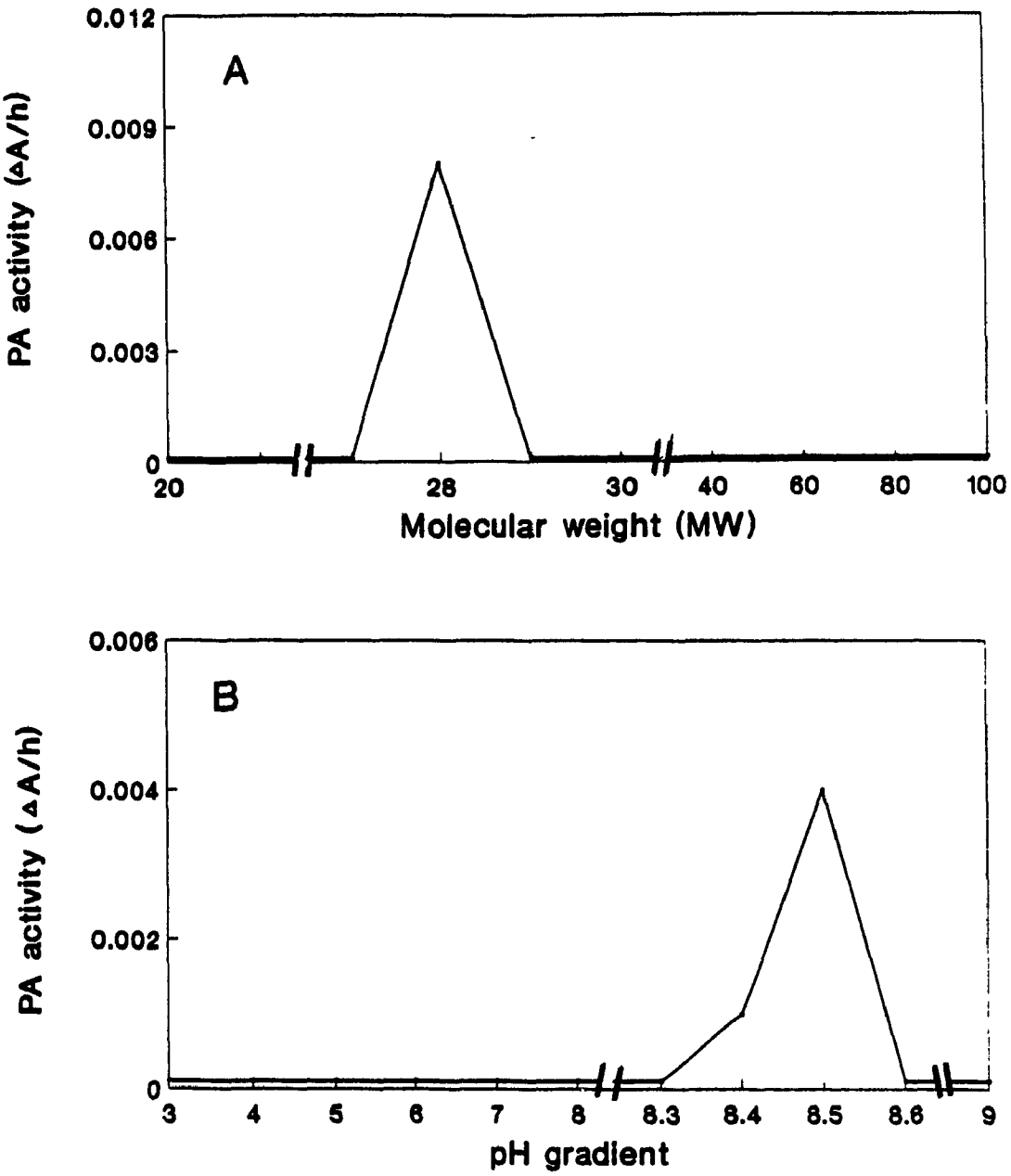


Figure 27. SDS-PAGE distribution of plasminogen activator (PA) activity of macrophage cell extracts (A). Distribution of PA activity of macrophage cell extracts in a pH gradient following electrofocussing (B). PA activity was assayed in 5 mm gel extracts as described in the materials and methods. Each point represents the average of two independent samples.

Macrophages do not produce plasminogen. This conclusion is based on the fact that no plasmin activity was detected in the determination of PA activity in macrophage cell extracts in the absence of exogenously supplied plasminogen.

Macrophages isolated from milk of mastitic cows had u-PA activity and u-PA content which was 2.5 and 2.4 fold greater respectively than that of macrophages from normal milk (Fig. 28). The parallel increase of PA activity and PA content strongly supports the concept of enhanced PA production responsible for the enhanced PA activity. Most of the previous studies, report PA activity values rather than PA content (Dano et al., 1985). This makes difficult to discriminate between changes in PA protein content and changes in PA activity due to the presence of inhibitors. Certain caution should be exercized in interpreting these data because the cows used in this study were not of the same genetic background.

Even though macrophages from normal and mastitic quarters produce PA, it is the secreted PA which is important with respect to its influence on the plasmin/plasminogen system in milk. Data in Fig. 29 show that milk macrophages from mastitic milk secrete higher amounts of PA (3.2 fold) than those of the normal milk, however in both cases the secreted PA represents only a minute amount of the cell-associated PA. In another experiment we investigated whether the limited secretion of u-PA by milk macrophages could be increased by longer culture time. Fig. 30 shows that prolonged culture does not modify u-

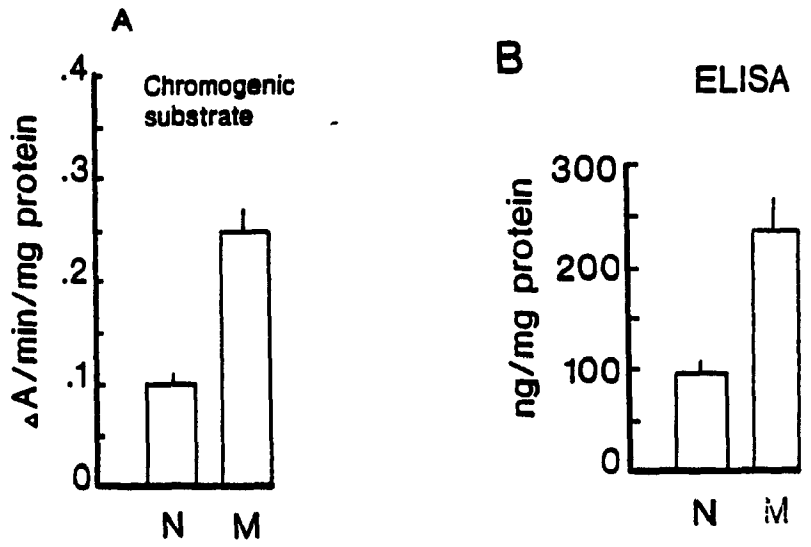


Figure 28 Comparison between intracellular plasminogen activator (PA) activity (A) and content (B) in macrophages isolated from normal (N) or mastitic milk (M). PA activity was determined by the rate of hydrolysis of the chromogenic substrate (Val-Leu-Lys-p-nitroanilide, S-2251) and the PA content was measured with an enzyme-linked immunosorbent assay (ELISA). Results are the mean ± SD of nine independent samples.

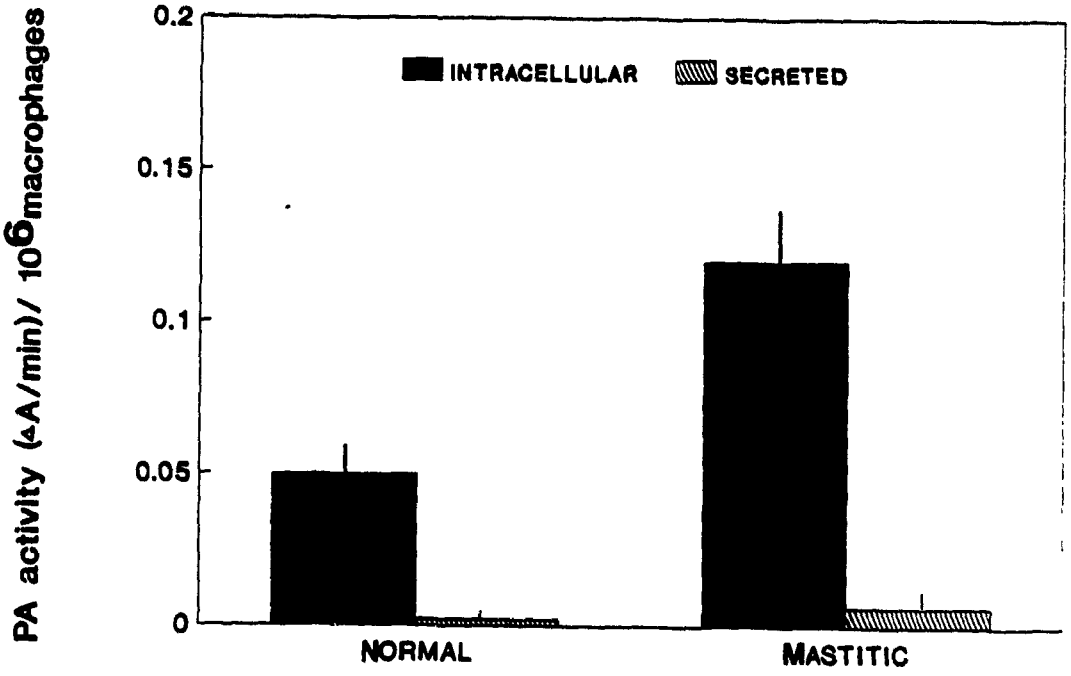


Figure 29. Plasminogen activator (PA) secretion by macrophages into the culture medium. A comparison between macrophages isolated from milk from normal (N) and mastitic (M) cows (■intracellular PA; ▨ secreted PA). Results are the mean ± SD of nine independent samples.

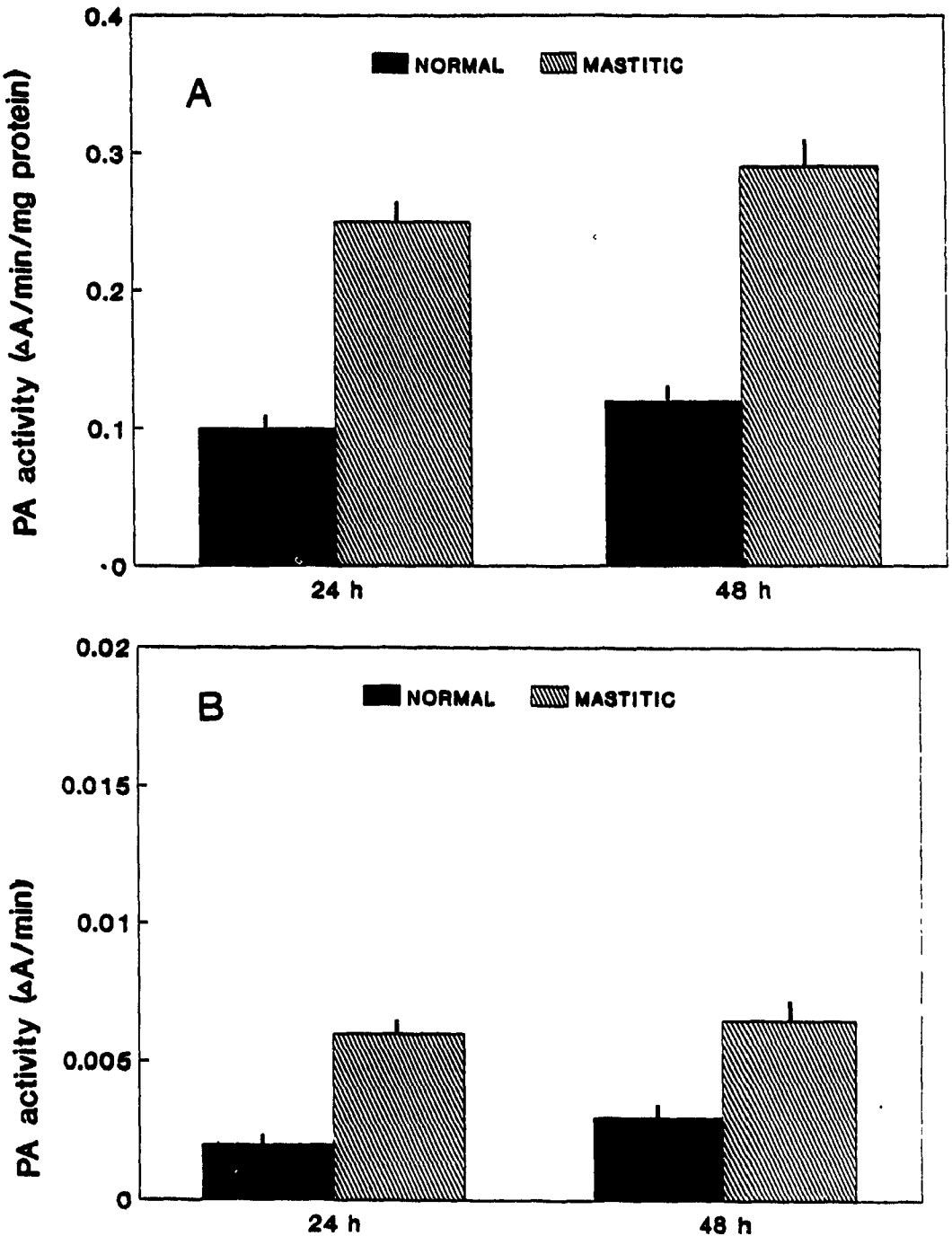


Figure 30 . Plasminogen activator activity of bovine macrophages at different culture times (A intracellular PA; B secreted PA). A comparison between macrophages isolated from normal and mastitic quarters. Results are the mean ± SD of three independent macrophage samples.

PA production by macrophages nor its secretion pattern. To eliminate the possibility that PA inhibitors present in the culture medium were affecting our PA activity measurements, the reaction was carried out in replicate tubes by adding known amounts of u-PA (Sigma) in addition to the 50 µl of macrophage cell extracts. The recovery was always more than 90% (Table 6).

