AN ABSTRACT OF THE THESIS OF

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Title: Factors Affecting Zona Pellucida Solubility and Hatching in Bovine Embryos in Vitro.

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This thesis examines mechanisms involved in hatching by bovine embryos and the role and regulation of plasminogen activator (PA) during this process. Three studies were conducted to determine: 1) the effects of blastocoelic expansion and PA suppression on zona pellucida solubility; 2) the effects of intracellular modulators of phosphorylation on PA production, hatching and zona pellucida solubility; and, 3) identify the PA produced by Day 12-14 bovine embryos.

The first study consisted of two experiments. In Experiment 1, relationships between the extent of blastocoelic expansion and zona pellucida solubility were determined. Bovine embryos were collected on Day 6 and cultured in Ham's F-12 containing 1.5% bovine serum albumin (BSA). Overall embryo diameters (OD), zona pellucida

thicknesses (ZPT) and blastocoelic diameters (BD), if present, were measured using an ocular micrometer. Embryos of lesser quality, PRE-embryos, were immediately placed in .2% SDS and the time required for complete dissolution of the zona pellucida (ZPDT) determined. Embryos with good quality grades, POST-embryos, were cultured for 192 h. At 24-h intervals, medium was recovered for PA analysis and OD, ZPT and BD were measured. The change in embryonic surface area (SA) occurring during hatching was computed by deducting the SA of the embryo prior to blastocoelic expansion from the maximal SA achieved prior to hatching. Mean OD was less ($P<.05$) and mean ZPT and ZPDT were greater ($P<.05$) in PRE-embryos compared to POST-embryos. Zona pellucida dissolution time was negatively correlated ($P<.01$) with OD, BD and the change in SA. In Experiment 2, the effects of suppressing PA and blastocoelic expansion on hatching and zona pellucida solubility were examined. Embryos were cultured in Ham's F-12 containing 1.5% bovine serum albumin (BSA) and 0, 10 or 100 IU/ml PA inhibitor-2 (PAI-2). After 24 h of culture, embryos were transferred to medium with 0, .1 or .5 nM ouabain for 20 h, washed and returned to their respective cultures. At 24-h intervals, OD, BD and ZPT were measured and medium was recovered for PA analysis. At the end of culture ZPDT was recorded for all embryos. Percentages of embryos hatching were not different ($P>.05$) among 0, 10 or 100 IU/ml PAI-2. More ($P<.05$) embryos hatched after exposure to 0 and .1 nM ouabain

compared to .5 nM oaubain. Embryos cultured in 0 IU/ml PAI-2 had reduced (P<.05) OD and BD and greater (P<.05) ZPT compared to 10 and 100 IU/ml PAI-2. No difference (P>.05) was observed in ZPT of embryos exposed to ouabain. Addition of PAI-2 suppressed (P<.05) PA activity in the culture medium in a dose-dependent fashion, whereas exposure to ouabain had no effect (P>.05) on PA activity. Zona pellucida dissolution time did not differ (P>.05) following culture in medium with PAI-2 or ouabain. However, zona pellucida dissolution time was negatively correlated (r=-.79; P=.06) with PA activity for embryos cultured in 0 IU/ml PAI-2 and exposed to .5 nM ouabain. These data suggest that when blastocoelic expansion is limiting, the amount of PA produced is correlated with solubility of the zona pellucida. However, zona pellucida solubility changes appear to be induced primarily by the physical distortion caused by the expanding blastocoel.

The second study examined the effects of okadaic acid (OA), a phosphatase inhibitor and 6-dimethylaminopurine (DMAP), a kinase inhibitor, on hatching and PA production. Day 6 bovine embryos were randomly assigned to one of five treatments: 0 (Control), 2.5 nM OA, 25 nM OA, 1 mM DMAP and 2 mM DMAP in Ham's F-12 with .15% BSA. At 24-h intervals medium was recovered and embryos were transferred to fresh microdrops and OD, BD and ZPT were measured. At the end of culture, zona pellucida enclosed embryos and shed zonae pellucidae were recovered and placed in .2% SDS and the time

required for complete dissolution (ZPDT) recorded. None of the embryos cultured in 2 mM DMAP medium developed to the blastocyst stage and only 13% of the embryos in 1 mM DMAP initiated hatching and, of those, none hatched. Embryos cultured in 25 nM OA possessed similar ($P>.05$) OD, BD and ZPT but PA production was greater ($P<.05$) compared to the Control. Embryos in 2.5 nM OA had reduced ($P<.05$) OD, BD and PA compared to Control embryos. Compared to the Control, embryos in DMAP had reduced ($P<.05$) OD, BD and PA production. Zona pellucida dissolution time did not differ ($P>.05$) among the Control, OA and DMAP treatments. Inhibition of kinases significantly inhibited PA production as well as having a deleterious effect on overall embryo viability. Okadaic acid modulates PA production, suggesting that phosphatases are working through a different signal transduction pathway or downstream of a second messenger stimulated pathway.

The third study characterized the type of PA produced by Day 12 and 14 bovine embryos in vitro. Day 12 and 14 embryos were cultured for 120 and 96 h, respectively, in Ham's F-12 with .15% BSA. Medium was recovered at 24-h intervals and embryos were extracted following culture for PA analysis. Characterization of the type of PA produced by bovine embryos was performed using SDS-PAGE and zymography with amiloride, a competitive inhibitor of urokinase-type PA (uPA), and immunoprecipitation. Medium and embryo extracts were treated with phosphate buffered saline (PBS),

nonspecific goat immunoglobulin (NSIgG) or goat antibodies to human uPA (anti-uPA), tissue-type tPA (anti-tPA) and PA inhibitors 1 and 2 (anti-PAI-1 and anti-PAI-2, respectively) and immunoprecipitated with a suspension of Protein G-bearing Streptococcus cells. Day 12 bovine embryos produced two plasminogen-dependent lytic zones (53.6 and 89.7 kD) whereas Day 14 embryos produced three plasminogen-dependent lytic zones (53.4, 82.0 and 90.5 kD). Total lytic area in the supernatant was lower ($P<.05$) when medium and tissue extracts were treated with anti-uPA compared to anti-PAI-1, anti-tPA and NSIgG. Treatment with anti-PAI-2 reduced ($P<.05$) total lytic area in the supernatant compared to anti-tPA and NSIgG and no differences ($P>.05$) in areas were detected among treatments with anti-PAI-1, anti-tPA and NSIgG. Total lytic area in the precipitates was greatest when medium and extracts were treated with anti-uPA. When amiloride was added to the zymographs, PA activity in the medium was completely inhibited. These results suggest Day 12-14 embryos possess an uPA and possibly a PAI which binds to the uPA and forms a high molecular mass complex.

FACTORS AFFECTING ZONA PELLUCIDA SOLUBILITY AND HATCHING IN BOVINE EMBRYOS IN VITRO

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Introduction

During early development, the mammalian embryo sheds the zona pellucida in a process known as hatching. For most species, hatching or loss of the zona pellucida is accomplished by Day 11 and is a prerequisite for the establishment of fetal maternal contact. The exact mechanism used by the embryo to hatch is not known. However, it has long been suggested that sublysis of the zona pellucida by an unknown protease in combination with the hydrostatic pressure from the expanding blastocoel facilitates hatching (Domon et al., 1973). Proteinases in a variety of species have been implicated as likely participants in the hatching process (Kirchner, 1972; Barrett et al., 1975; Perona and Wassarman, 1986; Menino and Williams, 1987; Menino et al., 1989; Sawada et al., 1990) but in the mammal the exact identity of an actual hatching enzyme remains elusive. Previous work in our laboratory has shown that bovine embryos produce the serine protease, plasminogen activator (PA). Plasminogen activator converts the serum zymogen, plasminogen, into the active serine protease, plasmin (Dano et al., 1985). The ability of an

embryo to convert plasminogen into the active enzyme plasmin, provides an important protease source that can be utilized for developmental events requiring membrane or tissue breakdown. Plasminogen activator production increases during blastocoelic expansion and hatching and decreases after hatching on Day 11 (Menino and Williams, 1987). Bovine embryos that hatch release more PA than embryos that fail to hatch and PA production is positively correlated to embryonic size, developmental stage and cell number (Kaaehuakiwi and Menino, 1991). Collectively, these observations suggest that in the bovine embryo, PA may be the proteinase involved in weakening the zona pellucida and allowing the embryo to hatch.

This thesis focuses primarily on investigating the mechanisms used by the bovine embryo to complete hatching. Studies were also undertaken to evaluate the effects of intracellular phosphorylation on PA production, development and hatching and further characterizing the type of PA produced by bovine embryos. The objective of the first study was to evaluate the effects of suppressing PA activity and blastocoelic expansion on zona pellucida solubility and hatching. It seemed reasonable that hydrostatic pressure from the expanding blastocoel could physically distort the zona pellucida and cause hatching. Ouabain, a NA^+/K^+ ATPase inhibitor was selected to block ion transport in the embryo and delay blastocoel expansion. Because the bovine

embryonic PA is of the urokinase-type (Dyk and Menino, 1991; Berg and Menino, 1992) PA inhibitor-2 (PAI-2) was selected to suppress PA activity. The objective of the second study was to examine the effects of okadaic acid (OA), a phosphatase inhibitor, and 6-dimethylaminopurine (6-DMAP), a kinase inhibitor, on hatching and zona pellucida solubility. This study was conducted to determine whether various developmental events in the early embryo, such as PA production and hatching, are regulated by kinase and phosphatase activities. The objective of the third study was to determine if the high molecular mass PA produced by bovine embryos is a PA-PAI complex.

Investigation of the hatching mechanism and the role and regulation of PA during hatching is important to further understanding of the processes occuring in early embryogenesis. Studies have shown that 30% of the embryonic death in livestock species occurs during the period of gestation that includes hatching. Understanding the hatching process and the significance of PA in development may provide valuable insights that can eventually be used to alleviate this high rate of embryonic death and improve reproductive efficiency.

The following literature review will focus on embryo development in the bovine, the hatching process and the biochemistry of PA and its involvement in reproduction and embryo development.

Review of Literature

Early Embryo Development in Cattle

Defining the day of detected estrus as Day 0, fertilization is completed on Day 1 at the ampullary-isthmic junction of the oviduct. Fertilization can be described as the process during which the male gamete, or spermatozoa, penetrates the oolemma of the oocyte causing spontaneous maturation and completion of meiosis as well as the release of the second polar body into the perivitelline space. Any corona radiata cells remaining after ovulation and fertilization are rapidly shed shortly thereafter leaving the embryo enclosed in the zona pellucida. The pronuclei remain distinct until their membranes break down and the male and female chromosomes meet for the first time during the first division of the zygote (Betteridge and Flechon, 1988). The zygote continues to undergo a series of mitotic divisions and morphological changes which eventually lead to the establishment of pregnancy.

The first cleavage of the zygote into two blastomeres, or cells, occurs within 24-28 hours after ovulation, or approximately one day post-fertilization. The embryo continues to divide although synchronization is eventually lost and blastomeres start to divide independently of each other. In addition to being asynchronous, early divisions

of the blastomeres are unequal, which is significant because it has been shown in mice (> 8-cell embryos) and cattle (16-32 cell embryos) that the larger cells become the outside cells and differentiate into trophectoderm while the smaller cells become inside cells and form the inner cell mass (Betteridge and Flechon, 1988). At this point (16-32 cell stage) the embryo enters the uterus where it will stay until parturition.

As blastomeres continue to divide in cattle embryos, it becomes progressively more difficult to count individual cells and by Day 6, when the cells compact into a morula, it is impossible to define individual blastomeres. The cells of the morula are wedge-shaped and become polarized with the nuclei localized to the basal portion. The morula possesses inner cell populations completely surrounded by other cells and an outer cell layer with free apical surfaces. The outer cells will eventually form trophectoderm and the inner cells will form the inner cell mass (ICM). By this time the embryo, still enclosed in the zona pellucida, has passed from the oviduct into the uterus.

The zona pellucida not only has the obvious role of keeping the blastomeres together, but may also function in playing a role in conserving the microenvironment of the perivitelline space (Betteridge and Flechon, 1988). The sperm recognition site is also contained in the zona pellucida and this site is critical for it is where the

sperm recognizes the egg and fertilizes it. The zona pellucida also plays an important role during fertilization when the zona reaction occurs. The zona reaction, caused by the fusion of the sperm to the oolemma, makes the zona pellucida impenetrable to other sperm thus preventing polyspermy, a lethal condition for the embryo.

While still enclosed within the zona pellucida, blastomeres of the compact morula develop tight intracellular junctions and accumulate fluid into a central cavity called the blastocoel. Onset of blastocoel formation is induced by activation of the Na^+/K^+ ATPase in the trophectodermal cells and usually occurs by Day 7 in cattle, marking the transformation from compact morula to early blastocyst.

Within a few days, the blastocyst becomes a thin-walled fluid-filled sac. At this time, the blastomeres have differentiated into two morphologically distinct populations, the ICM and trophectoderm. The endoderm cells spread out from beneath the ICM on Day 8 and by Day 10 completely line the trophectoderm to form the trophoblast. The action of the Na^+/K^+ ATPase increases the hydrostatic pressure in the blastocoel. By Day 10, the increased hydrostatic pressure, combined with the action of a protease, called the zona lysin, allows the blastocyst to erupt out of the zona pellucida in an event called hatching.

The hatched blastocyst continues to expand spherically as blastocoelic fluid accumulates. The ICM ruptures to the outside of the sphere by Day 12 and is clearly discernible as the embryonic disc. The shape of the blastocyst then changes from spherical to ovoid just prior to the obvious elongation stage which begins on Day 12-14. On Days 14-16, mesodermal cells, which differentiate from the embryonic disc, migrate between the trophectoderm and endoderm to form the trilaminar yolk sac. The mesoderm will eventually split into two layers. The outer layer of mesoderm and trophectoderm will collectively form the somatopleure which gives rise to the chorion and amnion. The inner mesodermal layer with the endoderm will give rise to the splanchnopleure which forms the yolk sac membrane and allantois. The space separating the somatopleure and splanchnopleure forms the extraembryonic coelum. The bovine blastocyst has undergone a morphological change at this point from a 3 mm sphere on Day 13 to a 25 cm filamentous form by Day 17. Due to continued hyperplasia of the trophectoderm and endoderm, by Day 18 the filamentous embryo has extended into the contralateral uterine horn.

Days 16-17 have been shown to be critical for maternal recognition of pregnancy in the cow (Betteridge and Flechon, 1988), presumably due to the release of an embryonic signal that prevents regression of the corpus luteum and allows pregnancy maintenance. Bovine trophoblast protein-1 (BTP-1)

is a protein secreted by the trophoblast between Days 16-24 and has been shown to suppress expression of $PGF_2\alpha$ from the uterus. It is probable that BTP-1 is the signal needed for maintenance of the corpus luteum.

Attachment begins at Day 28-32 in the cow and is completed by Day 40-45 (McLaren, 1980). Unlike rodents and primates, who experience invasive implantation where the blastocyst actually penetrates the endometrium, implantation in ruminants occurs through interdigitation of trophoblast and endometrial microvilli.

Hatching

Mammalian embryos are surrounded by a glycoprotein matrix, the zona pellucida, which is lost before implantation. The actual mechanism involved in this loss is a matter of much controversy. Loss of the zona has been attributed to an unknown protease, termed the "zona lysin" and/or hydrostatic pressure from the expanding blastocoel (Domon et al., 1973). McLaren (1970) proposed that both factors contribute to the loss of the zona pellucida in mice: a lytic factor, emanating from the estrogen-sensitized uterus, and the expansion of the blastocoel which causes a buildup in pressure until the embryo bursts from the zona pellucida. Bergstrom (1972) evaluated whether the volume of the mouse zona pellucida decreases as time of loss

approaches and found no difference. Bergstrom (1972) also fixed pregnant uteri in situ and examined blastocysts in various stages of expulsion from the zona pellucida. Zonae pellucidae showed minor cracks which seemed to provide additional indirect evidence in favor of mechanical hatching as the primary method whereby the zona pellucida is shed from the mouse blastocyst.

Mintz (1972) identified a uterine product which seemed to play an important role in triggering adhesion of the blastocyst to the uterine wall as well as possibly being the zonalytic agent involved in hatching. This factor was named the implantation-initiating factor (IIF) and was shown to be hormonally regulated. Pinsker et al. (1974) assayed for IIF levels in mouse uterine fluid and found that it was absent in non-pregnant mice. However, during pregnancy, levels were low on Day 1 but by Day 3 proteinase activity had increased 100-fold before declining on Day 4. Peak activity of IIF coincides with initiation of implantation.

Kirchner (1972) examined protease activity in rabbit uterine secretions and in blastocysts between 5 days 18 hours and 7 days 18 hours after mating. During this period the rabbit blastocyst is covered by a zona pellucida, mucoprotein layer, and attached uterine secretions. These blastocyst coverings start to disintegrate at Day 7 post-coitum. Kirchner (1972) reported that the blastocyst coverings contain an inherent protease and that this

protease is not identical with any of the proteases found in the uterine secretions. All proteolytic activity ceased when blastocyst coverings were completely dissolved suggesting a temporal association between proteolytic activity and degradation of the coverings.

Surani (1975) examined the effect of endogenous estradiol on zona pellucida denudation in ovariectomized rats. Control rats experienced an estrogen surge on Day 4 and because no empty zonae pellucidae could be found in the uterus, zona lysis was concluded to be complete by Day 5. In the ovariectomized animals, zona pellucida lysis was completed 30 h following estradiol treatment suggesting that the zonalytic process is estradiol-dependent. This study did not consider the actual mechanism of hatching but it does seem to point to the existence of a zonalytic enzyme which is responsible for complete zona pellucida degradation after hatching in utero and which may possibly weaken the zona prior to hatching. Rosenfeld and Joshi (1981) examined the effect of a rat uterine endopeptidase on in vitro lysis of the zonae pellucidae of unfertilized and fertilized rat, mouse and hamster ova. The endopeptidase lysed the zona of unfertilized rat and mouse ova but not of fertilized ova indicating that the zona pellucida modifications that occurred during fertilization is inducing resistance to proteolytic digestion. Lysis of the hamster zona pellucida occurred within 90 min regardless of the treatment

indicating some species specificity in the mode of action of the endopeptidase. Results obtained, however, do not preclude the possibility that the endopeptidase is involved in zona lysis by acting in conjunction with other uterine and/or blastocyst proteins being secreted at the time of implantation.

Wassarman et al. (1984) implicated the involvement of "strypsin", a trypsin-like protease, in the hatching process of mouse embryos. Wassarman et al. (1984) inhibited hatching by including various serine protease inhibitors in the culture medium. Using histochemistry, Wassarman et al. (1984) determined that strypsin activity first appeared at the 8-16 cell stage of development and was always associated with the trophectoderm. Perona and Wassarman (1986) further considered the role of strypsin as the zonalytic factor in mouse embryos. Strypsin was confined to the mural trophectoderm and detected only over a small region of the hatching blastocyst. Interestingly, inactive strypsin was detectable after hatching only at the opening of empty zonae pellucidae. These observations suggest the possible lytic generation of a single hole through which the embryo emerges when expansion of the blastocoel forces it out of the zona pellucida. Perona and Wassarman (1986) were unable to totally rule out the possibility that strypsin is actually a plasminogen activator (PA). However, there is contradictory evidence to the observations of Perona and Wassarman (1986).

Sawada et al. (1987) reported homogenous staining of the trophectoderm for trypsin-like proteases, indicating that the proteases are distributed uniformly. Yamazaki et al. (1989) observed the exact site of hatching of mouse embryos and found that there was no particular site destined for rupture and consequent shedding of the zona pellucida. They hypothesized that contraction and re-expansion of the blastocyst, in addition to rotary movement within the zona pellucida, may facilitate diffusion of the enzyme to an arbitrary site where hatching occurs.

Menino and O'Claray (1986) examined the effects of plasmin and plasminogen on in vitro mouse embryo development. They observed a greater incidence of hatching in mouse embryos cultured in plasmin- and plasminogen-supplemented medium compared to medium containing only bovine serum albumin. This observation suggests a possible role for the plasminogen system in early embryo development. Menino and Williams (1987) attempted to evaluate this mouse model in the bovine by culturing embryos in medium containing various concentrations of plasminogen and evaluating subsequent development. Peak PA production by bovine embryos was observed during blastocoelic expansion and initiation and completion of hatching. Little effect of level of plasminogen was detected on the percentage of embryos developing to a certain cell stage, but the timing at which a particular cell stage was reached was affected.

Plasminogen levels of 120 µg/ml accelerated initiation and completion of hatching, presumably because it was being activated to plasmin which was having a sublytic effect on the zona pellucida. Menino et al. (1989) evaluated the effects of PA on ovine embryo development and zona pellucida integrity. Plasminogen activator was found to be low until the morula stage, increased during the morula-blastocyst transition and remained elevated through blastocoelic expansion and hatching. Embryos in 60-120 µg/ml plasminogen had a higher hatching rate and their zonae pellucidae showed reduced resistance to solubilization. Menino et al. (1989) concluded that PA and plasmin seemed to have some effect on hatching but that other factors, such as blastocoelic expansion, play an important role. Kaaekuahiwi and Menino (1990) reported bovine embryonic PA production was greater by embryos that hatched in vitro and was positively correlated to cell number, cell stage and overall diameter. Although this does not provide evidence for the physiologic role of PA; it suggests that PA secretion may be a good parameter for embryo viability.

The escape of the blastocyst from the zona pellucida in vitro is at least partially due to the accumulation of fluid in the blastocoel and the resulting increase in pressure. Fluid accumulation is a result of active ion transport across the trophectoderm and is specifically mediated by Na^+/K^+ ATPase (Biggers et al., 1978). Since prostaglandins

of the E series are involved in the control of water movement across epithelia, the effect of prostaglandin antagonists on the hatching of mouse blastocysts has been examined by several investigators. Biggers et al. (1978) observed that prostaglandin antagonists inhibit hatching which provides circumstantial evidence that endogenous prostaglandins are involved in the swelling of the blastocyst prior to hatching. Baskar et al. (1981) also reported that hatching by mouse blastocysts in vitro was inhibited by other prostaglandin antagonists. The results obtained by Biggers et al. (1978) and Baskar et al. (1981) suggest that PGE_2 and $PGF_2\alpha$ may be involved in normal function of the mammalian blastocyst.

