To the Graduate Council:

I am submitting herewith a dissertation written by Scott Edward Smith entitled "Characterization of bovine uterine glandular epithelial and stromal cells." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

James D. Godkin, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate School

CHARACTERIZATION OF BOVINE UTERINE GLANDULAR EPITHELIAL AND STROMAL CELLS

A DISSERTATION

Presented for the

Doctor of Philosophy

Degree

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Scott Edward Smith

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ABSTRACT

Bovine endometrial glandular epithelial and stromal cells were isolated by enzymatic digestion from uteri of cows harvested on days 15 - 17 of the estrous cycle. Cell types were separated by slow speed centrifugation and grown a on collagen matrix. In a separate study, isolated gland cells were grown on millicell inserts coated with Matrigel and cultured in the presence of stromal cells. Response of isolated cells and co-cultured cells to bovine serum albumin (BSA), recombinant bovine trophoblast protein-1 (rbTP-1), recombinant bovine interferon (brIFN) and oxytocin were analyzed by measuring prostaglandin F2α (PGF) and E2 (PGE) and protein secretion. Regulation of gene expression of prostaglandin synthase (PGS) in each cell type was also evaluated.

Gland cells treated with rbTP-1 and brIFN produced less ($p < 0.01$) PGF (ng/μg DNA) than BSA treated cells. However, PGE was unaffected ($p > 0.05$) by rbTP-1 or brIFN. Oxytocin stimulated glandular cells to release significantly ($p < 0.01$) higher concentrations of prostaglandin F and E ($p < 0.01$). Recombinant bTP-1 and brIFN were ineffective in reducing ($p > 0.05$) oxytocin stimulated PGF secretion by gland cells. Isolated stromal cells released PGF in levels below the detection limits of the assay. Stromal cell

secretion of PGE was unaffected by rbTP-1 and brIFN. Oxytocin reduced ($P < 0.01$) PGE release by stromal cells when compared to cells cultured in the absence of oxytocin. Proteins synthesized and released in the medium were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and fluorography. Percent incorporation of ^{35}S methionine into radiolabeled proteins by isolated stromal cells was significantly ($p < 0.01$) higher than gland cells regardless of treatment. Patterns of proteins produced by glandular cells were different when compared to stromal cells. Glandular cells produced twenty unique proteins which were absent on fluorograms obtained from stromal cells.

Gland cells co-cultured with stromal cells released significantly ($p < 0.01$) higher concentrations of PGF (ng/μg DNA) than gland cells cultured in the absence of stromal cells. A significant ($p < 0.01$) reduction in PGF release occurred when co-cultured cells were incubated in the presence of rbTP-1 and brIFN. Only brIFN reduced ($p < 0.01$) PGE release by co-cultured cells. Secretion of PGF and PGE by co-cultured cells was unaffected ($p > 0.05$) by oxytocin treatment. Percent incorporation of radiolabeled amino acids into proteins were not affected by any treatment. However, protein secretion was significantly reduced as a result of glandular-stromal interactions. Differences were present in the array of proteins produced by co-cultured

cells and gland cells cultured alone. Cocultured cells produced an acidic protein (45 KDa, pI 5.5-6.8) which was absent on fluorograms from gland cells cultured alone.

Total RNA from glandular and stromal cells was isolated and subjected to Northern and slot blot analysis. Glandular cells contained a major PGS mRNA transcript of ≈ 2.8 Kb. Expression of PGS mRNA in stromal cells was evident but barely detectable. After correcting for total RNA loading by comparison with rat β-actin, quantitative slot blot analysis demonstrated no significant difference between treatments by either cell type.

These results indicate that the major effect of the trophoblast interferon is on PGF production by uterine glandular epithelial cells. Oxytocin stimulated prostaglandin production by gland cells and the IFN's diminished this response in some but not all cases. Patterns of proteins synthesized by isolated glandular epithelial and stromal cells were distinct. However, there was no effect of the IFN's on protein synthesis by either cell type. These data do not support the concept that trophoblast interferon modulates PGF production through alteration of PGS expression.

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PART 1: INTRODUCTION AND LITERATURE REVIEW

CHAPTER 1

INTRODUCTION

Early embryonic mortality is a major cause of reproductive inefficiency and economic loss in livestock production. Reproductive wastage as suggested by Hawk (1979) is a result of fertilization failure and embryonic death. It has been demonstrated by Gordon (1976) that 90-95% fertilization rates occur following artificial insemination. However, calving rates following artificial insemination have been reported to vary from 45-65% (Spalding et al., 1975; Roche et al., 1978). The majority of this embryonic loss occurs during early pregnancy.

Pregnancy failure may result from embryonic and uterine asynchrony (Pope, 1988). In order to maintain pregnancy, interactions between maternal tissue and the developing conceptus are essential to the establishment and maintenance of pregnancy. Uterine cell morphology, metabolism, maternal endocrinology and immunology are altered when a blastocyst is present in the uterus. These phenomena have been coined maternal recognition of pregnancy. However, little is known of what role isolated uterine cell types play in the establishment and maintenance of pregnancy.

The overall objectives of this study were to isolate and characterize glandular epithelial and stromal cells, the

two major cell types in the bovine uterine endometrium. In addition, isolated uterine cells were used as a model to study feto-maternal interactions in vivo.

The objectives of this study were to:

1) Determine the effects of rbTP-1, bovine recombinant interferon (brIFN) and oxytocin on prostaglandin and protein secretion by the isolated cell types.

2) Determine co-culture effects of glandular epithelial and stromal cells on secretion of prostaglandins and proteins.

3) Determine the regulation of gene expression of prostaglandin synthase in response to rbTP-1 in the isolated cell types.

CHAPTER 2

LITERATURE REVIEW

Maternal Recognition of Pregnancy

Maternal recognition of pregnancy in large domestic farm animals occurs before the trophoblast of the conceptus attaches to the maternal uterine epithelium. Progesterone from the corpus luteum is essential for the maintenance of early pregnancy in all placental mammals. If an embryo is not in the uterus by day 12-13 in sheep (Moor, 1968) or by day 16-17 in the cow (Northey and French, 1980; Betteridge et al., 1984) the corpus luteum will regress and progesterone production will decline in response to the episodic release of prostaglandin F2α (PGF).

Progesterone is essential for maintenance of early pregnancy. In beef cows, embryonic survival is greater in animals with higher serum progesterone levels than those with lower serum progesterone levels (Maurer and Echternkamp, 1982). In embryo transfer studies, cows with serum progesterone concentrations of 2 to 6 ng/ml prior to transfer had higher pregnancy rates than those with lesser or greater concentrations (Northey, et al., 1985). Exogenous progesterone treatments cause uterine secretion of products that are typical of more advanced stages of pregnancy. In ovine transfer studies, day 6 recipient ewes given progesterone, maintained day 10 transferred embryos (Lawson and Cahill, 1983). In ovine endometrial cultures,

secretory proteins were either augmented, inhibited, or unchanged in response to progesterone (Ashworth and Bazer, 1989).

During the estrous cycle, progesterone levels decline as a result of episodically released PGF (Wilson et al., 1972; Thornburn et al., 1973). This has been confirmed in studies by measuring 13,14-dihydro-15 keto prostaglandin F-2α (PGFM) a pulmonary metabolite of PGF (Kindahl et al., 1976; Thatcher et al., 1986). However, the presence of a viable embryo attenuated the release of PGF (Kindahl et al., 1976; Betteridge et al., 1984). Endometrium obtained from cows at day 17 of pregnancy produced less PGF than endometrium obtained from cows at day 17 of the estrous cycle (Thatcher et al., 1984; Gross et al., 1988a).

Interactions between progesterone, estradiol and oxytocin regulate PGF synthesis and secretion during the estrous cycle in cattle and sheep. According to studies by Louis et al., (1977), progesterone priming is necessary for the synthesis of prostaglandin (PG) by the uterus. During progesterone dominated phases of the estrous cycle, lipid and eicosanoid precursors are more abundant (Brinsfield and Hawk, 1973). However, during this time of increased plasma progesterone concentration, a significant reduction of progesterone receptor concentration in the endometrium occurs (Zelinski et al., 1982). As levels of progesterone decrease, estradiol and oxytocin receptors form in response

to increased estrogen secretion from the ovaries (Clark et al., 1977; McCracken et al., 1981, 1984). As a result of estrogen stimulation, lipid stores from the uterine epithelial cells are released and may be utilized in prostaglandin synthesis (Brinsfield and Hawk, 1973). Prostaglandin F is formed as a result of phospholipase A2 activity, which provides free arachidonic acid for the synthesis of prostaglandins (Dey et al., 1982).

Prostaglandins are synthesized from a common precursor, arachidonic acid which is a 20 carbon fatty acid. Arachidonic acid is found in the plasma membrane as a component of phospholipid, specifically, phosphatidylinositol (Holub et al., 1970). The enzyme phospholipase C hydrolyses phosphatidylinositol-bisphosphate into diacylglycerol (DAG) and inositol-triphosphate (IP-3) (for review see Berridge and Irvine, 1984). Diacylglycerol lipase can cleave arachidonic acid directly from DAG. Diacylglycerol can act as a second messenger to activate protein kinase C, which will phosphorylate specific protein substrates to activate or inactivate enzymes (Nishizuka et al., 1984). Inositol-triphosphate can also act as a 2nd messenger by causing an increase in calcium in the cytosol. Calcium is released from the endoplasmic reticulum and can activate phospholipase A2 which can directly cleave arachidonic acid from phopholipids (Van den Bosch, 1980). Once arachidonic acid is liberated from phosphatidylinositol

it is acted upon by prostaglandin G/H synthase (PGS). The overall rate of prostanoid synthesis is regulated by PGS (Smith and Lands, 1972). Prostaglandin synthase is a peripheral membrane protein which displays both cyclo-oxygenase and peroxidase activities (Ohki et al., 1979; Pagels et al., 1983). This hemoprotein is responsible for the conversion of arachidonic acid to prostaglandin (G) and (H), which are the common precursors for prostaglandins E, F, prostacyclin and thromboxane (Needleman et al., 1986). More specifically, cyclooxygenase catalyzes the formation of prostaglandin G from arachidonic acid while hydroperoxidase activity is involved in the reduction of prostaglandin G to prostaglandin H (Ohki et al., 1979; Pagels et al., 1983).

Luteolytic actions of oxytocin

Oxytocin is considered important in controlling episodic release of PGF from the ruminant uterus (Flint & Sheldrick, 1983; McCracken et al., 1984). In cattle, oxytocin infusion caused premature luteolysis when administered early in the estrous cycle (Oyedipe et al., 1984). Oxytocin also causes an increase in PGF production and luteolysis when injected during the late luteal phase of the cycle (Roberts and McCracken, 1976; Fairclough et al., 1984). Circulating oxytocin levels are highest during the mid-luteal phase (Schams et al., 1985) and decline prior to luteolysis (Wathes et al., 1984). These data support the idea that luteolysis is regulated by oxytocin receptor

levels rather than plasma oxytocin concentration.

Oxytocin administered to cows on days 14, 15 and 16 of the cycle caused an increase in PGFM levels and no extension of the cycle (Kotwica et al., 1988). However, oxytocin administered from day 10 after estrus caused no PGFM increases but a significant extension of the cycle was observed (Gilbert et al., 1989). Therefore, these results suggest that the bovine uterus becomes susceptible to oxytocin between days 10 and 14 of the cycle which may be due to up regulation of oxytocin receptor numbers.

In the cyclic cow, maximal luteolytic effect of oxytocin occurs early (up to day 5) (Armstrong and Hansel, 1959) or late (beginning on day 15) (McCracken et al., 1984). In the ewe, maximal responsiveness of the endometrium to oxytocin as determined by levels of PGF occurs on day 15 of the cycle (Roberts et al., 1976). These time periods of maximal responsiveness corresponds to periods of highest oxytocin receptor levels.

Oxytocin binding studies demonstrate that receptor concentrations begin to rise on day 15 of the cycle and then decline on day 3 after estrous in cattle (Jenner et al., 1991). It has been suggested that steroids may be responsible for fluctuating receptor levels (Sheldrick and Flint, 1985; Vallet et al., 1990). When progesterone was administered to sheep eight hours following estrus, PGF levels increased and cycle length was shortened (Ottobre et

al., 1980) In studies by McCracken et al., (1984) oxytocin receptor induction was influenced by estradiol. This is in agreement with Parkinson et al., (1990) findings that oxytocin receptor levels are negatively correlated with plasma progesterone concentrations and positively correlated with estradiol concentrations.

During early pregnancy, circulating oxytocin concentrations decline (Sheldrick and Flint, 1983) and oxytocin receptor levels are low at the expected time of luteolysis (Sheldrick and Flint, 1985; Jenner et al., 1991). It has been postulated that low levels of oxytocin receptors during pregnancy is one way in which uterine responsiveness to oxytocin is reduced. As a result of low oxytocin receptor levels, uterine PGF secretion is reduced, therefore, maintaining the corpus luteum.

Trophoblast protein-1

Maintenance of the corpus luteum is dependent upon the presence of viable embryo in the uterus. Protein secretion from the conceptus during early pregnancy attenuates production of PGF by the uterus (Godkin et al., 1984b; Fincher et al., 1986; Knickerbocker et al., 1986) . In sheep, the causative agent is ovine trophoblast protein-1 (oTP-1), a low molecular weight acidic polypeptide consisting of multiple isotypes (Mr 18 kDa, pI 5.4 - 5.7) and is the major secretory protein of the conceptus between days 13 - 21 of pregnancy (Godkin et al., 1982). Ovine TP-1

binds to uterine membrane receptors, prolongs estrous and maintains progesterone production by the corpus luteum (Godkin et al., 1984b; Vallet et al., 1988).

In cattle, bovine trophoblast protein -1 (bTP-1) has a similar role in the maintenance of early pregnancy. This glycoprotein (Anthony et al., 1988; Helmer et al., 1988) which consists of multiple isoforms (pI between 6.5 and 6.7) and two major molecular mass classes (22 kDa and 24 kDa) is secreted by the bovine conceptus between days 16-24 (Bartol et al., 1985). In goats, the trophoblast synthesizes at least three molecular weight forms (17, 21, 23 KDa) consisting of two isoelectric species (pI 5.2-5.7). This product is secreted between days 16 and 21 but not beyond day 23 (Gnatek et al., 1989) and is believed to be involved in maternal recognition of pregnancy.

High affinity low capacity binding sites for oTP-1 in ovine endometrium have been reported (Godkin et al., 1984a; Stewart et al., 1987). Studies by Knickerbocker and Niswender (1989) have characterized endometrial receptors during the estrous cycle and early pregnancy in sheep. In this study, unoccupied oTP-1 receptors in cyclic ewes were maximal on day 4 and declined to day 12 in cyclic and pregnant animals. Unoccupied receptors after day 12 decreased in pregnant animals while increasing in cyclic animals up to day 16. Declining receptor occupancy in pregnant ewes may be due to endogenous oTP-1 and/or receptor

down regulation. Receptors for bTP-1 in bovine endometrium have yet to be analyzed.

Prostaglandin secretion in response to trophoblast protein-1

Trophoblast protein-1 (TP-1) regulation of prostaglandin synthesis and/or secretion is not well understood. In sheep (Cherny and Findlay, 1990) and cows (Fortier et al,. 1988) isolated uterine glandular epithelial cells secrete primarily PGF while stromal cells secrete primarily PGE. In vitro, ovine TP-1 diminishes PG secretion by isolated ovine endometrial cells (Salamonsen et al., 1988). Endometrial explants obtained from cows between days 14 and 17 of the estrous cycle and cultured in the presence of bTP-1 decreased PGF secretion, while increasing PGE secretions (Helmer et al., 1989). In our laboratory, bTP-1 reduced PGF release by endometrial explants while PGE remained unchanged (Johnson, unpublished results).