The lack of secretion of u-PA by milk macrophages may partially explains why we don't observe more activation of plasminogen to plasmin in milk taken from mastitic quarters when compared to that obtained from normal quarters (Chapter, 3). Certain caution should be exercised in interpreting these data because it is not possible to predict by simply measuring secretion of PA into the culture medium the behaviour of macrophages in milk where different antigenic challenges exist. In chapter 3, however, we showed that isolated somatic cells which predominantly contain macrophages (Guidry, 1985) when added to milk (intact or lysed) were unable to influence to a significant extent plasminogen activation. The results of the <u>in vitro</u> and <u>in vivo</u> study taken together support the argument that macrophages play a minor role regarding the plasmin/plasminogen system.

In addition, to the inability of milk macrophages to secrete u-PA we have observed a limited ability of these cells to secrete interleukin-1 (IL-1) even after treatment with high levels (10 ng/ml) of lipopolysaccharide (LPS) (Appendix II). Impaired secretion of IL-1 has also been observed in human

Table 6. Evaluation of the presence of urokinase-plasminogen activator (u-PA) inhibitors ($\bar{X} \pm SD$, n=3) in culture medium.

Source	PA activity (ΔA/min/mg protein)
Control	0.21 ± 0.02
Control + u-PA[1]	0.39 ± 0.06
Control + u-PA[2]	0.57 ± 0.08

1 Activity of added u-PA 0.2 ΔA/min

2 Activity of added u-PA 0.4 ΔA/min

breast macrophages (Subiza et al., 1988). The high intracellular levels of PA and IL-1 may play a role once released in the newborn's digestive track but this possibility is difficult to be justified.

The u-PA and β-actin mRNA content of bovine macrophages isolated from normal and mastitic quarters were examined qualitatively and quantitatively by Northern analysis. The data in Fig. 31 show that in preparation of total RNA isolated from macrophages either from normal or mastitic milk, RNA that hybridized with ^{32}P-labeled u-PA and β-actin cDNA probes was 2,400 and 2,100 bases long, respectively. The sizes of u-PA and β-actin are consistent with sizes reported in the literature for u-PA (2,400) by Belin et al., (1985) and for β-actin (2,100) by Degen et al., (1983). A short nucleotide sequence which hybridize with the u-PA cDNA probe (Fig. 30A) cannot be identified at this point. Our data indicate that macrophages isolated from mastitic quarters were associated with higher (2.7 X) u-PA mRNA when compared to that of macrophages isolated from normal quarters (Fig. 32). It should be noted that the u-PA mRNA levels have been normalized against the corresponding amounts of β-actin mRNA levels that were found not to be influenced by mastitis. Changes in mRNA levels mirror those obtained at the protein level suggesting that the modulation of u-PA protein content during mastitis may occurs at the steady-state level of mRNA. At this point it is not possible to establish whether the variation of u-PA mRNA is due to changes in the gene transcriptional activity

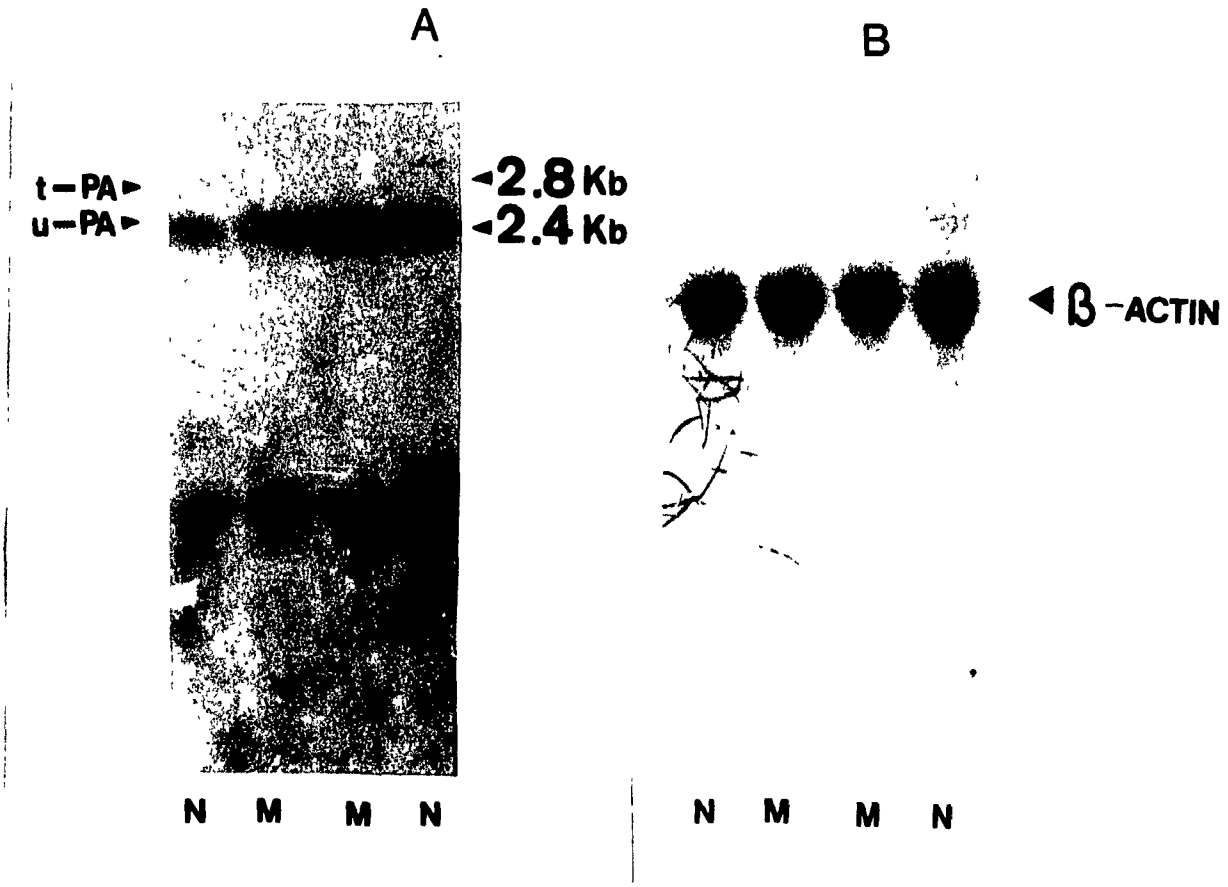


Figure 31. Northern blot analysis of bovine urokinase-plasminogen activator (u-PA) mRNA with a murine u-PA cDNA probe (A) and bovine β-actin mRNA with a bovine β-actin cDNA probe (B). A comparison between the levels of u-PA and β-actin mRNA obtained from macrophages isolated from milk of normal (N) and mastitic (M) cows. A very short nucleotide sequence hybridizing with the murine u-PA cDNA cannot be identified.

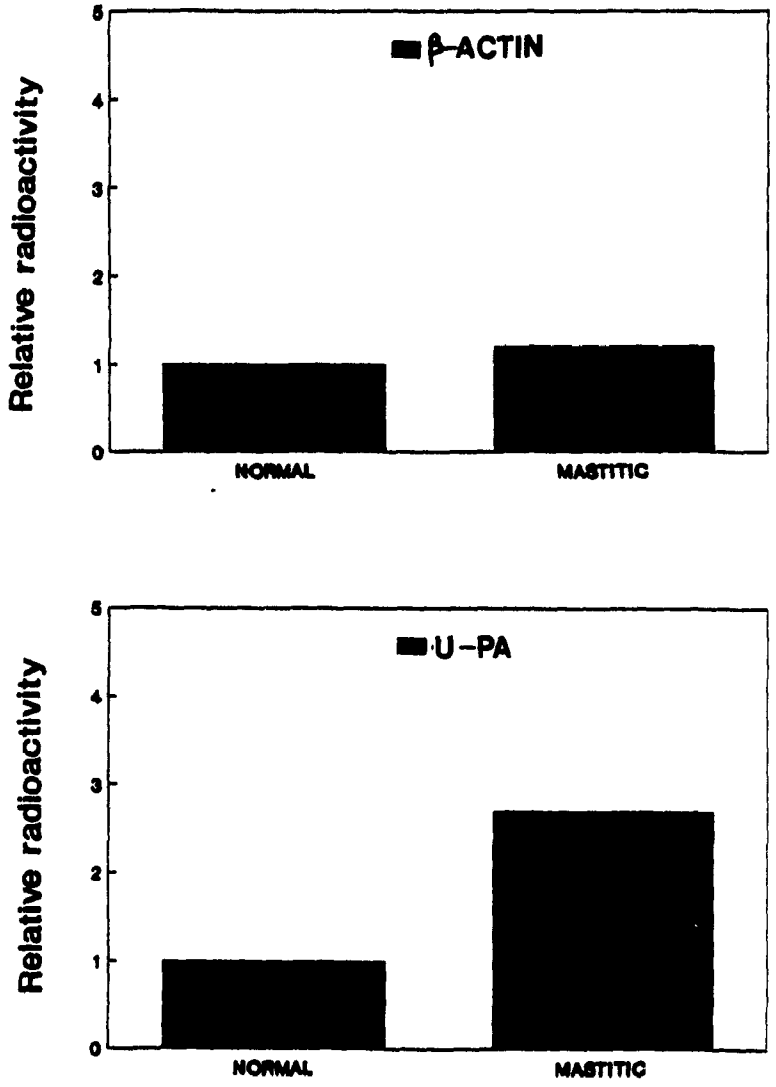


Figure 32. Relative hybridization as a measurement of mRNA abudance for β-actin (A) and urokinase-plasminogen activator u-PA (B). A comparison between the levels of mRNA obtained from macrophages isolated from normal (N) and mastitic (M) quarters. The level of mRNA for u-PA and β-actin in the case of normal macrophages was set arbitrarily to 1.

or in the mRNA stability. Elevated u-PA activity and uPA protein content (Fig. 28) together with the existence of u-PA mRNA (Fig. 31) strongly support the concept that bovine macrophages express the u-PA gene.

Macrophages exhibit considerable heterogeneity with respect to their morphology and functional capacities depending upon their origin and their state of activation. Macrophage activation usually occurs during the infection. We suggest that the increased expression of u-PA gene and increased u-PA enzymatic activity during mastitis is one of the many functional differences observed during this activation process. The current hypothesis suggests that PA's allow penetration of monocytes through capillary endothelium and the basement membrane of the alveolus during bacterial induced inflammation (Saksela, 1985). We believe that the expression of u-PA in milk macrophages represents the terminal stage of this process and thus can be considered as a residual function of a rather differentiated macrophage population. This corroborates with the observation (Fig. 29) of increased u-PA content within macrophages without concurrent secretion.

6. PLASMINOGEN ACTIVATOR AND PLASMINOGEN ACTIVATOR INHIBITOR DURING OVARIAN FOLLICULAR MATURATION IN THE PIG

Abstract

Plasminogen, plasmin, plasminogen activator (PA) activities, PA and PA Inhibitors (PAI) contents were measured in granulosa (GC) and theca interna (TIC) cell extracts and follicular fluid (FF) obtained from preovulatory follicles of prepubertal gilts treated with pregnant mares' serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) to induce follicular growth and ovulation. In FF, plasminogen levels remained relatively constant during follicular development whereas plasmin activity was detected only just prior to the time of expected ovulation. The increase in follicle plasmin levels was preceded by significant ($P<0.01$) increases in PA activity, PA content and decreases in PAI content in GC and FF. Immunoprecipitation and Western blot analysis showed that tissue-PA (t-PA) was the principal form of PA in the follicle. Two PAI-PA complexes with apparent molecular weights (MW) of 126,000 and 130,000 when treated with 0.5% sodium dodecyl sulfate and 1.5M NH_4OH were dissociated into t-PA and a common 52,000 MW PAI protein. The isoelectric point of this PAI form was 4.8. The ovarian PAI is distinct from nexin (another PAI inhibitor), being active at pH 2.7 and extremely stable in the presence of guanidine, urea or acetic acid. The above characteristics suggest that porcine ovarian PAI belongs to

the same class of inhibitors as bovine and human PAI-1. These data indicate that increased PA and decreased PAI production is temporally associated with ovulation and may be involved in follicular rupture.

Introduction

In the rat, several lines of evidence indicate that PA and PAI may be involved in the ovulatory process. Increases in PA activity are correlated with ovulation and the enzyme is induced only in follicles near ovulation (Beers et al., 1975; Strickland and Beers, 1976; Reich et al., 1985) while antibodies against t-PA and α_2-antiplasmin blocked ovulation (Tsafriri et al., 1986); both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) increase t-PA but not u-PA in ovarian granulosa (GC) and theca interna (TIC) cells in immature and cycling rats (Ny et al., 1985; Reich et al., 1985; Liu et al., 1987) suggesting that gonadotropin regulation of t-PA may be involved in follicular rupture. Ny et al., (1985) reported that cultured rat GC cells produce a PAI, the levels of which are suppressed by FSH and LH administration. The work of Smokovitis et al. (1988) demonstrated increased t-PA activity mainly at the area of rupture of the follicular wall at the time of ovulation in sows. Their conclusions are based on an experiment performed

using one animal. In addition, they determined PA activity and not PA content. It is obvious that the role of PA but more importantly PAI in ovulation in the pig remains to be elucidated.

In previous studies, the prepubertal gilt treated with pregnant mares' serum gonadotropin (PMSG, 750 IU) and, 72 h later, with human chorionic gonadotropin (hCG, 500 IU) has been used to produce groups of synchronously developing follicles which ovulate approximately 42 h after hCG treatment (Ainsworth et al., 1982). This model has been employed to study intrafollicular events leading to ovulation, particularly the contribution of the prostaglandins (Tsang et al., 1979; Ainsworth et al., 1980; Evans et al., 1983), and it offers a convenient and reproducible system for examining the biochemical links in the control of ovulation.

The objectives of the present study were to measure changes in PA and PAI content and activity in the preovulatory follicle, and to determine its cellular source using the PMSG/hCG-treated gilt model. In addition, the PA and PAI forms produced by follicular cells were biochemically characterized.