Researchers have had much success in isolating a hatching enzyme from the sea urchin. The sea urchin egg has a tough protective extracellular coat called the fertilization envelope from which it eventually hatches. Ishida (1936) was the first to propose that sea urchin hatching was controlled by a specific protease, termed the "hatching enzyme", which was synthesized and secreted by the embryo. Roe et al. (1990) showed that the enzyme is indeed synthesized de novo by the blastula just hours before hatching. Various laboratories have purified the hatching enzyme, cloned its cDNA and analyzed temporal expression of the gene. Lepage et al. (1992) investigated whether or not the activation of the hatching gene is related to the

foundation cell lineages by analyzing the distribution of the hatching enzyme gene products in developing embryos. The enzyme was detectable only between the 128-cell stage and hatching and is thus specific to the prehatching blastula. Lepage et al. (1992) also determined that the enzyme and its mRNA are present only in particular areas of the prehatching blastula embryo. Lepage et al. (1992) hypothesized that the control of this gene might be linked to the process of cell specification.

Clearly, the hatching mechanism of a free floating embryo is a little easier to study than that of an embryo inside a reproductive tract. Mammalian embryos have many uterine factors acting on the embryo as well as embryonic factors. Understanding the puzzle of how the hatching mechanism works is critical to understanding early embryonic development. Without hatching or some form of loss of the zona pellucida, implantation and growth of the embryo are limited; it is a critical stage of development.

Plasminogen Activator

History

Plasminogen activators are serine proteases that convert the inactive zymogen plasminogen to the active enzyme plasmin. Historically, interest in plasmin and PA originated with early researchers who were investigating the role of these enzymes in fibrinolysis. Research in this area began as early as the 18th century when it was first noted that the blood of animals and humans that had died violently remained fluid (Christman et al., 1977). By the early 20th century, it was clear this phenomenon was due to the proteolytic conversion of fibrinogen or fibrin to a form which was no longer able to clot blood. However, the enzyme capable of this conversion was not immediately identified. Christenson (1945) demonstrated the existence of an inactive proenzyme (profibrinolysin or plasminogen) which could be activated by a streptococcal factor to the active protease fibrinolysin, or plasmin, and was responsible for the degradation of fibrin. Christenson (1945) also demonstrated a method to control plasmin activity under physiologic conditions by removing plasma inhibitors of plasmin by either chloroform treatment or salt precipitation. It soon became obvious that there was an important alternate mechanism for regulating fibrinolysis or one that was

mediated through the activation of plasminogen to plasmin, and the search for PA was begun. Substances capable of activating plasminogen were found in almost all tissues and tissue extracts, vascular endothelium, urine and blood (Christman et al., 1977; Dano et al., 1985). The widespread distribution of PA led to the hypothesis that the breakdown of fibrin was crucial to many aspects of cellular maintenance, including: vascularization during wound healing, inflammation and tumor infiltration.

Investigators have postulated that fibrinolysis is related to tumor growth since the early 1900's; therefore much of the past and present research on PA has concentrated in this area. Investigators in the late 1950's and early 1960's noted that tumor tissues produced PA as well as other proteases (Christman et al., 1977). Unkeless et al. (1973) and Ossowski et al. (1973) demonstrated for the first time that primary cultures of chicken fibroblasts, cells which were not producing detectable levels of PA, could be induced to produce PA by transformation with an oncogenic virus. They also showed viral infection alone was not sufficient to induce PA production, but that morphological transformation was required. The release of PA from these transformed cells caused a significant increase in extracellular proteolytic activity (Ossowski et al., 1973 a,b; Unkeless et al., 1973, 1974). Since these reports, new purification techniques for PA have been developed and results obtained

reaffirm that PA plays a major role in tissue degradation in the normal organism as well as in cancer (Dano et al., 1985).

Classification

Two forms of PA exist and can be distinguished on the basis of molecular weights, fibrin affinity and immunological reactivity. It has been demonstrated by determining the amino acid nucleotide sequences that the two forms are completely different gene products (Dano et al., 1985). Plasminogen activators are classified as either urokinase-type (uPA) or tissue-type (tPA) and have MW of 50,000 and 70,000, respectively. The PA were named uPA and tPA because they were originally identified in urine and tissue extracts, respectively. In addition to these molecules, other forms of PA have been reported. However, in most cases these enzymes have not been fully characterized and it is unclear whether they are a complex or in fact, a distinct PA.

Biochemistry

An important step in classifying PA was accomplished by Deutsch and Mertz (1970) who developed an effective purification procedure utilizing lysine-Sepharose affinity chromatography and who determined the entire amino acid sequence for plasminogen. Undegraded human plasminogen, the first to be isolated by this method and studied in detail, was found to have a MW of 90,000 and is composed of a single polypeptide chain with a glutamine residue at the amino terminus and an asparagine residue at the carboxyl terminus. Plasmin is generated by two proteolytic cleavages of the zymogen (Robbins, 1973) resulting in an active enzyme composed of two peptide chains linked by a disulfide bond. The light chain is derived from the carboxyl terminus, has a MW of 25,000, contains the active site and has amino acid sequence homologies to other serine proteases such as trypsin and chymotrypsin. The heavy chain is derived from the amino terminus, has a MW of 60,000 and has no proteolytic capacity.

Homologous proteins from closely related species have been found to be similar in their primary structure. Plasminogens from a variety of mammalian species were compared and the amino acid residues at the amino terminus of both the heavy and light chains of all the plasmins studied were found to be homologous (Christman et al.,

1977). This suggests that similar proteolytic cleavages are involved in the activation of all plasminogens.

Two major forms of plasminogen have been used for investigating activation by PA: Glu-plasminogen and Lys-plasminogen (the Lys76-Lys77 bond of Glu-plasminogen is cleaved by plasmin and an activation peptide is liberated). Plasminogen activators can convert Glu-plasminogen to Glu-plasmin and Lys-plasminogen to Lys-plasmin by cleavage of the Arg560-Val561 bond resulting in the active two chain plasmin polypeptide.

Urokinase is the best understood of the plasminogen activators because of the relative ease of obtaining pure samples of it from human urine. Kinetic studies of how urokinase activates plasminogen to plasmin have been performed and the most likely sequence for activation is as follows:

Pathway 1:

(1) 92,000kD --(plasmin)--> 86,000kD + 7,000kD
Glu-plasminogen Lys-plasminogen activation peptide

(2) 86,000kD ----urokinase--------> (H)63,000-(L)25,000kD
Lys-plasminogen Lys-plasmin

There has been some controversy, however, on whether this pathway can be catalyzed by both urokinase and plasmin or only by plasmin and as to whether Reaction (1) always occurs before enzymatically active plasmin is formed. Therefore an alternate sequence was suggested:

Pathway 2:

(3) 92,000kD ----urokinase--------→ (H)70,000-(L)25,000kD
Glu-plasminogen Glu-plasmin

(4) (H)70,000-(L)25,000kD -(plasmin)→ (H)63,000-(L)25,000kD
Glu-plasmin Lys-plasmin
+ 7,000kD
activation peptide

Conversion of Lys-plasminogen to Lys-plasmin occurs much more rapidly than Glu-plasminogen to Lys-plasmin (Christman et al., 1977) prompting the hypothesis that removal of the 'activation peptide' regulates the rate of conversion of plasminogen to plasmin. There is also evidence that limited proteolysis of plasminogen by urokinase never results in detectable amounts of Glu-plasmin. Hence only a few molecules of Glu-plasmin may be formed that could catalyze the removal of the activation peptide from Glu-plasminogen and Glu-plasmin, making Glu-plasmin an extremely transient form. When this evidence is considered, it would seem that

once plasmin is formed, Reaction (1) would dominate and Pathway 1 would appear to be the major pathway of plasminogen activation.

However, Holvoet et al. (1985) characterized a murine monoclonal antibody (LPm1) that reacted specifically with Lys-plasminogen and Lys-plasmin-α2-antiplasmin complex, but not with Glu-plasminogen or Glu-plasmin-α2-antiplasmin complex. Holvoet (1985) demonstrated that activation of plasminogen in vivo yields Glu-plasmin-α2-antiplasmin complex, suggesting that physiological plasminogen activation does not occur via the Lys-plasminogen intermediate as previously proposed. Holvoet and his coworkers (1985) therefore support Pathway 2 as the most likely sequence of events for activation of plasminogen.

Urokinase-type Plasminogen Activator

Urokinase-type PA was originally discovered in high concentrations in human urine. It has since been identified in human seminal plasma, hyperplastic and malignant prostate tissue and plasma. Urokinase-type PA is a glycoprotein that is synthesized as a single chain zymogen (MW=55,000). Conversion to active uPA involves a proteolytic cleavage yielding a disulfide-linked two-chain molecule that expresses a high level of enzymatic activity. In 1982

Gunzler et al. reported the complete amino acid sequence of uPA.

The A chain, or light chain (MW=25,000), contains a 'kringle' structure in the C-terminal end (a kringle is a triple loop structure composed of approximately 80 amino acids with 3 disulfide bonds). The N-terminal end of the A-chain contains a sequence rich in cysteine and is homologous to murine epidermal growth factor (EGF). The A-chain is also the binding region for the cell surface uPA receptor.

The B-chain (MW=30,000) possesses the structurally conserved regions characteristic of other serine proteases, except that a four amino acid insertion is found in one of the conserved regions. The amino acid residues His, Asp and Ser constitute the catalytic site in uPA and are in homologous positions with other serine proteases.

Tissue-type Plasminogen Activator

Tissue-type PA is also a glycoprotein and has been purified from various sources, including tissue extracts from porcine heart, kidney and ovary and human uterus and plasma. Tissue-type PA appears to be the primary mediator of vascular fibrinolysis (Collen et al., 1980, 1985). Catalytically, these enzymes differ in one important aspect: the activity of tPA is enhanced by the presence of fibrin,

whereas that of uPA is not. This difference has been used as a basis to suggest that the physiological function of tPA is primarily fibrinolysis, whereas uPA may be involved in cell migration and tissue remodeling (Strickland et al., 1976; Sherman, 1980). Tissue-type PA is also synthesized as a single chain zymogen (MW=70,000) and must be proteolytically cleaved to yield an enzymatically active, two-chain molecule linked by a single disulfide bond. The light chain, or B chain, (MW=30,000) contains the catalytic site, while the heavy chain, or A chain, (MW=40,000) contains several domains relating to other activities of the molecule. The A chain consists of a fibronectin 'finger' domain, an EGF domain and two 'kringle' domains (Kringle I and II). The two kringles are located at the C-terminal end whereas the N-terminal contains a region rich in cysteine. The N-terminal 43 amino acids of tPA show a high degree of homology with the finger domains believed to be responsible for the fibrin affinity of fibronectin. The fibronectin finger and Kringle II domains are responsible for the interaction of tPA with fibrin. The B-chain contains the active site and is homologous with other serine proteases. Conversion of single chain tPA to the two chain form is catalyzed by plasmin.

Other Plasminogen Activators

Streptokinase is a bacterial protein which is capable of activating plasminogen. Its mode of action is much different than that of either uPA or tPA. Streptokinase has no enzymatic activity of its own; it is activated only after it complexes with plasminogen to form a streptokinase-plasminogen complex which is then capable of converting plasminogen to plasmin (Christman et al., 1977). It has been shown that streptokinase causes conformational changes in plasminogen and generates an active site without actually cleaving a peptide bond, converting, in effect, plasminogen into a PA.

Plasminogen Activator Inhibitors

Regulation of extracellular proteolysis is critical for the maintenance of homeostasis in every organism. In recent years a number of researchers have investigated how PA are regulated. Sites for PA regulation include: hormonal control of their biosynthesis and release, proteolytic conversion of single chain PA into two chain enzymes, interaction with cell surface receptors or with proteins that function as cofactors in the activation of plasminogen,

clearance of PA by the liver, or PA inhibitors (PAI) that may also be subject to hormonal regulation.
Four proteins have been identified as PAI and include PAI-1, PAI-2, protease nexin (PN) and PAI-3.

Plasminogen activator inhibitor-1 (MW=52,000) is the principal inhibitor found in plasma. It is derived primarily from endothelial cells and platelets and is believed to be involved in regulation of plasma tPA activity. A cDNA of PAI-1 has been isolated and its sequence demonstrates that it is a member of the serine protease inhibitor family (SERPINS) with arginine at its' active site (Kruithof, 1988). Plasminogen activator inhibitor-1 is a potent inhibitor of tPA and although its affinity for uPA is comparatively lower, it is still an effective inhibitor of uPA (Andreasen et al., 1990).

Plasminogen activator inhibitor-2 (MW=60,000) has been found in several tissues, including placenta, monocytes, U937 cells and polymorphonuclear leukocytes. Plasminogen activator inhibitor-2 is antigenically distinct from PAI-1 and is believed to play a role in the regulation of extracellular PA activity involved in tissue remodeling (Hart et al., 1988). Plasminogen activator inhibitor-2 cDNA has been isolated and it is also a member of the SERPIN family with arginine at it's active site (Kruithof, 1988). Plasminogen activator inhibitor-2 binds uPA with greater

affinity than tPA but is an effective inhibitor of both types of PA (Andreasen et al., 1990).

Plasminogen activator inhibitor-3 (MW=51,000) is present in human urine and plasma and has been shown to be antigenically distinct from PAI-1 and -2 (Kruithof, 1988). Little is known regarding the physiological role of PAI-3.

Protease nexin was first identified in the conditioned medium of fibroblasts and since its initial detection has been found in other cells. Protease nexin is a glycoprotein with a broad spectrum of inhibitory action rather than being PA specific. This has led to the suggestion that the function of PN is probably not only inhibition of PA but rather general control of local serine protease activity (Kruithof, 1988).

Male Reproduction

Lacroix et al. (1979) reported Sertoli cells in culture produce and secrete PA and that follicle stimulating hormone (FSH) and dibutrylcyclic adenosine monophosphate (dbcAMP) stimulates this process. It has been hypothesized that PA, synthesized and secreted by Sertoli cells, is involved in restructuring of the seminiferous tubule during the changes occuring in the cycle of the seminiferous epithelium. In 1981, Lacroix et al. demonstrated that PA activity in rat testis tubules at Stages VII and VIII (the cycle of the

seminiferous epithelium has 14 stages in the rat) is much greater than that released by tubule segments at any other stage of the cycle. Plasminogen activator was assayed using [^{125}-I]-labeled fibrin plates. Stages VII and VIII of the cycle are the regions in which spermiation occurs and in which movement of Sertoli cell cytoplasmic processes around leptotene spermatocytes also takes place. In 1985, Marzowski et al. purified Sertoli cell plasma membranes and characterized the glycoprotein components by two-dimensional SDS-PAGE. Her data suggest that two forms of PA are synthesized by cultured Sertoli cells: a secreted PA and a membrane-associated enzyme; each of which may have a different biological function. The three species of secreted PA which were detected included a higher MW form of 38,000-40,000 and two lower MW forms of 20,000 and 24,000. The PA associated with the partially purified membranes exhibited primarily the higher MW component (38,000-40,000). It is possible that the membrane associated PA may function to restructure discrete membrane components at the cell-cell interface and that the secreted form may function at more remote sites in the seminiferous epithelium (Marzowski et al., 1985). Many researchers have considered the possible roles that PA plays in initiating extracellular proteolysis. In 1986, Hettle et al. examined the type of PA synthesized and secreted by cultured rat Sertoli cells and by organ cultures of seminiferous tubules at defined stages of the

cycle of the seminiferous epithelium. Hettle et al. (1986) observed that Sertoli cells maintained under basal conditions secreted uPA. In contrast, Sertoli cells cultured in the presence of FSH or dbcAMP produced predominantly tPA. Results obtained with short-term organ cultures of tubule segments of defined stages of the cycle of the seminiferous epithelium indicate that cells at all stages within the seminiferous tubule secrete uPA under basal conditions. Tubule segments stimulated by FSH, however, respond with increased secretion of tPA. These findings by Hettle et al. (1986), show that levels and types of PA are modulated both by hormonal and by cell-mediated influences. The physiological significance of FSH stimulation of tPA remains to be assessed, and the functions of each type of PA in spermatogenesis needs to be elucidated. In 1988, Vihko et al. used immunohistochemistry to localize uPA and tPA in rat seminiferous tubule segments at various stages of the cycle. Urokinase-type PA immunoreactivity was detected only in Sertoli cells at stages VII - VIII of the cycle. No uPA immunoreactivity was detected in any spermatogenic cell at any stage of the cycle. Tissue type PA immunoreactivity was found in stages VII-XIII of the cycle of the seminiferous epithelium. Vihko et al. (1988) findings support the hypothesis that PA secreted from Sertoli cells at Stages VII and VIII promote the release of preleptotene spermatocytes from the basal

lamina. Vihko et al. (1988) findings also support the alternative hypothesis that PA facilitates movement of cytoplasmic extensions from Sertoli cells which leads to the formation of tight junctional complexes in the basal region. There continues to be many unanswered and interesting questions to be determined regarding the role of PA in the seminiferous tubules.

Female Reproduction

Beers (1975) identified the presence of plasminogen, PA and PAI in bovine follicular fluid. He used lysine-Sepharose chromatography to fractionate the follicular fluid and purify follicular proteolytic activity. These fractions were then analyzed for the presence of PA and PAI using a fibrin-agar overlay assay. Beers (1975) found the levels of PAI's present in follicular fluid are approximately the same as those found in serum. The concentration of plasminogen found was also approximately the same as that found in serum. This level represents a large source of protease, in the form of plasmin, that could be used for physiological purposes. Plasminogen activator was found in both the follicular fluid and wall which provides a potential mechanism for modulating the level of proteolytic activity within the follicle. Beers (1975) also showed that plasmin

can decrease follicle wall tensile strength suggesting that the PA-plasmin system may be involved in degrading the follicle wall during the ovulatory process. Beers et al. (1975) evaluated this possible relationship by examining levels of PA released by granulosa cells and correlating the changing levels of PA to the stages of the estrous cycle. Granulosa cells were collected from rats in various stages of the estrous cycle and PA content was measured. Plasminogen activator levels increased following LH administration and reached peak levels prior to ovulation. Beers et al.(1975) also collected granulosa cells from preovulatory follicles and nonpreovulatory follicles 8 hr after LH injection and tested each for PA. Only cells from preovulatory follicles showed significant activity, again suggesting that the appearance of PA is functionally correlated with ovulation. Beers et al. (1975) proposed the following explanation for the influence of PA on ovulation: in response to increasing levels of circulating LH, granulosa cells secrete increasing amounts of PA. This enzyme acts on the available plasminogen in follicular fluid generating plasmin in the follicle. Plasmin, acting on the basement membrane, proenzymes, and/or other substrates, catalyzes the disruption of follicular integrity necessary for the release of the ovum. In 1983, Shimada et al. showed that indomethacin blocked ovulation but did not prevent the normal increase in PA activity which seemed to indicate that

follicular rupture may not be induced primarily by PA. Shimada et al. (1983) suggested PA may have a role in cumulus detachment and/or granulosa cell proliferation during the ovulatory process. In 1985, Canipari and Strickland investigated the type of PA produced by the ovary and whether secretion of PA is under gonadotropin control. Using antibodies specific for tPA and uPA, they found granulosa cells produce tPA exclusively and thecal cells secrete uPA. Canipari and Strickland (1985) found that granulosa cells responded to FSH stimulation by producing increasing amounts of tPA. However, thecal cells were not stimulated by FSH whereas LH increased uPA secretion. Experiments by Canipari and Strickland (1985) indicate that uPA and tPA are involved in ovulation, but considering the observations of Shimada et al. (1983) they concluded that further insights into the granulosa:thecal interactions in the ovary need to be elucidated before any conclusions can be drawn. In 1985, Ny et al. published results that conflicted with those of Canipari and Strickland (1985). Using SDS-PAGE and fibrin autography techniques to characterize the fibrinolytic components secreted by granulosa cells, they found production of both tPA and uPA. Ny et al. (1985) showed FSH and LH stimulated granulosa tPA activity and suppressed PAI activity whereas uPA activity was not affected. Reich et al. (1985) examined the activity of PA in intact follicles when treated with gonadotropins

and inhibitors of steroidogenesis and arachidonic acid metabolism. They found LH to be as potent as FSH in stimulating PA activity and that LH-stimulated PA production was enhanced by estradiol-17ß but not by arachidonic acid metabolites. They also found that injection of serine protease inhibitors into the ovarian bursa of proestrus rats inhibited ovulation in the treated ovary. Reich et al. (1985) supported the hypothesis that PA is, in some way, involved in ovulation. In 1987, Liu et al. examined the activity of tPA and uPA in granulosa and theca-interstitial cells obtained from gonadotropin-treated immature rats during the periovulatory period. Liu et al. (1987) used SDS-PAGE and zymography to indicate the presence of PA. Their results demonstrated that tPA activity in both granulosa cells and theca-interstitial cells increased following treatment with PMSG and hCG, reaching a maximum prior to ovulation. These findings support those of Reich et al. (1985).

In 1988, Somokovitis et al. examined the PA, PAI and plasmin inhibitor (PI) levels in sow follicles at different stages of growth and observed PA, PAI and PI in all major components of the mature follicle and in ruptured follicles. Plasminogen activator activity in the walls of follicles at the time of ovulation was found to be much higher than in the walls of growing and mature follicles. The highest level of PA was found at the area of rupture and, in

follicles that had already ruptured, with their openings sealed, a marked decrease in PA was noted. The observations by Somokovitis et al. (1988) support the suggestion that PA plays an important role in ovulation.