It has been suggested that in sheep oxytocin stimulates endometrial prostaglandin synthesis by increasing the hydrolysis of phosphatidylinositol (Flint et al., 1986). Oxytocin stimulated endometrial phosphatidylinositol turnover and PGF release were inhibited in the presence of oTP-1 (Mirando et al., 1990; Vallet et al., 1989). Since oxytocin receptor numbers are low during early pregnancy (Flint and Sheldrick, 1986) it has been suggested that oTP-1 may interfere with oxytocin receptor synthesis. Other studies by Salamonsen et al., (1990;1991) have investigated

prostaglandin synthase (PGS) as a possible site for oTP-1 action.

In sheep, immunocytochemical studies showed changes in PGS localization throughout the estrous cycle. Stromal cells showed maximal staining during the follicular phase while in glandular cells maximal staining occurred during the luteal phase. However, localization of PGS in endometrium of early pregnant ewes (Day 15) was not different from that of cyclic animals (Day 15) (Salamonsen et al., 1990). Further studies, by Salamonsen et al., (1991), using ovariectomized (OVX) ewes have defined the roles of estradiol-17β and progesterone on endometrial PGS cellular localization and regulatory expression of PGS messenger ribonucleic acid (mRNA). Immunocytochemical location of endometrial PGS in OVX ewes treated with progesterone indicated that PGS enzyme is located in luminal and glandular epithelial cells while staining of stromal cells was less intense. Endometrial staining for PGS was present in stromal cells when tissue was obtained from estradiol-17β treated ewes. However, no staining was present in epithelial cells. The effect of estradiol-17β and progesterone administration together to OVX ewes showed no difference when compared to progesterone treated ewes.

Expression of uterine PGS mRNA in ewes treated with estradiol-17β was suppressed while mRNA levels in ewes treated with progesterone were slightly lower than in

untreated ewes. During the estrous cycle PGS mRNA expression showed no significant variation between days. In endometrium from pregnant ewes (Day 15), no significant differences in mRNA concentration for PGS were evident when compared to days 14 or 15 of the cycle. These studies have suggested that estrogen may be responsible for increased turnover rate of mRNA for PGS. Regulation may be due to influences by local factors which in turn may be modified by estrogen. The similarities between day 15 of pregnancy and day 14 and 15 of the cycle in PGS mRNA and cellular localization suggests that conceptus induced attenuation of prostaglandin may be indirect (Salamonsen et al., 1990; 1991). Isolated epithelial and/or stromal cells may provide an in vitro model for studying the effects of steroids and conceptus products on the expression of PGS.

In cattle, the presence of an endogenous endometrial inhibitor of prostaglandin synthesis was detected in higher amounts in endometrium from early pregnant cows than from cyclic cows (Basu and Kindahl, 1987). The inhibitor was further studied by Gross et al., (1988b) and found to be proteinaceous but it has yet to be isolated or defined. Bovine TP-1 decreased PGF secretion in bovine endometrial explants from day 17 cyclic animals and induced the putative intracellular inhibitor of PGF synthesis (Helmer et al., 1989). In cotyledonary microsomal preparations, the conversion of [^{3}H] arachidonic acid to prostaglandin was

reduced in the presence of the inhibitor. This indicates that the inhibitor may inactivate cyclooxygenase (Gross et al., 1988b). Other enzymes may be regulated by the inhibitor since bTP-1 reduced PGF secretion (Johnson, unpublished results) and increased PGE secretion in endometrial explants (Helmer et al., 1989).

Protein secretion in response to trophoblast protein-1

Another effect of TP-1 on uterine metabolism is its modulation of protein synthesis. Godkin et al., (1984a) and Vallet et al., (1987) have demonstrated that the incubation of endometrial explants with oTP-1 resulted in increased synthesis and release of specific proteins while other specific proteins were diminished. Addition of oTP-1 to ovine uterine epithelial endometrial cell cultures resulted in increased synthesis of specific uterine proteins (Salamonsen et al., 1988). Sharif et al., (1989) compared protein production by uterine tissue from early pregnant and non-pregnant ewes. Two proteins designated p70 and p15 were produced maximally between days 14 and 26 of pregnancy. In cyclic ewes production of p70 and p15 was low or absent. They concluded that uterine synthesis of p70 and p15 proteins coincides with oTP-1 production by the conceptus.

Previous studies have indicated that bTP-1 alters uterine protein production. Incorporation of radiolabeled amino acids into proteins by bovine endometrial explants were reduced in the presence of bTP-1 and bCSP (Helmer et

al., 1989; Johnson, unpublished results). In addition, no change in secretion of radiolabeled proteins were detected between pregnant and nonpregnant cows or explants treated with bCSP (Bartol et al., 1985; Geisert et al., 1988; Gross et al., 1988a). Specific bovine uterine proteins were described by Bartol et al., (1985). Comparisons of these proteins produced by endometrial explants obtained from pregnant and non-pregnant cattle revealed no differences in the array of proteins released. Geisert et al., (1988) reported an enhanced secretion of two low molecular mass basic proteins (14 - 16 KDa; pI 6.8 - 7.2) by endometrial explants obtained from day 17 pregnant cows. Endometrial explants cultured in the presence of bCSP also induced secretion of two low molecular weight basic proteins (10 KDa; 13 KDa; pI 7.8 - 8.4) (Gross et al., 1988a). Analysis of 1D-PAGE demonstrated bCSP, but not bTP-1 stimulated endometrial synthesis of a 15 KDa protein (Helmer et al., 1989).

In another study, two molecular weight classes of proteins (12 KDa; pI 7.0; 28 KDa, pI 5) were induced by endometrial explants in response to increasing doses of rbTP-1 (Reuda et al., 1991). Recently, we have shown that retinol binding protein (RBP) (Mr 23 kDa, pI 5.3-6.3) is synthesized by isolated stromal cells. This protein was present in isolated gland cells but at a barely detectable level. Retinol BP transports retinol (Vitamin A) from the

liver to its target tissues (Goodman, 1984). Vitamin A is essential in reproductive processes which include embryonic development (Wolf et al., 1984). Further studies are required to characterize the effects of rbTP-1 on proteins produced *in vitro* by isolated endometrial cells.

Trophoblast Interferons

Molecular cloning and protein sequencing of oTP-1 and bTP-1 have revealed evidence that TP-1 is a type I interferon (IFN) (Stewart et al., 1987; Charpigny et al, 1988). Type I IFN have three distinct subtypes which include α, β and ω. Both oTP-1 and bTP-1 have approximately 50% sequence identity to IFNα, less than 30% sequence identity to IFNβ and approximately 70% sequence identity to IFNω (Imakawa et al., 1989). Similarities between oTP-1 and bTP-1 to bovine IFNω occur throughout their polypeptide chains, which includes conserved signal sequences. Conserved regions which are found in most IFNα, include the cysteines involved in disulfide bridges (Imakawa et al, 1989). However, oTP-1 and bTP-1 are more closely related structurally to each other than they are to IFNω within their own species (Imakawa et al., 1989). This evidence suggests that trophoblast IFN could be considered as a distinct structural subtype. Therefore, oTP-1 and bTP-1 have recently been renamed ovine (o) and bovine (b) IFN tau (τ) (see Roberts et al., 1992).

Interferon effects on cells include antiviral and antiproliferative activation. Interferon binds cell surface receptors and are internalized by receptor-mediated endocytosis (Zoon et al., 1983). Experiments have demonstrated that interferons lack biological activity within the cells that produce them and they must first be secreted and receptor bound in order to cause a response (Vengris et al., 1975). Interferon synthesis, as a rule, does not occur in uninfected cells and production is induced by viral infection, double stranded RNA (dsRNA) and other activators and inhibitors (for review see Fields et al., 1990).

An antiviral state is initiated in cells exposed to interferon. This is accomplished by inducing the production of two enzymes. These enzymes are 2',5' oligoadenylate synthetase and P1/eIF-2α protein kinase. In order for 2',5'- oligoadenylate synthetase to become active it must first bind to dsRNA of greater than 30 base pairs (Minks et al., 1979). Following activation, this enzyme catalyzes production of a family of oligonucleotides with 2'-5' phosphodiester bonds (2,5 A) (Kerr and Brown, 1978). The function of 2-5 A is to activate a latent endoribonuclease (RNase L) (Dougherty et al., 1980). Once activated, RNase L catalyzes the cleavage of viral and cellular RNA (Slattery et al., 1979; Nilsen et al., 1981) thus inhibiting production of viral proteins. Since a 2',5'

phosphodiesterase linkage is unusual the 2,5 A does not respond to normal nucleases and must be cleaved by 2',5' phosphodiesterase, thus breaking down 2,5 A into ATP and AMP (Schmidt et al., 1979).

Recently, Short et al., (1991) demonstrated that bIFN ω enhanced 2',5'- oligoadenylate synthetase activity by isolated bovine uterine cells. In addition, oIFN and human IFN ω have identical actions on ovine uterine cell protein and prostaglandin metabolism (Salamonsen et al., 1988).

Another mechanism by which IFN induces an antiviral state is by induction of a protein kinase, known as P1. Normally this protein is inactive but when acted upon by a virus it autophosphorylates and then phosphorylates protein synthesis initiation factor (eIF-2α). This P1/eIF-2α kinase is dependent on dsRNA activation (Lebleu et al., 1976). In normal cells initial protein synthesis is dependent upon the binding of guanosine triphosphate (GTP) and guanine nucleotide exchange factor (GEF) to catalyze the exchange of GDP to GTP. Initiation factor eIF-2α will bind to GDP to form a met-$tRNA_{met}$-eIF-2α-GTP complex. In response to viral infection, the α unit of eIF-2α is phosphorylated, thus inhibiting protein synthesis (for review see Fields et al., 1990).

Biological properties of interferon ω and type I IFN are similar. For example, IFN ω has potential antiviral activity which falls in the general range for IFN α

antiviral activities (Betteridge et al., 1988; Godkin et al., 1988; Pontzer et al., 1988). In studies by Pontzer et al., (1988) it was demonstrated that oTP-1 expresses antiviral activity which falls in the general range of IFNα antiviral activity. Bovine TP-1 and total bovine conceptus secretory proteins also have antiviral activity (Godkin et al., 1988; Betteridge et al., 1988).

Another function of IFNs is inhibition of cell proliferation. Mitogen stimulation of ^{3}H thymidine incorporation into lymphocytes was highly reduced in the presence of IFN ω and IFNα (Roberts et al., 1989; Fillion et al., 1991). In another study by Kulesh et al., (1987) using human fibrosarcoma (HT 1080) cells and normal CUA foreskin fibroblast cells (CUA) demonstrated that HT1080 cells are highly sensitive to the antiproliferative effects of IFN, while CUA cells naturally stop cell proliferation due to contact inhibition. They determined by mRNA levels that the suppressed genes in HT 1080 cells in response to IFN treatment corresponded to genes suppressed in CUA cells during contact inhibition. Thus, interferons may regulate cell proliferation by affecting the activity of genes which normally are responsible for cell proliferation.

Receptor binding studies have been carried out to determine if IFN will bind oTP-1 receptors in the uterus. Initial studies were carried out by Stewart et al., (1987) using human interferon α, (Sigma,Poole, Dorset, UK) labelled

with ^{125}I. It was found that human IFNα bound to endometrial preparations from sheep in a displaceable manner. The binding of ^{125}I labelled human interferon was displaced by purified ^{125}I labelled oTP-1 and ^{125}I labelled bovine recombinant interferon αs (brIFNα-I). Labelled oTP-1 bound to sheep endometrial receptors and binding was blocked by unlabelled brIFNα-I (Stewart et al., 1989). The above studies have demonstrated that the biological activities of IFNτ are similar to other type I IFN.

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PART 2: THE EFFECTS OF RECOMBINANT BOVINE TROPHOBLAST PROTEIN-1, RECOMBINANT BOVINE INTERFERON AND OXYTOCIN ON PROSTAGLANDIN AND PROTEIN SECRETION BY ISOLATED BOVINE GLANDULAR EPITHELIAL AND STROMAL CELLS

CHAPTER 1

INTRODUCTION

A successful pregnancy is dependent upon communication between the uterine endometrium and the developing conceptus. In all placental animals, progesterone is essential for the maintenance of pregnancy. In order for progesterone secretion to continue, the corpus luteum must remain active and be protected from the luteolytic actions of prostaglandin F2α (PGF) (Wilson et al., 1972; Thornburn et al., 1973; Kindahl et al., 1976; Thatcher et al., 1986b). Prostaglandin F is episodically released by the uterus, which is believed to be under the control of oxytocin (Flint and Sheldrick, 1983; McCracken et al., 1984).

The maintenance of luteal function during early pregnancy is mediated through the actions of a protein released by trophoblast cells of the developing conceptus. The antiluteolytic signal in sheep and cattle is a trophoblast interferon (IFN) previously referred to as ovine (o) or bovine (b) trophoblast protein-1 (TP-1) and recently named IFN tau (τ) (see Roberts, 1992). This protein, first discovered in sheep and named oTP-1 (Godkin et al., 1982) attenuates production of luteolytic pulses of PGF by the uterus (Godkin et al., 1984b; Fincher et al., 1986; Knickerbocker et al., 1986a). In cattle, bTP-1 has a similar role in the maintenance of early pregnancy through diminished uterine PGF production. This glycoprotein

(Anthony et al., 1988; Helmer et al., 1988) consists of multiple isoforms (pI between 6.5 - 6.7), contains two molecular mass classes (22 kDa and 24 kDa) and is the major conceptus secretory protein between days 16 - 24 (Bartol et al., 1985b). Production of bTP-1 by the bovine conceptus is more prolonged than in sheep and continues through at least day 36 of pregnancy (Godkin et al., 1988). Bovine TP-1 immunologically cross reacts with oTP-1 (Helmer et al., 1987) and demonstrates high amino acid sequence homology with oTP-1 and bovine interferon alpha (Imakawa et al., 1989).

Infusion of bTP-1 into the uterine lumen of cows between days 14 and 17 of the cycle lengthens the lifespan of the corpus luteum (Helmer et al., 1989b). Prostaglandin F secretion was reduced in endometrial explants while prostaglandin E2 (PGE) levels increase as a result of bTP-1 actions (Helmer et al., 1989a). In a similar study, bTP-1 significantly reduced PGF secretions by endometrial explants and PGE secretions remained unchanged (Johnson, unpublished results). In cows (Fortier et al., 1988) and sheep, (Cherny and Findlay et al., 1990) isolated uterine glandular epithelial cells secrete primarily PGF while stromal cells secrete primarily PGE. Watson et al., (1992) have recently demonstrated that isolated glandular cells from endometrial tissue, obtained from mares secrete significantly more PGF than isolated stromal cells. These results agree with

studies performed in ruminants.

In cattle, attachment of the placenta is not complete until the third week of pregnancy (Amoroso 1952; King et al., 1979). During this time, endometrial secretions are believed to provide a proper environment for the growth, development and eventually implantation of the embryo. Analysis of uterine protein components, in the cow, were first derived from uterine flushings (Roberts and Parker, 1974; Bartol et al., 1981). These results indicate that serum or serumlike protein are a major component of the uterine environment. However, uterine specific proteins also contribute to the composition of flushings. Major and minor uterine-specific proteins have been identified and characterized by in vitro analysis in bovine (Bartol et al., 1985a) and ovine (Mofatt et al., 1980) endometrial tissue.

The developing conceptus produces products which interact with maternal endometrium and influence protein secretions. More specifically, in sheep, oTP-1 increased protein secretion by endometrial explants (Godkin et al., 1984a; Vallet et al., 1987). In isolated ovine endometrial glandular cells, oTP-1 caused increased production of specific-uterine proteins (Salamonsen et al., 1988). Sharif et al., (1989) described the presence of two proteins, secreted by ovine uterine tissue, which were maximally produced between day 14 and 26 of pregnancy. These proteins were barely detectable to absent in non-pregnant animals.

In the cow, the effects of pregnancy or, more specifically, the effects of bTP-1 on uterine protein secretion is less well understood. Helmer et al., (1989a) reported a decrease in protein synthesis and secretion when explants were treated with bTP-1 and bovine conceptus secretory proteins (bCSP). However, other studies report increases in uterine-specific proteins in tissues obtained from pregnant animals (Bartol et al., 1985a; Geisert et al., 1988), and in tissue prepared from non-pregnant animals treated with bCSP (Gross et al., 1988a).