Materials and Methods

Prepubertal Landrace gilts, 4-5 months in age and weighing 60-75 kg, were assigned randomly to experimental groups. They were housed in groups of 4-6 and were given access ad libitum

to a pelleted commercial finishing ration containing 14% protein. Feeding of the animals, injections with PMSG and hCG and isolation of granulosa and theca cells were kindly managed by A. Srikandakumar, Dep. of Animal Science as part of a collaborative effort. Gilts were injected IM with 750 IU PMSG (Equinex^{R}; Ayerst Labs., Montreal, PQ) and 72h later with 500 IU hCG (APL^{R}, Ayerst Labs) to induce follicular growth and ovulation which will occur 42 h later (Ainsworth et al., 1982). Pairs of gilts were laparotomized and ovaries removed at the time of hCG injection (time 0) and at 12, 24, 36, 38, 40 and 42h after hCG injection. Before laparotomy, gilts were sedated with Innovar^{R}-Vet (M.T.C. Pharmaceuticals, Mississauga, ON). General anaesthesia was induced and maintained with halothane in a semi-closed system.

The excised ovaries were placed in cold, phosphate-buffered saline (PBS, 0.1M NaCl, 0.1M sodium phosphate, pH 7.2) and follicles of uniform diameter (7-9 mm) were dissected out and freed of adherent stromal/interstitial tissue. The follicles were cut into halves or quarters in petri dishes and follicular fluid (FF) collected, centrifuged to remove cellular debris and the supernatant stored at -20°C until assayed. The follicle pieces were transferred into fresh PBS and GC were harvested under a dissecting microscope by gently scraping the inner surface of the follicle with a thin wire loop. The TIC layers were then peeled away from the theca externa with fine forceps. This methodology has been described by Tsang et al., (1985) and gives GC and TIC preparations with

a purity of 99 and 95% respectively as determined by the relative ability of the two cell types to secrete different steroids (Tsang et al., 1985). Isolated GC and TIC from a given gilt were pooled, washed with cold PBS and sonicated for 10 sec in 3 ml distilled water as suggested by Searls (1980). The sonicate was centrifuged at 2000 x g for 5 min to remove cellular debris and aliquots of the supernatant were stored at -20°C until assayed.

Determination of Plasminogen Activator Activity

Plasminogen activator activity was assayed by the spectrophotometric method of Searls (1980) described in chapter 4 with the following modifications. To measure PA activity in TIC and FF, an 10 µl sample of TIC extracts (400-600 µg protein/ml) or FF was mixed with 140 µl of 50 mM Tris buffer (pH 7.8) and was treated as described in Chapter 4. To assay PA activity in GC, an 80 µl sample of GC extracts (200-400 µg protein/ml) was mixed with 70 µl of Tris buffer and was also treated as for TIC and FF samples. In all assays, a sample without added plasminogen served as a control. Very low minimal activity ($\Delta A/h < 0.001$) was detected in the absence of exogenous plasminogen. Plasminogen activator activity was expressed as change in absorbance (545nm) per hour per µg of protein. The above assay system was validated for measuring PA activity in GC, TIC and FF by following the protocol suggested in Chapter 4. Protein content was determined by the

method of Bradford (1976) using bovine serum albumin as a standard.

Determination of Plasminogen and Plasmin in Follicular Fluid

Plasminogen in FF was determined by the colorimetric method of Searls (1980) described above, but modified to include the addition of urokinase (150 Plough Units/ml) to the reaction mixture to activate plasminogen. No exogenous plasminogen was added. Plasmin activity was expressed as change in absorbance at 545 nm per hour. Activity was converted to plasminogen concentration using a standard curve by plotting plasmin activity versus plasminogen concentration (Fig. 33).

Inhibition of plasminogen-derived activity was used to confirm the presence of plasminogen in FF. Replicate tubes containing 25μl FF were incubated for 90 min at room temperature, in 50 mM phosphate buffer (pH 7.3) solutions containing various concentrations of antisera against human plasminogen (American Diagnostica). The samples were centrifuged at 10,000 X g for 5 min and the plasmin activity in the supernatant after the addition of urokinase (150 Plough Units) was determined colorimetrically as above.

The presence of plasmin in FF was determined using a modification of the method of Searls (1980), omitting additions of plasminogen and urokinase. Ten μl samples of FF were mixed with 890 μl of 100mM L-lysine containing 0.75 mM of S-2251 and incubated at 37°C for 1h. At this concentration

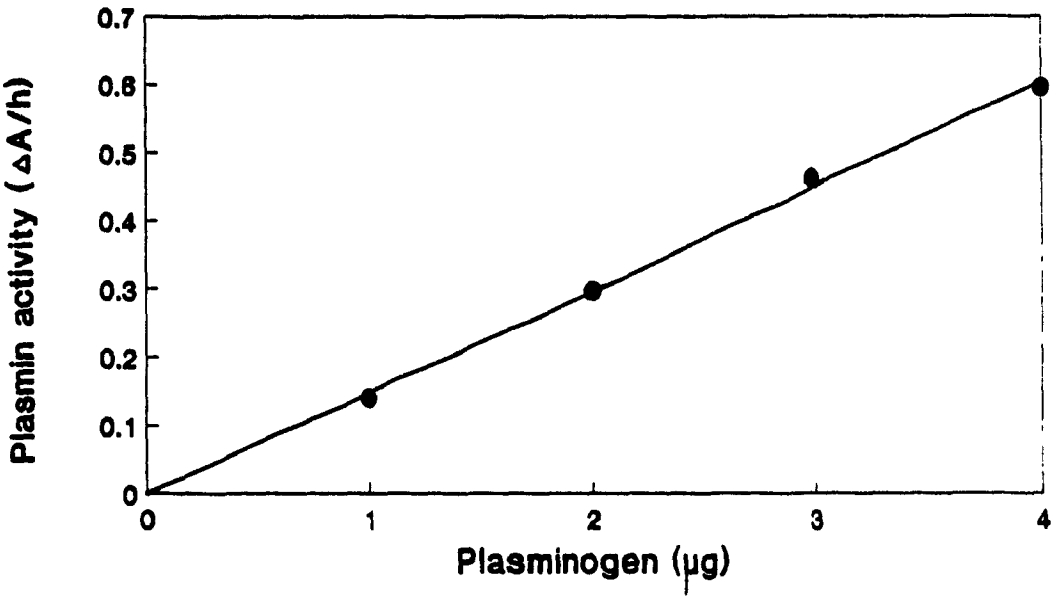


Figure 33. Standard curve for plasmin activity (ΔA/h) to plasminogen concentration (μl). Plasmin activity was determined in three independent samples for each plasminogen concentration.

L-lysine inhibits the activation of plasminogen to plasmin without affecting the rate of hydrolysis of the chromogenic substrate (S-2251) by plasmin (Searls, 1980). The reaction was terminated and diazotization and coupling of p-nitroanilide to NED were carried out as described above. Plasmin activity was expressed as change in absorbance at 545 nm per hour.

Immunological and Biochemical Characterization of PA, PAI Activity and Plasminogen in Porcine Follicle

In order to characterize the PA forms produced by porcine GC and TIC, three different approaches were used:

1) Immunoprecipitation of PA activity present in GC and TIC cell extracts

2) Western blot analysis using antibodies against human u-PA and t-PA

2) Localization of PA within the electrophoretic lane following SDS-PAGE of GC and TIC cell extracts, extraction of the protein from the gel, and determination of its activity, molecular weight and isoelectric point.

For the immunoprecipitation of PA activity, samples of GC and TIC extracts (5 μl) and FF (25 μl) were incubated for 90 min at room temperature in 50 mM phosphate buffers (pH 7.3) containing increasing concentrations (0 to 18 μg) of goat anti-human urokinase IgG, goat anti-human t-PA IgG, or goat anti-porcine t-PA IgG (American Diagnostica). We used small volumes 5 μl of TIC and FF and 25 μl of GC cell extracts to

minimize the quantity of antibody used in these reactions. The samples were centrifuged (8,000 X g, 10 min) and the PA activity remaining in solution was determined as described above.

Proteins in cell extracts were fractionated by SDS-PAGE as described by Laemmli (1970), using 10% acrylamide resolving and 4% acrylamide stacking gels and run at 40V at room temperature. Gel was processed either for Western blots or to localize PA or PAI activity within the gels slices. Western blots were prepared using nitrocellulose membranes and electrostatic transfer of the samples fractionated by SDS-PAGE. Membranes were incubated with purified goat anti-human urokinase IgG, goat anti-porcine t-PA IgG, goat anti-human PAI-1 IgG or goat anti-human PAI-1 IgG at a final concentration of 0.8 μg/ml and goat antiserum against human plasminogen or rabbit antiserum against bovine PAI-1 at a 1:1000 dilution. Immune complexes were detected enzymatically using anti-goat IgG-peroxidase at a 1:2000 dilution (Sigma). Other details of the Western blot were as described in Chapter 4. Localization of PA activity and isoelectric focussing was performed as described in Chapter 4.

An alternative approach to confirm that the measured PA activity is in fact attributable 100% to PA is to use different protease inhibitors which have a predictable effect on PA activity. We used a variety of protease inhibitors at concentrations suggested by Bartholomew and Woolley (1988).The influence of these exogenous serine protease inhibitors on PA

activity in GC and TIC extracts and FF was examined by the addition of aprotonin (2 mg/ml), soybean trypsin inhibitor (SBTI, 1 mg/ml), phenylmethylsulphonylfluoride (PMSF, 0.35 mg/ml), tosyl-methyl chloroketone (TLCK, 20 μg/ml) or leupeptin (1 mg/ml; Sigma) to the test samples.

Dissociation and Stability of PAI

In order to determine if the high MW forms of PAI present in GC and TIC (see results) could be dissociated into a lower MW form, aliquots of GC and TIC extracts prior to their storage at -20°C were treated for 30 min at 37°C in 0.5% sodium dodecyl sulphate (SDS) and 1.5M NH_4OH as described in Chapter 4. Samples were dialyzed overnight against 0.1 M Tris (pH 8.1), containing 0.5% Triton X-100, and then analyzed for PAI by Western blot and localization of PAI protein within the electrophoretic lane as described in Chapter 4.

In addition, the ability of the low MW form of PAI isolated from the electrophoretic lane to retain its activity (inhibitory capacity) was investigated under various conditions including 5% (v/v) β-mercaptoethanol, 1M acetic acid, 6M urea, 4M guanidine hydrochloride. Following the addition of an agent the gel extract containing PAI was incubated for 1 h at 37°C. The inhibitory capacity of the low MW form of PAI following these treatments was determined by incubating 50, 100, 200, and 250 μl of gel extract with 70 IU/ml of urokinase. Extracts from a blank gel were tested for

inhibitory capacity inherent in the gel itself and were used as a control.

To study the stability of PAI at acid pH, subsamples of the gel extracts were incubated for 1 h at 37°C in 0.005 M Tris-Glycine Buffer (pH 2.7) containing 0.1% gelatin. The sample was neutralized by the addition of 3 volumes of 0.1 M Tris (pH 7.8) and subsequently tested for the remaining inhibitory capacity as described in the previous paragraph.

Enzyme-Linked Immunosorbent Assay (ELISA) for t-PA and PAI:

Tissue-PA or PAI protein content was determined with an ELISA using goat anti-porcine t-PA IgG or goat anti-human PAI-1 IgG, respectively at a concentration 0.8 μg/ml as the primary antibody and peroxidase-conjugated anti-goat IgG at a dilution 1:2000 as the secondary antibody. Other details of this assay system were as in Chapter 4.

Statistical analysis

Data were analyzed by analysis of variance for significant effects of time on change in PA activity, PA content and PAI content in GC, TIC and FF (Steel and Torrie, 1980). Differences between the means were evaluated with Duncan's Multiple Range Test.

Results

Plasminogen concentration in FF of PMSG/hCG-treated gilts remained relatively constant at 200 μg/ml (range 160 to 270) for up to 42h after the hCG injection (Fig. 34). Plasmin was detectable in FF only 42h post-hCG, when 53% of the follicles had already ovulated. Addition of antisera against human plasminogen precipitated 100% of the plasminogen-derived activity in FF (Fig. 35). Western blot analysis of plasminogen in FF showed that the major form of the pro-enzyme has an apparent MW of 84,000 although small amounts of MW 64,000 and 62,000 were also observed at 42 h post-hCG (Fig. 36). Conclusive evidence that the low MW bands represent plasminogen forms is lacking. However, since plasmin and plasminogen are present at the 42 h post-hCG sample and that plasmin catalyzes the conversion of high to low MW forms of plasminogen (Dano et al., 1985) it is possible that these latter species may represent low MW forms of porcine plasminogen.

Plasminogen activator activity and content in GC extracts increased (4 x) significantly ($P<0.01$) as the time of expected ovulation approached while there was a small non-significant ($P=0.18$) increase in PA activity and content in TIC extracts during the same time period (Fig. 37, 38). Plasminogen activator activity and content in FF remained relatively low until about 40h, but increased (3 x) significantly ($P<0.01$) by 42h post-hCG. PAI content in GC and FF decreased

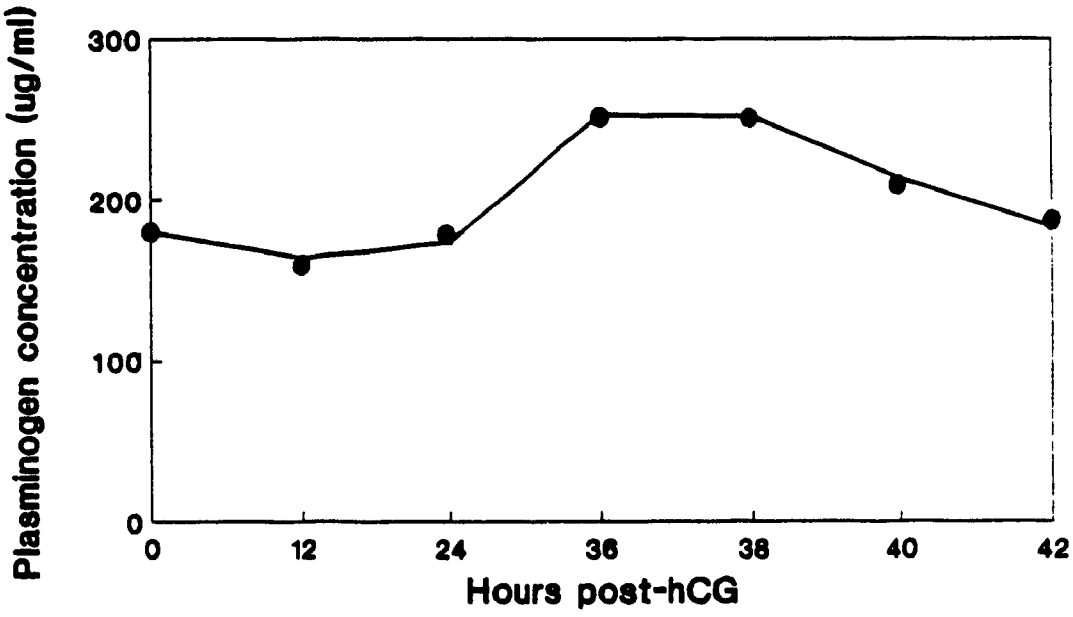


Figure 34. Plasminogen content in follicular fluid at various times after human chorionic gonadotropin (hCG) administration. Each point represent the average of two independent samples.