It has also been hypothesized that PA may have a role in embryo implantation. Strickland et al. (1976) examined the production of PA by trophoblast and parietal endoderm. It is well known that, in many species, trophoblast cells invade the uterine wall during embryo implantation and that the parietal endoderm cells begin to multiply and migrate until they form a continuous sheet of cells adjacent to the inner surface of the trophectoderm. In the mouse, the parietal endoderm cells are eventually responsible for the secretion of Reichert's membrane. Strickland et al. (1976) assayed for PA using the fibrin-agar overlay technique and found that PA is produced by both the trophoblast and parietal endodermal cells. The period during which the cultured trophoblast was secreting PA corresponded closely with the invasive period of the trophoblast (in the mouse this extends from the sixth to the tenth equivalent day of gestation). Parietal endodermal cells started to produce PA as soon as they had differentiated from the ICM and production continued throughout culture, suggesting that these cells may produce PA throughout their lifespan. It seems possible that PA produced by the parietal endoderm may function in the migration of cells along the trophectoderm

or may participate in the formation of Reichert's membrane. In 1980, Mullins et al. measured the levels of PA in uterine flushings from the pig through the estrous cycle and during early pregnancy. Production of PA by pig embryos cultured in vitro was also measured using the indirect ^{125}-I fibrin plate assay. The pig has a non-invasive trophoblast and it has been hypothesized that this may be due to either failure of the conceptus to produce PA or uterine production of PAI. Mullins et al. (1980) found that the elongating pig blastocyst does produce PA. Uterine flushings were found to secrete a protease inhibitor which is progesterone-induced and which is active against both day 12 pig conceptus PA and urokinase. It would therefore appear that the inhibitor plays a key role in preventing implantation in the pig. In 1985, Axelrod, used an implantation-defective mutant mouse to determine whether a correlation could be made between PA activity and invasiveness of the trophectoderm. On day 6 of gestation, the mutant embryos make poor contact with the uterine wall and penetration of the endometrium does not occur as it would in a normal animal. Axelrod (1985) measured PA levels by the labeled fibrin assay and found that the mutant embryos trophoblast produced less PA than that of the normal controls. Because these mutants are deficient only in their invasive properties, not in proliferative properties, there is a possible link between low PA and decreased invasive ability.

There has also been some speculation that PA is involved in the induction of meiotic maturation. In 1985, Huarte et al. identified a tPA produced by oocytes, the production of which was triggered by resumption of meiosis in the mouse and rat. Huarte et al. (1985) used SDS-PAGE and zymography to prove the presence of PA and then characterized the PA further using antibodies for uPA and tPA. Primary oocytes did not contain active tPA; however, oocytes that had undergone germinal vesicle breakdown (GVBD) contained tPA. Huarte et al. (1985) found that tPA activity was blocked by dbcAMP and cyclohexamide but not by α-amanitin suggesting tPA is present in primary oocytes and meiotic maturation induces translation of tPA mRNA. Interestingly, fertilized eggs denuded of their zona pellucida no longer contained PA, suggesting that tPA production stops somewhere around the time of fertilization. In 1987, Huarte et al. documented the accumulation of tPA mRNA in growing oocytes and its subsequent degradation during meiotic maturation. Total RNA of mouse oocytes was analyzed using Northern blot hybridization. More tPA mRNA was found in a single fully grown primary oocyte that in 500 primordial oocytes. Huarte et al. (1987) also determined that synthesis of tPA is triggered upon resumption of meiosis. Within 3 hr after GVBD the size of tPA mRNA progressively increased due to an apparent elongation of it's poly(A) tail at the same time that it was being

translated. Huarte et al. (1987) found that 10 hr after fertilization tPA mRNA had completely disappeared. This time course is in accord with the general pattern of utilization of maternal components during early embryogenesis. Liu et al. (1987) induced immature rats to ovulate using PMSG and hCG and determined PA activity in cumulus-oocyte complexes at various times following gonadotropin treatment. Plasminogen activator activity was measured using SDS-PAGE and the fibrin overlay technique. Liu et al. (1987) found that after hCG treatment, tPA showed a time-dependent increase in cumulus-oocyte complexes and denuded oocytes; an accompanying increase in cumulus cell expansion and dispersion was also noted. The level of tPA secreted by cumulus-oocyte complexes is elevated immediately prior to ovulation and decreases thereafter. In 1987, Ny et al. examined the effect of GnRH on tPA activity in rat granulosa cells and cumulus-oocyte complex's. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and the fibrin overlay technique were used to measure PA levels. Gonadotropin-releasing hormone, like gonadotropins, increased the level of tPA activity in granulosa cells. Gonadotropin releasing hormone also increased tPA content in the oocyte and had no effect on denuded oocytes. These data suggest that GnRH may act on cumulus cells to increase unidentified messenger molecules which then may be transported to the oocyte via gap junctions to signal an

increase in tPA level. Strickland et al. (1988) attempted to identify what molecular determinants specify translational recruitment of tPA mRNA in mouse oocytes during meiotic maturation by injecting different antisense RNA and evaluating which were able to block tPA synthesis. Strickland et al. (1988) found that injection of antisense RNA complementary to the 103 nucleotides of the extreme 3' untranslated region was sufficient to prevent the polyadenylation, translational activation, and destabilization of tPA mRNA. This demonstrated how important the 3' untranslated region of oocyte mRNA is in the control of its translation and subsequent degradation. This work was followed in 1989 by Vassalli et al. who found that injected RNA fragments that correspond to the 3' untranslated region of tPA mRNA are subject to regulated polyadenylation and that the presence of a long poly(A) tail is necessary and sufficient for translation. Vassalli et al. (1989) also found that an absent poly(A) tail renders mRNA silent in primary and maturing oocytes and that in vitro polyadenylation of a transcript allows its translation even in primary oocytes.

Rodent Embryos

Liedholm and Astedt (1975) were the first to report fibrinolytic activity in rat embryos. Rat embryos exhibited fibrinolytic activity during tubal passage on Days 1-4 but activity decreased on Day 5 and completely disappeared at implantation on Day 6. No fibrinolytic activity was observed in embryos treated with tranexamic acid, an established inhibitor of plasminogen activation. Liedholm and Astedt (1975) hypothesized that the fibrinolytic activity, probably induced by PA, prevented adhesion of the embryos to fibrin deposits on the tubal mucosa during transport. The subsequent disappearance of fibrinolytic activity upon entry into the uterus was suggested as a prerequisite for implantation.

Strickland et al. (1976) and Sherman et al. (1976) characterized a biphasic pattern of PA production by mouse embryos. Strickland et al. (1976) used a fibrin-agar overlay to directly assay fibrinolysis and found a complex pattern of PA secretion produced by blastocysts. The first phase of PA activity appeared on Day 6, rose to a peak by Day 8-9 and then decreased steadily to Day 11. The second phase of PA production started on Day 11 and increased to Day 15 at which point the enzyme activity was five-fold higher than on Day 8. Trophoblast cells were responsible for the first phase of PA production whereas parietal

endoderm contributed to the second phase (Strickland et al., 1976). Interestingly, trophoblast is recognized as one of the most invasive tissues known. Trophoblastic invasiveness is transitory and corresponds closely to the time when PA secretion is occuring, suggesting that this enzyme is a contributing factor in implantation (Sherman et al., 1976). Two possible roles for PA in the parietal endoderm have been hypothesized. Plasminogen activator may function in the initial migration of cells along the trophectoderm and in the cyclic breakdown and synthesis of Reichert's membrane. A common phenotypic property of cells that produce PA is the ability to migrate and degrade connective tissue. Sherman et al. (1976) suggested the possibility of using PA as a marker for identifying cell types as they acquire the ability to move within the embryo during development.

Bode and Dziadek (1979) performed detailed analysis of PA secretion on mouse embryonic tissues between Days 7-10 of gestation. Using casein and fibrin-agar overlay plates to measure PA secretion, Bode and Dziadek (1979) demonstrated that all tissues investigated (parietal endoderm, visceral yolk sac endoderm and mesoderm and amnion) eventually secreted PA, making it an unsuitable tissue-specific biochemical marker. However, they also concluded that PA may play a role in embryonic growth and morphogenetic movements.

Sherman (1980) reported that fibrinolytic activity associated with mouse embryos during preimplantation development is restricted to the zona pellucida and is most likely due to a PA. Sherman (1980) concluded it was unlikely that PA was involved in hatching because the zona remained intact despite substantial ongoing fibrinolysis. He proposed instead that the protease was involved in trophoblast invasiveness during implantation. Kubo et al.(1980) studied the effects of protease inhibitors on attachment and outgrowth of mouse blastocysts grown on mouse decidual monolayers. They found nitrophenol-p-guanidino benzoate (NPGB), a competitive inhibitor of serine proteases, to be the most potent inhibitor of peri-implantation development. ε-aminocaproic acid (EACA), a specific inhibitor of PA at low concentrations, did not interfere with attachment and outgrowth. However, EACA at higher concentrations, where it also interferes with plasmin and trypsin, resulted in inhibition of both attachment and outgrowth. Given these results, Kubo et al. (1980) proposed that there are two protease-dependent events in the mouse blastocyst: trophoblastic attachment requiring trypsin-like activity and trophoblastic outgrowth requiring trypsin like activity and PA. Plasminogen activator was one of the enzymatic parameters investigated by Sellens and Sherman (1980) in mouse embryos cultured in various media. In this study PA was secreted continuously by embryos but the amount

secreted decreased substantially in suboptimal media. Apparently, PA is secreted only by those embryos which are going to survive.

Marotti et al. (1982) examined parietal endoderm, visceral endoderm and extraembryonic mesoderm from the mouse embryo for production of PA. Three criteria were used: (1) MW (2) interaction with serum inhibitor(s) and (3) antigenic properties to determine the type of PA being produced. At least two forms of PA were present in the early mouse embryo. Marotti et al. (1982) found that PA activity from parietal endoderm was evident in cultures both with and without serum, however for visceral and extraembryonic mesoderm the enzyme was only detectable in serum-free medium. Parietal endoderm produced a tPA (MW=79,000) which was only partially affected by acid-treated serum and inhibited by anti-tPA antibodies. Visceral and extraembryonic mesoderm were producing an uPA (MW=48,000) which was inhibited by acid-treated serum and immunoprecipitated by anti-uPA antibodies. Marroti et al. (1982) suggested that PA may facilitate the movement of cells during embryogenesis.

Menino and O'Claray (1986) examined the effects of plasmin and plasminogen on hatching, attachment and trophoblastic outgrowth in mouse embryos developing in vitro. Plasmin- and plasminogen-supplemented Whitten's medium supported greater blastocyst formation, hatching and

trophoblastic outgrowth then medium lacking enzyme supplementation. Although these results indicate that plasmin can induce and support trophoblastic outgrowth, some additional serum factors, e.g. fibronectin, must also be involved since the extent of outgrowth was reduced compared to serum-free medium.

Porcine Embryos

Attachment of the conceptus to the uterine wall occurs gradually in pigs. There is no actual penetration of the uterus and contact with the maternal organism is thru interdigitation of microvilli on opposing surfaces of the trophoblast and uterine wall. Researchers have questioned whether this is due to failure of the conceptus to produce PA or due to a protease inhibitor secreted by the uterus that inhibits the invasive potential of the trophoblast. Mullins et al. (1980) observed that expanding Day 12 pig embryos secreted significant amounts of PA into the culture medium. Mullins et al. (1980) also observed that the endometrium released a PA or plasmin inhibitor under the influence of maternal progesterone which could explain how the uterine epithelium resists trophoblastic invasion.

Fazleabas et al. (1983) found that Day 10-16 pig embryos released PA in a time-dependent fashion in vitro.

Plasminogen activator production occurred in a biphasic pattern similar to the profile observed in the mouse. The first phase, between Days 10-12, coincided with blastocyst elongation and the second phase, between Days 14-16, occurred at a time when embryonic DNA was significantly increasing. Fazleabas et al. (1983) confirmed the report by Mullins et al. (1980) and observed that the endometrium was secreting a PI into the lumen with embryonic PA production. Nonpregnant gilts injected with estradiol valerate on Day 11 of the estrous cycle also produced PI. These data suggest that initiation of estrogen production by the elongating blastocyst triggers endometrial PI production which acts to inhibit blastocyst-induced proteolysis by PA and prevents endometrial invasion.

Bovine Embryos

It has long been suggested that sublysis of the zona pellucida by an unknown protease and the hydrostatic pressure from the expanding blastocoel facilitate hatching (Domon et al, 1973). Menino and Williams (1987) explored this hypothesis in bovine embryos by culturing morulae in various concentrations of plasminogen and evaluating subsequent development, PA production and plasminogen activation. Embryos were cultured in Ham's F-12 containing

0, 15, 30, 60 or 120 µg/ml plasminogen and recovered medium was assayed for PA or plasmin using a caseinolytic assay. Menino and Williams (1987) observed no significant difference in the timing of blastocyst formation due to plasminogen level; however, plasminogen accelerated initiation and completion of hatching. For all plasminogen levels, embryonic activation was lowest during the first 48-72 h, peaked at 168 h and plateued thereafter (Menino and Williams, 1987). These data suggest that plasminogen activation may be effecting sublysis of the zona pellucida because peak PA production occurred during hatching. Kaaekuahiwi and Menino (1990) investigated the relationship of PA production to cell stage, cell number and changes in overall diameter and zona pellucida thickness in bovine embryos. Late morulae to early blastocysts were cultured in Hams F-12. At 24-h intervals, changes in overall diameter and zona pellucida thickness were recorded and medium was recovered and stored at -20°C until assayed for PA. Kaaekuahiwi and Menino (1990) observed that total PA production was positively correlated to embryonic size, developmental stage and cell number and negatively correlated to zona pellucida thickness, suggesting that PA secretion may be an indicator of embryo viability. Dyk and Menino (1991) used SDS-PAGE and zymography to determine the tissue source and type of PA produced by Day 12-14 bovine blastocysts. Bovine blastocysts secreted light (47.0 ± 1.0

kDa) and heavy (86.1 ± 0.7 kDa) forms of PA. Dissected trophoblasts also produced both forms of PA but PA was not detected in embryonic discs. These results suggested that Day 12-14 bovine blastocysts produced a uPA (41.5 - 47.0 kDA) and a high molecular mass form (86.1 - 92.2 kDa) that was either a novel tPA or a PAI complexed with the uPA. Berg and Menino (1992) cultured intact embryos, embryonic discs and trophoblastic vesicles from Day 12-14 bovine blastocysts for 5 d in αMEM. Culture medium was recovered daily and at the end of culture blastocoelic fluid and embryonic tissues were recovered. Plasminogen activator concentration in medium, tissues and blastocoelic fluid were determined with a caseinolytic assay. Antibodies to uPA (anti uPA) and tPA (anti tPA) and amiloride (a uPA inhibitor) were used to identify the type of PA being produced. Berg and Menino (1992) observed that intact embryos and trophoblastic vesicles produced more PA then embryonic discs, and tissues with expanded blastocoels released less PA than those with collapsed blastocoels. Treatment with anti uPA decreased PA activity and amiloride completely eliminated PA activity, suggesting that Day 12-14 bovine embryos secrete only uPA. Although several aspects of bovine PA production have been elucidated, many questions still remain in determining this enzyme's role in embryogenesis.

Ovine Embryos

Menino et al. (1989) measured levels of PA production by ovine embryos and evaluated the effects of PA on ovine embryo development and zona pellucida integrity. Eight to 16 cell embryos were collected and cultured in Whitten's medium containing 0, 60 or 120 µg/ml plasminogen. Medium was recovered and stage of development was recorded daily. Changes in zona pellucida solubility were measured by recording the time required for zona dissolution at room temperature when treated with acidified Dulbecco's Phosphate Buffered Saline (DPBS; pH 2.5). Menino et al. (1989) observed that more blastocysts hatched in medium with 60 and 120 µg/ml plasminogen than in 0 µg/ml plasminogen. Plasminogen activator production was low until the morula stage, increased during the morula-blastocyst transition and remained elevated through blastocoelic expansion and hatching. These events may reflect transcriptional and translational events occuring during activation of the embryonic genome in sheep. Zona pellucida solubility increased as embryonic stage advanced and the zona pellucida of embryos cultured in medium with plasminogen also showed reduced resistance to solubilization in acidified DPBS. The authors concluded that PA and plasmin could participate in hatching, but other factors, e.g. blastocoelic hydrostatic

pressure, are also involved in changing the solubility of the zona pellucida.

EFFECTS OF BLASTOCOELIC EXPANSION AND PLASMINOGEN ACTIVATOR ACTIVITY ON HATCHING AND ZONA PELLUCIDA SOLUBILITY IN BOVINE EMBRYOS

Abstract

Two experiments were conducted to evaluate factors affecting zona pellucida solubility in bovine embryos in vitro. In Experiment 1, relationships between the extent of blastocoelic expansion and zona pellucida solubility were determined. Day 6 embryos (n = 42) were collected nonsurgically and graded for quality and stage of development. Overall embryo diameters (OD), zona pellucida thicknesses (ZPT) and diameters of the blastocoel (BD), if present, were measured with an ocular micrometer. Embryos with good or excellent quality grades (POST-embryos) were cultured for 192 h in a humidified atmosphere of 5% CO_2 in air at 39° C in Ham's F-12 containing 1.5% bovine serum albumin (BSA). Embryos of lesser quality (PRE-embryos) were immediately placed in .2% SDS and the time required for complete dissolution of the zona pellucida (ZPDT) determined by observation at 200x magnification. At 24-h intervals for POST-embryos, medium was recovered for plasminogen activator (PA) analysis and OD, ZPT and BD were measured with an ocular micrometer. The change in embryonic surface area (SA) that occurred during hatching was computed by deducting the SA of the embryo prior to blastocoelic expansion from the maximal SA achieved prior to hatching. Twenty-two of 27 (81%) embryos completed hatching in vitro. Compared to POST-embryos, mean OD was less ($P<.05$) and mean

ZPT and ZPDT were greater (P<.05) in PRE-embryos (159.1μ vs 234.7μ, 13.5μ vs 10.9μ and 9.2 min vs 7.1 min, respectively). Zona pellucida dissolution time was negatively correlated (P<.01) with OD, BD and the change in SA. In Experiment 2, effects of suppressing PA and blastocoelic expansion on hatching and zona pellucida solubility in bovine embryos were examined. Day 6 embryos (n = 99) were randomly assigned to a 3 x 3 factorial experiment. Embryos were cultured for 192 h in a humidified atmosphere of 5% CO_2 in air at 39° C in Ham's F-12 containing 1.5% BSA and 0, 10 or 100 IU/ml human PA inhibitor-2 (PAI-2). After 24 h of culture, embryos were transferred to medium with 0, .1 or .5 nM ouabain for 20 h, washed and returned to their respective cultures. At 24-h intervals, OD, BD and ZPT were measured with an ocular micrometer, medium was recovered for PA analysis, stage of development was recorded and embryos were transferred to fresh microdrops. At the end of culture, zona pellucida-enclosed embryos and shed zonae pellucidae were recovered and placed in .2% SDS and ZPDT was determined. Percentages of embryos hatching were not different (P>.05) among 0, 10 and 100 IU/ml PAI-2 (56, 68 and 47%, respectively). More (P<.05) embryos hatched after exposure to .1 nM (83%) than 0 (59%) or .5 nM (33%) ouabain and more (P<.05) embryos hatched in 0 nM compared to .5 nM ouabain. Exposure to .1 nM ouabain increased (P<.05) OD and BD compared to 0 and .5

nM ouabain, however, embryos exposed to .5 nM ouabain had reduced ($P<.05$) BD compared to 0 nM ouabain. Embryos cultured in 0 IU/ml PAI-2 possessed reduced ($P<.05$) OD and BD compared to 10 and 100 IU/ml PAI-2. Zona pellucida thickness was greater ($P<.05$) in embryos cultured in 0 IU/ml PAI-2 compared to 100 IU/ml PAI-2, however no difference ($P>.05$) in ZPT was observed due to exposure to ouabain. Addition of 0, 10 and 100 IU/ml PAI-2 suppressed ($P<.05$) embryonic PA activity in the culture medium (102.0, 43.6 and 6.5×10^{-3} IU/d, respectively) and exposure to ouabain had no effect ($P>.05$) on subsequent PA activity. Zona pellucida dissolution time did not differ ($P>.05$) following culture in medium with PAI-2 or exposure to ouabain. Zona pellucida dissolution time was negatively correlated ($r=-.79$; $P=.06$) with PA activity for embryos cultured in 0 IU/ml PAI-2 and exposed to .5 nM ouabain. These results suggest that the degree of physical distension induced in the zona pellucida by the expanding blastocoel correlates significantly with its solubility at the time of hatching. Although hatching was reduced following exposure to .5 nM ouabain, neither ouabain nor PAI-2 treatment effected changes in zona pellucida solubility. However, these conditions may have induced compensatory mechanisms in the embryo that overcame potential effects on the zona pellucida. The negative correlation between zona pellucida dissolution time and PA

activity when blastocoel expansion is limited supports a zonalytic role for PA.

Introduction

A critical stage in mammalian embryo development is when the embryo sheds its protective covering, the zona pellucida, in the process known as hatching. It has long been suggested that hatching results from sublysis of the zona pellucida by an unknown protease or zona lysin and the hydrostatic pressure generated from the expanding blastocoel (McLaren, 1970; Domon et al., 1973). Proteases have been implicated as participants in the hatching process during development in a variety of animal species, including, sea urchins (Barrett et al., 1975) and mice (Perona and Wassarman, 1986; Sawada et al., 1990), as well as remodeling of the extracellular coverings in rabbits (Kirchner, 1972; Denker, 1977).