In the present study, uterine glandular epithelial and stromal cells, obtained from cows between days 15 and 17 of the cycle (estrus = 0) were isolated and grown on a collagen matrix derived from rat-tail collagen. Secretion of PGF and PGE by isolated cell types were measured in response to rbTP-1, brIFN and in the presence and absence of oxytocin. In a separate but related study, characterization of proteins produced <u>in vitro</u> by the isolated cell types, in response to rbTP-1, brIFN and oxytocin were also analyzed.

CHAPTER 2

MATERIAL AND METHODS

Materials

Cell culture medium, Hank's balanced salt solution (HBSS), HBSS without (Ca^{+2}) and (Mg^{+2}), Dulbeccos modified Eagle's medium (low glucose) (DMEM), DMEM methionine free, Minimal essential media (MEM), fetal calf serum (FCS) (charcoal treated; 5 g/100 ml), amino acids and basal medium Eagle vitamin solution were purchased from Gibco Laboratories, Grand Island, NY. Insulin was obtained from Eli Lilly and Co. Indianapolis, IN. Enzymes, collagenase A and dispase were purchased from Boehringer Mannheim, Indianapolis, IN. Rat tail type I collagen, bovine serum albumin (BSA), human interferon (huIFN), (specific activity 1.67×10^6 U/mg), oxytocin and glucose were obtained from Sigma Chemical Co., St. Louis, MO. Trans ^{35}S- label (specific activity 1066 ci/mmole) was purchased from ICN Biochemicals, Inc. Irving, Ca. Recombinant bovine trophoblast protein-1 (rbTP-1), specific activity 1.5×10^7 U/mg (GBK-1/VSV) was a gift from R. M. Roberts. Recombinant bovine interferon (rbIFN), specific activity 2.6×10^7 U/mg was donated by Ciba-Geigy Corp. Supplies for electrophoresis were as described previously (Godkin et al., 1988).

Animals and collection of uterus

In this study, two cell types were isolated from the

bovine uterine endometrium. These include glandular epithelial and stromal cells. Since actions of bTP-1 critical to pregnancy maintenance are believed to occur between days 15 and 17, endometrium was obtained from cows between days 15 and 17 of the cycle (estrus=0). Uteri from 4 cows were utilized in this study. The estrous cycles of cows were synchronized with a single I.M. injection of Lutalyse (25 mg; Upjohn Co., Kalamazoo, MI). Cows were observed twice daily for estrus behavior. Reproductive tracts were collected at slaughter within 40 minutes of death, placed on ice and transported to the laboratory where they were trimmed, the exterior washed in water, then ethanol and placed in a laminar flow hood for dissection.

Isolation and culture of glandular epithelial and stromal cells.

Endometrial strips obtained from above were placed in a 150 x 25 mm petri dish, containing sterile HBSS without Ca^{+2} and Mg^{+2} and minced into small fragments (2-4 mm^2) using two scalpel blades. Minced material was placed into a sterile 500 ml Erylenmyer flask containing 0.1% collagenase and 0.5 U/ml dispase in 250 ml of HBSS without Ca^{+2} and Mg^{+2}. The Erylenmyer flask containing the digestion media was placed in a 37° C shaking water bath for approximately 2 hours, during which the tissue was checked periodically to determine the progress of digestion. After digestion, HBSS without Ca^{+2} and Mg^{+2} was added to digesta and transferred to

a 150 x 25 mm petri dish. Undigested material and cellular debris was removed manually and the remainder was passed through a 200 μm sterile nylon mesh (Spectrum Medical Industries, Inc., Los Angeles, CA) through which small glandular fragments, isolated cells and small clumps of cells passed through. Fragments were isolated by gravity sedimentation and/or slow speed centrifugation (30 x g). Following centrifugation, glandular fragments were dispersed through a sterile pipette producing smaller fragments. Stromal cells were obtained by high speed centrifugation (500 x g) of the supernatant from glandular cell washings.

Isolated cell types were seeded onto 0.01% rat tail collagen coated 24 well plates, following resuspension in DMEM supplemented with 10% heat inactivated FCS, 4x glucose, 2x ABAM, 1x insulin, 1x basal medium Eagle vitamin solution and 1x non-essential amino acids. Cells were incubated at 38° C in a humidified atmosphere of 5% CO_2 : 95% air. Following 48 hours of incubation glandular cells were treated briefly with 0.05% trypsin/ 0.04% EDTA to remove contaminating stromal cells. Media was changed every 48 hours until confluent. Once confluent, cells were washed 3x in HBSS prior to treatment. Cells were assigned treatments in a randomized block design. Treatments for prostaglandin analysis were prepared in MEM (1x) while treatments for protein analysis were prepared in methionine free - DMEM with ^{35}S methionine (5.0 μci/ml). In order to determine a

specific range of activity for rbTP-1 and huIFN a dose response study was performed. These cells received the following treatments in triplicate. 1) Control/ no treatment 2) BSA (0.3, 3, 30, 250 ng/ml) 3) huIFN (5, 50, 500 IU/ml) 4) rbTP-1 (0.3, 3, 30 ng/ml). Following 36 hours of culture medium was harvested and stored frozen for PGF, PGE and protein analysis. From the dose - response study it was determined that a dose of 30 ng/ml gave the best response and was used in the remainder of the study. Bovine recombinant interferon alpha (brIFN) was used instead of huIFN for the remainder of the study, since huIFN was used only to aid in the determination of a dose range for interferons in the isolated cell types.

In the remainder of the study, cells received the following treatments in triplicate. 1) control (no treatment) 2) BSA (30 ng/ml) 3) rbTP-1 (30 ng/ml) 4) brIFN (30 ng/ml) 5) oxytocin (50 mU) 6) BSA (30 ng/ml) + oxytocin (50 mU) 7) rbTP-1 (30 ng/ml) + oxytocin (50 mU) 8) brIFN (30 ng/ml) + oxytocin (50 mU). For treatments involving oxytocin, cells were cultured for 30 hours in the absence of oxytocin at which time oxytocin was added. At 36 hours, media was harvested, subjected to centrifugation in an eppendorf centrifuge and supernatant was stored at -20° C.

Radioimmunoassay procedure

Prostaglandin F and E2 were analyzed with RIA kits

purchased from Advanced Magnetics (Cambridge, Massachusetts). Intra- and interassay coefficient of variation (CV) were 4.14% and 6.75% for PGF and 8.67 % and 14.68% for PGE. Sensitivity of the assay for PGF and PGE was 8 pg/assay tube.

Protein Synthesis

Percent incorporation of radiolabeled ^{35}S methionine into protein was measured by using trichloroacetic acid (TCA) precipitation (Mans and Novelli, 1961). Briefly, duplicate samples of media were placed on a sampling manifold (Millipore Corporation, Bedford, MA) containing Whatman glass microfibre filters (Whitman International LTD. Maidstone, England) presoaked with 5% TCA. Unincorporated radioactivity was removed by serial washings of filters with 10% and 5% TCA followed by 95% ethanol. Whatman filters containing precipitated proteins were collected and analyzed by scintillation counting.

Protein Electrophoresis

Media from isolated cell types was dialysed against 1mM Tris-HCl (4 changes at 8 hr intervals). After dialysis an aliquot containing 100,000 cpm was lyophilized and dissolved in a volume of 5 mM K_2CO_3 containing 9.4M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) procedure was described by Roberts et al., (1984). Briefly, following electrophoresis, gels were stained with Coomassie

Brillant Blue R-250 impregnated with 1M sodium salicylate (Chamberlain, 1979) dried and exposed to Kodak XAR film for analysis of radioactive proteins.

DNA measurement

Prostaglandin and protein (% incorporation) data was based on cell DNA content, which was quantitated using 4', 6- diamidine-2-phernylindole (DAPI) (Kapuscinski and Skoczylas, 1977; Kapuscinski and Yanagi, 1979). Briefly, after removal of media from cultured cells, one milliliter of DAPI buffer (150 mM NaCl; 5mM Hepes, pH 7.0) was added to each well and froze at -20° C. After thawing, cells were further disrupted by sonication (10 - 20 second intervals). Unknown cellular DNA was measured in a cytofluor 2300 using calf thymus DNA as a standard.

Statistical Analysis

Prostaglandin and protein data are expressed as generalized least squared means +/- SE. When necessary data was log transformed to stabilize the variance. Data were analyzed using general linear mixed models methodology using GLMM (Blouin and Saxton, 1990). The analysis of variance considered treatments as a fixed factor and cows as a random factor. Differences between treatments were separated by orthogonal contrast. P values of less than 0.05 were considered significant.

CHAPTER 3

RESULTS

Effects of BSA, rbTP-1 and huIFN on PGF secretion by isolated glandular epithelial cells - dose response.

Bovine serum albumin (0.3 - 250 ng/ml) had no significant ($p > 0.05$) effect on PGF secretion when compared to non-treated cells (Figure 1). Recombinant bTP-1 (0.3 - 30 ng/ml) dose-dependently decreased PGF levels below control values. Significant ($p > 0.05$) decreases were not obtained with doses of 0.3 or 3 ng/ml. However, the highest concentration of 30 ng/ml did cause a significant ($p < 0.01$) decline in PGF release. The maximal reduction was obtained at 30 ng/ml (Figure 2A).

Human IFN alpha (5 - 500 IU/ml) did not effect PGF secretion in a dose dependent manner. Samples obtained and assayed for PGF from control and 5 IU/ml were not significantly different ($p > 0.05$). However, a significant ($p < 0.01$) rise in PGF levels above control values was seen at 50 IU/ml, while a significant ($p < 0.01$) decrease occurred at a dose of 500 IU/ml (Figure 2B).

Effects of BSA, rbTP-1 and huIFN on PGE secretion by isolated glandular epithelial cells - dose response.

Media from glandular epithelial cells assayed for PGE following 36 hours of culture was unaffected by BSA at all concentrations (0.3 - 250 ng/ml) (Figure 3).

Figure 1. PGF production by uterine glandular epithelial cells in response to treatment with increasing concentrations of BSA. Media was collected after 36 hours of treatment and assayed for PGF by RIA. There was no significant effects in PGF secretion after stimulation with BSA. Log transformed data was expressed as means (SE = 0.56) of three experiments prepared in triplicate.

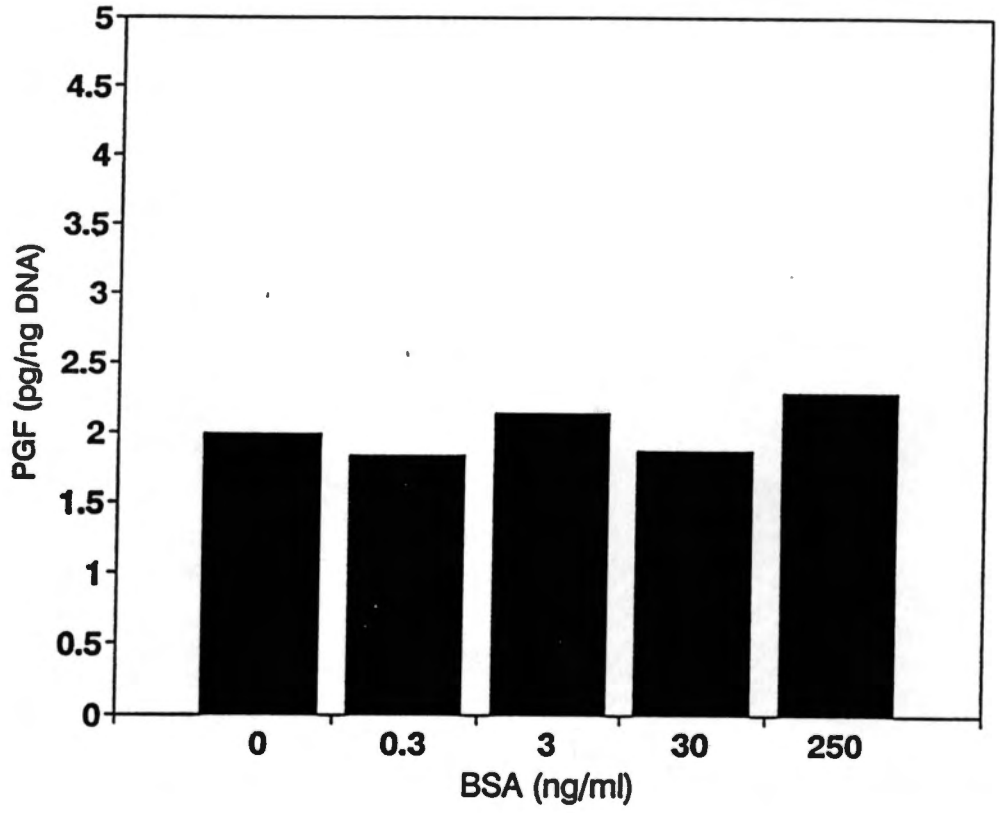
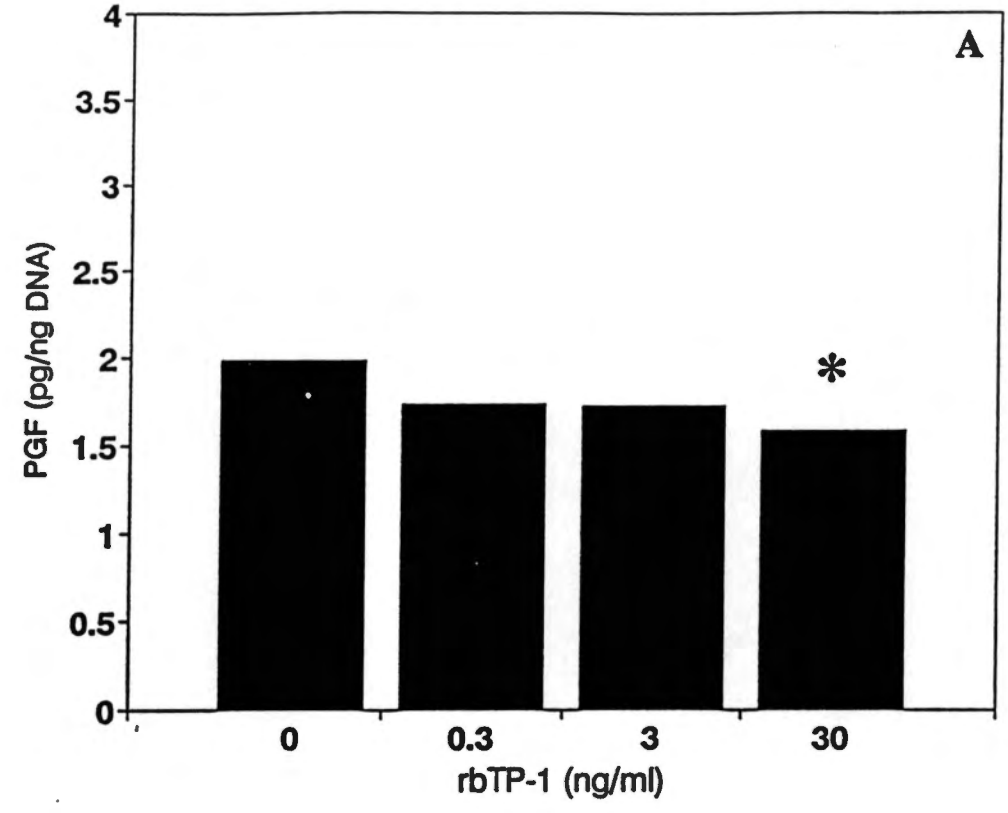

PGF (pg/ng DNA)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
0.3
3
30
250
BSA (ng/ml)

Figure 2. PGF production by uterine glandular epithelial cells in response to treatment with increasing concentrations of rbTP-1 (A) and huIFN (B). Media was collected after 36 hours of treatment and assayed for PGF by RIA. Significantly ($p < 0.01$) lower levels of PGF were seen with rbTP-1 (30 ng/ml) and huIFN (500 IU/ml). Log transformed data was expressed as means (SE = 0.56) of three experiments prepared in triplicate; all significant differences denoted by *.