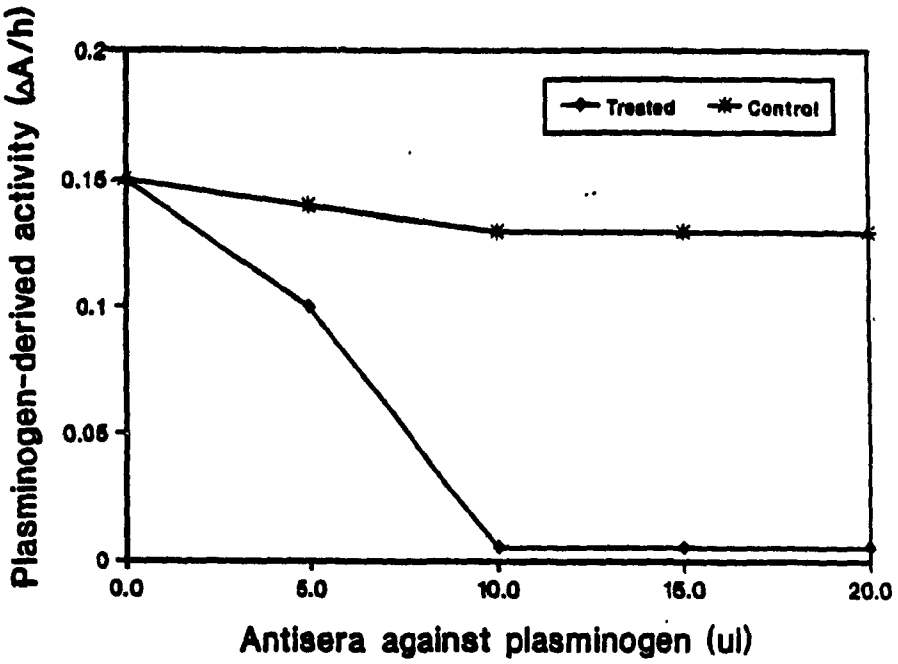


Figure 35. Inhibition of plasminogen-derived activity in follicular fluid (FF) by the addition of antiserum against human plasminogen. Incubation of a duplicate sample of FF with the same quantity of a nonimmune serum served as the control. (Plasminogen-derived activity = plasmin activity generated by addition of urokinase). Each point represents the average of two independent samples.

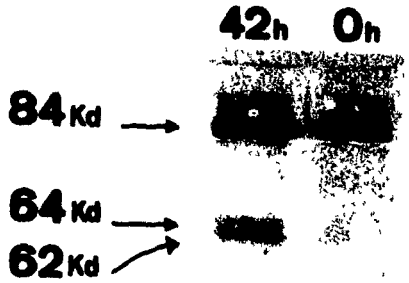


Figure 36. Western blot analysis of plasminogen in follicular fluid (40 µl) at 0h and 42h after administration of human chorionic gonadotropin (hCG).

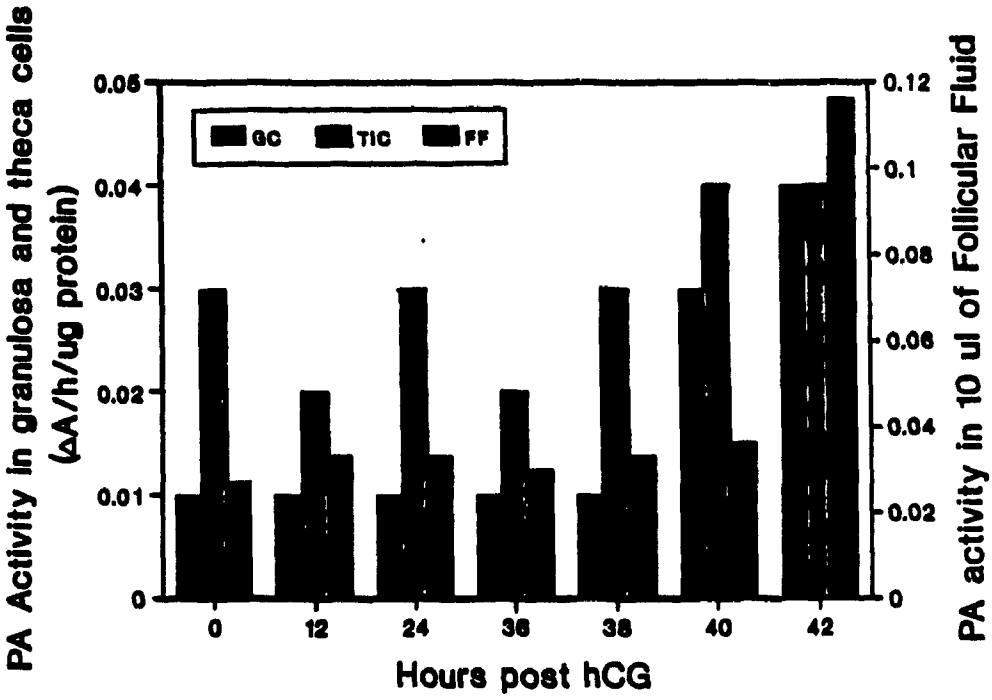


Figure 37. Plasminogen activator activity of granulosa (GC) and theca interna (TIC) cell extracts and follicular fluid (FF) at various times after human chorionic gonadotropin (hCG) administration. Each bar represents the average of two independent samples.

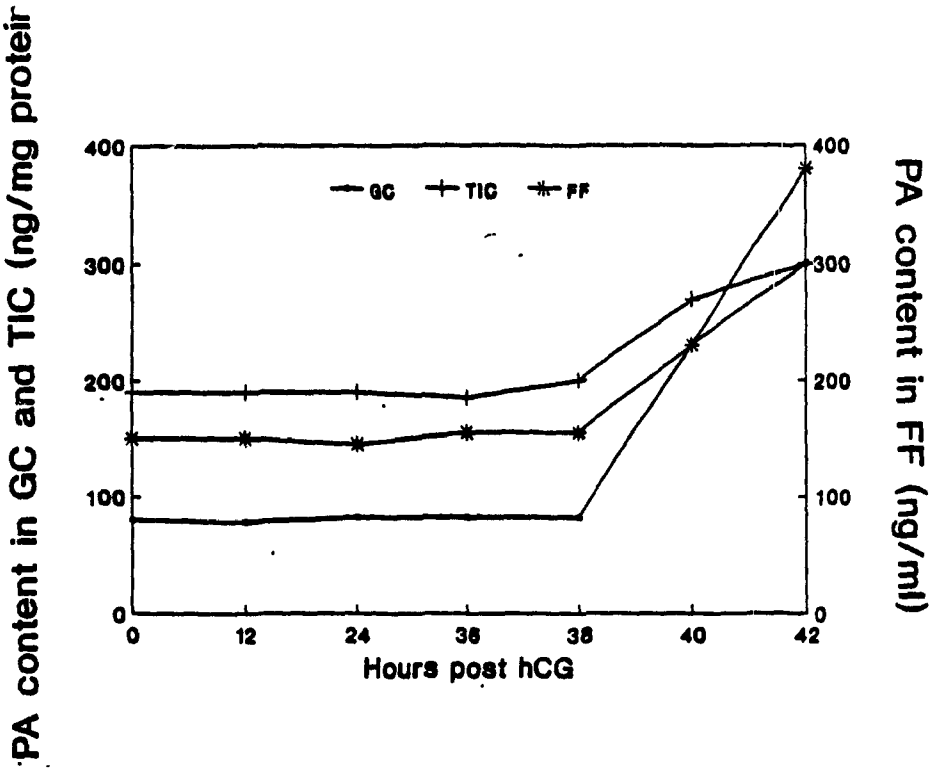


Figure 38. Tissue-plasminogen activator content of granulosa (GC) and theca interna (TIC) cell extracts and follicular fluid (FF) at various times after human chorionic gonadotropin (hCG) administration. Each point represents the average of two independent samples.

significantly ($p < 0.01$) as the time of expected ovulation (42 h post-hCG) approached (Fig. 39).

Exogenous leupeptin and PMSF, but not TLCK and SBTI, inhibited PA activity (Table 7). Aprotonin, another protease inhibitor, was moderately inhibitory at a concentration of 2 mg/ml. Higher concentrations of aprotonin resulted in no further inhibition of PA activity.

To determine the type of PA present, the effect of specific antibodies against the two forms of PA, t-PA and u-PA, on the activity in the cell extracts and FF was examined. Antibodies directed against human t-PA and u-PA IgG gave no insight as cross-reactivity between these antibodies was observed and both precipitated 100% of the PA activity. Anti-porcine t-PA IgG inhibited PA activity in GC and TIC (100%) and FF (90%) (Fig. 40) indicating that t-PA is the predominant PA in the porcine follicle. The remaining 10% activity in FF was inhibited by anti-human u-PA IgG. Western blot analysis of GC and TIC extracts indicated the presence of PA in two main forms with apparent MWs of 78,000 and 72,000 (Fig. 41). Following SDS-PAGE, PA activity in gel slices appeared in two major bands, corresponding to these molecular weights (Fig. 42A). If our recovery of PA activity after SDS-PAGE was 100%, we should recover a total of PA activity 0.6 (0.04 X 15) for both GC and TIC where 0.04 is the PA activity (ΔA/h/μg protein) of GC and TIC and 15 μg is the quantity of protein loaded to each gel channel. The actual recoveries were (0.097 + 0.252)/ 0.6= 58.2% for GC and (0.117 + 0.273)/0.6= 65% for

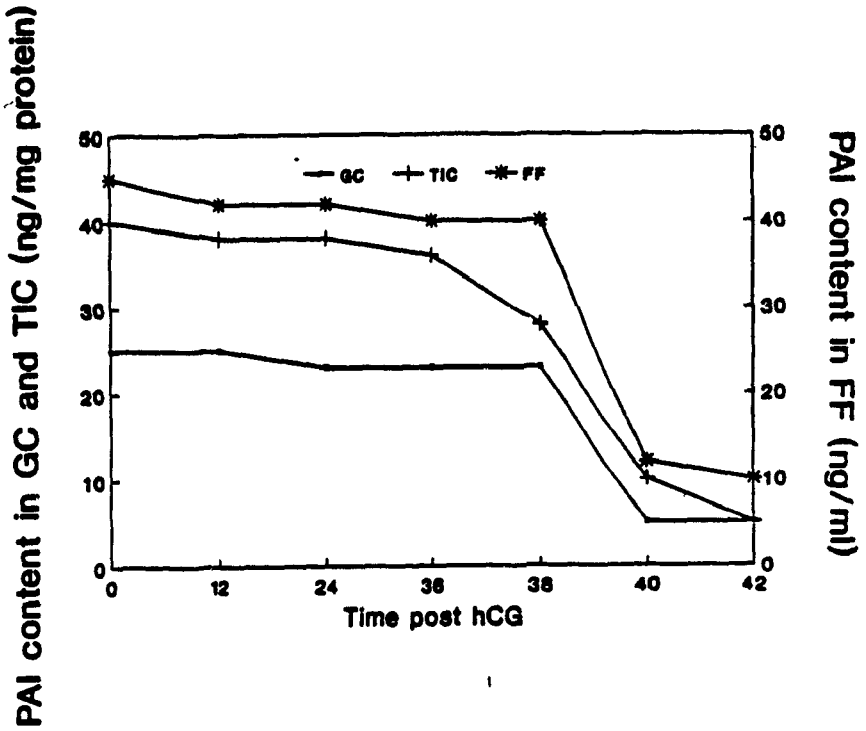


Figure 39 . Plasminogen activator inhibitor content of granulosa (GC) and theca interna (TIC) cell extracts and follicular fluid (FF) at various times after human chorionic gonadotropin (hCG) administration. Each point represents the average of two independent samples.

Table 7. Effect of various protease inhibitors on PA activity of granulosa (GC), theca cell extracts (TIC) and follicular fluid (FF) from all samples obtained after hCG treatment

Inhibitor	Inhibition of PA activity (%)		
	GG	TIC	FF
Leupeptin (1 mg/ml)	100	100	100
PMSF[1] (0.35 mg/ml)	100	100	100
Aprotonin (2 mg/ml)	38	35	40
TLCK[2] (20 µg/ml	6	3	6
SBTI[3] (1 mg/ml)	3	2	5

[1]PMSF: phenylmethylsulphonylfluoride

[2]TLCK: tosyl-methyl chloroketone

[3]SBTI: soybean trypsin inhibitor

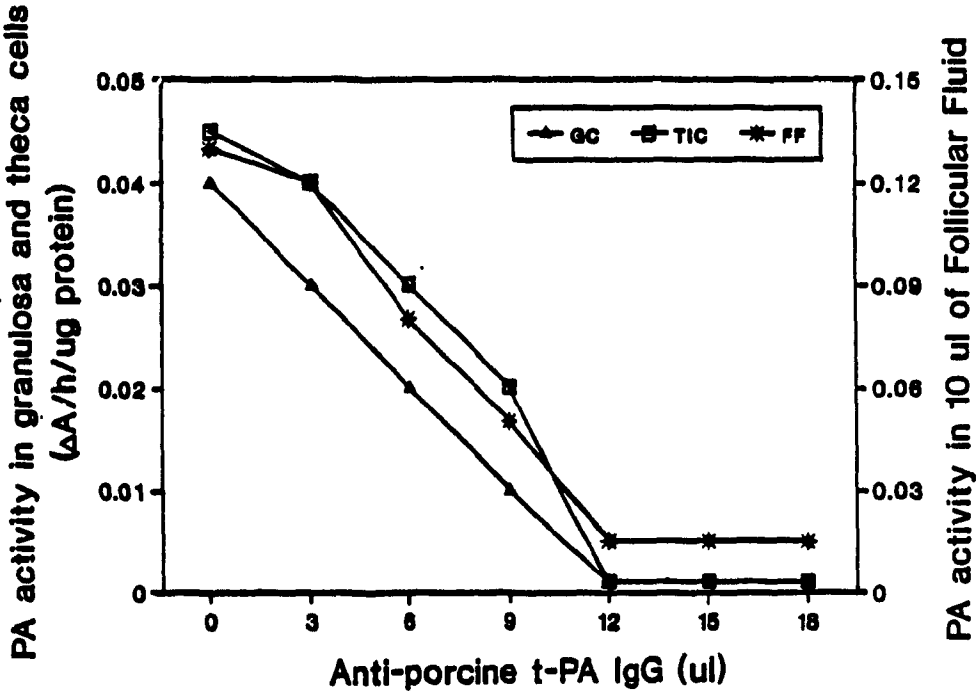


Figure 40. Inhibition of plasminogen activator (PA) activity of granulosa (GC) and theca interna (TIC) cell extracts and follicular fluid (FF) by purified antibodies against porcine tissue-PA (t-PA).