Embryos from several species, including mice (Strickland et al., 1976), pigs (Fazleabas et al., 1983), cattle (Menino and Williams, 1987) and sheep (Menino et al., 1989) produce the serine protease plasminogen activator (PA). Menino and Williams (1987) reported that bovine embryos liberated PA during blastocoelic expansion and hatching and that addition of plasminogen to the culture

medium accelerated hatching. Kaaekuahiwi and Menino (1990) observed that hatching embryos released more PA than embryos that failed to hatch. These observations suggest that embryonic PA may be either directly or indirectly involved in inducing sublytic changes in the zona pellucida to facilitate hatching. However, hatching is not a unilateral process facilitated only by production of a purported zona lysin. Blastocoelic expansion contributes to hatching through the hydrostatic pressure generated in the expanding blastocoel (McLaren, 1970). Therefore two experiments were conducted to evaluate factors affecting solubility of the bovine zona pellucida and hatching. The objective of the first experiment was to evaluate the relationship between changes in embryonic dimensions and solubility of the zona pellucida in vitro. The objective of the second experiment was to determine the effects of transiently suppressing blastocoelic expansion and inhibiting PA activity on hatching and zona pellucida solubility in vitro.

Materials and Methods

Animals and Embryo Collection. Seven (Experiment 1) and 29 (Experiment 2) crossbred beef cows were estrous synchronized with prostaglandin $F_2\alpha$ ($PGF_2\alpha$; Lutalyse, The Upjohn Co., Kalamazoo, MI, U.S.A.) and superovulated with

porcine follicle stimulating hormone (pFSH; Schering Corp., Kenilworth, NJ, U.S.A.). Cows received two 25 mg injections of $PGF_2\alpha$ administered i.m. 12 days apart (Day 0 = first $PGF_2\alpha$ injection). Cows received once-daily injections of pFSH at dosages of 12, 10, 8 and 6 mg on Days 10, 11, 12 and 13, respectively. Estrus detection was initiated 24 h after the second injection of $PGF_2\alpha$. Cows were handmated using 1 of 4 bulls at onset of estrus and thereafter at 12-h intervals for as long as the cow would accept the bull.

Embryos were collected from cows nonsurgically 6 d after mating by flushing the uterus with Dulbecco's phosphate buffered saline (DPBS) containing 2 ml/l heat-treated cow serum (HTCS) and 10 ml/l of antibiotic-antimycotic solution (Sigma Chemical Co., St Louis, MO, U.S.A.). Embryos were recovered from the flushes by aspiration and washed three times in microdrops of Ham's F-12 with 1.5% bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO, U.S.A.) under paraffin oil (Fisher Scientific Co., Tustin, CA, U.S.A.). Embryos were morphologically evaluated at 100-200x magnification with an inverted-stage phase-contrast microscope and overall diameters (OD), zona pellucida thicknesses (ZPT) and, if present, blastocoel diameters (BD) were measured using an ocular micrometer.

Embryo Culture. In Experiment 1, good and excellent quality embryos (POST-embryos) were cultured in Ham's F-12 with 1.5% BSA whereas embryos of lesser quality (PRE-

embryos) were immediately evaluated for zona pellucida solubility. In Experiment 2, embryos were randomly assigned to a 3 x 3 factorial design. Embryos were cultured in Ham's F-12 with 1.5% BSA containing 0, 10 or 100 IU/ml human PA inhibitor-2 (PAI-2; American Diagnostica, Greenwich, CT, U.S.A.). After 24 h of culture, embryos were transferred to medium with 0, .1 or .5 nM ouabain for 20 h, washed three times and returned to their respective cultures. In both experiments, embryos were cultured for 192 h in microdrops (25 µl, Experiment 1; 35 µl, Experiment 2) of Ham's F-12 with 1.5% BSA under paraffin oil in a humidified atmosphere of 5% CO_2 in air at 39°C. At 24-h intervals embryos were observed for stage of development and OD, ZPT and BD were measured using an ocular micrometer. Beginning 24 h after initiation of culture and continuing through 192 h of culture, medium was recovered and frozen at -20°C until assayed for PA and embryos were transferred to fresh microdrops. To evaluate plasmin contamination and to detect any nonspecific proteases in the culture medium, medium without embryos were incubated and stored under identical conditions. After 192 h of culture, zona pellucida-enclosed embryos and shed zonae pellucidae were recovered and evaluated for solubility in .2% SDS.

Evaluation of Zona Pellucida Solubility. Zona pellucida-enclosed embryos and shed zonae pellucidae were washed three times in DPBS with .1% BSA (DPBS + BSA). Five

microliters of DPBS + BSA containing one embryo or shed zona pellucida were transferred to a 50-µl microdrop of .2% SDS. Zonae pellucidae were continually observed for dissolution at room temperature with an inverted-stage phase-contrast microscope at 200x magnification. The time between placement in .2% SDS and when the zona pellucida was no longer visible at 200x magnification was designated as the zona pellucida dissolution time (ZPDT).

Plasminogen Activator Assay. Plasminogen activator concentrations in the culture medium were determined using the caseinolytic assay described by Kaaekuahiwi and Menino (1990) for bovine embryos with urokinase (American Diagnostica, Greenwich, CT, U.S.A.) as the standard.

Measurements of Zona Pellucida Distortion. As a measure of the physical changes occurring in the zona pellucida in Experiment 1, zona pellucida thicknesses for each embryo were plotted over time and the total area of change from the initial measurement was determined using a polar planimeter (Figure 1). Total area was divided into two sections corresponding to a peak and panhandle. Peak area followed the change in the zona pellucida during blastocoelic expansion and completion of hatching. Panhandle area represented the difference in thickness after hatching and the period of time the zona pellucida remained in culture before determining solubility. The total area is the total amount of distortion in the zona pellucida that

occurred throughout the entire culture. The change in embryonic surface area (SA) that occurred during hatching was also computed by deducting the SA of the embryo prior to blastocoelic expansion from the maximal SA achieved prior to hatching. Surface area was calculated using the formula $4\pi r^2$ where r is equal to half the OD.

Electrophoresis and Zymography. One-dimensional SDS-PAGE under non-reducing conditions and zymography (Granelli-Piperno et al., 1978; Vassali et al., 1984) were used for evaluation of the PA produced by bovine embryos. Frozen culture medium was thawed and combined with one-half volume of 2x sample buffer. Urokinase standards were prepared with 1x sample buffer to final concentrations of .5 and .1 IU/ml. Each polyacrylamide gel included one lane containing molecular mass standards (Bio Rad Laboratories, Richmond, CA, U.S.A.). Aliquots of 75 µl of urokinase, molecular mass markers and culture medium were placed in castellated wells in a 4.0% acrylamide stacking gel with a 12.0% separating gel.

Following electrophoresis, gels were washed for 30 min in 2.5% Triton X-100 followed by a 30 min wash in DPBS. A 4% casein-2% agar gel containing 20 µg/ml purified human plasminogen (Sigma) supported on a glass plate (the zymogram) was applied to the surface of the polyacrylamide gel. Polyacrylamide gels with zymogram underlays were incubated for 24-36 h at 39°C. Incubation was terminated by

separating the zymogram from the gel. Zymograms were fixed with 3% acetic acid for 15 minutes, dried and stained for permanent storage.

Statistical Analysis. Differences in the percentages of embryos developing to a particular cell stage due to treatment were determined by Chi-square procedures. Differences in the times required for development to a particular cell stage and ZPDT due to concentration of PAI-2 or ouabain were determined by two-way analysis of variance (ANOVA). Differences in OD, BD, ZPT and PA production due to concentrations of PAI, ouabain and time in culture were determined using three-way ANOVA. Differences between means were determined by Duncan's new multiple range test. Correlation-regression analysis were used to determine the relationships between PA, OD, ZPT, BD and ZPDT for Experiments 1 and 2 and the relationships between ZPDT, peak, panhandle and total areas of zona pellucida distortion and the change in embryonic SA for Experiment 1. All analysis were performed using the NCSS Statistical Software Program (Number Cruncher Statistical System, version 4.1, 1984, Hintze, J.L., Kaysville, UT, U.S.A.).

Results

In Experiment 1, 75 ova were recovered from seven cows for a mean recovery rate of 10.7 embryos per cow. Of the embryos recovered, 15 were assayed for ZPDT immediately (PRE-embryos), 27 were cultured in Ham's F-12 with 1.5% BSA (POST-embryos) for 192 h and 12 were assigned to another experiment. Twenty-two of 27 embryos (81%) completed hatching in vitro. Mean ZPDT and ZPT were less and OD was greater ($P<.05$) for POST-embryos compared to PRE-embryos (Table 1). Correlation coefficients among OD, BD, ZPT, PA and ZPDT for PRE-embryos, POST-embryos and all embryos are presented in Table 2. Negative correlations ($P<.10$) were observed for ZPT with ZPDT for PRE-embryos and for OD with ZPT and ZPDT for all embryos. For POST-embryos, positive correlations ($P<.05$) were observed for OD with BD and PA and for BD with PA. Negative correlations ($P<.05$) were observed for ZPDT with OD, BD, panhandle and total areas of distortion and changes in SA.

In Experiment 2, a total of 246 ova were collected from 29 cows and, of those, 99 had normal morphology and were randomly assigned to the 3 x 3 factorial design. In vitro bovine embryo development was not affected ($P>.05$) by the concentration of PAI-2 in the culture medium (Table 3). Fewer ($P<.05$) embryos expanded and completed hatching following exposure to .5 nM ouabain compared to 0 and .1 nM

ouabain. More (P<.05) embryos commenced and completed hatching after treatment with .1 nM ouabain compared to 0 nM ouabain. No differences (P<.05) were detected due to PAI-2 or ouabain treatment and no significant interaction was observed in the times required for embryos to initiate blastocoelic expansion and hatching and complete hatching (Table 4).

Overall embryonic diameter and BD attained by bovine embryos were affected (P<.05) by the main effects of PAI-2, ouabain and time in culture and an interaction between ouabain and time in culture persisted (P<.05). Embryos cultured in 0 IU/ml PAI-2 had reduced (P<.05) OD and BD compared to 10 or 100 IU/ml PAI-2. Embryos cultured in .1 nM ouabain attained greater (P<.05) OD and BD than 0 or .5 nM ouabain and BD was greater (P<.05) in embryos cultured in 0 than .5 nM ouabain. Overall embryonic diameter (Figures 2 and 3) and BD (Figures 4 and 5) progressively increased (P<.05) with time in culture. Embryos cultured in .1 nM ouabain experienced greater (P<.05) OD and BD than 0 and .5 nM ouabain later in culture (Figures 2 and 4) and BD (Figure 4) decreased (P<.05) following exposure to ouabain at 24 h of culture thereby contributing to the ouabain and time in culture interaction. Zona pellucida thickness was affected (P<.05) by the main effects of PAI-2 and time in culture. No difference (P>.05) was observed in ZPT due to ouabain treatment and embryos cultured in 0 IU/ml PAI-2 had greater

(P<.05) ZPT than 100 IU/ml PAI-2. Zona pellucida thickness typically decreased (P<.05) with time in culture (Figures 6 and 7). Plasminogen activator activity in the culture medium was suppressed (P<.05) by 10 and 100 IU/ml PAI-2 and no differences (P>.05) were observed due to ouabain treatment. Plasminogen activator production was greater (P<.05) at 144 h compared to 24, 48 and 72 h of culture (Figures 8 and 9). Zona pellucida dissolution times were similiar (P>.05) following culture in PAI-2 or exposure to ouabain. Zymographic analysis revealed only a single band in medium recovered from embryos cultured in 0, 10 or 100 IU/ml PAI-2 (Figure 10). No high molecular mass PA were observed that would indicate complex formation between PAI-2 and embryonic PA.

Correlation coefficients for OD, BD, ZPT, ZPDT and PA for embryos treated with various concentrations of PAI-2 and ouabain are reported in Tables 6, 7 and 8. Consistent positive correlations (P<.06) were observed between OD and BD in all treatment groups. When embryos were cultured in 0 IU/ml PAI-2 and exposed to .1 nM ouabain, negative correlations (P<.10) were observed for ZPDT with BD and OD and ZPT with PA (Table 6). Zona pellucida dissolution time was positively correlated (P=.07) to ZPT and negatively correlated (P=.06) to PA when embryos were cultured in 0 IU/ml PAI-2 and exposed to .5 nM ouabain. Plasminogen activator activity was positively correlated with OD and BD

(P<.04) and negatively correlated with ZPDT (P=.04) for embryos cultured in 10 IU/ml PAI-2 and exposed to 0 nM ouabain (Table 7). Zona pellucida dissolution time was positively correlated (P<.09) with OD and ZPT for embryos cultured in 10 IU/ml PAI-2 and exposed to .1 nM ouabain. Zona pellucida thickness was positively correlated (P<.09) with OD and BD and negatively correlated (P=.02) with PA for embryos cultured in 10 IU/ml PAI-2 and exposed to .5 nM ouabain. Under these culture conditions, ZPDT was negatively correlated (P<.05) with OD and BD and BD was negatively correlated with PA (P=.01). For embryos cultured in 100 IU/ml PAI-2 and exposed to 0 or .1 nM ouabain, negative correlations between OD and ZPDT and BD and ZPDT approached significance (P<.16). Plasminogen activator activity was positively correlated (P=.10) with OD for embryos exposed to .5 nM ouabain.

Discussion

Results from Experiment I demonstrate changes in zona pellucida solubility during culture and that the decreased ZPDT is highly correlated with the distortion induced by the expanding blastocoel. The greater the degree of distension occurring in the zona pellucida throughout culture, the lower the observed ZPDT. Embryos can hatch in vitro which

suggests that the forces involved may originate from within the embryo. It has long been hypothesized that there are two factors which facilitate hatching; sublysis of the zona pellucida by an unknown protease and hydrostatic pressure from the expanding blastocoel (Domon et al., 1973). Results of the correlation analyses strongly support the role for blastocoelic expansion as the major force driving hatching and altering the solubility of the zona pellucida. The physical forces that lead to the expansion of the blastocoel and subsequent thinning of the zona pellucida are a direct result of the accumulation of fluid in the blastocoel (McLaren, 1970; Bergstrom, 1972). This accumulation depends upon the development of mechanisms that allow water transport across the trophectoderm. Biggers et al. (1978) reported that Na/K ATPase mediates transtrophectodermal fluid transport in preimplantation mouse embryos. In the rabbit blastocyst, studies using tritium labeled ouabain have demonstrated that the Na/K ATPase is localized to the basolateral surfaces of the trophectodermal cells (Benos et al., 1990). The nonsignificant correlation between PA and ZPDT suggests that under these conditions PA production has little impact on zona pellucida solubility. Similar correlations among OD, ZPT and PA were observed by Kaakuahiwi and Menino (1990).

Peak PA production by bovine embryos occurs during hatching (Menino and Williams, 1987) implicating PA as an

attractive candidate for the zona lysin. With this premise, Experiment II was designed to evaluate the effects of transiently limiting blastocoelic expansion and inhibiting PA activity on solubility of the zona pellucida in vitro. Ouabain has been used successfully to inhibit blastocoelic expansion in both mouse (Wiley, 1984) and rabbit embryos (Biggers et al., 1978). Plasminogen activator inhibitor-2 binds urokinase-type PA (uPA) with greater affinity than tissue-type PA (tPA; Andreasen, 1990) and therefore, because bovine embryos produce uPA (Dyk and Menino, 1991; Berg and Menino, 1992) PAI-2 was selected. Although zymographic analysis did not reveal PA-PAI complex formation, PA activity was suppressed in medium with PAI-2. Typically, PAI-2 forms a covalent complex with uPA (Andreasen et al., 1990), however, the heterologous interaction between human PAI-2 and bovine embryonic PA may differ.

No delays in development were observed due to PAI-2 or ouabain treatment and the number of embryos hatching was not affected by PAI-2 concentration. However, .1 nM ouabain increased the number of embryos completing hatching when compared to either 0 nM or .5 nM ouabain. It is possible a compensatory mechanism was elicited by embryos exposed to .1 nM ouabain causing an enhanced rate of expansion. A similar change was not observed with .5 nM ouabain; embryos exposed to this concentration encountered reduced rates of expansion and hatching. Although ZPDT did not differ by ouabain

treatment, a trend persisted where embryos exposed to .1 nM ouabain attained the largest dimensions and had the shortest ZPDT and those exposed to .5 nM ouabain had the smallest dimensions and the greatest ZPDT.

A totally unexpected result was the increased OD and BD encountered in embryos cultured with PAI-2. Level of PAI-2 had no effect on ZPDT despite potent inhibition of PA activity by 10 and 100 IU/ml PAI-2. The increase in embryonic size experienced in PAI-2 coupled with the lack of difference in ZPDT, the reduction in PA activity and the similar hatching rates may point to PA having a zonalytic effect that was masked by the increased zona pellucida distortion. Thinning of the zona pellucida, or equivalent membrane, because of protease action has been demonstrated in other species. Barrett et al. (1975) purified the hatching enzyme of the sea urchin and found that this protease attacks native rather than denatured proteins found in the fertilization envelope. Kirchner (1972) examined proteases secreted by the rabbit uterus and blastocyst and suggested participation of these enzymes in digestion of the zona pellucida and remodeling of the extra-embryonic membranes. Perona and Wassarman (1986) reported histochemical and autoradiographic evidence that a trypsin-like protease was localized to the opening in the mouse zona pellucida through which the embryo emerged and that this protease activity was localized to the mural trophectoderm

prior to hatching. Sawada et al. (1990) supported this conclusion by observing that when this trypsin-like protease was inhibited, hatching was strongly inhibited. Sawada et al. (1990) suggested that this enzyme does not completely dissolve, but only partially weakens or digests, the zona pellucida immediately prior to hatching. Subsequent characterization of this enzyme has not been conducted and its identity as a PA has not been eliminated (Perona and Wassarman, 1986). Plasminogen activator secretion by mouse embryos also begins about the time of hatching (Strickland et al., 1976). Although PA are fairly specific for their substrate, plasminogen, reports have indicated alternative substrates. Keski-Oja and Vaheri (1982) have demonstrated cleavage of a 66 kD protein in pericellular matrices obtained from cultured human lung fibroblasts by urokinase. Baron-Van Evercooren et al. (1987) were unable to detect plasmin or plasminogen in developing cerebellar tissue however, cerebellar neurons secrete PA during migration thru the extracellular matrix.

Good evidence for a potential zonalytic role for bovine embryonic PA is in the negative correlation coefficient between ZPDT and PA for embryos cultured in 0 IU/ml PAI-2 and exposed to .5 nM ouabain. Embryos in this group had the lowest embryonic dimensions, therefore the least physical distortion exerted on the zona pellucida, and high PA activity. These data suggest that when blastocoelic

expansion is limiting, the amount of PA produced correlates well with solubility of the zona pellucida. However, zona pellucida solubility changes appear to be induced primarily by the physical distortion provided by the expanding blastocoel. Further research is necessary to clarify the role of PA in zona lysis and evaluate the zona pellucida as a substrate for the embryonic PA.

Table I-1. Mean zona pellucida dissolution times (ZPDT), zona pellucida thickness (ZPT) and overall diameters (OD) for bovine embryos cultured in Ham's F-12 with 1.5% BSA for 192 h (POST-embryos) or assayed for ZPDT at the time of collection (PRE-embryos).

	ZPDT		ZPT		OD	
		(min)		(μ)		(μ)
	n	$\bar{x}$ ± S.E.	n	$\bar{x}$ ± S.E.	n	$\bar{x}$ ± S.E.
PRE	15	9.2 ± 7[a]	15	13.5 ± .3[a]	15	159.1 ± 9.3[a]
POST	26	7.1 ± .6[b]	27	10.9 ± .3[b]	27	234.7 ± 7.0[b]

[a,b] Values in the same column without common superscripts are different (P<.05)

Table I-2. Correlation coefficients for overall diameters (OD), blastocoel diameters (BD), zona pellucida thicknesses (ZPT), plasminogen activator production (PA), zona pellucida dissolution times (ZPDT), areas of zona pellucida distortion (Peak, Panhandle and Total) and changes in surface areas (SA) for bovine embryos assayed immediately for ZPDT (PRE-embryos), embryos cultured in Ham's F-12 with 1.5% BSA (POST-embryos) and both PRE- and POST-embryos (All embryos).

X-value	Y-value	PRE	POST	All
OD	BD	----	.89 (.00)	----
	ZPT	.42 (.12)[a]	-.09 (.65)	-.51 (.00)
	ZPDT	.06 (.83)	-.64 (.00)	-.59 (.00)
	PA	----	.53 (.00)	----
BD	ZPT	----	-.26 (.18)	----
	ZPDT	----	-.55 (.00)	----
	PA	----	.54 (.00)	----
ZPT	ZPDT	-.45 (.09)	.23 (.27)	.18 (.27)
	PA	----	-.13 (.51)	----
ZPDT	PA	----	-.17 (.40)	----
	Peak	----	-.28 (.17)	----
	Panhandle	----	-.41 (.04)	----
	Total	----	-.42 (.03)	----
	SA	----	-.60 (.00)	----

[a] Value in parenthesis indicates the probability

Table I-3. Development of bovine embryos in medium with 0, 10 or 100 IU/ml plasminogen activator inhibitor-2 (PAI-2) and after exposure to 0, .1 or .5 nM ouabain.

Treatment	n	Number (%) of embryos developing to: Blastocyst	Expanded blastocyst	Hatching blastocyst	Hatched blastocyst
PAI-2					
0 IU/ml	32	30 (94)[a]	26 (81)[a]	23 (72)[a]	18 (56)[a]
10 IU/ml	31	31 (100)[a]	29 (94)[a]	22 (71)[a]	21 (68)[a]
100 IU/ml	36	35 (97)[a]	29 (81)[a]	21 (58)[a]	17 (47)[a]
Ouabain					
0 nM	34	34 (100)[a]	32 (94)[a]	25 (74)[a]	20 (59)[a]
.1 nM	29	29 (100)[a]	28 (97)[a]	27 (93)[b]	24 (83)[b]
.5 nM	36	33 (92)[a]	24 (67)[b]	14 (39)[c]	12 (33)[c]

[a,b,c] Values in the same column within a treatment without common superscripts are different ($P<.05$).