A
PGF (pg/ng DNA)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
0
0.3
3
30
rbTP-1 (ng/ml)

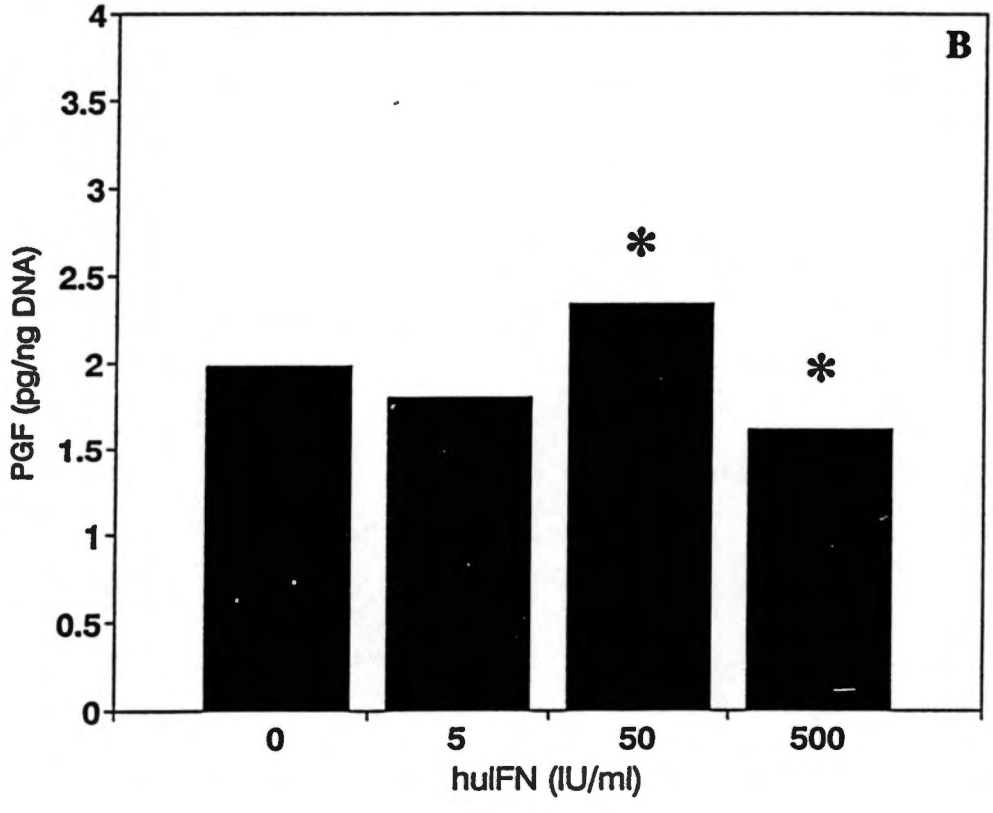
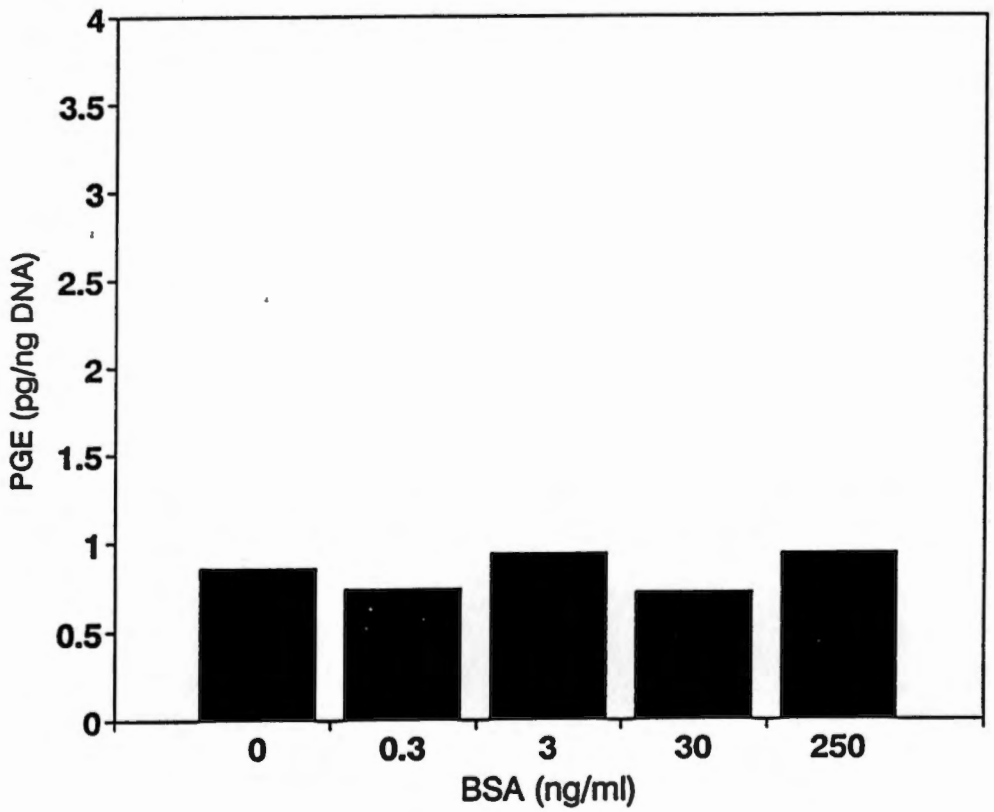

B
PGF (pg/ng DNA)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
0
5
50
500
hulFN (IU/ml)

Figure 3. PGE production by uterine glandular epithelial cells in response to treatments with increasing concentrations of BSA. Media was collected after 36 hours of treatment and assayed for PGE by RIA. There was no significant effect in PGE secretion after stimulation with BSA. Log transformed data was expressed as means (SE = 0.42) of three experiments prepared in triplicate.


4
3.5
3
2.5
2
1.5
1
0.5
0
PGE (pg/ng DNA)
0
0.3
3
30
250
BSA (ng/ml)

As shown in Figure 4A, rbTP-1 significantly decreased ($p < 0.01$) PGE secretions at all doses with maximal reduction occurring at 0.3 ng/ml.

When compared to control PGE-2 levels, huIFN did not inhibit secretion of PGE in a concentration dependent manner. A significant ($p < 0.01$) reduction occurred at doses of 5 and 500 IU/ml whereas, 50 IU/ml was not significant ($p > 0.05$) from control treatments. Maximal reduction of PGE secretion occurred at 500 IU/ml (Figure 4B).

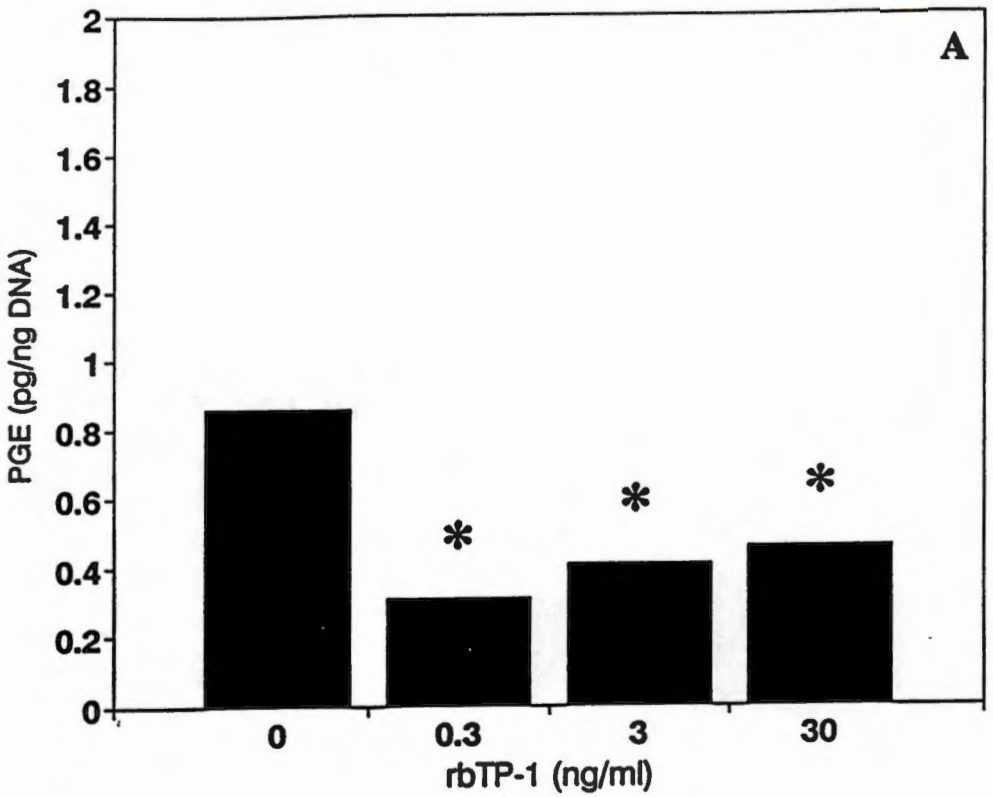
Effects of BSA, rbTP-1 and huIFN on PGF and PGE secretion by isolated uterine stromal cells - dose response.

The effects of all treatments on PGF secretion by stromal cells were undetectable, since measurements were below detectable levels of the assay. Bovine serum albumin and huIFN did not significantly affect release of PGE at any concentration. Secretory levels of PGE, although not significant, were greater for rbTP-1 at all doses (0.3 - 250 ng/ml) when compared to control (Table 1).

Effects of rbTP-1 and brIFN on PGF and PGE production by isolated uterine glandular epithelial cells cultured in the absence or presence of oxytocin.

Recombinant bTP-1 and brIFN, significantly ($p < 0.01$) reduced PGF secretion when compared to BSA treated cells. In the presence of oxytocin, uterine glandular epithelial cells released significantly ($p < 0.01$) higher amounts of PGF than those cultured in the absence of oxytocin.

Figure 4. PGE production by uterine glandular epithelial cells in response to treatments with increasing concentrations of rbTP-1 (A) and huIFN(B). Media was collected after 36 hours of treatment and assayed for PGE by RIA. In response to rbTP-1, a significant ($p < 0.01$) decrease in PGE secretion was evident at all doses when compared to controls. Human IFN significantly ($p < 0.01$) decreased PGE at doses of 5 and 500 IU/ml. Log transformed data was expressed as means (SE = 0.42) of three experiments prepared in triplicate; all significant differences denoted by *.


A
PGE (pg/ng DNA)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
0
0.3
3
30
rbTP-1 (ng/ml)

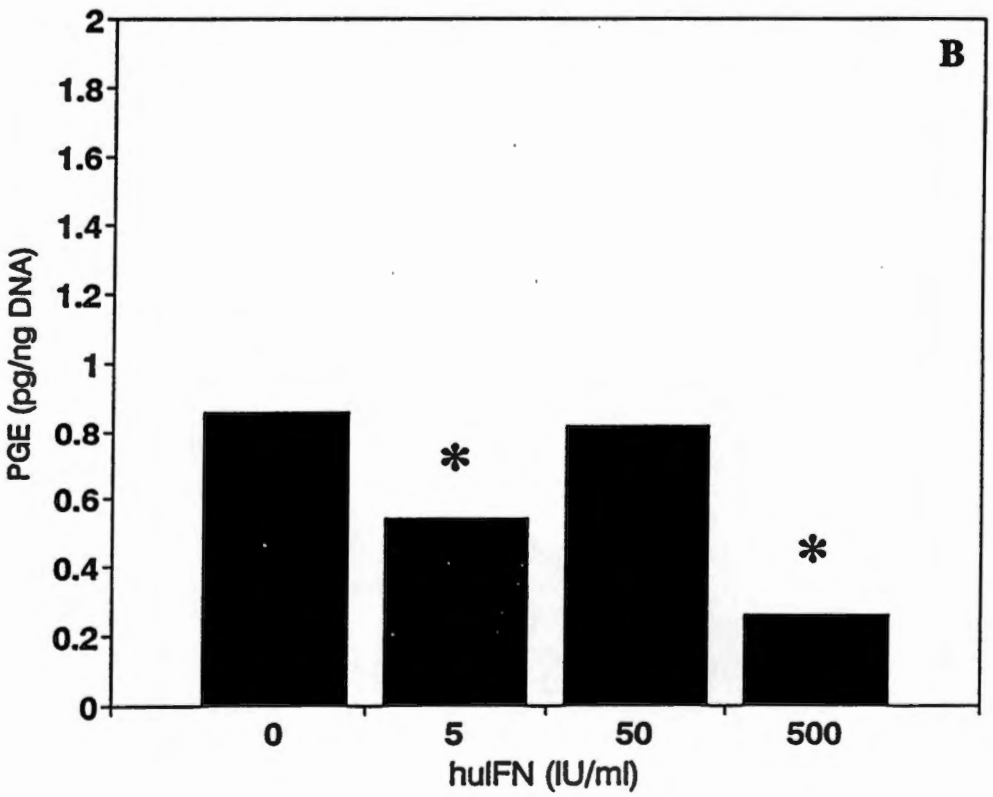

B
PGE (pg/ng DNA)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
0
5
50
500
hulFN (IU/ml)

TABLE 1. Effects of increasing concentrations of BSA, rbTP-1 and huIFN on PGE production by uterine stromal cells.*

Treatment	PGE (pg/ng DNA)
Control	-1.01 ± 0.73
BSA (ng/ml)	
0.3	-1.49 ± 0.73
3	-1.55 ± 0.73
30	-1.44 ± 0.69
250	-0.87 ± 0.69
rbTP-1 (ng/ml)	
0.3	-0.61 ± 0.69
3	-0.48 ± 0.69
30	-0.53 ± 0.69
huIFN (IU/ml)	
5	-1.18 ± 0.73
50	-0.90 ± 0.69
500	-0.97 ± 0.69

*Log transformed data expressed as mean ± SEM of three experiments performed in triplicate.

Recombinant bTP-1 or brIFN in combination with oxytocin did not have any effect on PGF secretion when compared to cells treated with oxytocin alone (Figure 5). In the absence of oxytocin, rbTP-1 and brIFN tended to reduce PGE secretion when compared to BSA treated cells, but not significantly ($p > 0.05$). In the presence of oxytocin, uterine glandular cells released significantly ($p < 0.01$) higher amounts of PGE than cells cultured in the absence of oxytocin. BSA had no effect on PGE secretion when cultured in the presence of oxytocin. However, uterine glandular cells treated with rbTP-1 or brIFN in the presence of oxytocin reduced PGE secretion significantly ($p < 0.01$) (Figure 6).

Effects of rbTP-1 and bovine rIFN on PGF and PGE production by isolated uterine stromal cells cultured in the absence or presence of oxytocin.

Treatment of stromal cells with rbTP-1 or brIFN had no effect on PGE secretion. Production of PGE by cells treated with oxytocin was significantly ($p < 0.01$) reduced when compared to cells cultured in the absence of oxytocin. Stromal cells treated with rbTP-1 and brIFN for 30 hours prior to oxytocin treatment secreted significantly ($p < 0.01$) higher levels of PGE then cells treated with oxytocin alone. Isolated stromal cells released PGF in low levels of which were below the limits of detection of the assay (Figure 7).

Figure 5. PGF production by uterine glandular epithelial cells cultured in the absence or presence of oxytocin. Media was collected after 36 hours of treatment and assayed for PGF by RIA. Significant ($p < 0.01$) decreases in PGF secretion occurred in cells treated with rbTP-1 and brIFN. In the presence of oxytocin cells released significantly ($p < 0.01$) higher levels of PGF than cells cultured in the absence of oxytocin. rbTP-1 and brIFN had no effect on PGF output by cells treated with oxytocin. Data was expressed as means +/- SEM of four experiments prepared in triplicate. (* significantly different from control; BSA) (ª significantly different than control; no treatment).