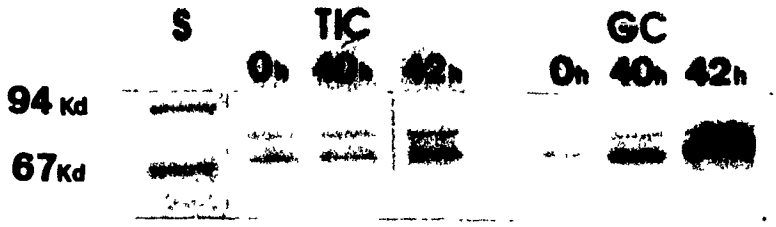


Figure 41. Western blot analysis of theca interna (TIC, 15 μg protein) and granulosa (GC, 25 μg protein) cell extracts using purified antiserum against porcine tissue plasminogen activator (t-PA). Times refer to post-hCG administration.

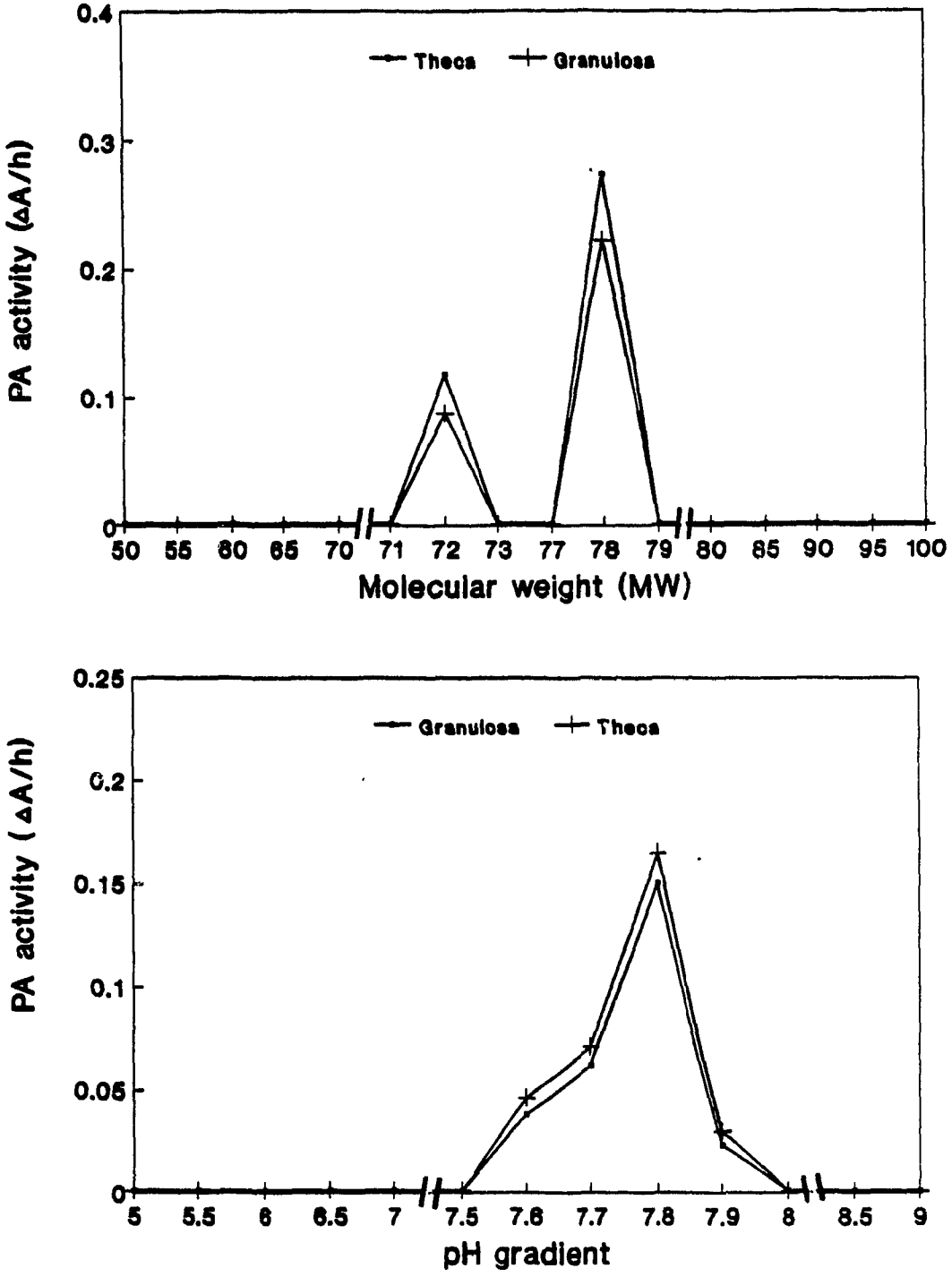


Figure 42. SDS-PAGE distribution of plasminogen activator (PA) activity of granulosa and theca cell extracts (A). Plasminogen activator activity distribution in a pH gradient during electrofocussing (B). PA activity was assayed in 5 mm gel slices extracts as described in materials and methods. (— granulosa; + theca).

TIC (Fig. 42A). The low MW form (72,000) represented 70% of the total PA activity recovered from the gel, while the high MW form (78,000) accounted for the remainder.

The isoelectric points (pI) of both forms of PA occurred between 7.6 to 7.9 with a peak at pH 7.8 (Fig. 42B). The recoveries of PA activities after electrofocussing were 0.272/(0.097 + 0.252)= 78% for GC and 0.297/(0.117 + 0.252)= 76% for TIC (Fig. 43B). All these recoveries are overestimated because the PA activity is determined in the gel extracts without the influence of PAI which is not the case for the original determination of PA in the GC and TIC cell extracts.

To determine the type of PAI present in GC, TIC and FF, Western blot analysis was performed. Antibodies directed against human and bovine PAI-1 and human PAI-2 gave no insight with regard to PAI nature due to cross reactivity between them. However, analysis with either anti PAI-1 IgG or anti PAI-2 IgG indicated the presence of two protein bands with apparent MWs of 126,000 and 130,000 (Fig. 43). Treatment of cell extracts with 0.5% SDS and 1.5 M NH_4OH led to a partial dissociation of the high MW proteins and the appearance of a band at 52,000. Following SDS-PAGE the 52,000 protein band was allowed to elute from the gel slices and its ability to inhibit PA was tested. Urokinase-PA was inhibited by the elutant before or after acid treatment at pH 2.7 in a dose dependent-manner with 100 and 150 μl of gel extract resulting in 50 and 100% inhibition, respectively. (Fig. 44). In addition, the inhibitory capacity of the porcine ovarian PAI

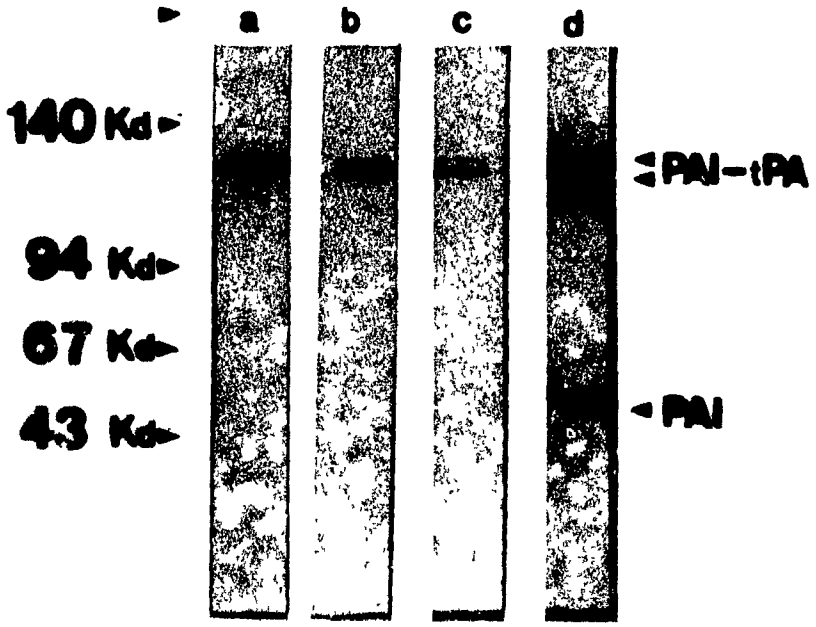


Figure 43. Western blot analysis of (a) granulosa (15 μg protein), (b) theca interna (15 μg protein) cell extracts, (c) follicular fluid (25 μl) and (d) granulosa cell extracts (15 μg protein) after being treated with 0.5% sodium dodecyl sulfate and 1.5 M NH_4OH with purified antibodies against human plasminogen activator inhibitor 1.

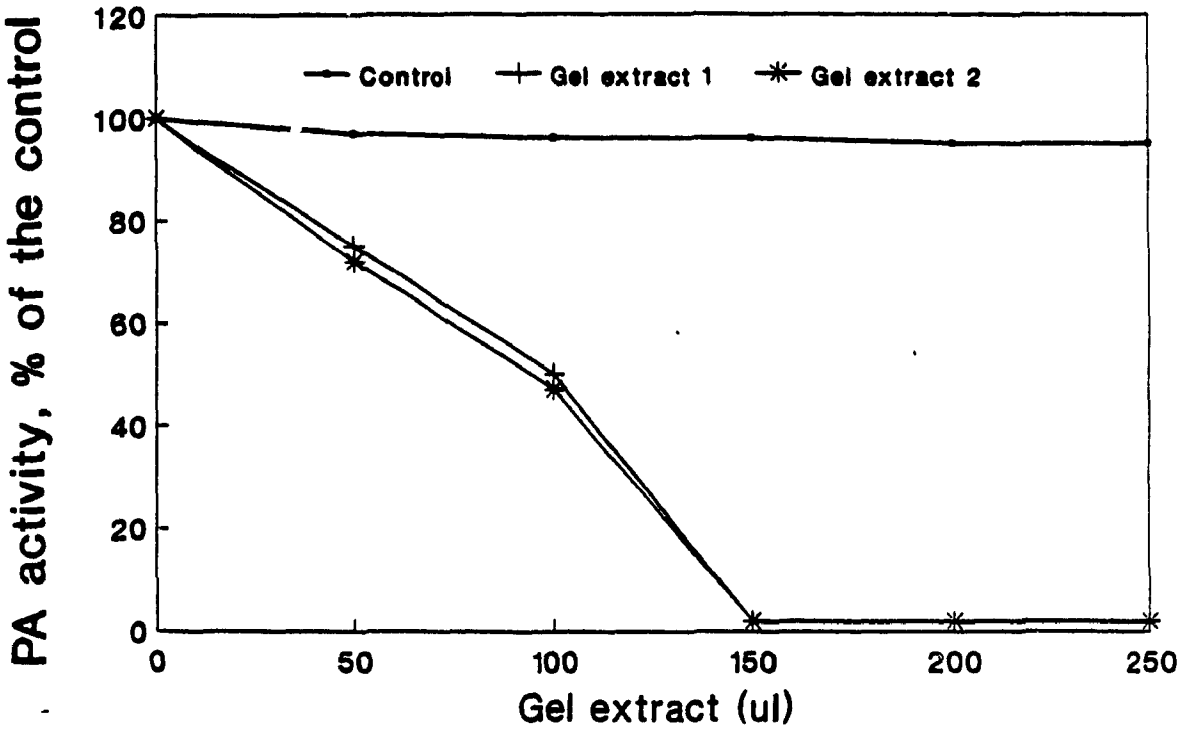


Figure 44. Plasminogen activator inhibitory capacity of gel extracts. The active fractions were pooled (0.6 ml of cell extracts) and tested for inhibitory capacity before (+) and after (*) acidification at pH 2.7. PA activity in replicate tubes after the addition of extracts prepared from a blank gel was determined to serve as a control. Each point represents the average of two independent samples.

was not affected by treatment with 5% β-mercaptoethanol or 1M acetic acid (Fig. 45A) and 4M guanidine hydrochloride or 6M urea (Fig. 45B). The isoelectric point of PAI was also determined and occurred between 4.7 and 4.9 with the peak at 4.8 (Fig. 46).