Table I-4. Time (h) to blastocoelic expansion, onset of hatching and completion of hatching for bovine embryos cultured in medium containing 0, 10 or 100 IU/ml plasminogen activator inhibitor-2 (PAI-2) and after exposure to 0, .1 or .5 nM ouabain.

	Development to the following stages:					
	Expanded blastocyst		Hatching blastocyst		Hatched blastocyst	
Treatment	n	$\bar{x}$ ± SE	n	$\bar{x}$ ± SE	n	$\bar{x}$ ± SE
PAI-2						
0 IU/ml	27	68.6 ± 6.5	23	102.2 ± 8.9	18	120.8 ± 8.3
10 IU/ml	29	55.7 ± 6.3	22	93.4 ± 9.1	21	116.4 ± 7.6
100 IU/ml	29	53.6 ± 6.3	21	99.7 ± 9.3	18	134.0 ± 8.3
Ouabain						
0 nM	32	60.6 ± 6.0	25	92.6 ± 8.5	20	118.5 ± 7.0
.1 nM	28	60.0 ± 6.4	27	97.3 ± 8.2	25	127.6 ± 7.0
.5 nM	25	56.7 ± 6.7	14	104.4 ± 11.4	12	125.1 ± 10.1

Table I-5. Mean overall diameters (OD), blastocoel diameters (BD), zona pellucida thicknesses (ZPT), plasminogen activator production (PA) and zona pellucida dissolution times (ZPT) for bovine embryos cultured in 0, 10 or 100 IU/ml plasminogen activator inhibitor-2 (PAI-2) and after exposure to 0, .1 or .5 nM ouabain.

	OD (μ)	BD (μ)	ZPT (μ)	PA (IU x 10^{-3})	ZPDT (min)
Treatment	x ± S.E.	x ± S.E.	x ± S.E.	x ± S.E.	x ± S.E.
PAI-2					
0 IU/ml	214.4 ± 4.4[a]	143.1 ± 5.5[a]	13.8 ± .3[a]	102.0 ± 9.3[a]	9.8 ± .8[a]
10 IU/ml	233.5 ± 4.4[b]	162.4 ± 5.5[b]	13.5 ± .3[a,b]	43.6 ± 18.3[b]	8.4 ± .8[a]
100 IU/ml	227.2 ± 4.7[b]	166.0 ± 5.9[b]	12.8 ± .3[b]	6.5 ± 18.6[b]	10.0 ± .8[a]
Ouabain					
0 nM	218.8 ± 5.0[a]	154.9 ± 6.2[b]	13.2 ± .3[a]	49.3 ± 17.0[a]	9.4 ± .7[a]
.1 nM	241.2 ± 3.9[b]	176.8 ± 4.9[c]	13.4 ± .2[a]	59.6 ± 18.0[a]	8.5 ± .8[a]
.5 nM	206.3 ± 4.8[a]	128.9 ± 6.0[a]	13.6 ± .3[a]	33.8 ± 22.3[a]	10.2 ± .9[a]

[a,b,c] Values within a treatment without common superscripts are different ($P<.05$)

Table I-6. Correlation coefficients (r) for overall diameters (OD), blastocoel diameters (BD), zona pellucida thicknesses (ZPT), plasminogen activator production (PA) and zona pellucida dissolution times (ZPDT) for bovine embryos cultured in medium with 0 IU/ml plasminogen activator inhibitor-2 (PAI-2) and exposed to 0, .1 or .5 nM ouabain.

		Level of ouabain (nM):		
X	Y	0	.1	.5
OD	BD	.95 (.02)[a]	.90 (.00)	.99 (.00)
	ZPT	-.43 (.48)	.15 (.71)	-.27 (.61)
	ZPDT	-.53 (.36)	-.63 (.07)	-.50 (.31)
	PA	-.02 (.98)	.13 (.73)	.43 (.39)
BD	ZPT	-.64 (.25)	-.24 (.53)	-.25 (.63)
	ZPDT	-.36 (.55)	-.58 (.10)	-.56 (.25)
	PA	.03 (.96)	.48 (.20)	.48 (.33)
ZPT	ZPDT	-.06 (.93)	-.04 (.92)	.77 (.07)
	PA	-.52 (.37)	-.79 (.01)	-.42 (.40)
ZPDT	PA	-.32 (.45)	-.24 (.50)	-.79 (.06)

[a] Value in parenthesis indicates the probability

Table I-7. Correlation coefficients (r) for overall diameters (OD), blastocoel diameters (BD), zona pellucida thicknesses (ZPT), plasminogen activator production (PA) and zona pellucida dissolution times (ZPDT) for bovine embryos cultured in medium with 10 IU/ml plasminogen activator inhibitor-2 (PAI-2) and exposed to 0, .1 or .5 nM ouabain.

		Level of ouabain (nM):		
X	Y	0	.1	.5
OD	BD	.90 (.02)[a]	.97 (.00)	.80 (.06)
	ZPT	-.11 (.83)	.21 (.62)	.74 (.09)
	ZPDT	-.18 (.77)	.75 (.09)	-.89 (.04)
	PA	.84 (.04)	.10 (.82)	-.57 (.24)
BD	ZPT	-.34 (.51)	.02 (.96)	.89 (.02)
	ZPDT	-.07 (.91)	.59 (.22)	-.88 (.05)
	PA	.90 (.01)	-.01 (.97)	-.90 (.01)
ZPT	ZPDT	-.11 (.86)	.79 (.06)	-.62 (.27)
	PA	-.08 (.88)	.52 (.18)	-.89 (.02)
ZPDT	PA	-.62 (.04)	.43 (.33)	-.24 (.64)

[a] Values in parenthesis indicates the probability

Table I-8. Correlation coefficients (r) for overall diameters (OD), blastocoel diameters (BD), zona pellucida thicknesses (ZPT), plasminogen activator production (PA) and zona pellucida dissolution times (ZPDT) for bovine embryos cultured in medium with 100 IU/ml plasminogen activator inhibitor-2 (PAI-2) and exposed to 0, .1 or .5 nM ouabain.

		Level of ouabain (nM):		
X	Y	0	.1	.5
OD	BD	.99 (.00)[a]	.99 (.00)	.96 (.01)
	ZPT	-.51 (.39)	-.44 (.27)	-.55 (.33)
	ZPDT	-.75 (.15)	-.56 (.15)	-.56 (.44)
	PA	.44 (.56)	.50 (.20)	.81 (.10)
BD	ZPT	-.47 (.42)	-.42 (.30)	-.38 (.52)
	ZPDT	-.76 (.13)	-.54 (.16)	-.72 (.28)
	PA	.46 (.54)	.49 (.22)	.63 (.26)
ZPT	ZPDT	.64 (.24)	.25 (.55)	.52 (.48)
	PA	.59 (.41)	.22 (.61)	-.71 (.18)
ZPDT	PA	.33 (.36)	-.19 (.61)	-.28 (.65)

[a] Values in parenthesis indicates the probability

Figure I-1. Representative graph depicting changes in thickness of the zona pellucida during culture. The areas measured are delineated by a horizontal line starting at 0 h of culture, where zona pellucida thickness was greatest, and a vertical line at 192 h, at the end of culture. Peak area is measured to the left of the vertical line designating the time of hatching (H) and panhandle area is measured to the right of the hatching line. For embryos that expanded and/or initiated hatching but did not completely hatch a vertical line was drawn at the first interval where the thickness of the zona pellucida no longer changed following expansion.

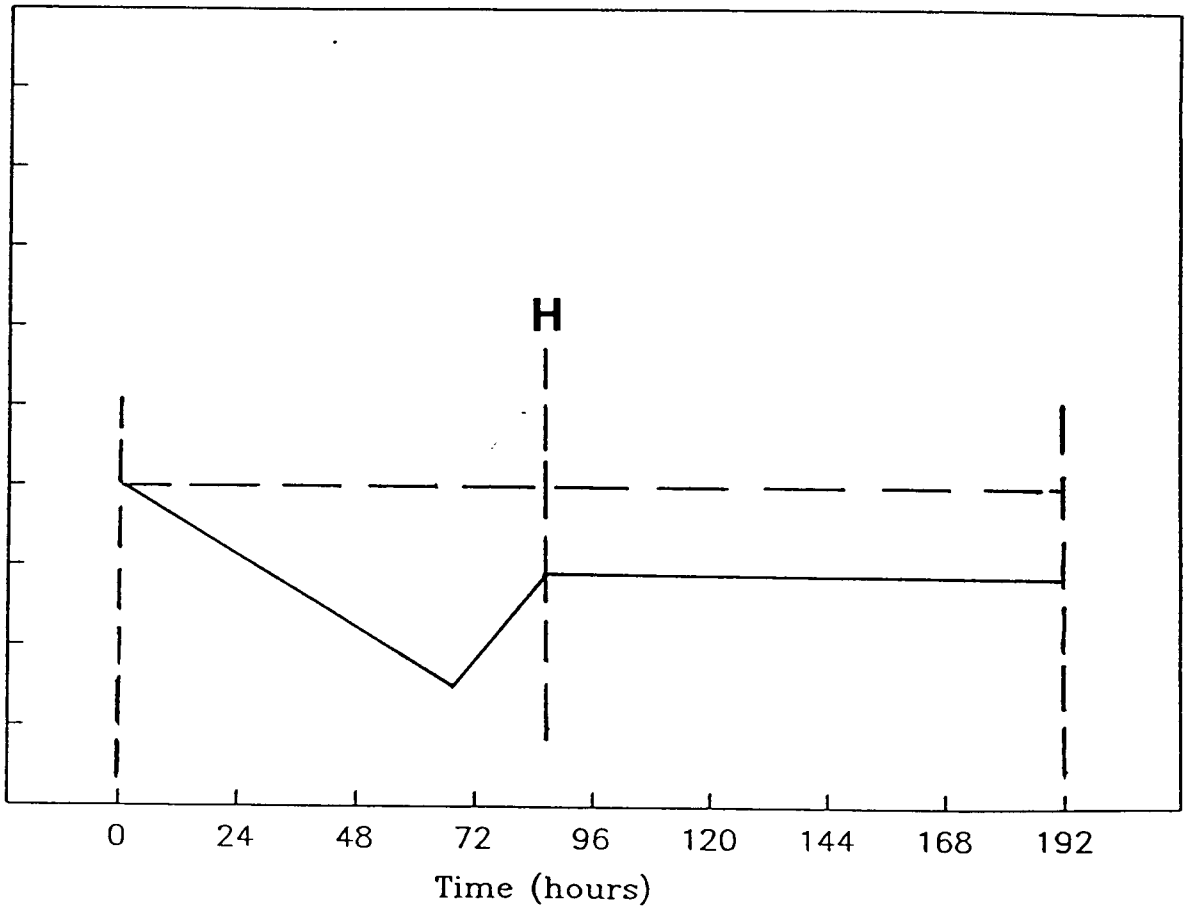


Figure I-2. Changes in overall diameters (OD) for bovine embryos cultured in 0 (○), .1 (●) or .5 (▽) nM ouabain by time.

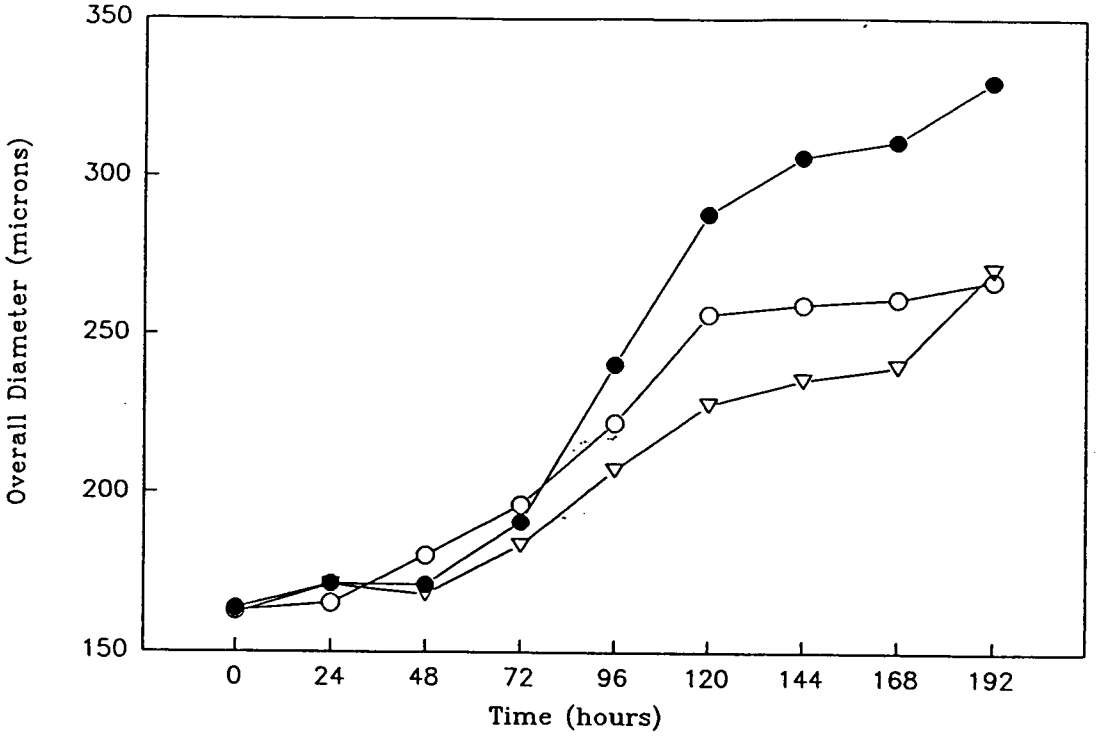


Figure I-3. Changes in overall diameters (OD) for bovine embryos cultured in 0 (○), 10 (●) or 100 (▽) IU/ml PAI-2 by time.

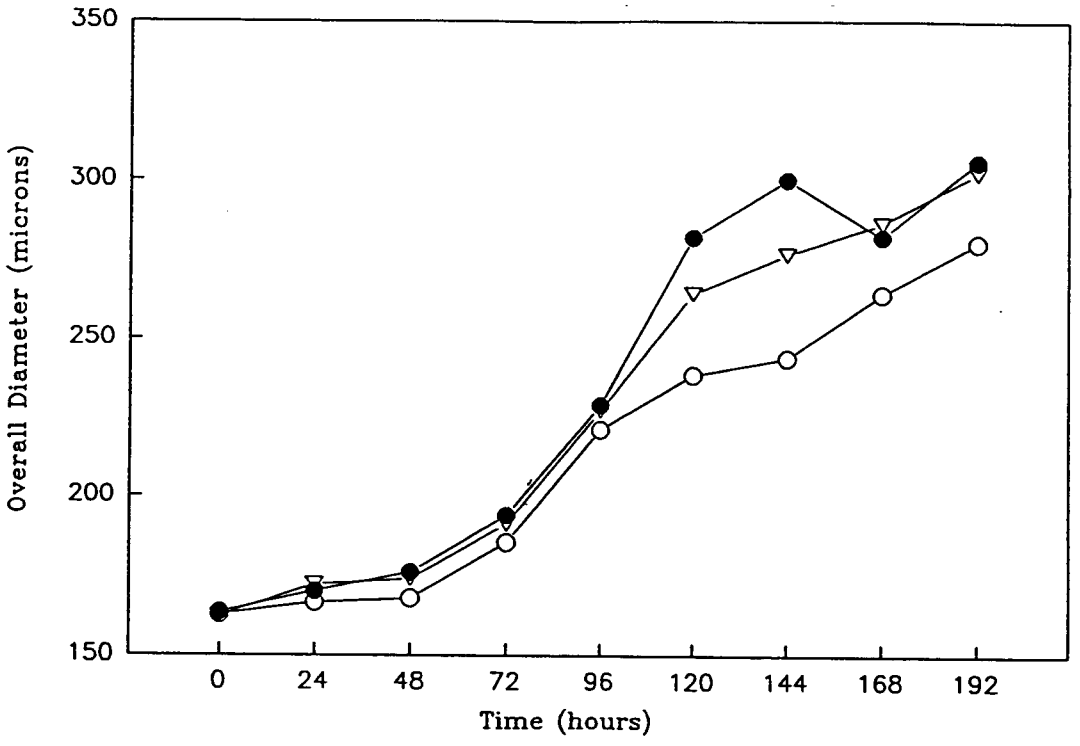


Figure I-4. Changes in blastocoel diameters (BD) for bovine embryos cultured in 0 (○), .1 (●) or .5 (▽) nM ouabain by time.

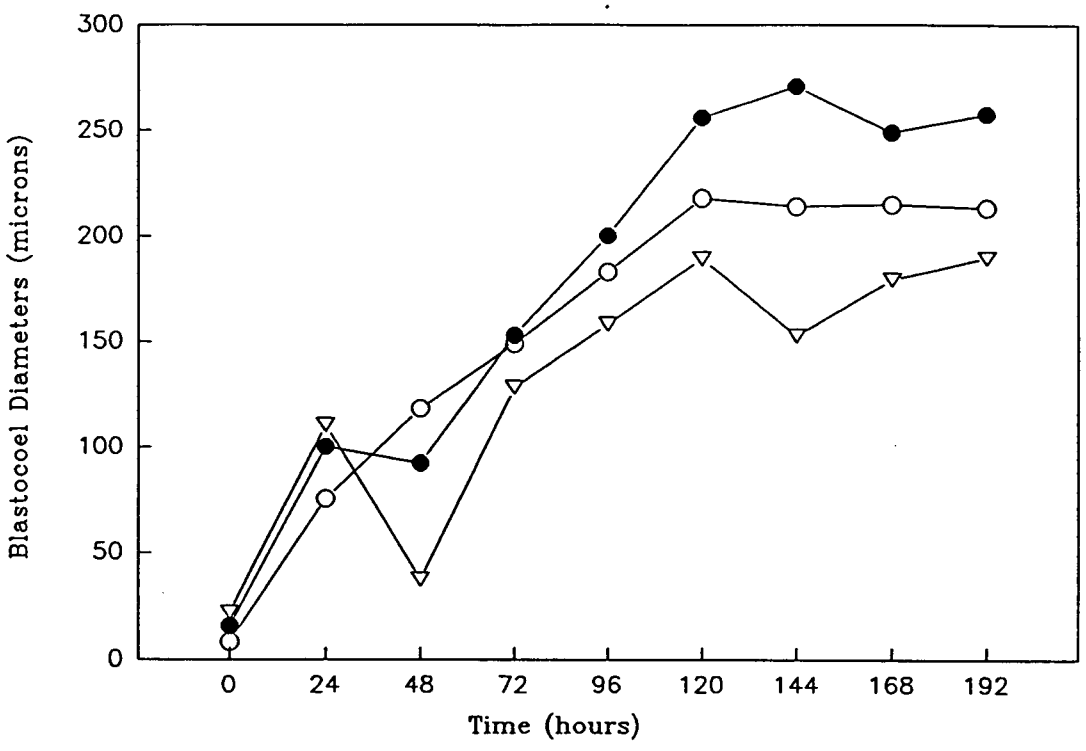


Figure I-5. Changes in blastocoel diameters (OD) for bovine embryos cultured in 0 (○), 10 (●) or 100 (▽) IU/ml PAI-2 by time.

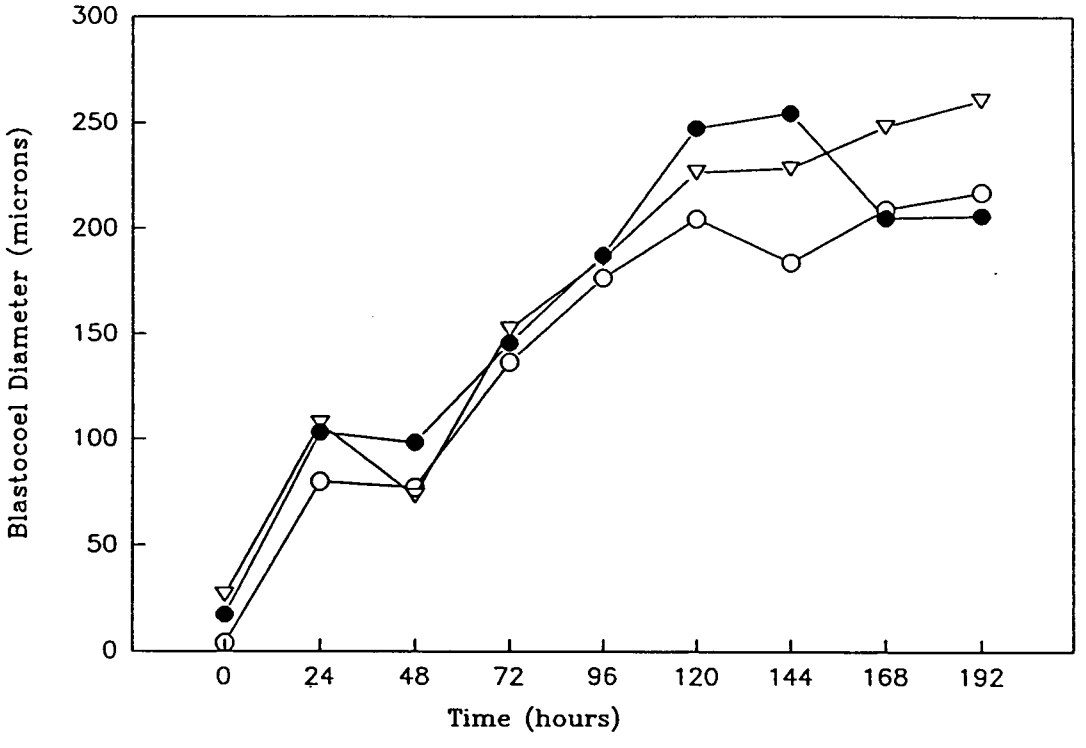


Figure I-6. Changes in zona pellucida thicknesses (ZPT) for bovine embryos cultured in 0 (○), .1 (●) or .5 (▽) nM ouabain by time.