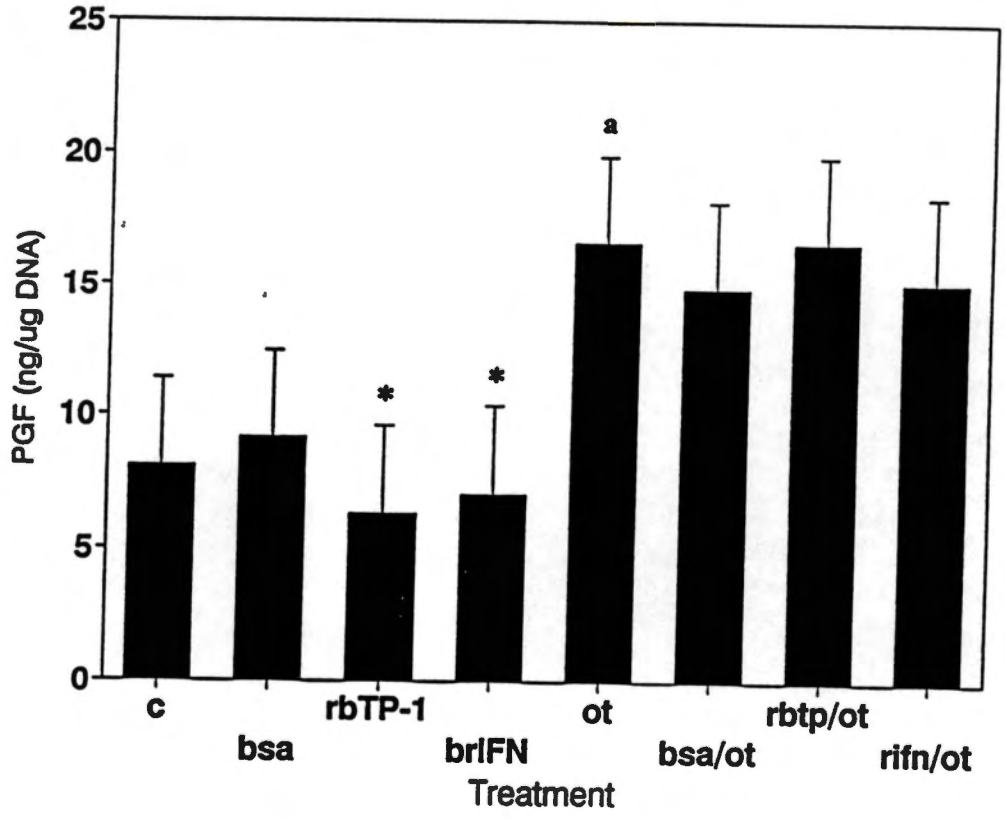

25
20
15
10
5
0
PGF (ng/ug DNA)
a
*
*
c
bsa
rbTP-1
brIFN
ot
bsa/ot
rbtp/ot
rifn/ot
Treatment

Figure 6. PGE production by uterine glandular epithelial cells in the absence or presence of oxytocin. Media was collected after 36 hours of treatment and assayed for PGE by RIA. Significant ($p > 0.05$) differences in PGE release was not detected in cells treated with rbTP-1 or brIFN when compared to BSA control. However, a significant increase in PGE output occurred in cells treated with oxytocin alone. rbTP-1 and brIFN significantly ($p < 0.01$) reduced PGE release when compared to cells treated with oxytocin alone. Log transformed data was expressed as means (SE = 0.40) of four experiments prepared in triplicate. (* significantly different from control; no treatment) (^{a} significantly different from oxytocin (ot).

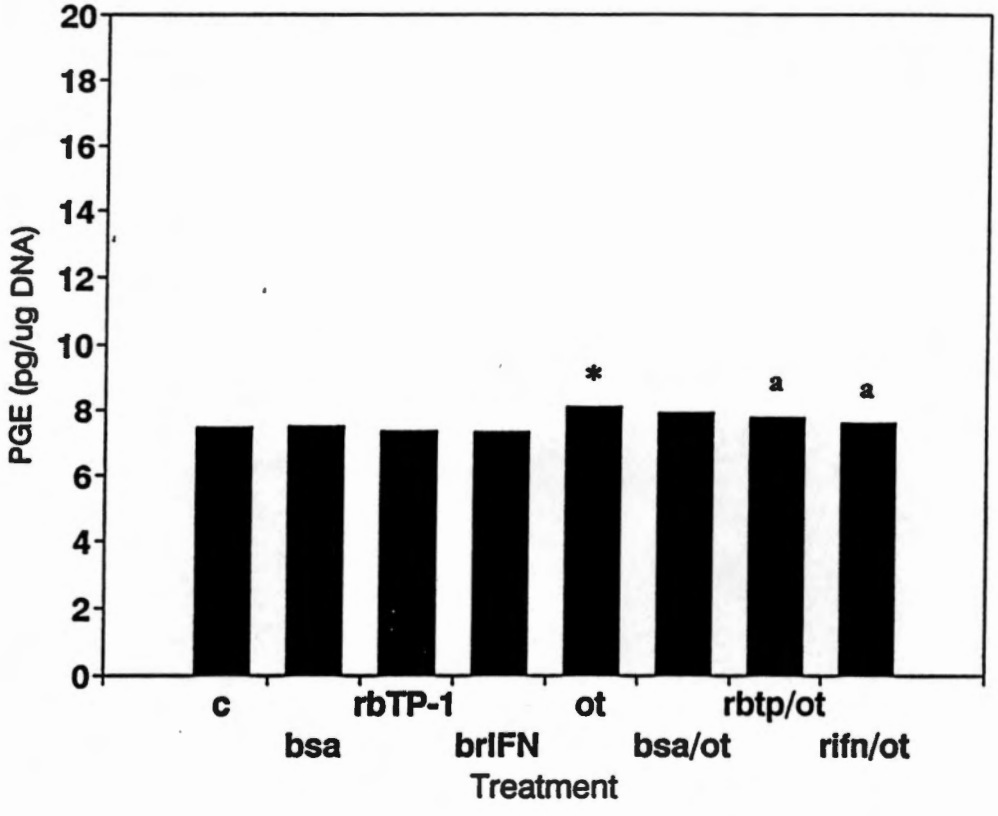
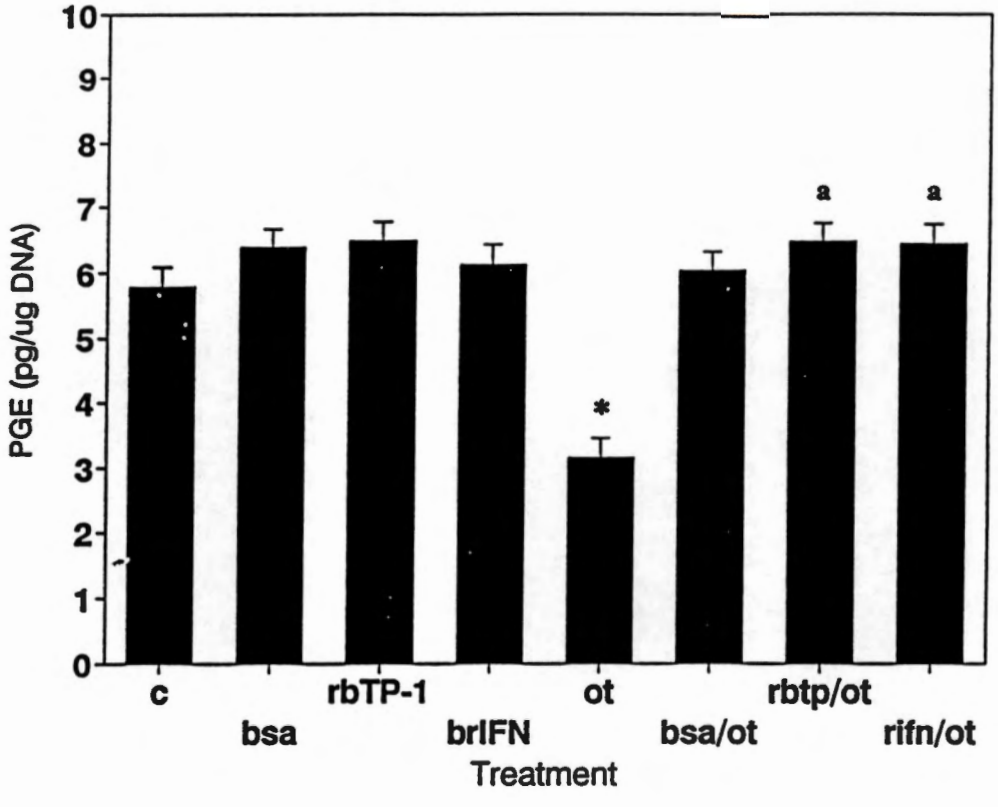

20
18
16
14
12
10
8
6
4
2
0
PGE (pg/ug DNA)
*
a
a
c
bsa
rbTP-1
brIFN
ot
bsa/ot
rbtp/ot
rifn/ot
Treatment

Figure 7. PGE secretion by uterine stromal cells cultured in the presence or absence of oxytocin. Media was collected after 36 hours of treatment and assayed for PGE by RIA. rbTP-1 and brIFN did not significantly ($p > 0.05$) affect PGE release. A significant ($p < 0.01$) reduction in PGE release was caused by oxytocin, while rbTP-1 and brIFN caused cells treated with oxytocin to significantly increase PGE output. PGE secretion was not altered by BSA addition. Log transformed data was expressed as means +/- SE of four experiments prepared in triplicate. (* significantly different from control; no treatment) (ª significantly different from oxytocin (ot).


10
9
8
7
6
5
4
3
2
1
0
PGE (pg/ug DNA)
a
a
*
c
bsa
rbTP-1
brIFN
ot
bsa/ot
rbtp/ot
rifn/ot
Treatment

Comparison of PGE secretion by glandular epithelial and stromal cells.

Gland cells secreted significantly ($p < 0.01$) higher levels of PGE than stromal cells (Figure 8).

Comparison of percent incorporation by gland and stromal cells.

Percent incorporation of radiolabelled amino acids into proteins was significantly ($p < 0.01$) higher in stromal cells than gland cells, regardless of treatment (Figure 9).

Effect of BSA, rbTP-1 and huIFN on percent incorporation of ^{35}S methionine into secreted proteins by isolated gland cells and stromal cells - dose response.

The effects of BSA, rbTP-1 and huIFN on glandular cell incorporation of radiolabeled amino acid into proteins was variable. Cells cultured in the presence of 30 ng/ml of BSA were observed to synthesize more radiolabeled protein than cells cultured in media alone. Human IFN significantly ($p < 0.05$) increased protein synthesis at a dose of 500 IU/ml but was not dose dependent. Cells cultured in the presence of rbTP-1 (all doses) showed no dose dependent response. The highest dosage of rbTP-1 (30 ng/ml) caused a significant ($p < 0.05$) increase in percent incorporation when compared to cells cultured in media alone (Table 2).

Figure 8. Comparison of PGE release by glandular epithelial and stromal cells. After 36 hours of incubation media was collected and assayed for PGE by RIA. Measurements of PGE obtained from cells cultured in media alone. Isolated glandular cells release significantly ($p < 0.01$) higher levels of PGE than isolated stromal cells. Log transformed data was expressed as mean +/- SEM of four experiments prepared in triplicate. All significant differences denoted by *.

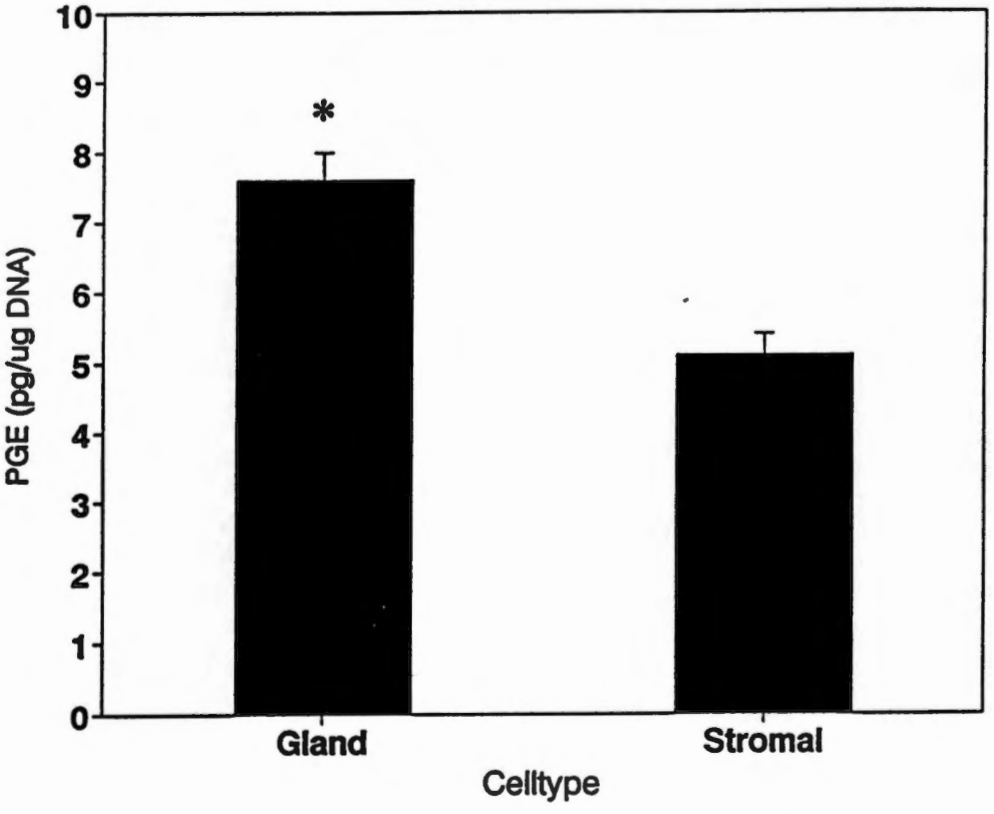

10
9
8
7
6
5
4
3
2
1
0
*
PGE (pg/ug DNA)
Gland
Stromal
Celltype

Figure 9. Comparison of percent incorporation of ^{35}S label into secreted proteins by glandular and stromal cells. Media was collected at 36 hours and analyzed by the method of TCA as described in material and methods. Media was analyzed from cells cultured in the absence of treatment. Glandular cells secrete significantly ($p < 0.01$) less proteins than stromal cells. Data expressed as means (SE = 0.22) from four experiments prepared in triplicate. Significant difference denoted by *.

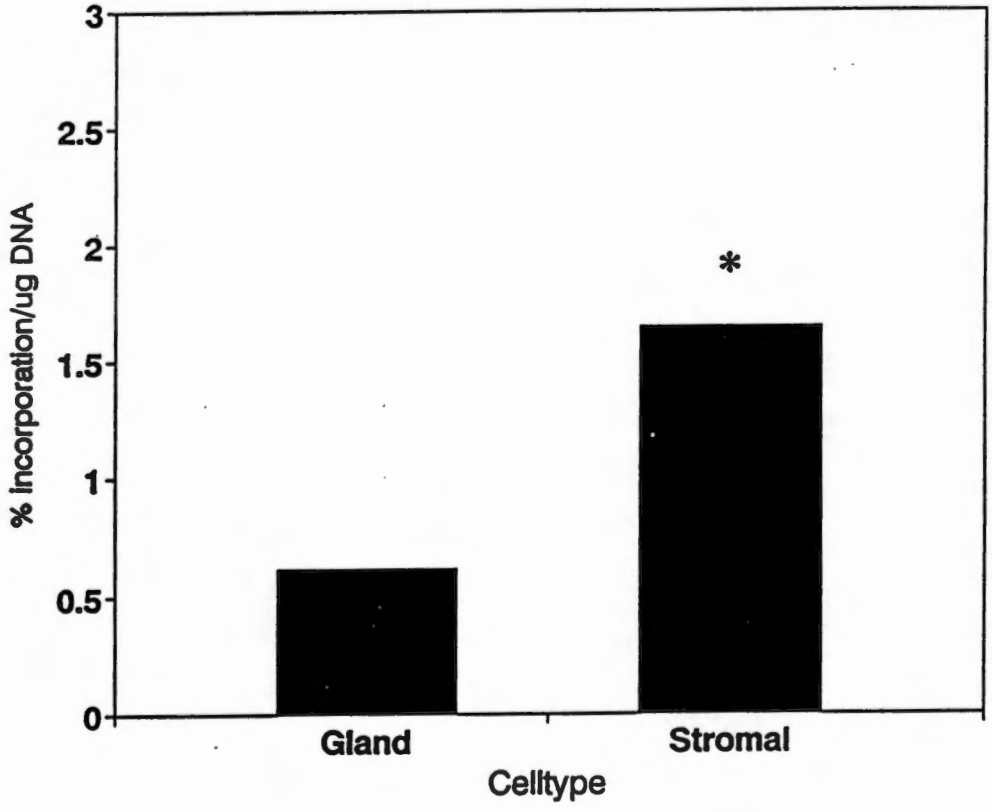

3
2.5
2
1.5
1
0.5
0
% Incorporation/ug DNA
*
Gland
Stromal
Celltype

TABLE 2. Effects of increasing concentrations of BSA, rbTP-1 and huIFN on percent incorporation of radiolabeled ^{35}S methionine in secreted proteins by uterine glandular epithelial cells.*

Treatment	Percent Incorporation
Control	0.59
BSA (ng/ml)	
0.3	0.57
3	0.71
30	1.05[a]
250	0.62
rbTP-1 (ng/ml)	
0.3	0.59
3	0.59
30	0.89[b]
huIFN (IU/ml)	
5	0.62
50	0.56
500	0.80[b]

*Log transformed data expressed as mean percent incorporation (SE = 0.31) per ng DNA of three experiments performed in triplicate.
[a]Significantly different from control; no treatment ($p<0.01$).
[b]Significantly different from control; no treatment ($p<0.05$).