Discussion

The present study shows that plasminogen is present at relatively constant levels (200 μg/ml) (Fig. 34) in all FF samples from the time of hCG administration until ovulation. However, plasmin was present in FF only at 42h post-hCG, when 53% of the follicles had already ovulated. This suggests that the activation of plasminogen to plasmin coincides with follicle rupture and this conversion is limited only by the availability of PA and PAI. This concept is supported by our findings that ovulation is preceded by marked increases (4x) in GC PA activity and PA content and a decrease in PAI content beginning 4h prior to ovulation. These results are consistent with those obtained in follicular tissues from cycling sows (Smokovitis et al., 1988) who studied changes only in PA activities and not in PA contents. Their experiment was also performed using only one sow which may limits the reliability of their findings. Our finding that PAI is decreased just prior to ovulation is an original contribution to scientific knowledge. The association between increased PA and decreased PAI contents to achieve an overall enhancement of PA activity

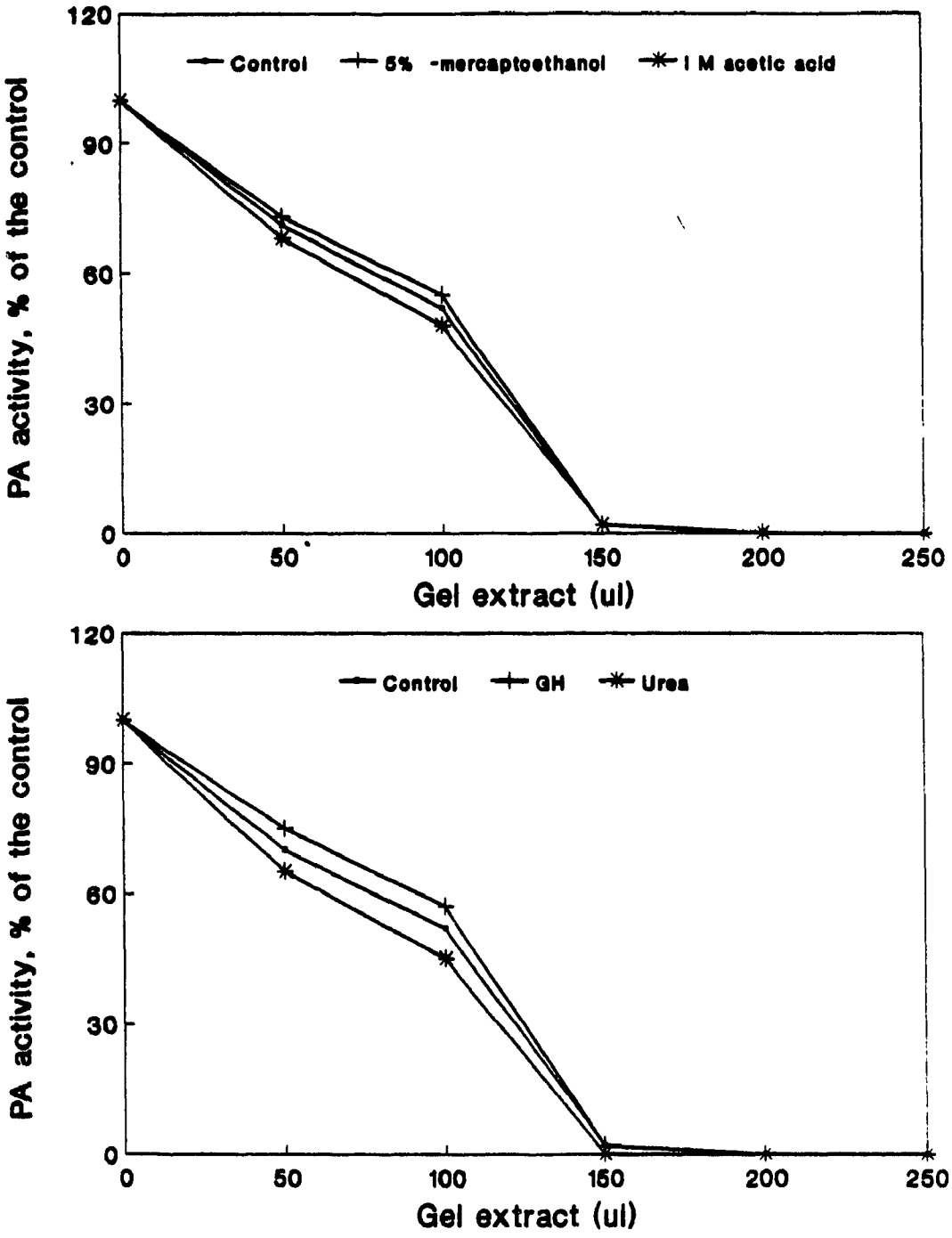


Figure 45 .Plasminogen activator inhibitory capacity of gel extracts (A) after treatment with 5% β-mercaptoethanol and 1 M acetic acid and (B) after treatment with 1 M guanidine hydrochloride (GH) and 6M urea. PA activity of untreated gel extracts served as a control. Each point represents the average of two independent samples.

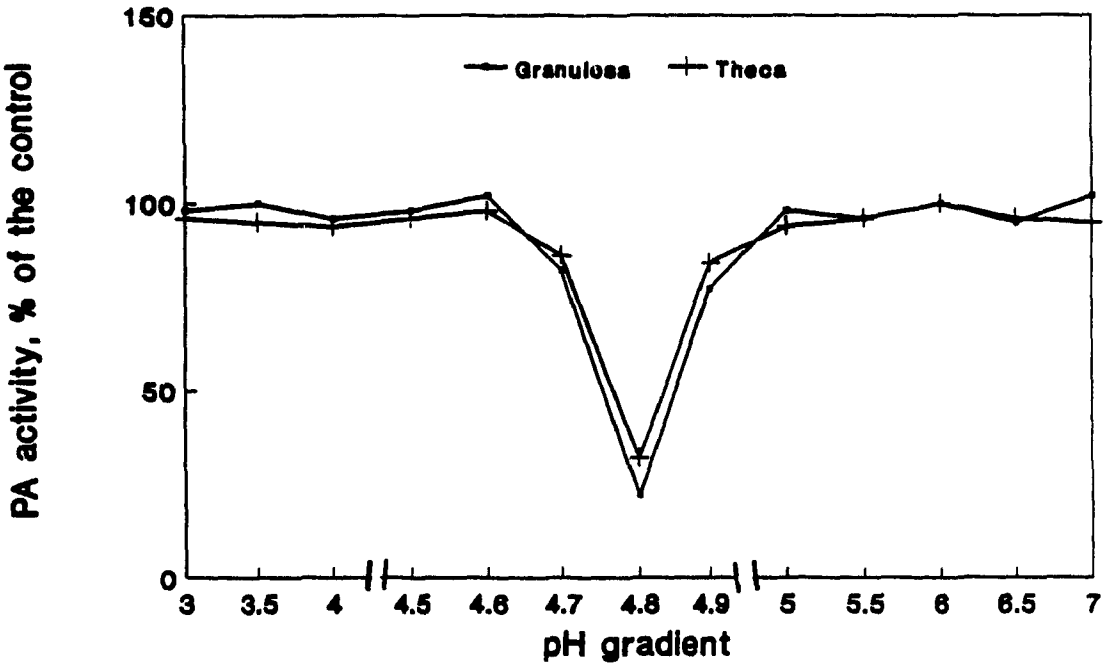


Figure 46 . Plasminogen activator inhibitor activity distribution (PAI) in a pH gradient during electrofocussing. PAI activity was assayed by the ability of the 5 mm gel extracts to inhibit 70 IU/ml of urokinase-PA (—— granulosa; —+— theca). Each pont represents the average of two independent samples.

and the presence of plasmin in FF provides further support for the suggestion that follicular changes in PA activity are associated with the processes leading to follicular rupture.

The effectiveness of various exogenous protease inhibitors on PA activity was assessed. Leupeptin and PMSF were very effective inhibitors while TLCK and SBTI failed to inhibit PA activity (Table 7). These inhibitory activities are consistent with that previously reported (Bartholomew and Wooley, 1988) for PA inhibitors, providing more evidence that the measured activity is attributable to PA.

Existing forms of PA have been classified into two groups, u-PA and t-PA, distinguishable on the basis of primary structure (MW and pI) and antigenic nature (Dano et al., 1985). The present study shows that the major form of PA in porcine follicles is t-PA as demonstrated by specific immunoprecipitation using anti-porcine t-PA IgG. In addition, MW in the range of 72,000 to 78,000 and pI of 7.8 are consistent with the characteristics reported for t-PA (Dano et al., 1985). Species differences do exist inasmuch as Canipari et al. (1987) found that rat GC produce t-PA while mouse GC produce u-PA.

It is of interest to note that there are two forms (78,000 and 72,000) of t-PA in GC and TIC extracts (Fig. 42 and 43). A reasonable explanation of our findings is that the low MW form represents a partially degraded product which retains enzymatic activity. An alternate hypothesis is that there are two subpopulations of GC and TIC, each producing a different

form of t-PA. Dano et al. (1985) reported that the variants of porcine t-PA may be due to differences in glycosylation or amino acid composition. A definite conclusion depends on purification of both molecules. It is noteworthy that FF contains both u-PA and t-PA. The source of t-PA is probably GC and TIC. Urokinase-PA likely entered the FF compartment from the blood since, having a MW of only 50,000 (Dano et al., 1985), it could easily cross the blood-FF barrier.

Our study also demonstrated the existence of a previously undetected PAI in the porcine ovary which occurs in two main high MW forms (126,000 and 130,000) (Fig. 43). We suspected that the high MW forms represent enzyme-inhibitor complexes since most of the PAI have a MW in the range of 45,000 to 55,000 (Sprengers and Kluft, 1987). Furthermore, we demonstrated the existence of two forms of t-PA in the porcine ovary (Fig. 41). Thus, one should expect two enzyme-inhibitor complexes in the range of 120,000 tO 135,000 which, indeed, was the case. Treatment with a nucleophilic agent (NH_4OH) led to a partial dissociation of the two high MW forms and the appearance of a common 52,000 protein (Fig. 43) which was shown to inhibit u-PA (Fig. 44). The use of antibodies against human PAI-1 and PAI-2 gave no insight as to ovarian PAI nature due to cross-reactivity between these antibodies. The ovarian PAI was shown to retain its enzymatic activity after acidification at pH 2.7. In contrast, protease nexin I, another PAI, which has a similar MW but different pI with PAI-1 and PAI-2, is irreversibly inactivated by treatment at low

pH (Van Mourik, et al., 1984). Thus, the ovarian PAI and protease nexin I are biochemically unrelated molecules. Furthermore, MW of 52,000 and pI 4.8 are very different from the well-characterized inhibitors like α_2-antiplasmin and α_2-macroglobulin (Van Mourik et al., 1984). Antibodies against PAI-1 and PAI-2 recognize the ovarian PAI, suggesting that all these molecules are immunologically related. However, the primary structure of PAI (MW and pI) as well as its ability to retain inhibitory capacity after treatment with 5% β-mercaptoethanol, I M acetic acid, 4 M guanidine hydrochloride and 6 M urea are consistent with characteristics previously reported for bovine and human PAI-1 (Loskutoff et al., 1983; Van Mourik et al., 1984; Sprengers and Kluft, 1987) suggesting that they belong to the same class if indeed are not identical.

In addition to the involvement of PA and PAI in the breakdown of the follicular wall, the t-PA produced by porcine granulosa and theca cells may have some additional functions. The first may involves breakdown of the proteoglycans which are responsible for the high viscosity of the follicular fluid. This breakdown results in a liquidation of follicular fluid allowing the liberation of the ovum during follicular rupture. The second possible role for the t-PA may be during angiogenesis, apparent during the conversion of the avascular to highly vascular granulosa cell layer after ovulation. This possibility was suggested by Mullins and Rohrlich (1983) and Ny et al., (1985). The third possible role for t-PA is related

to the formation of the blood clot after the liberation of the ovum. t-PA may act on a fibrinolytic capacity preventing a disasterous premature blood clot formation that might trap the ovum in the follicle.

Tsang et al. (1979) demonstrated that follicular growth and development are associated with changes in the levels of cAMP and prostaglandins in porcine FF. These changes are controlled at least in part by gonadotropins. Based on these observation and the results of the present study, the following sequence of events is postulated for the control of t-PA during ovulation. Gonadotropins induce t-PA production in two ways: directly via their receptors located on GC and TIC or indirectly via prostaglandins produced by the stimulated GC and TIC. Canipari and Strickland (1986) reported a very similar model for the control of t-PA by gonadotropins in rats and they emphasized that the effect of prostaglandins is magnified in vivo because their secretion into the limited volume of the follicle allows effective concentrations to be reached very quickly.

In conclusion, our results show that an increase in PA activity and content and a decrease in PAI is temporally correlated with ovulation in the pig suggesting an involvement of this protease in follicular rupture. The follicular PA activity appears to be principally of the t-PA type and, while both GC and TIC are involved, production by GC predominates. Biochemical characterization of porcine ovarian PAI indicates that it belongs to the same class of inhibitors as bovine and

human PAI-1.

7. GENERAL CONCLUSIONS-SPECULATIONS

The role of plasmin and plasminogen activator in bovine lactation

The role of plasmin and PA in bovine lactation was examined from three different perspectives. In the first two perspectives, we used an *in vivo* approach. First, we investigated variations in plasmin and plasminogen concentrations related to health status of the udder and the stage of lactation. The second perspective, was the active manipulation of the lactational performance by exogenous somatotropin administration. The third perspective was an *in vitro* approach to identify cells within the mammary gland which produce plasminogen, PA and PAI. The possibility that mammary gland cells actively regulate plasmin production was examined.

Mastitis, a wide-spread inflammation of mammary gland, resulted in greater concentrations of plasmin, plasminogen and serum albumin in milk. There is a highly significant ($p < 0.01$) correlation between plasminogen and serum albumin contents of milk ($r=0.74$). Serum albumin is the major blood-originated component present in milk (Philippy and McCarthy, 1974). These results, collectively, led us to suggest the following hypothesis explaining the increased plasmin in milk during mastitis: plasminogen and serum albumin cross the mammary epithelium barrier which during mastitis is

significantly compromised. Increased concentration of plasminogen in milk, despite the fact that the ratio of (plasminogen + plasmin)/plasmin is unaffected, results in greater concentration of plasmin in milk.

Milk obtained from mastitic quarters contains high levels of somatic cells. These cells are mostly macrophages and polymorphonuclear leucocytes (Guidry, 1985). Both cell types have the potential to produce PA (Saksela, 1985). Thus, someone should expect changes in the activation pattern of plasminogen to plasmin in milk taken from mastitic quarters. Contrary to this expectation, we observed that the ratio of (plasminogen + plasmin)/ plasmin which serves as an index of the activation process was not altered in this kind of milk. In an attempt to give an answer to this apparent paradox, we used two different <u>in vitro</u> approaches. In the first, we assessed the ability of macrophages isolated from normal and mastitic quarters to produce and secrete PA. We found that macrophages isolated from mastitic quarters produce higher (2.4 X) levels of u-PA when compared to that of macrophages isolated from normal quarters. However, in both cases macrophages are unable to release the PA already produced. The lack of PA secretion may explain why we don't observe more activation of plasminogen to plasmin in mastitic milk <u>in vivo</u>. In the second approach, somatic cells were isolated from milk samples and were added (intact or lysed) to the original milk in high numbers (>1,000,000/ml). We did not observe any differences in milk plasmin and plasminogen concentrations

even though these milk samples were incubated at 37°C for 24 h. These results suggest that even in the case that macrophages were able to secrete their intracellular PA, the impact on the milk plasmin and plasminogen would have been minor. Therefore, somatic cells by themselves have a limited ability to influence the plasmin/ plasminogen system in milk. The results of the in vivo and in vitro study, collectively, suggest the association between SCC and plasmin and plasminogen in milk is not a "cause:effect" relationship but rather the outcome of increased transport of both somatic cells and plasminogen from blood to milk.