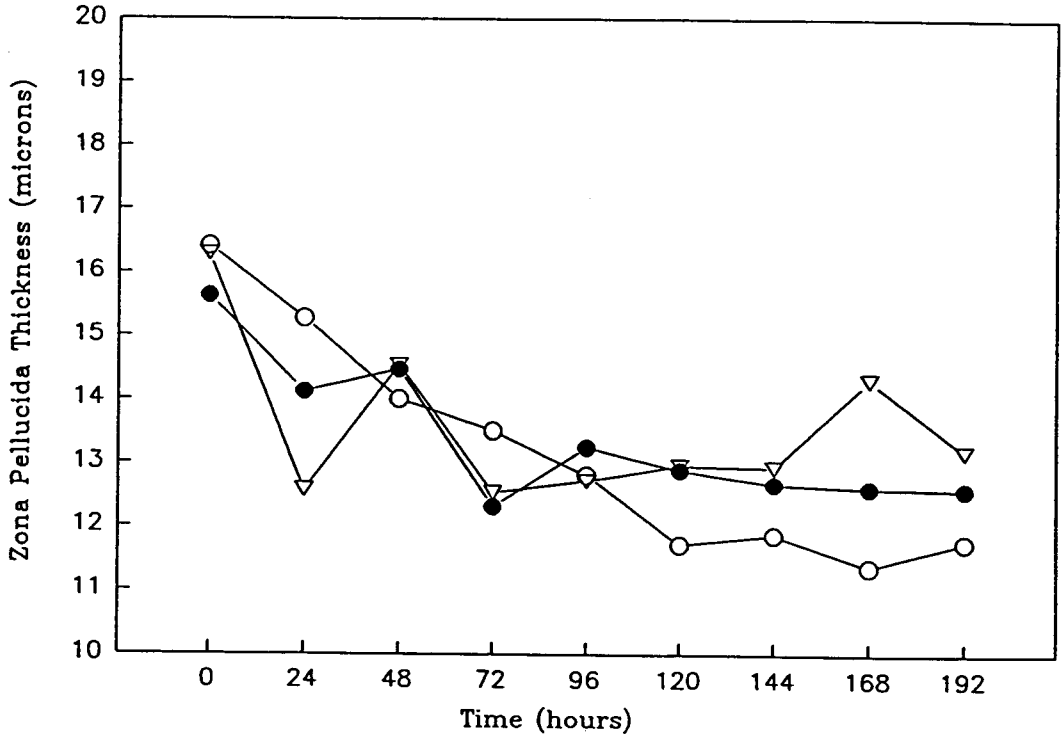


Figure I-7. Changes in zona pellucida thicknesses (ZPT) for bovine embryos cultured in 0 (○), 10 (●) or 100 (▽) IU/ml PAI-2 by time.

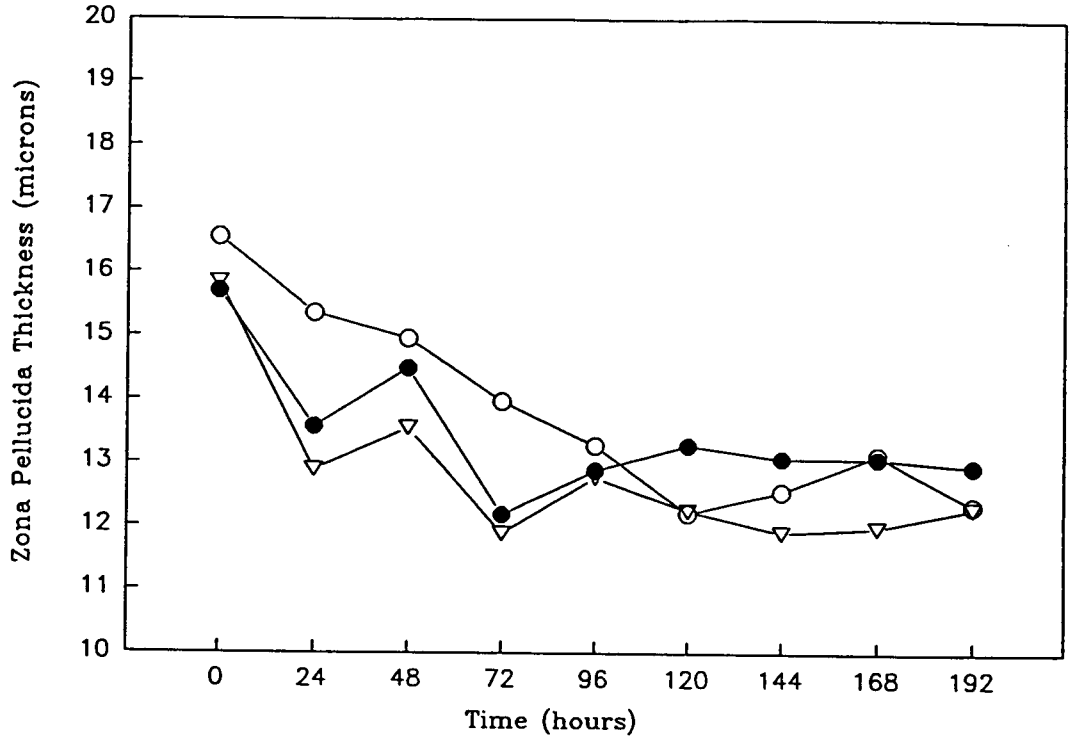


Figure I-8. Changes in plasminogen activator activity (PA) for bovine embryos cultured in 0 (○), .1 (●) or .5 (▽) nM ouabain by time.

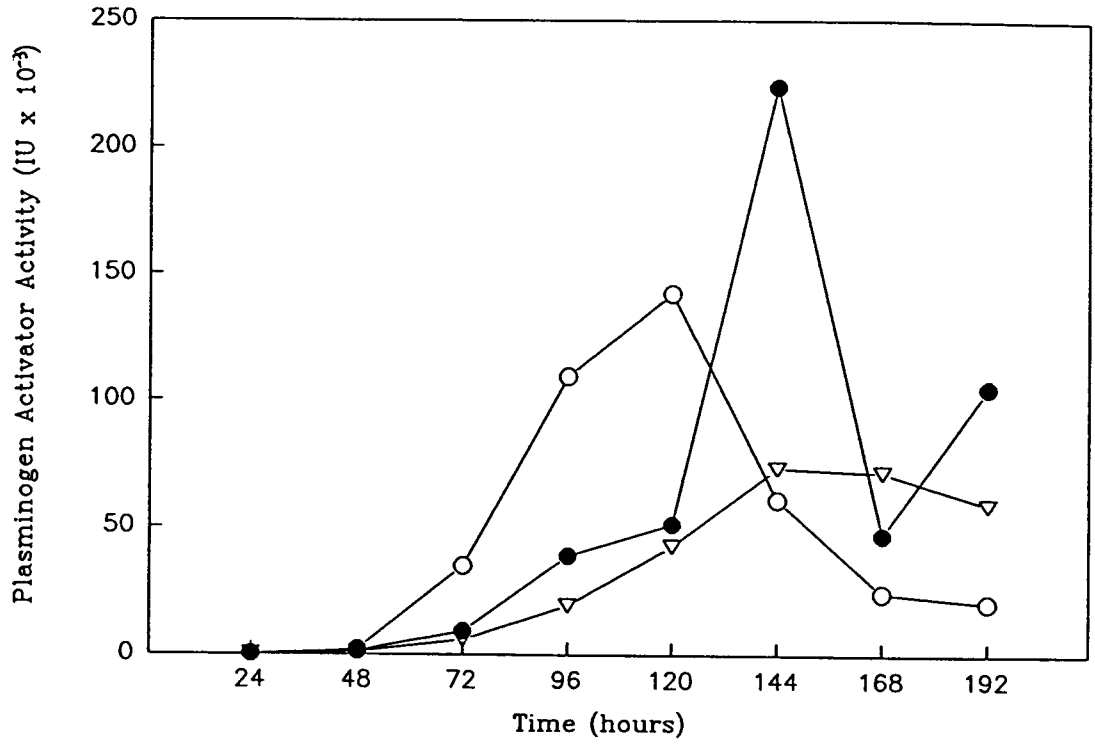


Figure I-9. Changes in plasminogen activator activity (PA) for bovine embryos cultured in 0 (○), 10 (●) or 100 (▽) IU/ml PAI-2 by time.

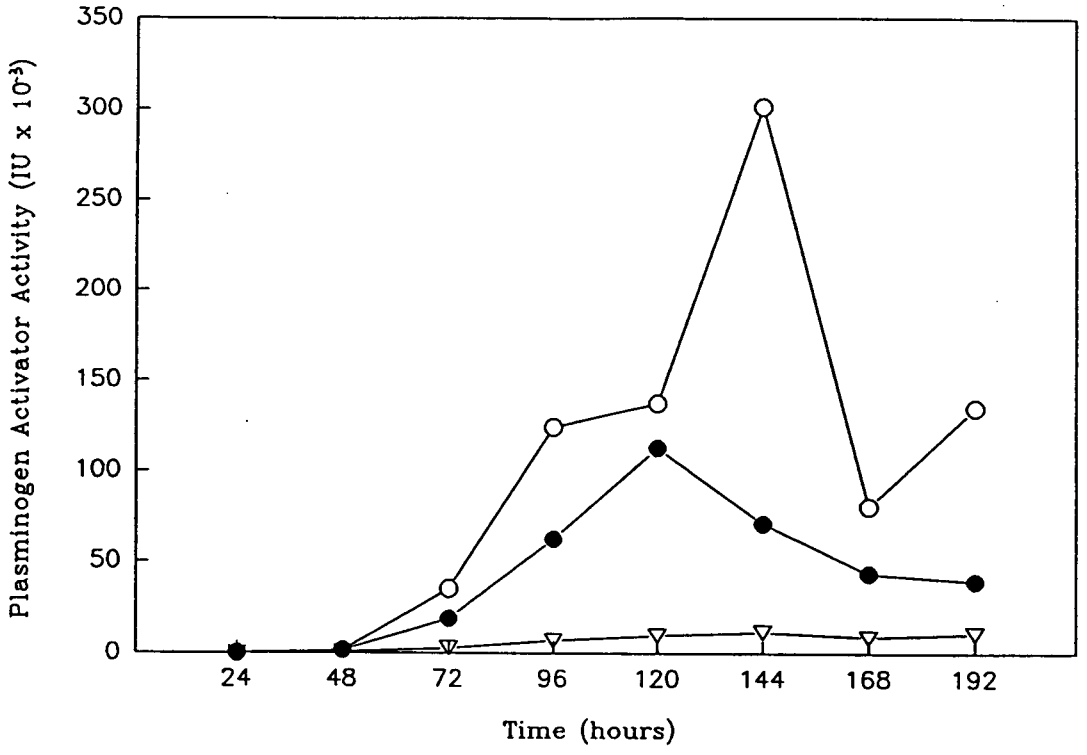
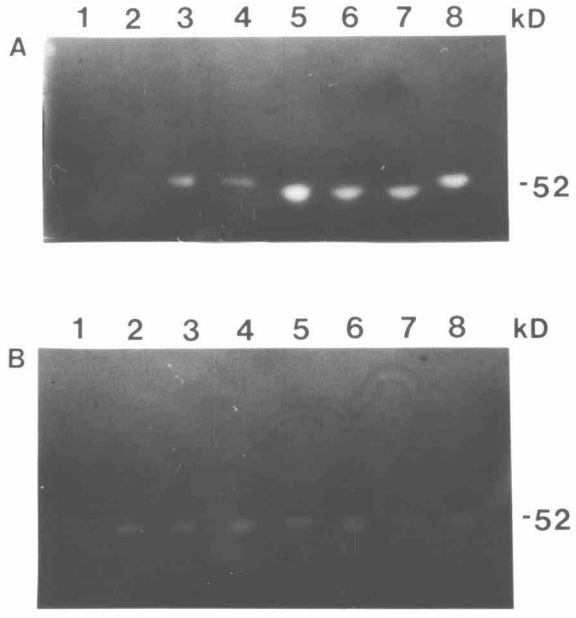


Figure I-10. Zymographic analysis of medium recovered from bovine embryos cultured in (A) 0 IU/ml or (B) 100 IU/ml plasminogen activator inhibitor-2 (PAI-2). Lanes 1, 2, 3, 4, 5, 6, 7 and 8 contain medium collected at 24, 48, 72, 96, 120, 144, 168 and 192 h, respectively.



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EFFECTS OF MODULATORS OF INTRACELLULAR PHOSPHORYLATION ON BOVINE EMBRYO DEVELOPMENT IN VITRO

Abstract

Effects of okadaic acid (OA), a phosphatase inhibitor and 6-dimethylaminopurine (DMAP), a kinase inhibitor, on hatching, plasminogen activator (PA) production and zona pellucida solubility in bovine embryos in vitro were examined. Day 6 embryos were nonsurgically collected and randomly assigned to one of five treatments: 0 (Control), 2.5 nM OA, 25 nM OA, 1 mM DMAP or 2 mM DMAP in Ham's F-12 with .15% bovine serum albumin (BSA). Embryos were cultured in 35-µl microdrops under paraffin oil for 192 h in a humidified atmosphere of 5% CO2 in air at 39°C. At 24-h intervals medium was recovered, embryos were transferred to fresh microdrops and overall embryo diameters (OD), zona pellucida thicknesses (ZPT) and blastocoel diameters (BD) were measured with an ocular micrometer. At the end of culture, zona pellucida-enclosed embryos and shed zonae pellucidae were recovered and placed in .2% SDS and the time required for complete dissolution of the zona pellucida (ZPDT) recorded. Plasminogen activator activities in the medium were determined using a caseinolytic assay. None of the embryos cultured in 2 mM DMAP medium developed to the blastocyst stage and although 50% developed into blastocysts in 1 mM DMAP, only 13% initiated hatching and none hatched. Embryos cultured in OA did not differ (P>.05) in development compared to the Control embryos. Embryos cultured in 25 nM

OA possessed similar (P>.05) OD, BD and ZPT but PA production was greater (P<.05) compared to the Control. Embryos cultured in 2.5 nM OA had reduced (P<.05) OD, BD and PA than Control embryos. Compared to the Control, embryos cultured in DMAP had reduced (P<.05) OD, BD and PA production. Zona pellucida dissolution time did not differ (P>.05) among the Control, OA and DMAP treatments. These results suggest that kinase activity is vital to processes in early embryo development and phosphatases may have a role in regulating PA production by bovine embryos.

Introduction

Plasminogen activators (PA) are serine proteases that convert the inactive zymogen, plasminogen, into the active protease, plasmin. Two types of PA have been identified on the basis of molecular weight, fibrin affinity and immunological reactivity: urokinase-type (uPA) and tissue-type (tPA) with molecular masses of 55,000 kD and 70,000 kD, respectively (Dano et al., 1985). It has been suggested that uPA participates in localized proteolysis of the extracellular matrix which accompanies tissue remodeling and cellular migration (Vassalli et al., 1976). Tissue-type PA has a high affinity for fibrin and is therefore considered to be a key enzyme in fibrinolysis (Astrup, 1978).

Plasminogen activators have been found in a wide variety of tissues, as well as in the embryos of mice (Strickland et al., 1976), rats (Liedholm and Astedt, 1975), pigs (Mullins et al., 1980), cattle (Menino and Williams, 1987) and sheep (Menino et al., 1989). The exact role of PA in early embryonic development is not well understood although it has been implicated in the migration of parietal endoderm cells and implantation in the mouse (Strickland et al., 1976; Axelrod, 1985; Sappino et al. 1989), hatching in cattle and sheep (Menino and Williams, 1987; Menino et al., 1989) and induction of meiotic maturation in the mouse and rat (Huarte et al., 1985).

Extracellular proteolytic activity of the PA system is regulated by levels of PA, plasminogen and PA inhibitors (PAI). There are several different agents which have been shown to modulate PA activity in a variety of cells (Dano et al, 1985). However, in bovine embryos, Al-Hozab and Menino (1992) reported that the pattern of PA production was unaffected following culture with several hormones or inducers of intracellular messengers, despite changes in embryonic dimensions. These results suggest that PA production cannot be influenced extrinsically through cell-signalling pathways. It is conceivable that PA is modulated by a signal transduction pathway but during early development the regulation cannot be externally modified. Therefore, the objective of this study was to determine if

modulation of intracellular phosphorylation by kinases and phosphatases would alter PA production and affect development of cultured Day 6 bovine embryos.

Materials and Methods

Embryo Collection and Culture. Eleven crossbred beef cows were estrous synchronized with prostaglandin $F_2\alpha$ ($PGF_2\alpha$; Lutylase, The UpJohn Co., Kalamazoo, MI, U.S.A.) and superovulated with porcine follicle stimulating hormone (pFSH; Schering Corp., Kenilworth, NJ, U.S.A.). Cows received two 25 mg injections of $PGF_2\alpha$ administered i.m. 12 d apart (Day 0 = first PGF2α injection). Cows received once-daily injections of pFSH at dosages of 12, 10, 8 and 6 mg per d on Days 10, 11, 12 and 13, respectively. Estrous detection was initiated 24 h after the second injection of $PGF_2\alpha$. Cows were handmated using 1 of 4 bulls at the onset of estrus and thereafter at 12-h intervals for as long as the cow would accept the bull.

Cows were flushed nonsurgically 6 d after mating with Dulbecco's phosphate buffered saline (DPBS) containing 2 ml/l heat-treated cow serum (HTCS) and 10 ml/l of antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, MO, U.S.A.). Embryos were recovered from the flushes by aspiration and washed three times in microdrops of Ham's

F-12 with .15% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO, U.S.A.) under paraffin oil (Fisher Scientific Co., Tustin, CA, U.S.A.) and morphologically evaluated at 100-200x magnification with an inverted-stage phase-contrast microscope.

Morphologically normal morulae and early blastocysts were cultured in Ham's F-12 with .15% BSA containing 2.5 nM or 25 nM okadaic acid (OA) or 1 mM or 2 mM 6-dimethylaminopurine (6-DMAP). Ham's F-12 with .15% BSA served as the Control for all treatments. Embryos were cultured for 192 h in 35-µl microdrops of medium under paraffin oil in a humidified atmosphere of 5% CO_2 in air at 39° C and observed daily for stage of development. At onset of culture and at 24-h intervals thereafter, embryos were transferred to fresh microdrops and overall embryo diameters (OD), zona pellucida thicknesses (ZT) and blastocoel diameters (BD) were measured using an ocular micrometer. Beginning 24 h after initiation of culture and continuing through 192 h of culture, medium was recovered and frozen at -20° C until assayed for levels of PA. To detect any nonspecific proteases and/or plasmin contamination in the culture medium, medium without embryos were incubated and stored under identical conditions.

Evaluation of Zona Pellucida Solubility. After 192 h of culture, zona pellucida-enclosed embryos and shed zonae pellucidae were recovered and washed three times in DPBS

with .1% BSA (DPBS + BSA). Five µl of DPBS + BSA containing one embryo or shed zona pellucida were transferred to a 50-µl microdrop of .2% SDS. Zonae pellucidae were continually observed for dissolution with an inverted-stage phase-contrast microscope at 200x magnification. The time between placement in .2% SDS and when the zona pellucida completely disappeared at 200x magnification was designated as the zona pellucida dissolution time (ZPDT).

Plasminogen Activator Assay. Plasminogen activator concentrations in the culture medium were determined using the caseinolytic assay as described by Kaaekuahiwi and Menino (1990) for bovine embryos with urokinase (American Diagnostica, Greenwich, CT, U.S.A.) as the standard.

Electrophoresis and Zymography. One-dimensional SDS-PAGE under non-reducing conditions and zymography (Granelli-Piperno et al., 1978; Vassalli et al., 1984) were used for evaluation of the PA produced by bovine embryos. Frozen culture medium was thawed and combined with one-half volume of 2x sample buffer. Urokinase standards were prepared with 1x sample buffer to final concentrations of .5 and .1 IU/ml. Each polyacrylamide gel included one lane containing molecular mass standards (Bio Rad Laboratories, Richmond, CA, U.S.A.). Aliquots of 75 µl of urokinase, molecular mass markers and culture medium were placed in castellated wells in a 4.0% acrylamide stacking gel with a 12.0% separating gel.

Following electrophoresis, gels were washed for 30 min in 2.5% Triton X-100 followed by a 30 min wash in DPBS. A 4% casein-2% agar gel containing 20 µg/ml purified human plasminogen (Sigma) supported on a glass plate (the zymogram) was applied to the surface of the polyacrylamide gel. Polyacrylamide gels with zymogram underlays were incubated for 24-36 h at 39°C. Incubation was terminated by separating the zymogram from the gel. Zymograms were fixed with 3% acetic acid for 15 min, dried and stained for permanent storage.

Statistical Analyses. Differences in the percentages of embryos developing to a particular cell stage due to treatment were determined by Chi-square procedures. Treatment effects on the times required for development to a particular cell stage and ZPDT were determined by one-way analysis of variance (ANOVA). Differences in OD, BD, ZPT and PA production due to treatment were determined using two-way ANOVA. Differences between means were evaluated using Duncan's new multiple range test. All analyses were performed using the NCSS Statistical Software Program (Number Cruncher Statistical System, version 4.1, 1984, Hintze, J.L., Kaysville, UT, U.S.A.).

Results

A total of 62 ova were collected from eleven cows and 46 embryos of good or excellent morphology were randomly assigned to the treatments.

Development to the hatched blastocyst stage did not differ (P>.05) among embryos cultured in 2.5 and 25 nM OA and the Control (Table 1). Compared to the Control, embryo development to the blastocyst, expanding blastocyst and hatched blastocyst stages was inhibited (P<.05) by DMAP. Time to completion of hatching was not significantly different for embryos cultured in either level of OA when compared to the Control (Table 2).