The effects of treatments on protein synthesis by stromal cells was inconsistent. BSA at two concentrations (3 ng/ml & 250 ng/ml) caused a significant increase ($p < 0.05$) in % incorporation of radiolabeled amino acid into proteins. Human IFN at a dose of 5 IU/ml significantly ($p < 0.05$) increased percent of radiolabeled amino acid incorporation into proteins. Treatment of stromal cells with rbTP-1 (all doses) had no significant affects on percent radiolabel amino acid incorporation. A dose dependent decrease in protein secretion did occur in cells treated with huIFN and rbTP-1, however, this effect was not significant (Table 3).

Effect of rbTP-1 and brIFN on percent incorporation of ^{35}S methionine into secreted proteins by isolated uterine glandular and stromal cells cultured in the presence and absence of oxytocin.

Percent incorporation of ^{35} S-methionine into radio-labeled proteins by isolated glandular cells was not affected by any treatment. Protein synthesis by stromal cells was increased with the combined treatments of BSA + oxytocin and rbTP-1 + oxytocin. This increase was significant ($p < 0.01$) when compared to cells treated with oxytocin alone (Table 4).

TABLE 3. Effects of increasing concentrations of BSA, rbTP-1 and huIFN on percent incorporation of ^{35}S methionine into secreted proteins by stromal cells.*

Treatment	Percent Incorporation
Control	4.44
BSA (ng/ml)	
0.3	4.89
3	6.49[a]
30	4.76
250	8.65[a]
rbTP-1 (ng/ml)	
0.3	6.36
3	4.10
30	3.79
huIFN (IU/ml)	
5	6.40[a]
50	5.28
500	3.87

*Log transformed data expressed as mean percent incorporation (SE = 3.16) per ng DNA of three experiments performed in triplicate.
[a]Significantly different from control; no treatment ($p<0.05$).

TABLE 4. Effects of BSA, rbTP-1 and brIFN on percent incorporation of ^{35}S methionine into secreted proteins by isolated uterine glandular epithelial and stromal cells in the presence and absence of oxytocin.*

Treatment	Percent Incorporation	
	Gland cells	Stromal cells
Control	0.68	1.65
BSA	0.61	1.91
rbTP-1	0.49	1.89
brIFN	0.53	1.87
OT	0.66	1.68
BSA + OT	0.56	2.25[a]
rbTP-1 + OT	0.66	1.99[a]
brIFN + OT	0.80	1.90

*Data expressed as mean percent incorporation (SE = 0.22) per μg DNA of four experiments performed in triplicate.
[a]Significantly different from oxytocin (OT); ($p < 0.05$).

Two - Dimensional PAGE of synthesized proteins released by isolated endometrial gland and stromal cells.

Pattern of proteins produced by isolated glandular epithelial cells (Figure 10A) were different when compared to isolated stromal cells (Figure 10B). Glandular cells produced twenty proteins which were absent on fluorograms from stromal cell preparations. Four proteins were specifically produced by stromal cells which were not present on fluorograms obtained from gland cells (Table 5). All fluorograms within celltype were similar regardless of teatment.

Figure 10. Fluorograms of 2D-SDS PAGE gels of ^{35}S methionine labeled proteins synthesized and released by uterine glandular epithelial cells (A) and stromal cells (B). One hundred thousand cpm were loaded on each gel and fluorographs were exposed to dried gels for 14 days. Twenty specific proteins were released by isolated glandular cells while four specific proteins were released by isolated stromal cells.

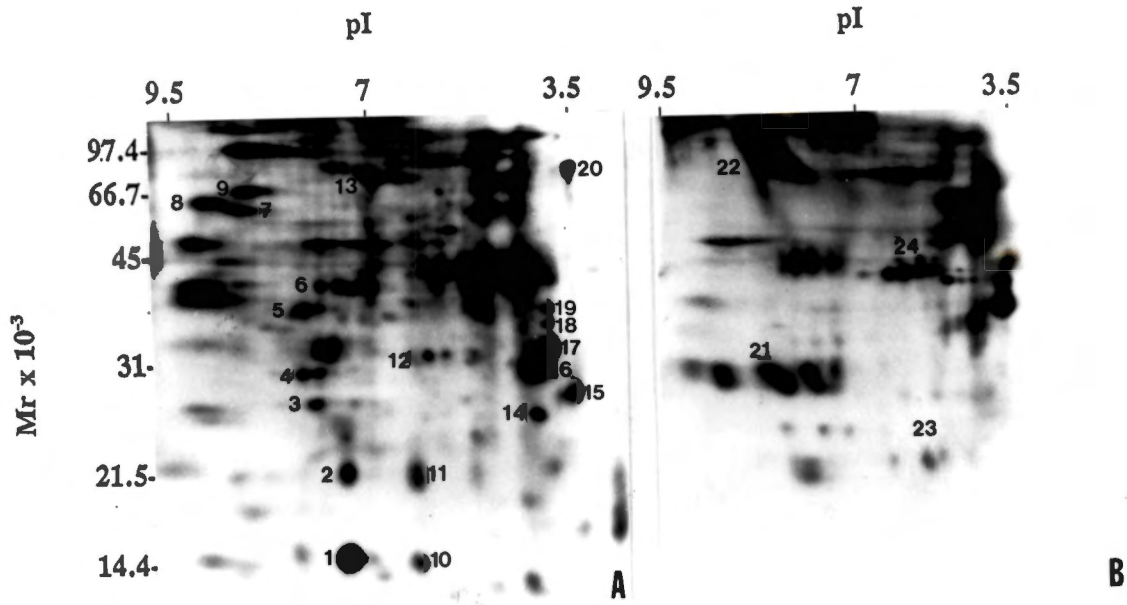

pI
9.5
7
3.5
97.4
66.7
45
31
21.5
14.4
Mr x 10-3
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
A
pI
9.5
7
3.5
21
22
23
24
B

TABLE 5. Specific proteins secreted by isolated bovine uterine glandular epithelial and stromal cells.

Cell type	Protein	Approximate Mole Wt. ($x10^3$)	Approximate pI
Gland[a]	1	14.4	7.5
	2	22.5	7.5
	3	25	8.0
	4	28	8.0
	5	37.5	8.5
	6	41.5	7.0-8.0
	7	63	9.0-9.4
	8	66	9.3-9.5
	9	75	8.3-9.3
	10	14.4	6.5
	11	22.5	6.5
	12	32	6.3
	13	80	7.0
	14	26	5.0
	15	29	3.5
	16	30	5.0
	17	32	4.0
	18	35	4.0
	19	38	4.0
	20	82	3.5
Stromal[b]	21	30	7.7-9.5
	22	>66.7	7.7-8.7
	23	23	5.3-6.3
	24	41.5	5.5-6.8

[a]Protein numbers as identified in Figure 10A.
[b]Protein numbers as identified in Figure 10B.

CHAPTER 4

DISCUSSION

Bovine endometrial luminal epithelial and stromal cells have previously been isolated and grown on plastic (Fortier et al., 1988). Results from the above study has indicated that luminal epithelial cells secrete greater quantities of PGF than stromal cells while stromal cells secrete more PGE than luminal epithelial cells. In the present study, we demonstrated that glandular epithelial cells secrete significantly more PGF and PGE than stromal cells. One reason for the different results between the present and former study may include the presence of an extracellular matrix. Cells in the present study were grown on a collagen matrix while cells in the former study were cultured on plastic.

Extracellular matrices have been known to cause induction of epithelial cell growth, differentiation and polarization in epithelial cells. Ghosh et al., (1991) using isolated mouse uterine epithelial cells describe differences which occurred between cells grown on basement membrane extract (BME) and type I collagen. Functional as well as morphological differences were observed. Thymidine incorporation in cells treated with epidermal growth factor (EGF) plus insulin was greater in cells grown on collagen type I than cells cultured on BME. Morphological differences included a more columnar shaped cell and more

extensive junctional complexes in cells grown on BME as compared to those on collagen. Differentiation in other epithelial cell types including rat small intestine (Carroll et al., 1988) and sertoli cells (Hadley et al., 1985) has been shown to be influenced by extracellular matrices.

In the study by Fortier et al., (1988) luminal epithelial cells were isolated from cows during early to mid luteal phase (days 2 to 12) while the present study obtained and isolated glandular epithelial cells from animals during the time period of maternal recognition of pregnancy (days 15 - 17). Therefore, the presence of an extracellular matrix, the time of the cycle when tissue was collected and the type of epithelial cell isolated may be reasons for the differences observed in prostaglandin secretion between the present study and the study by Fortier et al., (1988).

Episodic release of PGF by the uterus is thought to be responsible for luteolysis (see, Thatcher et al., 1986a). Uterine infusion of bCSP and bTP-1 attenuated the release of uterine PGF and extended the interestrous interval (Knickerbocker et al., 1986b; Helmer et al., 1989b). In addition, endometrial explants treated with bCSP and bTP-1 released reduced amounts of PGF (Gross et al., 1988a; Helmer et al., 1989a; Johnson, unpublished results). In sheep, isolated glandular epithelial cells also release reduced amounts of PGF when treated with oTP-1 (Salamonsen et al., 1988). In the present study, rbTP-1 and brIFN reduced PGF

secretion by isolated glandular epithelial cells which supports the data of previous studies. It has been demonstrated that an inhibitor of prostaglandin secretion exists in the endometrium from pregnant cows (Basu and Kindal, 1987; Gross et al., 1988b) and is induced by bTP-1 and bCSP (Helmer et al., 1989a). The inhibitor may function to inhibit the conversion of arachidonic acid to PGF and PGE (Gross et al., 1988b).

The effects of conceptus proteins on PGE release by bovine endometrial explants have been inconsistent. Secretion of endometrial PGE was unaltered when explants were incubated in the presence of bCSP and bTP-1 (Gross et al., 1988a; Johnson, unpublished results). In contrast, Helmer et al., (1989a) reported that bTP-1 and bCSP both increased PGE secretion by endometrial explants. In sheep, isolated glandular epithelial cells cultured in the presence of oTP-1 and recombinant human interferon release reduced amounts of PGE. In the present study, PGE release by isolated glandular epithelial and stromal cells was unaltered in response to rbTP-1 and brIFN. Therefore, supporting the above findings that conceptus proteins do not alter endometrial PGE secretion. A reason why conceptus proteins do not diminish PGE release by the endometrium may be due to the luteal protective role of PGE (Silvia et al., 1984).

The episodic release of PGF by the ruminant uterus is

believed to be under the control of oxytocin (Flint and Sheldrick, 1983; McCracken et al., 1984; Oyedipe et al., 1984). Oxytocin, in the present study, stimulated isolated uterine glandular cells to significantly increase both PGF and PGE release. The present *in vitro* data support an *in vivo* study by Kotwica et al., (1988) in which administration of oxytocin on days 14, 15 and 16 of the cycle caused an increase in PGF secretion. The present study also supports an *in vitro* study by Roberts et al., (1976) in which oxytocin stimulation caused increased PGF output by endometrial explants. Therefore, the responsiveness of the isolated glandular epithelial cells to oxytocin indicates that these cells respond in a manner similar to uterine cells in situ. In contrast to the effects of oxytocin on prostaglandin secretion by gland cells, stromal cells secrete PGF in barely detectable levels even under the influence of oxytocin. Low PGF secretion by isolated stromal cells maybe due to the absence of ovarian steroids in the culture media or to the lack of epithelial-stromal cell interactions. The overall effect of oxytocin on PGE secretion by stromal cells was highly variable between animals and thus complicates data interpretation. These data suggest a larger population of animals is needed to further study the effects of oxytocin on stromal cell PGE secretion.

Results from the present study suggest that oxytocin

receptors are located on both glandular and stromal cells since a response occurred in each cell type. Oxytocin receptor concentration as determined by oxytocin binding studies begin to rise on day 15, peak at day 18 and then decline by day 3 after estrus (Jenner et al., 1991). Fluctuation in receptor levels maybe due to changing concentration of steroids (Sheldrick and Flint, 1985; Vallet et al., 1990).

It has been proposed by McCracken et al., (1984) that during the estrous cycle the binding of progesterone to its endometrial receptors blocks the synthesis of oxytocin receptors. The present study concludes that rbTP-1 and brIFN were unable to inhibit oxytocin stimulated PGF release. These data support an earlier study in which oTP-1 was unable to inhibit oxytocin induced PGF release in the absence of progesterone (Ott et al., 1992). Perhaps the absence of progesterone in the culture media may have prevented down regulation of oxytocin receptors. However, PGE secretion was significantly reduced by isolated glands in the presence of rbTP-1, brIFN and oxytocin. These results are surprising since rbTP-1 and brIFN did not suppress PGE by gland cells in the absence of oxytocin. Further investigation of ovarian steroids on isolated uterine cell types is needed to elucidate their roll in oxytocin binding of prostaglandin metabolism.

Previous studies have indicated that bTP-1 alters

uterine protein production. Incorporation of radiolabeled amino acids into proteins by bovine endometrial explants were reduced in the presence of bTP-1 and bSCP (Helmer et al., 1989a; Johnson, unpublished results). In addition, no change in secretion of radiolabeled proteins were detected between pregnant and nonpregnant cows or explants treated with bCSP (Bartol et al., 1985a; Geisert et al., 1988; Gross et al., 1988a).

In the present study, secretion of radiolabeled proteins by gland cells were unresponsive to all treatments. Isolated stromal cells, reacted differently than gland cells when cultured in the presence of rbTP-1 (30 hours) + oxytocin (6 hours) and BSA (30 hours) + oxytocin (6 hours). These were the only treatments which caused significant increases in the incorporation of radiolabel into protein. The combined affect of brIFN and oxytocin also increased protein secretion, although not significantly. This data may suggest that isolated stromal cells require the additive effect of BSA rbTP-1, brIFN and oxytocin to increase protein secretion. Stromal cells may be responsive to these effects since they secrete a significantly larger amount of protein when compared to gland cells.

Uterine flushings obtained from pregnant cows have been analyzed for protein content and compared to serum proteins (Roberts and Parker 1974; 1976). Results from these studies indicate that several proteins (8 KDa to 48 KDa) were

present in flushings from pregnant cows but were absent in serum proteins. In a study by Bartol et al., (1981), four proteins (15, 307, 322, 343 KDa) were identified in uterine flushings from pregnant cows that were absent in flushings from non-pregnant cows. However, it was unknown if these uterine specific proteins were contributions of the uterus or conceptus.