At the end of lactation period, plasmin levels are increased. This increase is not the outcome of increased plasminogen as plasminogen slightly increased during the same time period. The ratio of (plasminogen + plasmin) / plasmin decreased throughout the lactation and reached the minimum value just prior to the cessation of milk removal. These results made us to suggest an alternate mechanism for modulating milk plasmin i.e. increased activation of plasminogen to plasmin during late lactation. The kinetics of plasminogen activation are difficult to be interpreted. The simplest scenario would suggest that plasminogen activators secretion in milk increases which results in reciprocal increases in plasmin. This possibility implies that mammary gland, itself, may actively regulate milk plasmin. This would be possible, if cells within the mammary glang produce plasminogen, PA or PAI. We have demonstrated that mammary

epithelial and fibroblasts in culture do not produce plasminogen but they produce t-PA. Mammary epithelial cells produce PA-1 while fibroblasts produce an acid-labile PAI with biochemical characteristics similar to that of protease nexin I. At this point, however, it is not possible to establish whether the production of these proteins is a property of cells under culture conditions or whether these proteins are also produced in vivo.

The increased levels of plasmin in milk to reach the peak value just prior to the cessation of milking coincide with the process of gradual involution. This process is characterized by a regression of the lactation function during the declining phase of lactation (3rd to 10th mo) and peaks during late lactation. The strong inverse relationship between increasing plasmin and decreasing milk production during the gradual involution may not be a simple correlation but rather a "cause:effect" relationship. Clarification of this possibility must await further experiments where selective inhibition of plasmin or PA should inhibit the gradual involution, thus allowing a persistence of milk production.

Exogenous somatotropin (STH) administration has a major impact on the lactation function of dairy cows. Bauman et al., (1983) reported increases (15-40%) in milk yield. The mechanism by which somatotropin increases milk production remains unknown. In the light of the findings that the effect of STH predominates at the end of lactation, we have suggested that STH may exert its lactogenic effects through retarding

gradual involution, thus allowing a persistence of milk production. The relationship between milk plasmin and plasminogen and exogenous somatotropin administration supports this interpretation. We have shown that STH administration resulted in a significant suppression of plasmin levels while the plasminogen levels were unaffected. We also showed that abrupt cessation of somatotropin resulted in significant increases in plasmin levels in milk within 48 h following cessation of STH administration. Strickly speaking, our data to this point suggest only a simple correlation. Plasmin, however, is the only known milk component the concentration of which is altered by STH administration. It is also striking that plasmin changes occur very quickly and these changes coincide with the change in milk production. Based on these results we suggested a more complete hypothesis regarding the action of somatotropin on the bovine mammary glang: STH suppresses directly or indirectly plasmin production, thereby suppressing involution, thus creating conditions permissive for lactation.

The action of rSTH on the mammary gland can be predicted to be indirect. There exists no high affinity receptors for STH within the mammary gland (Gertler et al., 1983; Akers, 1985). Exogenous STH administration does, however, increase circulating levels of the peptide hormone, insulin-like growth factor-I (IGF-I). Elevated serum IGF-I levels are a result of augmented hepatic production while, at the cell level, paracrine mechanisms for IGF-I production exist (Turner et

al., 1988). Mammary gland receptors for IGF-I have been identified in the ovine (K_d 0.5-1.5 X 10^9) (Disenhaus et al., 1988) and bovine (Glimm et al., 1988). Further, IGF-I can exert its effect in vitro at physiological concentrations. Typically in serum, IGF-I levels respond to increasing STH levels in blood until a three fold increase is obtained (Collier et al., 1988). Our results showed that IGF-1 inhibited an increase in t-PA production by mammary epithelial cells which was observed when these cells were cultured in the absence of IGF-1. If, indeed, IGF-1 is able to maintain t-PA production by mammary epithelial cells at their basal levels in vivo, this would provide more support for the hypothesis that somatotropin through elevated levels of IGF-1 suppresses plasminogen activator production by mammary epithelial cells which results in a reciprocal decrease in plasmin levels in milk.

Exogenous rSTH administration in our experiments was sufficient to achieve this maximal stimulation of IGF-I levels (Collier et al., 1988). Why then does milk yield eventually decline in spite of elevated IGF-I concentrations in the blood? Our hypothesis includes the involvement of insulin. At the onset of lactation, serum insulin levels are typically low (Hart et al., 1978; 1980) and gradually increase as lactation progresses. This increase is unaffected by exogenous STH administration. Thus, the ratio of insulin to STH or more correctly, insulin to IGF-I align themselves with the level of plasmin in milk and, inversely, milk yield. The structural

similarities between the hormones IGF-I and insulin themselves but also between their cell surface receptors is striking. It is intriguing to suggest that competition between these hormones for receptors is the physiological reality of the insulin/IGF-I ratio. Typically insulin competes poorly for IGF-I receptors, except at supraphysiological levels (Disenhaus et al., 1988). On the other hand, IGF-I competes for insulin receptors at its typical serum levels (Disenhaus et al., 1988). Therefore, as insulin levels rise during lactation insulin may displace IGF-I from insulin receptors and this could initiate involution marked by increased plasmin production.

The role of plasmin in porcine ovulation

The importance of the plasmin/plasminogen system in porcine ovulation was examined. First, we were able to detect PA and PAI activity in granulosa, theca cell extracts and follicular fluid. We showed using immunochemistry and different biochemical criteria that t-PA is the major form of PA present in the porcine ovary. The PAI has similar biochemical properties with human and bovine PAI-1. In the next step, we investigated variations in plasmin, PA and PAI during follicular development. We showed that plasmin and PA

increased while PAI decreased in granulosa, theca cell extracts and follicular fluid as the time of expected ovulation approached. The increase in PA and the decrease in PAI are consistent with the augmented proteolytic activity required for the rupture of the follicle wall (Espey, 1980; Lipner, 1988). It appears that PA and PAI are regulated in a concerted manner which promotes an overall activation of enzymatic activity. These findings support the validity of the hypothesis that PA may be a prerequisite in the sequence of events leading to follicle rupture. The complete hypothesis has as follows: the preovulatory follicle in the pig stimulated by gonadotropin and PGF_{2a} produce and secrete PA; PA converts plasminogen to plasmin; plasmin activate latent collagenase; collagenase breaks down collagen giving rise to telopeptide-free collagen which is degraded by non-specific proteases.

Our observation that t-PA is associated with ovulation in pigs together with the fact that mammary epithelial cells and fibroblasts produce t-PA has considerable implications in the current thinking on the role of t-PA. Tissue-PA and u-PA cleave the same peptide bond in plasminogen and convert it to active plasmin (Dano et al., 1985). However, t-PA, unlike u-PA, shows a fibrin dependency (Saksela, 1985) which explains its high efficiency as a thrombolytic agent. The role of u-PA, however, is unclear. It has been suggested that u-PA plays

an important role in processes requiring extracellular proteolysis (Saksela, 1985), and one would expect u-PA rather than t-PA to be involved in the process of mammary gland involution and ovulation. Several types of tissue produce t-PA and many of these are not related to thrombolytic systems. Examples include pituitary cells, pancreatic β-cells, secondary oocytes and many cells of neuronal origin (Rickles et al., 1988). This research effort adds in above list follicular cells such as granulosa and theca and cells of the bovine mammary gland such as epithelial and fibroblasts. The role of t-PA in these cells remains to be elucidated.

8.STATEMENT OF ORIGINALITY

To the author's knowledge, the following information contained in this thesis constitutes an original contribution to the scientific knowledge.

1) There is no difference in the activation pattern of plasminogen to plasmin throughout the whole range of somatic cell counts (SCC) and from third to ninth month in lactation period. The ratio of (plasminogen + plasmin)/ plasmin was 7.27 during early lactation (first and second month) and 4.23 during late lactation (tenth month) and both values are significantly different from all the other ratios throughout the whole range of SCC and from third to ninth month in lactaction. This suggests limited and increased activation of plasminogen to plasmin during early and late lactation, respectively.

2) Somatotropin administration in dairy cattle resulted in a suppression of plasmin levels in milk while the plasminogen levels were unaffected.

4) Bovine macrophages produce urokinase type plasminogen activator (u-PA). Secretion of u-PA in the culture medium is minimal (2%) when compared with the intracellular u-PA content.

5) Cloned bovine mammary epithelial cells produce tissue-PA (t-PA) and PA inhibitor-1 (PAI-1) while fibroblasts produce t-PA and a PAI with biochemical characteristics similar to that of protease nexin 1.

6) Insulin-like growth factor-1 (IGF-1) inhibited the induction of t-PA production of mammary epithelial cells which is oberved when these cells are cultured in absence of IGF-1.

7) Porcine granulosa and theca cells extracts contain low levels of a PAI which has similar structural and enzymatic properties with bovine and human PAI-1. A decrease in PAI levels of granulosa and theca cells is correlated with ovulation.

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APPENDIX I

RELATIVE CONTRIBUTION OF PLASMIN AND SOMATIC CELL PROTEASES ON MILK CASEIN PROTEOLYSIS DURING COLD STORAGE OF RAW MILK

Introduction

It is common practice for milk industries to store the incoming raw milk at low temperatures for several days before using it. The objective of cold storage is the preservation of the physico-chemical properties of the milk prior to use. Ali et al., (1980) and Reimerdes (1982) have shown that cold storing of milk alters the state of many milk components, the most important being the migration of casein from micelles to the soluble phase. Storage of milk at low temperatures also allows the growth of psychrotrophic bacteria which produce proteases which would contribute to the total proteolytic activity. These enzymes have high heat stabilities and they could possibly survive during pasteurization (Verdi and Barbano, 1988). The cumulative effects of such a system on the manufacturing behaviour of milk would be harmful. To measure the contribution of somatic cell proteases on milk proteolysis, it is necessary to selectively inhibit the activity of plasmin (Verdi and Barbano, 1988). There are no studies concerning relative contribution of plasmin and somatic cell proteases to milk proteolytic activity.

This study was undertaken to determine: 1) the effect of cold storage on the plasmin/plasminogen system in milk, 2) differences in the casein profiles during cold storage, 3) the relative contribution of plasmin and somatic cell proteases on casein proteolysis.

Material and Methods

Forty eight milk samples from individual Holstein cows with somatic cell count (SCC) less than 100,000/ml were collected and designated "Low SCC milk". Another, set of forty-eight samples was collected with SCC that ranged from 750,000 to 1,300,000/ml and was designated "High SCC milk". These milk samples were centrifuged to obtain skim milk (800 X g, 4°C, for 20 min), divided into three aliquots and then stored for up to 96 h at 4°C. Subsamples were taken every 24 h.

The first aliquot was analyzed for plasmin and plasminogen-derived activities by the colorimetric methods of Korycka-Dahl et al., (1983). Plasminogen-derived activity is the proteolytic activity measured after activation of plasminogen by urokinase.

The second aliquot was centrifuged at 38,000 X g for 2 h at 4°C. The casein pellet was separated from the supernatant (serum fraction) and was reconstituted to the original volume in 50 mM Tris buffer (pH 8.0) containing 110 mM NaCl. Individual caseins were determined by PAGE as described by

Anderson and Andrews (1977). The running gel was made from a mixture of 7 g of acrylamide and 150 mg of N', N'-methylenebisacrylamide and 27 g urea dissolved in 100 ml 0.38 M Tris running buffer containing 50 mg ammonium persulfate and 50 µl TEMED that were added immediately prior to their use. Ten µl of sample were mixed with 290 µl of 10 M Urea containing 0.1 M β-mercaptoethanol. Electrophoresis was performed at 150 V for 1.5 h. Gels were stained for 1 h in 0.25% (w/v) Coomassie Blue, 10% (w/v) TCA in methanol:H_2O (1:1, v/v). Gels were destained for 2 h in 2% (w/v) TCA in methanol:H_2O (1:4, v/v) and then overnight in 5% (v/v) acetic acid. Peak areas were quantified by reference to peak areas of standards containing known amounts of protein. Protein standards for α_s-casein, β-casein and k-casein with a purity of 95% were obtained from Sigma. Total casein was computed as the sum of the individual caseins.

Tyrosine value (TV) was determined by the method of Juffs (1973) as modified by Senyk et al., (1985) to serve as an additional index of proteolysis. This method is based on TCA precipitation of milk proteins and determination of the protein content (mostly free aminoacids) in the supernatant. Ten ml of milk sample were mixed with 10 ml of 15% (w/v) TCA. After 10 min the mixture was filtered through Whatman No 4 filter paper. Two ml of TCA filtrate were mixed with 9.02 ml of 0.1 N NaOH containing 2% (w/v) Na_2CO_3 and 0.98 ml of 1% (w/v) sodiun tartate containing 0.5% (w/v) $CuSO_4.5H_2O$. After 10 min, 1 ml of of 0.067 N Folin-Ciocalteau reagent (Fisher

Scientific) were added. The mixture was again filtered through Whatman No 4 filter paper and the extinction at 700 nm was measured against a reagent blank. Extinction was converted to tyrosine concentration (TV) by using a standard curve.

ϵ-amino caproic acid (ϵ-ACA) at a concentration of 120 mM was added to the third portion before the storage at 4°C for 96 h. Verdi and Barbano (1988) have shown that ϵ-ACA at a concentration of 120 mM will selectively inhibit plasmin without affecting the activity of other proteases present in milk. Samples were also taken every 24 h. Individual caseins and TV were determined as above.

An analysis of variance was performed to examine significant effects of storage time on changes in plasmin, plasminogen-derived activities and the content of individual caseins. Mathematical model fitted to the data included storage time and SCC as fixed classification effects. Dependent variables in this model were plasminogen and plasmin activities and contents of individual caseins (α_s-, β-, k-). When the impact of adding ϵ-ACA on casein profiles was examined, a third classification effect was added to the model, treatment. There were four subclasses for storage time (day 1, 2, 3 and 4). SCC were consisted of two subclasses; low and high as described in material and methods. Treatment consisted of two subclasses; control (no added ϵ-ACA) and treated. All the analyses were conducted using the General Linear Model procedure of the Statistical Analysis System (SAS, 1982).