Overall diameter, BD, ZPT and PA were affected (P<.05) by the main effects of treatment and time and a treatment by time interaction (P<.05) persisted for BD and PA. Overall diameter, BD and ZPT of embryos cultured in 25 nM OA did not differ (P>.05) from the Control. However, 25 nM OA increased (P<.05) PA production (Table 3). Treatment with 2.5 nM OA reduced (P<.05) OD, BD and PA but had no effect (P>.05) on ZPT compared to the Control. Overall diameter, BD and PA production were reduced (P<.05) in both levels of DMAP. Although ZPDT was greater for treatments with poorer developing embryos, no differences (P>.05) were observed. Overall diameter and BD increased (P<.05) and eventually plateaued with time in culture (Figures 1 and 2), whereas

ZPT decreased (P<.05) and remained constant (Figure 3). Plasminogen activator production (Figure 4) increased (P<.05) to a peak at 96 h and eventually declined. Significant interactions for BD and PA are due to the divergent patterns observed for these parameters by embryos cultured in DMAP vs OA.

Zymographic analysis of medium recovered from the various cultures are reported in Figure 5. Only a single lytic band (52 kD) was detected in medium from the Control and 2.5 and 25 nM OA, confirming production of a uPA. No high molecular mass species was observed. No lytic bands were observed in medium recovered from cultures containing 1 or 2 mM DMAP.

Discussion

Intracellular signal transduction pathways are believed to regulate biological processes through a series of phosphorylations and dephosphorylations of proteins involved in the biological response (Cohen and Cohen, 1989). Two well-known signal transduction pathways are those that utilize adenylate cyclase and phospholipase C. Activation of adenylate cyclase increases intracellular cyclic adenosine monophosphate (cAMP) levels, a second messenger which in turn activates cAMP-dependent protein kinase A

(PKA). Activated phospholipase C hydrolyses phosphoinositides resulting in the production of diacylglycerol (DAG) and phosphoinositol triphosphate (IP-3) which activate protein kinase C (PKC) and mobilize Ca^{+2} stored in the endoplasmic reticulum. Once a kinase (PKC or PKA) has been activated it will phosphorylate certain proteins, having the effect of either activating or deactivating them. It is conceivable therefore, that modulation of protein phosphatase and kinase activity may reverse the effect of proteins on a given biological process by changing it's state of activity.

In this study, PA activity was effectively inhibited and development also completely blocked when embryos were exposed to 2 mM DMAP. Embryos cultured in 1 mM DMAP also had reduced PA production and poor embryo development. 6-dimethylaminopurine blocks phosphorylation and cell division without affecting protein synthesis (Neant et al., 1988). Neant et al. (1988) demonstrated in vitro that DMAP inhibited cAMP and Ca^{+2}-dependent protein kinases. Rime et al. (1989) reported that DMAP may be interfering with the burst of phosphorylation associated with maturation promoting factor (MPF) activity in the first meiotic cell division in the mouse embryo. The poor development and limited PA production may have resulted from the effects of 6-DMAP on cell division.

Important modulators of PA activity are PA inhibitors. It is possible that some treatments were increasing the production of PAI resulting in a decrease in measurable levels of PA. Liu et al. (1991) reported injection of hCG caused an increase in tPA activity in the ovarian extracellular fluid (OEF) which reached a peak immediately prior to ovulation. Plasminogen activator inhibitor-1 was also found in the OEF and the level was inverse in relation to the tPA level. These observations would suggest that neutralization by PAI occurs before the PA surge can take place. Plasminogen activator inhibitors have been identified in the bovine (third manuscript of this thesis) but their role in regards to the regulation of PA production and hatching has not been elucidated. Okadaic acid is a potent inhibitor of both protein phosphatases 1 and 2A. In cell free extracts, OA inhibits protein phosphatase 2A at low concentrations (.1 nM) and protein phosphatase 1 at high concentrations (125 nM) (MacKintosh et al., 1989). In this study, 2.5 nM OA decreased PA production whereas embryos cultured in 25 nM OA increased PA production. Because high molecular mass lytic bands, indicative of PA-PAI complexes, were not detected in the zymograms, it is unlikely that 2.5 nM OA elicited PAI production. Development of embryos in both levels of OA did not differ from the Control, hence OA did not have a deleterious effect on embryo viability.

Nagamine et al. (1983) reported that in the porcine renal

epithelial line, LLC-PK cells, the uPA gene is induced independently of PKA and PKC. Both of these kinases phosphorylate proteins at serine or threonine residues in specific sequences that are dephosphorylated by protein phosphatases 1 and 2A (Cohen and Cohen, 1989). Nagamine et al. (1991) examined uPA gene expression in LLC-PK cells which had been induced by PKA and PKC in the presence or absence of OA. High levels of OA by itself strongly induced uPA mRNA accumulation. This implies that a significant level of phosphorylation of a factor that is involved in uPA gene regulation occurs constitutively; this factor is normally dephosphorylated by the action of OA-sensitive protein phosphatases. Nagamine et al. (1991) also reported that OA potentiated the effect of PKA and PKC induced uPA mRNA accumulation but only at high concentrations. They tested the hypothesis that OA-dependent uPA mRNA induction required protein synthesis by treating cells with OA in the presence and absence of cycloheximide, an inhibitor of eukaryotic protein synthesis. Six h after treatment, 90% of uPA mRNA accumulation was suppressed whereas PKA- and PKC-induced uPA mRNA accumulation was enhanced. This provides support for the possibility that OA activates a different pathway than that used to mediate PKA or PKC in uPA gene expression. The observation that PA activity was reduced by 2.5 nM OA and increased by 25 nM OA suggests that

phosphatases 1 and 2A may be having different effects on PA production.

Our study supports the hypothesis that regulation via intracellular phosphorylation is important in PA production. It would also seem that inhibition of kinases has a deleterious effect on overall embryo viability as well as significantly inhibiting the ability of the embryo to produce PA. Okadaic acid can modulate uPA production suggesting that phosphatases are working either through a different signal transduction pathway or downstream of a second messenger stimulated pathway.

Table II-1. Development of bovine embryos in Ham's F-12 with .15% BSA containing okadaic acid (OA) or 6-dimethylaminopurine (DMAP).

		Number (%) of embryos developing to the:			
Treatment	n	blastocyst	Expanded blastocyst	Hatching blastocyst	Hatched blastocyst
Control	14	13 (93)[a]	12 (86)[a]	12 (86)[a]	9 (64)[a]
2.5 nM OA	7	5 (71)[a,b]	4 (57)[a,b]	4 (57)[a,b]	4 (57)[a]
25 nM OA	9	9 (100)[a]	9 (100)[a]	8 (89)[a]	7 (78)[a]
1.0 mM DMAP	8	4 (50)[b]	2 (25)[b]	1 (13)[b,c]	0 (--)[b]
2.0 mM DMAP	8	0 (--)[c]	0 (--)[c]	0 (--)[c]	0 (--)[b]

[a,b,c] Values without common superscripts are different ($P<.05$)

Table II-2. Time to blastocyst formation, blastocoelic expansion, hatching and completion of hatching for bovine embryos cultured in medium containing okadaic acid (OA) or 6-dimethylaminopurine (DMAP).

	Development to the following stages:					
	Expanded blastocyst		Hatching blastocyst		Hatched blastocyst	
Trtment	n	$\bar{x}$ ± SE	n	$\bar{x}$ ± SE	n	$\bar{x}$ ± SE
Control	12	62.0 ± 8.9	11	87.3 ± 10.5	9	98.7 ± 9.7
2.5 OA	4	48.0 ± 15.4	4	72.0 ± 17.5	4	90.0 ± 14.6
25 OA	9	50.7 ± 10.3	8	63.0 ± 12.4	7	78.9 ± 11.0
1.0 DMAP	2	72.0 ± 21.8	1	96.0 ± 35.0	0	-----
2.0 DMAP	0	-----	0	-----	0	-----

Table II-3. Mean overall diameters (OD), blastocoel diameters (BD), zona pellucida thicknesses (ZPT), plasminogen activator production (PA) and zona pellucida dissolution times (ZPDT) for bovine embryos cultured in medium containing okadaic acid (OA) or 6-dimethylaminopurine (DMAP).

	OD (μ)	BD (μ)	ZPT (μ)	ZPDT (min)	PA (IU x 10^{-3}/d)
Trtmnt	$\bar{x}$ ± S.E.	$\bar{x}$ ± S.E.	$\bar{x}$ ± S.E.	$\bar{x}$ ± S.E.	$\bar{x}$ ± S.E.
25 OA	198.8 ± 4.7^{b,c}	135.5 ± 7.8^{c}	11.6 ± .3^{a,b}	8.9 ± .4^{a}	4.1 ± .3^{c}
2.5 OA	196.3 ± 5.4^{b}	97.5 ± 8.8^{b}	11.3 ± .3^{a}	10.8 ± .5^{a}	0.7 ± .4^{a}
2.0 DMAP	155.1 ± 5.0^{a}	8.7 ± 8.3^{a}	13.0 ± .3^{c}	12.1 ± .5^{a}	0.0 ± .3^{a}
1.0 DMAP	168.6 ± 5.0^{a}	28.6 ± 8.3^{a}	12.1 ± .3^{b}	11.7 ± .5^{a}	0.1 ± .3^{a}
Control	211.4 ± 3.9^{c}	142.4 ± 6.5^{c}	11.6 ± .2^{a,b}	8.9 ± .4^{a}	2.2 ± .3^{b}

^{a,b,c} Values without common superscripts are different ($P<.05$)

Figure II-1. Changes in overall diameters (OD) for bovine embryos cultured in Control (□), 2.5 nM OA (●), 25 nM OA (○), 2 mM DMAP (▽) or 1 mM DMAP (▼).

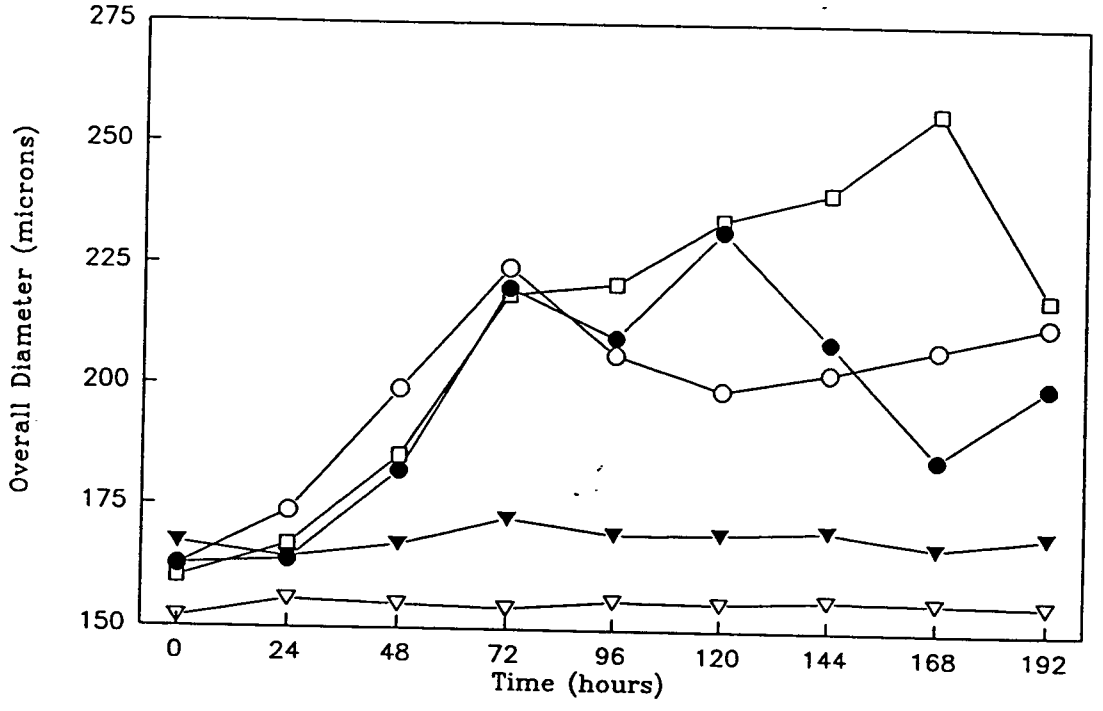


Figure II-2. Changes in blastocoel diameters (BD) for bovine embryos cultured in Control (□), 2.5 nM OA (●), 25 nM OA (○), 2 mM DMAP (▽) or 1 mM DMAP (▼).

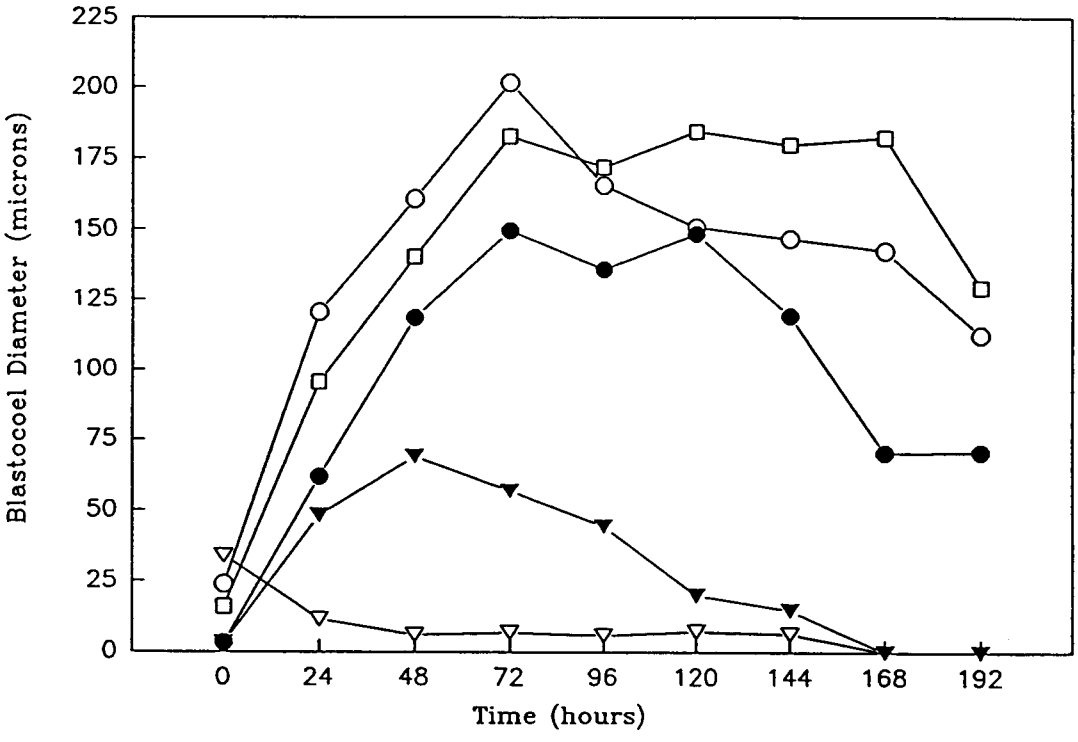


Figure II-3. Changes in zona pellucida thicknesses (ZPT) for bovine embryos cultured in Control (□), 2.5 nM OA (●), 25 nM OA (○), 2 mM DMAP (▽) or 1 mM DMAP (▼).

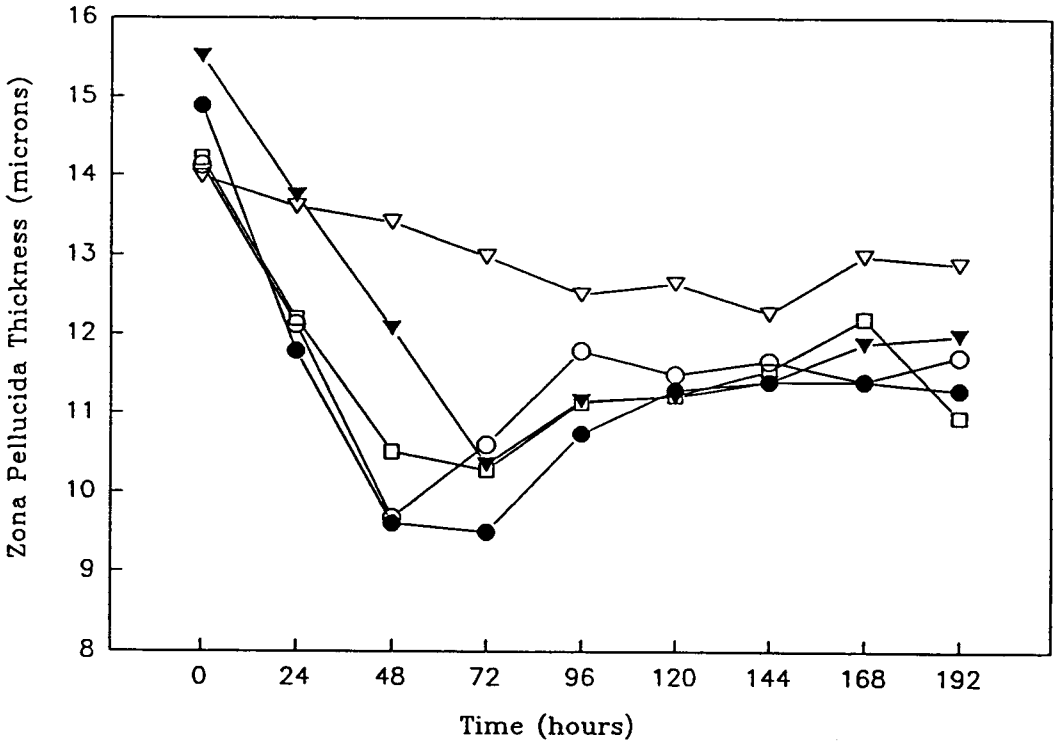


Figure II-4. Changes in plasminogen activator activity for bovine embryos cultured in Control (□), 2.5 nM OA (●), 25 nM OA (○), 2 mM DMAP (▽) or 1 mM DMAP (▼).

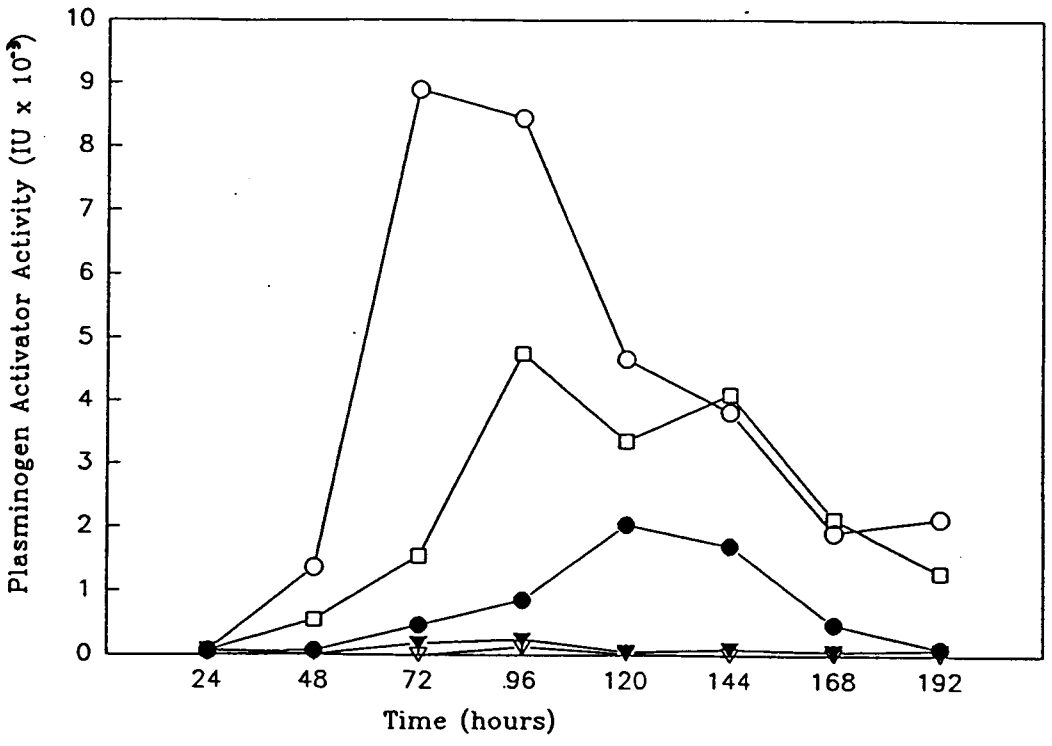
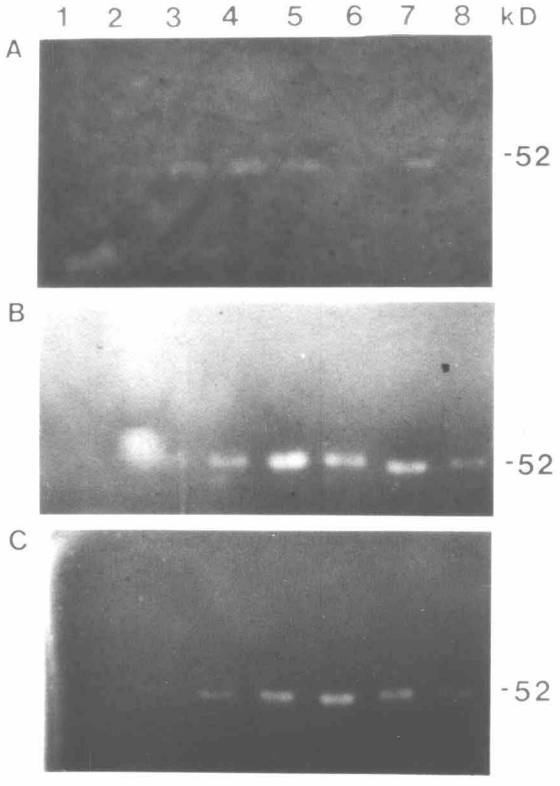


Figure II-5. Zymographic analysis of medium recovered from bovine embryos cultured in (A) 2.5 nM or, (B) 25 nM okadaic acid. Panel C is a zymograph of medium from embryos cultured in the Control. Lanes 1, 2, 3, 4, 5, 6, 7 and 8 contain medium collected at 24, 48, 72, 96, 120, 144, 168 and 192 h, respectively.