Specific bovine uterine proteins were described by Bartol et al., 1985. Comparisons of these proteins produced by endometrial explants obtained from pregnant and non-pregnant cattle revealed no differences in the array of proteins released. In the present study, low molecular weight acidic and basic proteins (14 - 22 KDa) were produced mainly by isolated gland cells. These proteins have similar electrophoretic mobility to proteins enhanced by pregnancy, bCSP and rbTP-1. Geisert et al., (1988) reported an enhanced secretion of two low molecular mass basic proteins (14 - 16 KDa; pI 6.8-7.2) by day 17 endometrial explants obtained from pregnant cows. Endometrial explants cultured in the presence of bCSP also induced secretion of two low molecular weight basic proteins (10 KDa; 13 KDa; pI 7.8-8.4) (Gross et al., 1988a). Analysis of 1D-PAGE demonstrated bCSP, but not bTP-1 stimulated endometrial synthesis of a 15 KDa protein (Helmer et al., 1989a). In another study, two molecular weight classes of proteins (12 KDa; pI 7.0; 28 KDa, pI 5) were induced by endometrial explants in response

to increasing doses of rbTP-1 (Reuda et al., 1991). In the present study, no differences in amount or pattern of proteins between treated and untreated cell types were observed. However, a group of proteins were present on fluorograms from gland cell preparations with a similar electrophoretic mobility as the 28 KDa protein described by Rueda et al., (1991).

Recently, immunoprecipitation studies using an antibody to bovine placental retinol binding protein (RBP) followed by SDS and fluorography revealed that RBP (Mr 23 KDa; pI 5.3 - 6.3) was synthesized by bovine endometrium (Liu and Godkin, 1992) and isolated stromal cells. This protein was present in medium from gland cells at a barely detectable level (Smith and Godkin, 1992). A group of proteins with similar electrophoretic mobility (Mr 19 - 24; pI 5.4 - 6.3) have also been described by Bartol et al., (1985a). In sheep, the effects of oTP-1 on endometrial protein secretion is more clear than in the cow. Endometrial explants, cultured in the presence of oTP-1 stimulated the release of a 70 KDa protein (Godkin et al., 1984a; Sharif et al., 1989). In the present study, glandular cells produced twenty proteins which were absent on fluorograms from stromal cell preparations. In addition, four specific proteins were released by stromal cells which were not released by glandular cells. Physical contact between glandular epithelial and stromal cells may be necessary for

bTP-1 to cause alterations in protein secretion as was evident in earlier studies.

In summary, isolated glandular cells when grown on a collagen matrix are the major prostaglandin producing cells when compared to isolated stromal cells. Recombinant bTP-1 attenuates PGF and PGE production by gland cells whereas in stromal cells rbTP-1 has no effect on PGE release. Distinct protein patterns are evident between the two cell types. Stromal cells secrete RBP while gland cells secrete low molecular weight proteins which are absent in stromal cell preparations. Therefore, this study has demonstrated that isolated glandular epithelial and stromal cells have different biological functions.

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PART 3: THE EFFECTS OF RECOMBINANT BOVINE TROPHOBLAST PROTEIN-1, BOVINE RECOMBINANT INTERFERON AND OXYTOCIN ON COCULTURED BOVINE UTERINE GLANDULAR EPITHELIAL AND STROMAL CELLS

CHAPTER 1

INTRODUCTION

In order for a successful pregnancy to occur the corpus luteum must be protected from the luteolytic effects of prostaglandin F2α (PGF). During the estrous cycle in cattle and sheep, pulsatile secretion of PGF by the endometrium is regulated by ovarian steroids and oxytocin. During pregnancy, the pulsatile secretion of PGF is diminished and the corpus luteum is maintained. The antiluteolytic signal in sheep and cattle is a trophoblast interferon (IFN) previously referred to as ovine (o) or bovine (b) trophoblast protein-1 (TP-1) and recently named IFN tau (τ) (see, Roberts et al., 1992). Interferon τ is the major secretory protein of ovine and bovine blastocyst during early pregnancy. In sheep, oTP-1 is secreted by the trophoblast between days 13 and 21 (Godkin et al., 1982) and is the major translation product of day 16 conceptus RNA (Hansen et al., 1985; Anthony et al., 1988). Maximal secretion of bTP-1 by the bovine trophoblast is between days 16 and 24 of pregnancy (Bartol et al., 1985).

Trophoblast IFN mediates luteal maintenance through interactions with uterine endometrium where they alter prostaglandin and protein metabolism. Effects of IFN τ on uterine prostaglandin and protein metabolism have been evaluated <u>in vitro</u> but are not well understood. Studies agree that PGF secretion by bovine endometrial explants is

reduced in the presence of bTP-1 and bovine conceptus secretory protein (bCSP) (Gross et al., 1988a; Helmer et al., 1989; Johnson, unpublished results). However, reports on prostaglandin E-2 (PGE) metabolism by endometrial explants are contradictory. Prostaglandin E secretion is either increased or unaltered in response to bTP-1 or bCSP (Gross et al., 1988a; Helmer et al., 1989; Johnson, unpublished results). Isolated ovine endometrial cells release reduced amounts of PGF in the presence of oTP-1 (Salamonsen et al., 1988).

Another effect of trophoblast proteins on uterine metabolism is its modulation of protein secretions. Increased secretions of specific uterine proteins by bovine and ovine endometrial explants occurred in the presence of IFN τ (Godkin et al., 1984a; Vallet et al., 1987; Rueda et al., 1991). Presently, the effects of IFN τ on isolated endometrial cell secretions of prostaglandin and proteins are not well understood. In sheep (Cherny and Findlay, 1990) and cows (Fortier et al., 1988) isolated uterine glandular epithelial cells secrete primarily PGF while stromal cells secrete primarily PGE. Isolated ovine endometrial cells increase synthesis of specific uterine proteins as a result of incubations with oTP-1 (Salamonsen et al., 1988). Secretory activity of isolated epithelial cells is associated with the development of polarity (Parry et al., 1987; Glasser et al., 1988). Prostaglandin

secretion by isolated uterine epithelial cells obtained from rat and sheep have a preferential basal secretion as opposed to apical secretion (Jacobs et al., 1990; Cherny and Findlay, 1990). Preferential protein secretory differences also occur by polarized bovine uterine epithelial cells (Godkin et al., 1988).

Interactions between stromal and epithelial cells in vitro have demonstrated that the culture of stromal cells with epithelial cells mediates normal morphology and estrogen responsiveness of uterine and mammary epithelial cells (Cooke et al., 1986; Inaba et al., 1988). Presently the effects of IFNτ on polarized bovine uterine epithelial cells co-cultured with stromal cells is unknown.

This study was designed to determine if stromal interactions with polarized bovine glandular epithelial cells alter secretion of prostaglandin and proteins in response to rbTP-1, brIFN and oxytocin.

CHAPTER 2

MATERIAL AND METHODS

Materials

Cell culture medium, Hank's balanced salt solution (HBSS), HBSS without (Ca^{+2}) and (Mg^{+2}), Dulbeccos modified Eagle's medium (low glucose) (DMEM), DMEM methionine free, Minimal essential media (MEM), fetal calf serum (FCS) (charcoal treated; 5 g/100 ml), amino acids and basal medium Eagle vitamin solution were purchased from Gibco Laboratories, Grand Island, NY. Insulin was obtained from Eli Lilly and Co. Indianapolis, IN. Enzymes, collagenase A and dispase were purchased from Boehringer Mannheim, Indianapolis, IN and Matrigel from Collaborative Research Inc. (Bedford, MA). Culture plate inserts, Millicell CM were supplied by Millipore (Bedford, MA) and 24 well plates were obtained from Corning Glass Works, Corning, NY. Rat tail type I collagen, bovine serum albumin (BSA), oxytocin and glucose were obtained from Sigma Chemical Co., St. Louis, MO. Trans ^{35}S- label methionine (specific activity 1066 ci/mmole) was purchased from ICN Biochemicals, Inc. Irving, Ca. Recombinant bovine trophoblast protein-1 (rbTP-1), specific activity 1.5 x 10^7 U/mg) (GBK-1/VSV) was a gift from R. M. Roberts. Recombinant bovine interferon (rbIFN), specific activity 2.6 x 10^7 U/mg was donated by Ciba-Geigy Corp. Supplies for electrophoresis were as described previously (Godkin et al., 1988).

Animals and collection of uterus

In this study, two cell types were isolated from the bovine uterine endometrium. These include glandular epithelial and stromal cells. Since actions of bTP-1 critical to pregnancy maintenance are believed to occur between days 15 and 17, endometrium was obtained from cows between days 15 and 17 of the cycle (estrus=0). Uteri from 3 cows were utilized in this study. The estrous cycles of cows were synchronized with a single I.M. injection of Lutalyse (25 mg; Upjohn Co., Kalamazoo, MI). Cows were observed twice daily for estrus behavior. Reproductive tracts were collected at slaughter within 40 minutes of death, placed on ice and transported to the laboratory where they were trimmed, the exterior washed in water, then ethanol and placed in a laminar flow hood for dissection.

Isolation and culture of glandular epithelial and stromal cells.

Endometrial strips obtained from above were placed into a 150 x 25 mm petri dish, containing sterile HBSS without Ca^{+2} and Mg^{+2} and minced into small fragments (2-4 mm^2) using two scalpel blades. Minced material was placed into a sterile 500 ml Erylenmyer flask containing 0.1% collagenase and 0.5 U/ml dispase in 250 ml HBSS without Ca^{+2} and Mg^{+2}. The Erylenmyer flask containing the digestion media was placed in a 37° C shaking water bath for approximately 2 hours, during which the tissue was checked periodically to

determine the progress of digestion. After digestion, HBSS without Ca^{+2} and Mg^{+2} was added to digesta and transferred to a 150 x 25 mm petri dish. Undigested material and cellular debris was removed manually and the remainder was passed through a sterile 200μ nylon mesh (Spectrum Medical Industries, Inc., Los Angeles, CA) through which allowed small glandular fragments, small clumps of cells and isolated cells passed through. Fragments were isolated by slow speed centrifugation (30 x g). Dispersion of glandular fragments through a sterile pipette produced smaller fragments. Stromal cells were obtained by high speed centrifugation (500 x g) of the supernatant from glandular cell washings.

Prior to seeding, with isolated gland cells, Millicell inserts were coated with 100 μl of diluted Matrigel (1:7) solution in DMEM and air dried overnight in a sterile hood. Isolated stromal cells were seeded onto 0.01% rat tail collagen coated 24 well plates. Both cell types were resuspended in DMEM supplemented with 10% heat inactivated FCS, 4x glucose, 2x ABAM, 1x insulin, 1x basal medium Eagle vitamin solution and 1x non-essential amino acids prior to seeding. Cells were incubated at 38° C in a humidified atmosphere of 5% CO_2 : 95% air. Following 48 hours of incubation glandular cells were treated briefly with 0.05 % trypsin/ 0.04 % EDTA to remove contaminating stromal cells. Electrical resistance was measured with a Millicell ERS

meter (Millipore, Bedford, MA). In three experiments resistance was measured from seeding to confluency. Unseeded millicell inserts were used as controls. Media was changed every 48 hours until confluent. Once confluent, Millicell inserts were placed over stromal cells for 24 hours before treatment. After 24 hours cells were washed 3x in HBSS and received the following treatments in triplicate. 1) control (no treatment) 2) BSA (30 ng/ml) 3) rbTP-1 (30 ng/ml) 4) brIFN (30 ng/ml) 5) oxytocin (50 mU) 6) BSA (30 ng/ml) + oxytocin (50 mU) 7) rbTP-1 (30 ng/ml) + oxytocin (50 mU) 8) brIFN (30 ng/ml) + oxytocin (50 mU). For treatments involving oxytocin, cells were cultured for 30 hours in the absence of oxytocin at which time oxytocin was added. At 36 hours, media from apical secretions were harvested, subjected to centrifugation in an Eppendorf centrifuge and supernatant was stored at -20° C.

Radioimmunoassay procedure

Prostaglandin F and E2 were analyzed with RIA kits purchased from Advanced Magnetics (Cambridge, Massachusetts). Intra- and interassay coefficient of variation (CV) were 4.14% and 6.75% for PGF and 8.67 % and 14.68% for PGE. Sensitivity of the assay for PGF and PGE was 8 pg/assay tube.

Protein synthesis

Percent incorporation of radiolabeled ^{35}S methionine into protein was measured by using trichloroacetic acid

(TCA) precipitation (Mans and Novelli, 1961). Briefly, duplicate samples of media were placed on a sampling manifold (Millipore Corporation, Bedford, MA) containing Whatman glass microfibre filters (Whitman International LTD. Maidstone, England) presoaked with 5% TCA. Unincorporated radioactivity was removed by serial washings of filters with 10% and 5% TCA followed by 95% ethanol. Whatman filters containing precipitated proteins were collected and analyzed by scintillation counting.

Protein Electrophoresis

Media from isolated cell types was dialysed (molecular weight cutoff 6,000 - 8,000) against 1mM Tris-HCl, pH 7.6 (4 changes at 8 hr intervals). After dialysis an aliquot containing 100,000 cpm was lyophilized and dissolved in a volume of 5 mM K_2CO_3 containing 9.4M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) procedure was described by Roberts et al., (1984). Briefly, following electrophoresis, gels were stained with Coomassie Brillant Blue R-250 impregnated with 1M sodium salicylate (Chamberlain, 1979) dried and exposed to Kodak XAR film for analysis of radioactive proteins.

DNA measurement

Prostaglandin and protein (% incorporation) data was based on cell DNA content, which was quantitated using 4', 6- diamidine-2-phenylindole (DAPI) (Kapuscinski and

Skoczylas, 1977; Kapuscinski and Yanagi, 1979). Briefly, after removal of media from cultured cells, 0.5 ml of DAPI buffer (150 mM NaCl; 5mM Hepes, pH 7.0) was added to each millicell and froze at -20° C. After thawing, cells were further disrupted by sonication (10 - 20 second intervals). Unknown cellular DNA was measured in a cytofluor 2300 using calf thymus DNA as a standard.

Statistical Analysis

Prostaglandin and protein data are expressed as the mean +/- SE. When necessary data was log transformed to stabilize the variance. Data were analyzed using general linear mixed models methodology using GLMM (Blouin and Saxton, 1990). The analysis of variance considered treatments as a fixed factor and cows as a random factor. Differences between treatments were separated by orthogonal contrast. P values of less than 0.05 were considered significant.

CHAPTER 3

RESULTS

Transepithelial Resistance

Polarity, a result of functional tight junctions was measured by transpithelial resistance. Electrical resistance of glandular epithelial cells steadily increased to day 7 at which time microscopic evaluation of confluence was observed. Maximal resistance occurred on day 11 of culture (Figure 1).

Effects of rbTP-1, brIFN and oxytocin on PGF production by isolated uterine glandular epithelial cells co-cultured with stromal cells.

Recombinant bTP-1 and brIFN significantly ($p < 0.01$) reduced secretion of PGF by co-cultured glandular cells when compared to BSA treated cells. When oxytocin was added to co-cultured cells, PGF release was lower in cells cultured in the absence of oxytocin, however, no significance ($p > 0.05$) was detected. Bovine serum albumin, rbTP-1 and brIFN in addition to oxytocin did not have a significant ($p > 0.05$) effect on PGF secretion by gland cells (Figure 2). As seen in Figure 3, gland cells co-cultured with stromal cells released significantly ($p < 0.01$) larger amounts of PGF than gland cells cultured in the absence of stromal cells.

Figure 1. Development of transepithelial resistance in isolated bovine glandular epithelial cells cultured on matrigel coated millicell-cm inserts. Transepithelial resistance was measured using a Millicell-ERS voltohmmeter. Resistance was calculated by subtracting the blank resistance (Millicells without cells) from millicells containing cells. The unit area was corrected for by multiplying by 0.6 cm^2 (area of 12 mm insert) thus giving resistance-area (Ω - cm^2).