Results and Discussion

The effect of cold storage on plasmin and plasminogen-derived activities in milk is presented in Table 1. There were non significant ($p < 0.05$) increases in plasmin activity by 6.8 and 12.5%, respectively for the low and high SCC milk during the 4-day storage period. Several explanations could be offered for the absence of plasminogen activation during cold storage. Plasminogen will be converted to plasmin by the action of PA. As PA exert their function extracellularly, they are accessible to inhibitors which are present in the soluble phase (Korycka-Dahl et al., 1983). Equally important factors are pH and temperature which affect plasminogen activation. The low temperature (4°C) is expected to have an adverse effect due to slow kinetics. The pH of milk was in the range of 6.63 to 6.59. PA has an optimum pH of 7.8 (Sakella, 1985). Thus, low pH values could decrease the conversion of plasminogen to plasmin.

The extent of proteolysis during cold storage can be quantified by the decrease in the individual casein content of milk. Individual casein contents were not significantly ($p < 0.05$) changed throughout the 4-day storage period for the low or the high SCC milk although there was a trend for lower values (Table 2). Casein values reported in this table are those of the casein pellet fraction of the milk as the supernatant was found to contain low casein levels (< 3% when

ble 1. Effect of cold storage (4°C) on plasmin[1] and plasminogen-derived[1,2] activities of raw milk

	Storage time (d)				
	0	1	2	3	4
	Activity (Unit/ml)				
w SCC[3]					
asmin	6.8 ± .54	6.8 + .54	7.0 ± .63	7.3 ± .65	7.3 ± .66
asminogen	41.0 ± .38	40.9 ± .38	40.7 ± .40	40.5 ± .40	40.5 ± .40
gh SCC					
asmin	8.4 ± .73	8.4 ± .73	8.8 ± .79	9.2 ± .84	9.4 ± .86
asminogen	44.9 ± .38	44.8 ± .38	44.5 ± .36	44.3 ± .36	44.0 ± .35

alues are expressed as means ± SD

lasminogen-derived is the proteolytic activity measured after activation by urokinase

CC = somatic cell counts

One unit of plasmin is defined the amount of enzyme that will
ause a Δ_{405nm} of 0.001 in 1 min when p-nitroanilide is measured
n our reaction mixture (see materials and methods).

compared to that present in the casein fraction). These data suggest that limited levels of proteolysis occurred during the 4-day cold storage period. Low levels of proteolysis in milk stored at 4°C for 96 h were reported by Kang and Frank (1988).

A new approach for proving a causative role for plasmin in casein proteolysis was originally suggested by Verdi and Barbano (1988) where the use of ϵ-ACA, a lysine analogue, at concentrations higher than 120 mM specifically inhibited plasmin. As plasmin is inactivated, any remaining proteolysis will be attributed to the somatic cell proteases. Casein profiles during the 4-day storage period after the addition of 120 mM ϵ-ACA are presented in Table 3. It should be noted that the casein values of the storage time (0 d) are the same with those of Table 2. The casein content reported in this table is the content in the casein pellet fraction of milk. Addition of ϵ-ACA led to a non significant ($p < 0.05$) change in the overall pattern of casein breakdown in the low or high SCC milk. Despite the fact that in both cases: low and high SCC milk, the addition of ϵ-ACA did not result in significant changes in the pattern of casein breakdown, there was a trend towards a suppression of proteolysis.

Tyrosine value was determined as an additional index of proteolysis (Juffs, 1973). In this case, proteins are removed by TCA precipitation. The protein content (mostly amino acids) determined in the supernatant serves as an index of proteolyis. Unfortunately, the results were very similar to those obtained from the breakdown of the caseins. Addition of

able 2. **Casein profiles[1] of raw milk during cold storage (4°C)**

Casein	Storage time (d) 0	1	2	3	4
			(mg/ml)		
ow SCC[2]					
- casein	16.32 ± 1.50	16.26 + 1.50	16.20 ± 1.48	16.12 ± 1.48	16.13 ± 1.47
- casein	11.80 ± 1.08	11.78 ± 1.08	11.68 ± 1.06	11.54 ± 1.04	11.54 ± 1.04
- casein	3.68 ± .35	3.69 ± .35	3.62 ± .35	3.56 ± .32	3.56 ± .32
tal - casein	31.80 ± 3.00	31.69 ± 2.98	31.50 ± 2.96	31.22 ± 2.90	31.23 ± 2.90
gh SCC					
- casein	16.26 ± 1.48	16.00 ± 1.47	15.70 ± 1.33	15.22 ± 1.33	14.96 ± 1.30
- casein	11.71 ± 1.08	11.42 ± 1.06	11.06 ± 1.03	10.82 ± 1.01	10.56 ± 1.00
- casein	3.50 ± .35	3.52 ± .35	3.38 ± 0.33	3.26 ± .31	3.18 ± .31
tal - casein	31.57 ± 3.00	30.94 ± 2.96	30.14 ± 2.92	29.30 ± 2.81	28.70 ± 2.74

alues are expressed as mean ± SD

CC = somatic cell count

ble 3. Impact of adding ε-ACA[1](120 mM) on casein profiles[2] during cold storage (4°C)

Casein	Storage time (d) 0	1	2	3	4
			(mg/ml)		
SCC[3]					
- casein	16.32 ± 1.50	16.26 + 1.50	16.26 ± 1.50	16.20 ± 1.49	16.20 ± 1.49
- casein	11.80 ± 1.08	11.78 ± 1.08	11.70 ± 1.08	11.66 ± 1.07	11.66 ± 1.07
casein	3.68 ± .35	3.65 ± .35	3.61 ± .35	3.56 ± .33	3.54 ± .33
al - casein	31.80 ± 3.00	31.75 ± 2.98	31.67 ± 2.98	31.50 ± 2.96	31.48 ± 2.96
h SCC					
- casein	16.26 ± 1.48	16.19 ± 1.48	16.03 ± 1.46	15.83 ± 1.44	15.71 ± 1.43
casein	11.71 ± 1.08	11.67 ± 1.08	11.50 ± 1.06	11.32 ± 1.07	11.22 ± 1.03
casein	3.60 ± .35	3.55 ± .35	3.40 ± .34	3.30 ± .33	3.20 ± .32
al - casein	31.57 ± 3.00	31.36 ± 2.94	30.89 ± 2.90	30.35 ± 2.78	29.90 ± 2.64

ACA: ε-amino-caproic acid

lues are expressed as means ± SD

C: somatic cell count

ϵ-ACA to inhibit plasmin led to no changes ($p < 0.05$) in the tyrosine value over the 4-day period (Table 4). Strickly speaking, these data should be interpreted as evidence that plasmin contribute to no proteolysis in milk. The alternative interpretation is that the sample size was inadequate. Thus, a much greater sample size may be required for this kind of experiment. In any case, the major obstacle will be the limited proteolysis occuring in the non-treated with ϵ-ACA milk.

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Table 4. Effect of cold storage (4°C) on milk proteolysis as measured by tyrosine value (TV)[1]

	Storage time (d)				
	0	1	2	3	4
	(mg/L)				
Low SCC[2]					
TV	43.53 ± 4.31	44.06 + 4.41	44.95 ± 4.46	45.21 ± 4.46	45.89 ± 4.51
TV - ε-ACA[3]	—	43.80 ± 4.42	44.14 ± 4.45	44.78 ± 4.45	45.00 ± 4.48
High SCC					
TV	44.02 ± 4.45	44.79 ± 4.45	45.51 ± 4.49	46.27 ± 4.53	47.19 ± 5.01
TV - ε-ACA	—	44.40 ± 4.44	45.00 ± 4.53	45.68 ± 4.59	46.00 ± 4.72

[1]Values are expressed as mean ± SD

[2]SCC = somatic cell count

[3]ε-ACA = ε-amino-caproic acid

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APPENDIX II

LACK OF SECRETION OF INTERLEUKIN 1 BY BOVINE MILK MACROPHAGES

Introduction

Interleukin 1 (IL-1) is a protein produced and secreted by activated mononuclear phagocytes and function to signal the onset of a disease, triggering defensive mechanisms such as lymphocyte activation vital for localization and the ultimate elimination of the disease (Dinarello, 1988). Increased production and secretion of IL-1 by activated macrophages could contribute to an enhancement of the immune responce (Dinarello, 1988). We have shown (Chapter 5) that bovine milk macrophages have a limited ability to secrete PA. It was then of interest to know if this impairment was a characteristic shared by IL-1.

The objective of the following study was to compare the IL-1 production and secretion by bovine milk macrophages isolated from normal and mastitic quarters.

Materials and Methods

Macrophages isolation and measurements of IL-1 were performed by M. Denis. Inst. of Parasitology as part of a collaborative effort. Number of cows used in this study, milk samples and isolation of macrophages are described in Chapter

5. A portion of these macrophages were used in the PA and the remainder in the IL-1 study. For IL-1 determination, cells collected from the interface of the Ficoll-Hypaque cushion (see Chapter 5) were plated overnight at 10^6 macrophages/well in 24-well plates (Nunc, Oxnard, CA). Following incubation, macrophages were washed 10 times with warm Hanks balanced salt solution (HBSS, Gibco). Macrophages were stimulated <u>in vitro</u> with 10 ng/ml lipopolysaccharide (LPS, from <u>E. Coli</u> 055:B5, Sigma) in 250 μl RPMI-1640 (Gibco) supplemented with 5% fetal calf serum. After 24 h incubation at 37°C in humidified air with 5% CO_2, the culture fluid was removed, cleared of particulate matter by filtration and was frozen. In order to recover the intracellular IL-1, 250 μl of distilled water were added to each individual well. After 30 min the lysates containing the intracellular IL-1, were removed and frozen. The assay system for measuring IL-1 is based on the unique property of IL-1 to stochiometrically induce the proliferation of thymic lymphocytes (Newton, 1985). Calf thymus from a single animal was obtained from a local abattoir and thymic lymphocytes were obtained through gentle physical dissociation. The same pool of thymocytes were used throughout this experiment. Thymocytes were washed twice and resuspended at 2 X 10^6 cell/ml in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum, phytohemogglutinin (PHA-P_1 0.1%, w/v) and 20% (v/v) of test supernatant. After 24 h incubation at 37°C, cultures were pulsed with [^{3}H]-thymidine (1 μCi/well, New England, Boston, MA) for another 24 h and then harvested

with a cell harvester. Assays were prepared in quadruplicate sets with the mean counts per minute (CPM) determined in each assay.

All data were expressed as means + standard deviation. Differences between means were evaluated using student's t-test ($p < 0.01$).

Results and Discussion

This study shows that bovine milk macrophages have a limited ability to secrete IL-1 in responce to LPS. IL-1 activity in 24 h supernatants was higher (2.4 x) in macrophages isolated from mastitic quarters when compared to that of macrophages isolated from normal milk. To discriminate between absence of production or secretion, we considered the possibility that IL-1 may be produced intracellularly but not secreted. Macrophages isolated from mastitic quarters had higher (2.1 x) IL-1 activity when compared to macrophages isolated from normal quarters. However in both cases milk macrophages were unable to secrete the IL-1 already produced. The lack of IL-1 secretion by bovine milk macrophages, also a characteristic of human breast macrophages (Subiza et al., 1988) suggests that they may be unable to promote lynphocyte activation. We suggest that lymphocyte activation may not be the primary function of macrophages in bovine milk. Pittard et al., (1977) have suggested that human breast macrophages play an important role as a vehicle for immunoglobulin

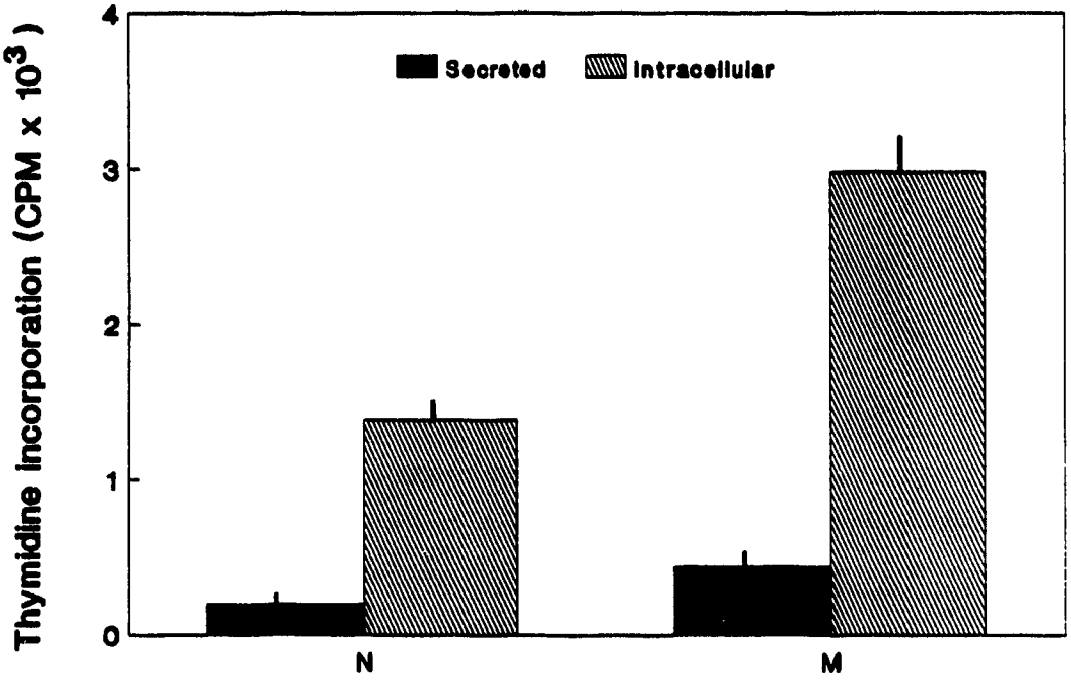

Figure 1 . Comparison between intracellular (▨) and secreted (■) interleukin-1 activity in macrophages isolated from normal (N) and mastitic (M) quarters after the addition of 10 ng/ml of lipopolysaccharide (LPS).

All values represent means ± SD.

transport. A role for bovine milk macrophages as a vehicle transport could explain the secretory defects in these cells with respect to IL-1 and PA.

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