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IDENTIFICATION OF THE PLASMINOGEN ACTIVATORS PRODUCED BY BOVINE EMBRYOS IN VITRO

Abstract

Characterization of the plasminogen activators (PA) produced by bovine embryos in vitro was conducted. Day 12 and 14 embryos were cultured for 120 and 96 h, respectively, in Ham's F-12 containing .15% BSA in a humidified atmosphere of 5% CO_2 in air at 39° C. Medium was recovered at 24-h intervals and embryos were extracted following culture for PA analysis. Characterization of the type of PA produced by bovine embryos was performed using SDS-PAGE and zymography with amiloride, a competitive inhibitor of urokinase-type PA (uPA), and immunoprecipitation. Medium and embryo extracts were treated with phosphate buffered saline (PBS), nonspecific goat immunoglobins (NSIgG) or goat antibodies to human uPA (anti-uPA), tissue-type PA (anti-tPA) and PA inhibitors 1 and 2 (anti-PAI-1 and anti-PAI-2, respectively) and immunoprecipitated with a suspension of Protein G-bearing Streptococcus cells. For the immunoprecipitation experiments, lytic zone areas in the zymographs were quantified densitometrically and expressed relative to the PBS treatment. Day 12 bovine embryos produced two plasminogen-dependent lytic zones (53.6 and 89.7 kD) whereas Day 14 embryos produced three plasminogen-dependent lytic zones
(53.4, 82.0 and 90.5 kD). Total lytic area in the supernatant was lower ($P<.05$) when medium and embryo

extracts were treated with anti-uPA compared to anti-PAI-1, anti-tPA and NSIgG. Treatment with anti-PAI-2 reduced ($P<.05$) total lytic area in the supernatant compared to anti-tPA and NSIgG and no differences ($P>.05$) in areas were detected among treatments with anti-PAI-1, anti-tPA and NSIgG. Total lytic area in the precipitate was greatest when medium and extracts were treated with anti-uPA. When 10 mM amiloride was included in the zymograph, PA activity in the medium and embryo extracts were completely inhibited. These results suggest that Day 12-14 bovine embryos possess an uPA and possibly a PAI which binds the uPA and forms a high molecular mass PA complex.

Introduction

Plasminogen activators (PA) are serine proteases that convert the inactive zymogen, plasminogen, to the active enzyme plasmin (Christman et al., 1977). Two types of PA have been identified and well-characterized: urokinase-type PA (uPA) with a molecular mass of 30-55 kD and tissue-type PA (tPA) with a molecular mass of approximately 72 kD. Plasminogen activators have been implicated in a variety of reproductive functions, including; spermatogenesis (Hettle et al., 1986), implantation (Strickland et al., 1976; Sappino et al., 1989) and ovulation (Beers, 1975). Menino

and Williams (1987) observed in bovine embryos that PA production could not be detected until the blastocyst stage but increased during blastocoelic expansion and remained elevated after hatching. Kaaekuahiwi and Menino (1990) found bovine PA to be positively correlated to embryonic size, developmental stage and cell number and observed that hatching embryos released more PA than embryos that failed to hatch. Dyk and Menino (1991) using SDS-PAGE and zymography, observed that Day 12-14 bovine embryos produced two lytic bands, a low molecular mass form (41.5 - 47 kD), suspected as a uPA, and a higher molecular mass form (86.1 - 92.2 kD). Berg and Menino (1992) used antibodies to uPA and tPA and the urokinase inhibitor, amiloride, to identify the type of PA produced by bovine embryos. All PA activity was eliminated in amiloride, confirming that Day 12-14 embryos produced uPA and possibly a binding protein or plasminogen activator inhibitor (PAI) that complexed with the uPA.

The objective of this experiment was to investigate whether the high molecular mass lytic band reported is a uPA-PAI complex.

Materials and Methods

Embryo Collection and Culture. Six crossbred beef cows were estrous synchronized with prostaglandin $F_2\alpha$ ($PGF_2\alpha$;

Lutalyse, The Upjohn Co., Kalamazoo, MI, U.S.A.) and superovulated with porcine follicle stimulating hormone (pFSH; Schering Corp., Kenilworth, NJ., U.S.A.). Two 25 mg injections of $PGF_2\alpha$ were administered i.m. 12 days apart (Day 0 = first $PGF_2\alpha$ injection). Cows received once daily injections of pFSH at dosages of 12, 10, 8 and 6 mg on Days 10, 11, 12 and 13, respectively. Estrous detection was initiated 24 h after the second injection of $PGF_2\alpha$. Cows were handmated using 1 of 2 bulls at the onset of estrus and thereafter at 12-h intervals for as long as the cow would accept the bull. Cows were slaughtered 12 or 14 days after mating and the reproductive tracts flushed with Ham's F-12 (Sigma Chemical Co., St. Louis, MO, U.S.A.). Flushings were examined with a dissecting microscope and embryos were collected from the flushings by aspiration. Embryos were washed three times in microdrops of Ham's F-12 with .15% bovine serum albumin (BSA; Sigma) under paraffin oil (Fisher Scientific Co., Tustin, CA, U.S.A.) and morphologically evaluated at 100x magnification with an inverted-stage phase-contrast microscope. Morphologically normal blastocysts were cultured for 96 and 120 h respectively, in 100 µl microdrops of Ham's F-12 with .15% BSA under paraffin oil in a humidified atmosphere of 5% CO_2 in air at 39°C. At 24-h intervals, 50 µl of medium were recovered from the microdrops and replaced with an equivalent volume and embryos were observed for stage of development. At the end

of culture all embryos were recovered and frozen at -20°C until electrophoresis. Medium without embryos were incubated and stored under identical conditions in order to detect any nonspecific proteases in the culture medium.

Electrophoresis and Zymography. One-dimensional SDS-PAGE under non-reducing conditions with zymography (Granelli-Piperno et al., 1978; Vassalli et al., 1984) was used for evaluation of the PA produced by bovine embryos. Frozen embryonic tissues were thawed and solubilized in SDS-solubilization buffer (Huarte et al., 1985). Extracted embryo tissues and culture medium were combined with equal volumes of 2X sample buffer. Urokinase standards were prepared with 1X sample buffer to final concentrations of 1.0 and .5 IU/ml. Each polyacrylamide gel included one lane containing molecular mass standards (Bio Rad Laboratories, Richmond, CA, U.S.A.). Seventy-five microliter aliquots of urokinase and molecular mass markers and 100 µl aliquots of embryonic tissue and medium samples were placed in castellated wells in a 4.0% acrylamide stacking gel with a 12.0% separating gel. Electrophoresis was conducted at 15 mA/gel through the stacking gel and 30 mA/gel through the separating gel for 2-4 h until the dye reached the bottom of the separating gel.

Following electrophoresis, gels were washed in 2.5% Triton X-100 for 30 min and in phosphate-buffered saline (PBS) for 30 minutes. A casein-agar gel containing purified

human plasminogen (20 µg/ml; Sigma) supported on a glass plate (the zymogram) was applied to the surface of the polyacrylamide gel. Zymograms were also prepared without plasminogen to detect non-specific proteases. Zymograms were prepared with 4% non-fat dry milk (Carnation Co., Los Angeles, CA, U.S.A.) and 2% agarose. Amiloride, a competitive inhibitor of uPA (Vassalli et al., 1987) was also added to some zymograms at a fixed concentration of 10 mM. Polyacrylamide gels with zymogram underlays were incubated for 24-48 h at 39°C. Zymogram incubation was terminated by separation from the polyacrylamide gel and fixing the zymogram with 3% acetic acid for 15 minutes. Zymograms were dried and stained for permanent storage.

Immunoprecipitation. For the immunoprecipitation experiments, 15 µl of embryonic tissue extracts were combined with 30 µl of PBS or 1 mg/ml non-specific goat immunoglobulins (NSIgG) (Sigma) or goat immunoglobulins raised against human uPA (anti-uPA), tPA (anti-tPA), PAI-1 (anti-PAI-1) or PAI-2 (anti-PAI-2) (American Diagnostica Inc., Greenwich, CT, U.S.A.) and incubated overnight at 4°C. Thirty µl of Protein G bearing Streptococcus cells were added to the samples and incubated for 2.5 h at room temperature. Samples were centrifuged with a Beckman microfuge and the supernatant was recovered. The pellet was rinsed three times with PBS by vortexing and centrifugation. Supernatants and precipitates were combined with SDS sample

buffer and evaluated for PA using SDS-PAGE and zymography. Lytic zones in the zymogram were quantified densitometrically and lytic areas were expressed relative to the PBS treatment. All experiments were replicated at least three times.

Statistical Analysis. Molecular masses of urokinase standards and medium and embryonic tissue samples were calculated from the equation of the line for log molecular mass by relative mobility. Differences among the relative densitometric areas of the lytic bands in the supernatants and precipitates were determined using ANOVA and least significant procedures. All analysis were performed using the NCSS Statistical Software Program (Number Cruncher Statistical System, Version 4.1, 1984, Hintze, IL, Kaysville, UT, U.S.A.).

Results

Thirty five Day 12 and 33 Day 14 embryos with normal morphology were collected from six cows for a mean recovery rate of 5.8 blastocysts per cow. One to seven embryos were cultured in each 100-µl microdrop.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and zymographic analysis of Day 12 and 14 bovine embryos exhibited 2 and 3 lytic bands, respectively.

Day 12 embryos produced a light (53.6 ± 0.9 kDa) and heavy (89.7 ± 1.3 kDa) band whereas Day 14 embryos produced a light (53.6 ± 1.1 kDa) and two heavy (82.0 ± 1.4; 90.5 ± 1.6 kDa) bands (Table 1; Figure 1). Addition of amiloride to bovine embryonic tissues abolished PA activity in both the low and high molecular mass bands (Figure 2).

Relative densitometric areas of the lytic zones were reduced ($P<.05$) in the supernatant fraction when samples were treated with anti-uPA and anti-PAI-2 (Table 2; Figure 3, Panel A). When samples were treated with anti-uPA and anti-PAI-1 relative densitometric areas in the pellet fraction, although greater, did not differ from treatments with NSIgG, anti-tPA or anti-PAI-2 (Table 3; Figure 3, Panel B).

Discussion

Zymographic analysis revealed that Day 12 embryos produce 2 plasminogen-dependent lytic zones and Day 14 embryos produce 3 plasminogen-dependent lytic zones (53.4, 82.0 and 90.5). These results are similar to those reported by Dyk and Menino (1991) although in this report only one high molecular mass band was observed. According to Dano et

al. (1985) the 53 kD species would be classified as uPA. The heavy form could be a tPA but the present experiments indicate that it is more likely a PAI complexing with the uPA. Two PAI have been particularly well-characterized, plasminogen activator inhibitor-1 (PAI-1; MW=52,000) a potent inhibitor of tPA and plasminogen activator inhibitor-2 (PAI-2; MW=60,000) which binds to uPA with greater affinity than tPA (Andreasen et al., 1990).

Vassalli et al. (1987) reported that amiloride is a specific and competitive inhibitor of uPA and has no inhibitory effect on tPA. When amiloride was added to the zymogram, PA activity was completely abolished, indicating that PA activity in all the lytic zones was due to uPA. Medium and embryo extracts treated with anti-uPA had reduced activity in the supernatant and increased activities in the pellets whereas anti-tPA had little effect. Treatment with anti-PAI-2 reduced total lytic area in the supernatant compared to NSIgG, suggesting that a PAI-2-like molecule may be complexing with the uPA and forming a high molecular mass complex. Interestingly, PA-PAI complexes retain PA activity in zymogram (Andreasen et al., 1990).

The role of PA in early bovine embryo development is not known. Strickland et al. (1976) and Sherman et al. (1976) characterized a biphasic pattern of PA production by mouse embryos. The first phase was produced by trophoblast cells whereas parietal endoderm produced the second phase.

Sappino et al. (1989) proposed that the first phase of PA production by mouse embryos is involved in trophoblast invasiveness during implantation and that the second phase is involved with migration of the parietal endoderm cells along the yolk sac cavity and through Reichert's membrane.

Unlike rodents and primates, who experience invasive implantation where the blastocyst actually penetrates the endometrium, implantation in ruminants, and in swine, occurs through interdigitation of trophoblast and endometrial microvilli. Fazleabas et al. (1983) reported that Day 10-16 pig embryos also release PA in a biphasic pattern. The first phase (10-12 days) coincides with blastocyst elongation and the second phase (14-16 days) occurs when embryonic DNA is significantly increasing. Mullins et al. (1980) reported that the pig endometrium releases a PA or plasmin inhibitor under the influence of maternal progesterone which could explain how the uterine epithelium resists trophoblastic invasion.

Because trophoblast from species with noninvasive placentation have invasive potential (Samuel, 1971), regulation of PA may be a key determinant in preventing implantation. This is supported by Axelrod (1985) who observed reduced PA activity and invasive ability in implantation-defective mutant mouse embryos. Bovine embryos do not start to attach until Day 28-32 (Betteridge and Flechon, 1988). It is possible that the bovine embryonic PA

we have observed is involved with penetration of the embryonic disc through Rauber's layer of trophectoderm or mesodermal migration between the trophectoderm and endoderm. Both events occur between Days 12 and 14 in the bovine. Further investigation into the role of PA and PAI in the developing blastocyst is needed to determine its physiological significance in early bovine embryogenesis.

Table III-1. Molecular masses[a] (kD) of plasminogen-dependent lytic zones produced by cultured Day 12 and 14 bovine embryos.

	Embryo Age	
Form	Day 12	Day 14
High	89.7 ± 1.3	90.5 ± 1.6
High	------	82.0 ± 1.4
Low	53.6 ± 0.9	53.4 ± 1.1

[a] Values reported are means ± standard errors of the means

Table III-2. Relative densitometric areas[a] of caseinolytic zones produced by the supernatant fraction of immunoprecipitated Day 12 and 14 bovine embryonic samples.

	Mean relative densitometric area		
Treatment	High	Low	Total
NSIgG	1.14[c]	.94[c]	.96[d]
Anti-uPA	.46[b]	.49[b]	.51[b]
Anti-tPA	1.06[c]	.85[c]	.87[d]
Anti-PAI-1	.81[b,c]	.75[b,c]	.79[c,d]
Anti-PAI-2	.80[b,c]	.55[b]	.61[b,c]
S.E.	.13	.09	.07

[a] Relative densitometric areas were computed by dividing the densitometric area (cm^2) of each treatment by the PBS treatment for a corresponding lytic zone.

[b,c,d] Means in the same column without common superscripts are different ($P<.05$).

Table III-3. Relative densitometric areas[a] of caseinolytic zones produced by the pellet fraction of immunoprecipitated Day 12 and 14 bovine embryonic samples.

	Mean relative densitometric area		
Treatment	High	Low	Total
NSIgG	1.18	.92	.88
Anti-uPA	1.47	1.89	1.62
Anti-tPA	.71	.92	.79
Anti-PAI-1	1.86	1.19	1.32
Anti-PAI-2	1.10	.83	.86
S.E.	.36	.36	.31

[a] Relative densitometric areas were computed by dividing the densitometric area (cm^2) of each treatment by the PBS treatment for a corresponding lytic zone.

Figure III-1. Zymographic analysis of culture medium recovered from Day 14 bovine embryos. Lane N contains control medium, Lane U contains 1.0 IU/ml human urokinase.

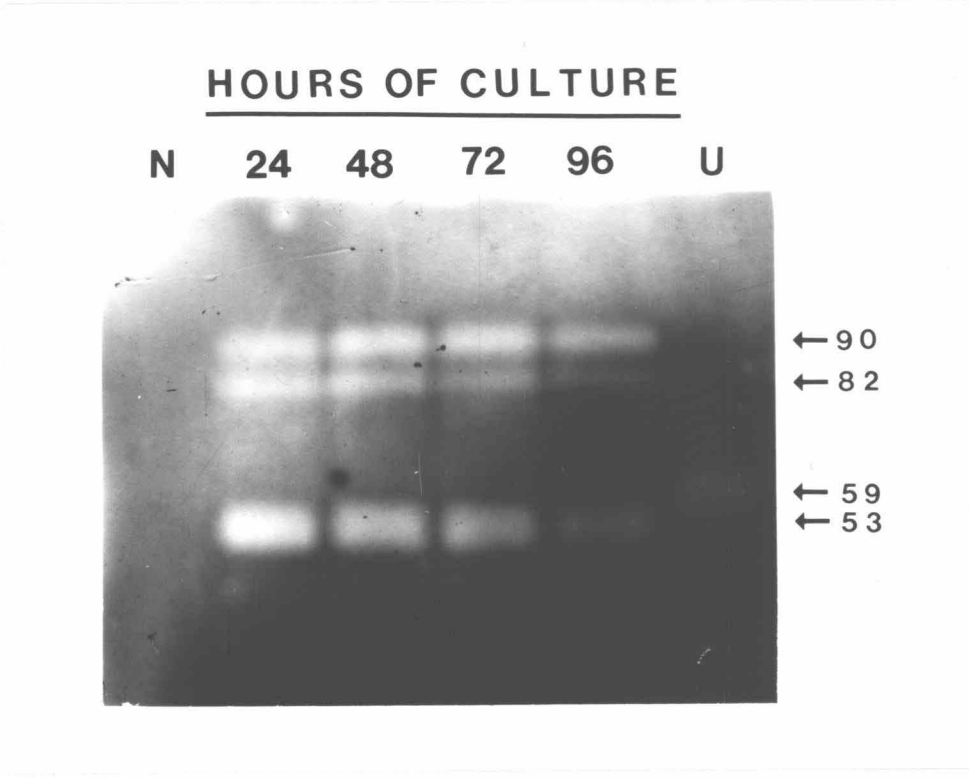


Figure III-2. Zymographic analysis of bovine embryonic plasminogen activator. Lanes 1 and 3 contain control medium, lanes 2 and 4 contain Day 14 bovine embryonic tissues. Amiloride (AMR; 10 mM) inhibited PA activity in the embryonic extracts.

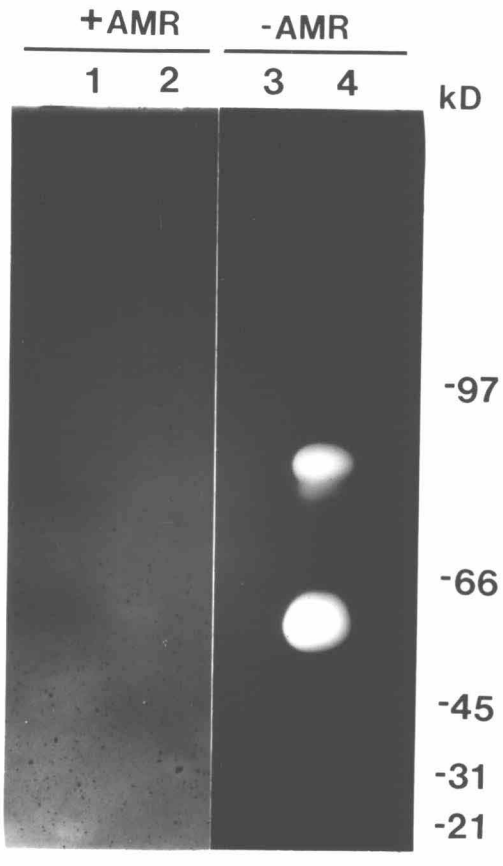
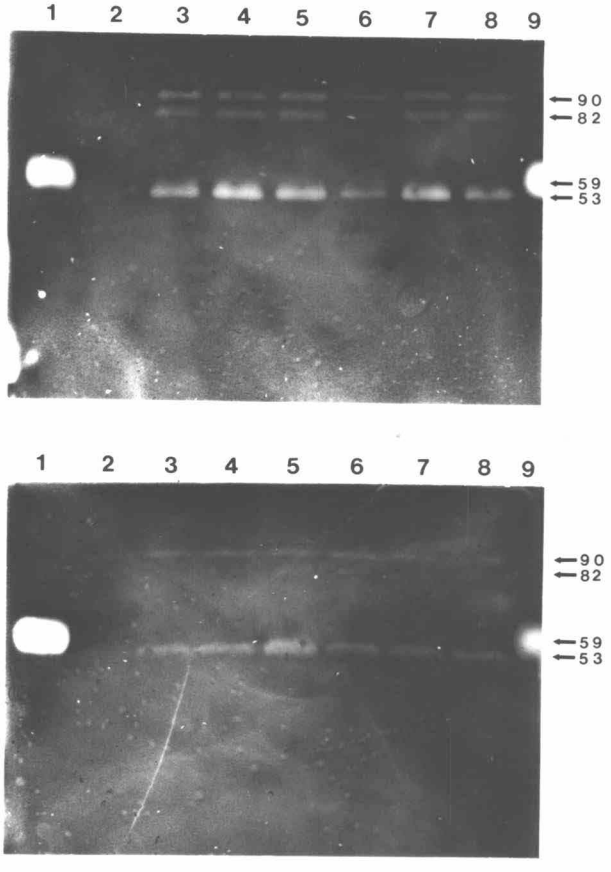


Figure III-3. Zymographic analysis of supernatant (Panel A) and pellet (Panel B) fractions of immunoprecipitated Day 14 bovine embryonic tissues. Panel A: lane 1, 0.5 IU/ml urokinase; lane 2, control medium; lane 9, 1 IU/ml urokinase; lanes 3, 4, 5, 6, 7 and 8 are Day 14 embryonic tissues treated with anti-PAI-2, PBS, anti-tPA, anti-uPA, NSIgG and anti-PAI-1, respectively. Panel B: lane 1, 1 IU/ml urokinase; lane 2, control medium; lane 9, 0.5 IU/ml urokinase; lanes 3, 4, 5, 6, 7 and 8 are Day 14 bovine embryonic tissues treated with NSIgG, PBS, anti-uPA, anti-PAI-2, anti-PAI-1 and anti-tPA, respectively.



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