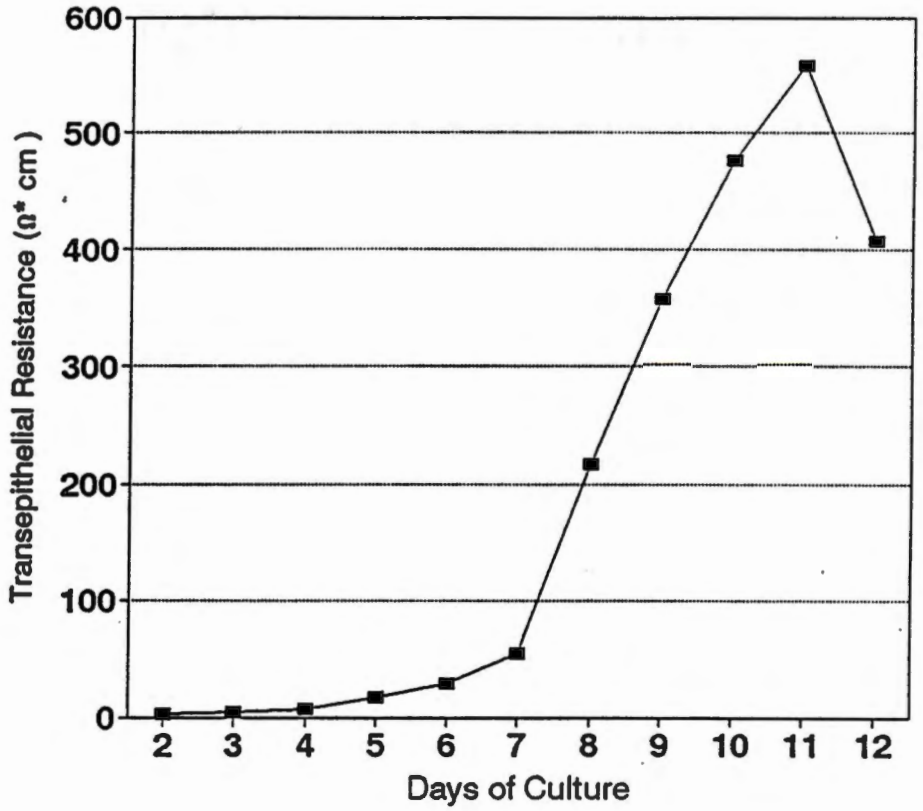

600
500
400
300
200
100
0
Transepithelial Resistance (Ω* cm)
2
3
4
5
6
7
8
9
10
11
12
Days of Culture

Figure 2. PGF production by glandular epithelial cells co-cultured with stromal cells in the presence or absence of oxytocin. Media was collected after 36 hours of treatment and assayed for PGF by RIA. Significant ($p < 0.01$) decreases in PGF secretion occurred in cells treated with rbTP-1 and brIFN. Oxytocin had no significant ($p > 0.05$) effect on PGF release. Data was expressed as mean +/- SEM of three experiments prepared in triplicate. (* significantly different from BSA).

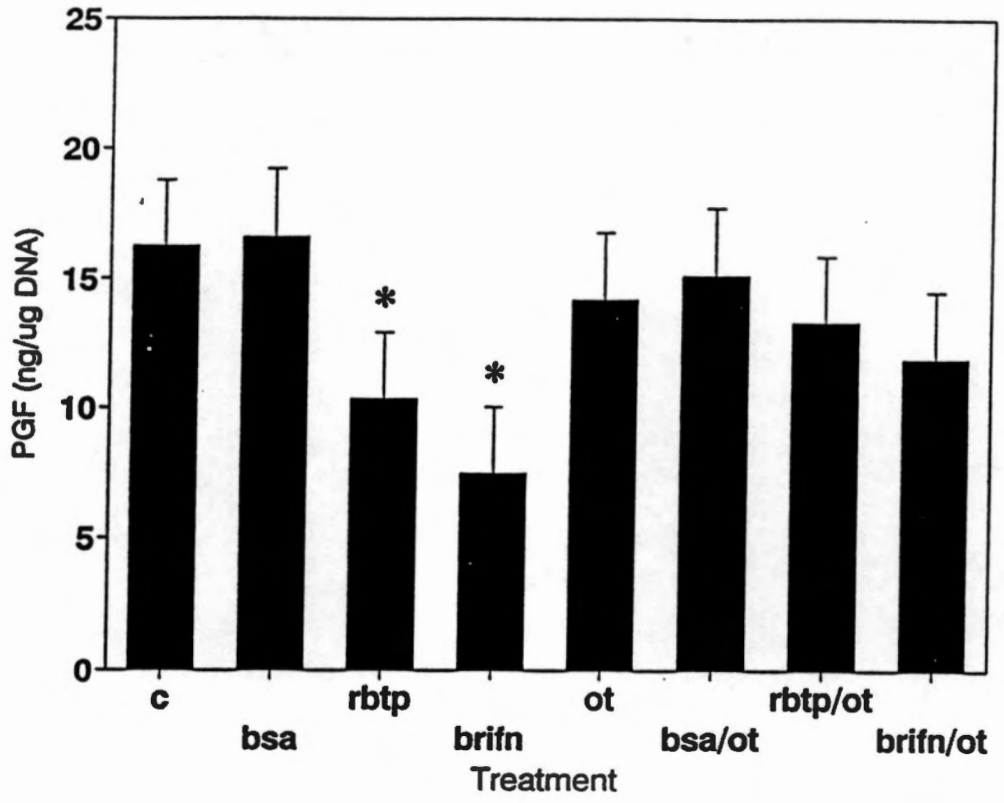
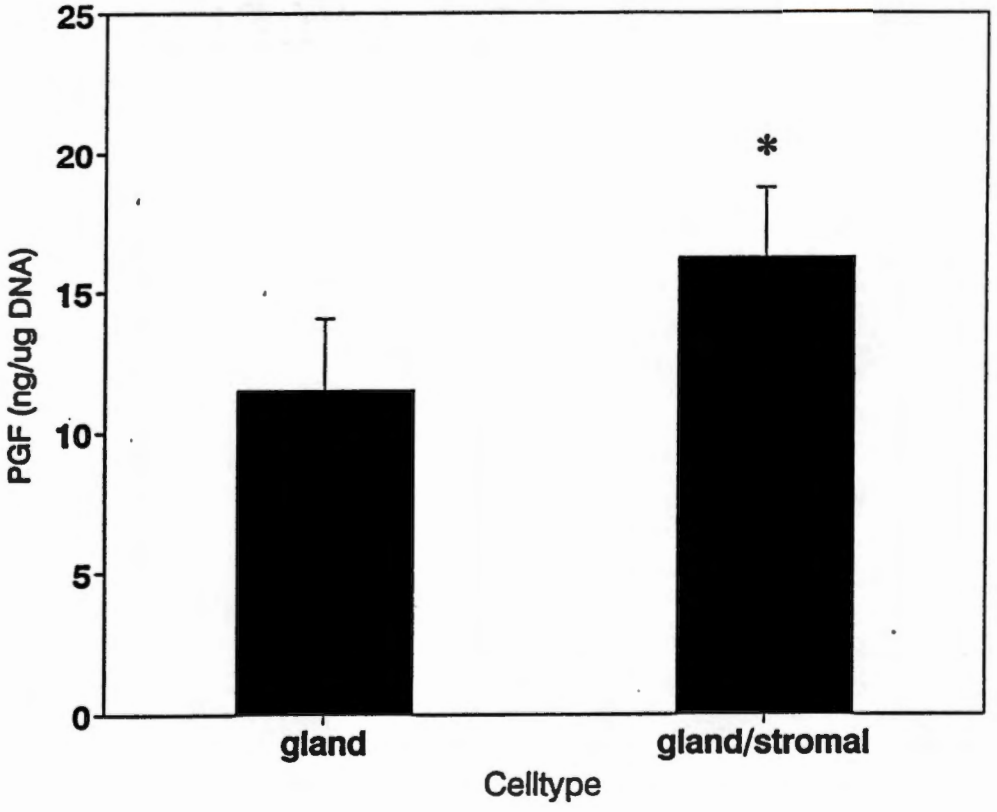

25
20
15
10
5
0
PGF (ng/ug DNA)
*
*
c
bsa
rbtp
brifn
ot
bsa/ot
rbtp/ot
brifn/ot
Treatment

Figure 3. Effect of epithelial-stromal interactions on PGF release. Media was collected after 36 hours of treatment and assayed for PGF by RIA. A significant ($p < 0.01$) increase in PGF release occurred as a result of stromal cell interaction. Data was expressed as mean +/- SEM of three experiments prepared in triplicate (significant difference denoted by *).


25
20
15
10
5
0
PGF (ng/ug DNA)
*
gland
gland/stromal
Celltype

Effect of rbTP-1, brIFN and oxytocin on PGE production by isolated uterine glandular epithelial cells co-cultured with stromal cells.

A reduction in PGE release occurred when co-cultured cells were incubated in the presence of rbTP-1 and brIFN. However, brIFN, but not rbTP-1 significantly ($p < 0.01$) reduced PGE secretion when compared to BSA treated cells. Prostaglandin E secretion by co-cultured gland cells cultured in the presence of oxytocin was not different than cells cultured in the absence of oxytocin. Co-cultured cells treated with brIFN, but not rbTP-1, for 30 hours prior to oxytocin treatment secreted significantly ($p < 0.05$) lower levels of PGE than cells treated with oxytocin alone (Figure 4). Glandular cells cultured without stromal cells released significantly ($p < 0.01$) less PGE than gland cells cultured with stromal cells (Figure 5).

Effects of rbTP-1, brIFN and oxytocin on % incorporation of ^{35}S- methionine into radiolabeled proteins by co-cultured glandular epithelial and stromal cells.

Percent incorporation of ^{35}S-methionine into radiolabeled proteins by co-cultured cells was not affected by any treatment. However, when compared to gland cells cultured alone, protein secretion was significantly ($p < 0.05$) reduced as a result of glandular-stromal interactions (Table 1).

Figure 4. PGE production by glandular epithelial cells co-cultured with stromal cells in the presence or absence of oxytocin. Media was collected after 36 hours of treatment and assayed for PGE by RIA. Bovine rIFN significantly reduced PGE release in the presence ($p < 0.05$) and absence ($p < 0.01$) of oxytocin. Data was expressed as mean +/- SEM of three experiments prepared in triplicate. (* significantly different from control; BSA) (^{a} significantly different from oxytocin).

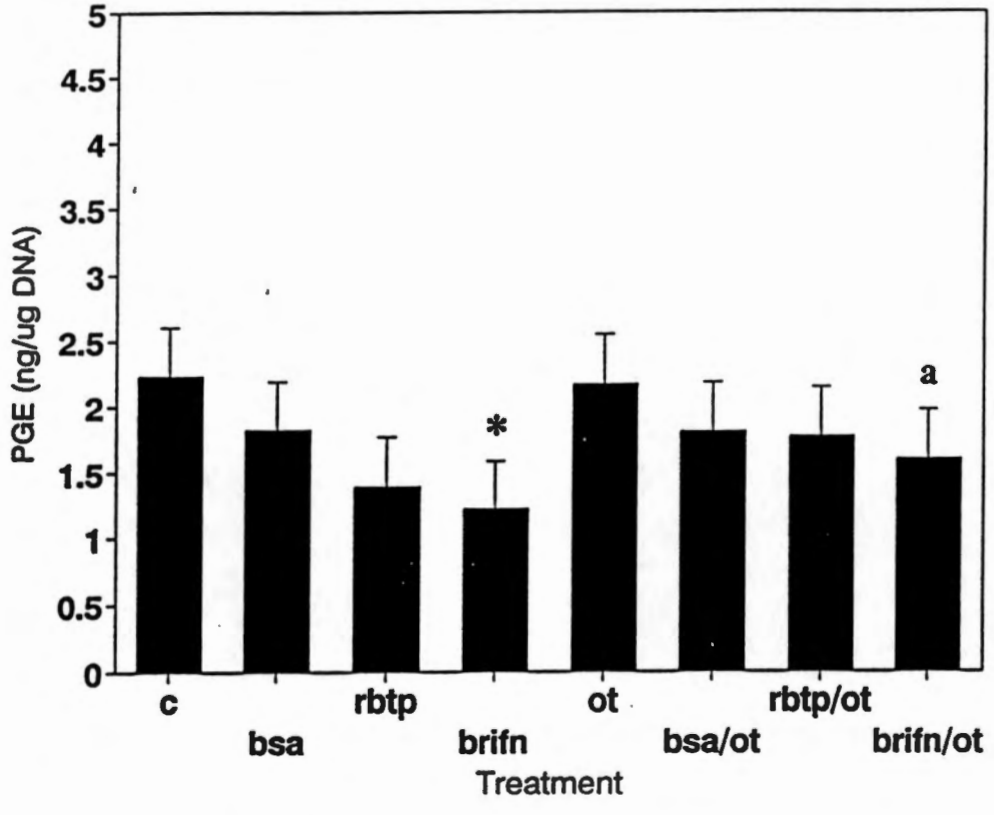

PGE (ng/ug DNA)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
a
c
bsa
rbtp
brifn
ot
bsa/ot
rbtp/ot
brifn/ot
Treatment

Figure 5. Effect of epithelial-stromal interactions on PGE release. Media was collected after 36 hours of treatment and assayed for PGE by RIA. A significant ($p < 0.01$) increase in PGE release occurred as a result of stromal interaction. Data was expressed as means (SE = 0.37) of three experiments prepared in triplicate (significant differences denoted by *).

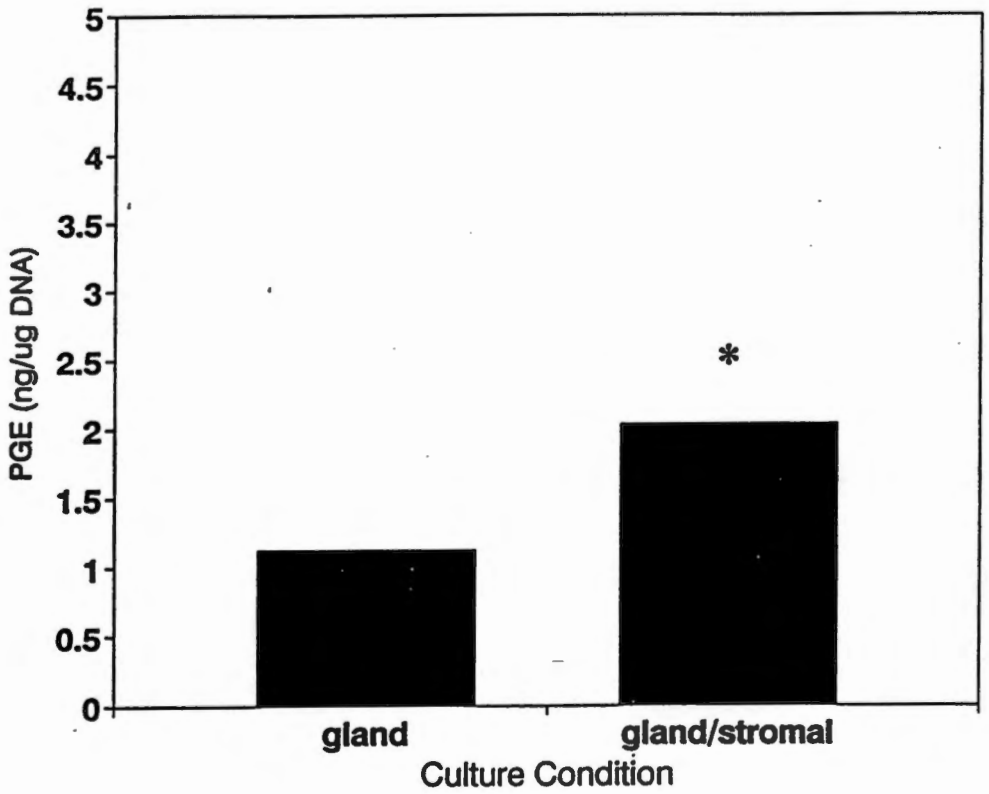

5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
PGE (ng/ug DNA)
*
gland
gland/stromal
Culture Condition

TABLE 1. Effects of BSA, rbTP-1 and brIFN on percent incorporation of ^{35}S methionine into secreted proteins by co-cultured uterine glandular epithelial and stromal cells.*

Treatment	Percent Incorporation
Control	-1.24
BSA	-1.09
rbTP-1	-1.11
brIFN	-1.04
OT	-1.32
BSA + OT	-1.33
rbTP-1 + OT	-1.31
brIFN + OT	-1.50
No stromal	-0.79[a]

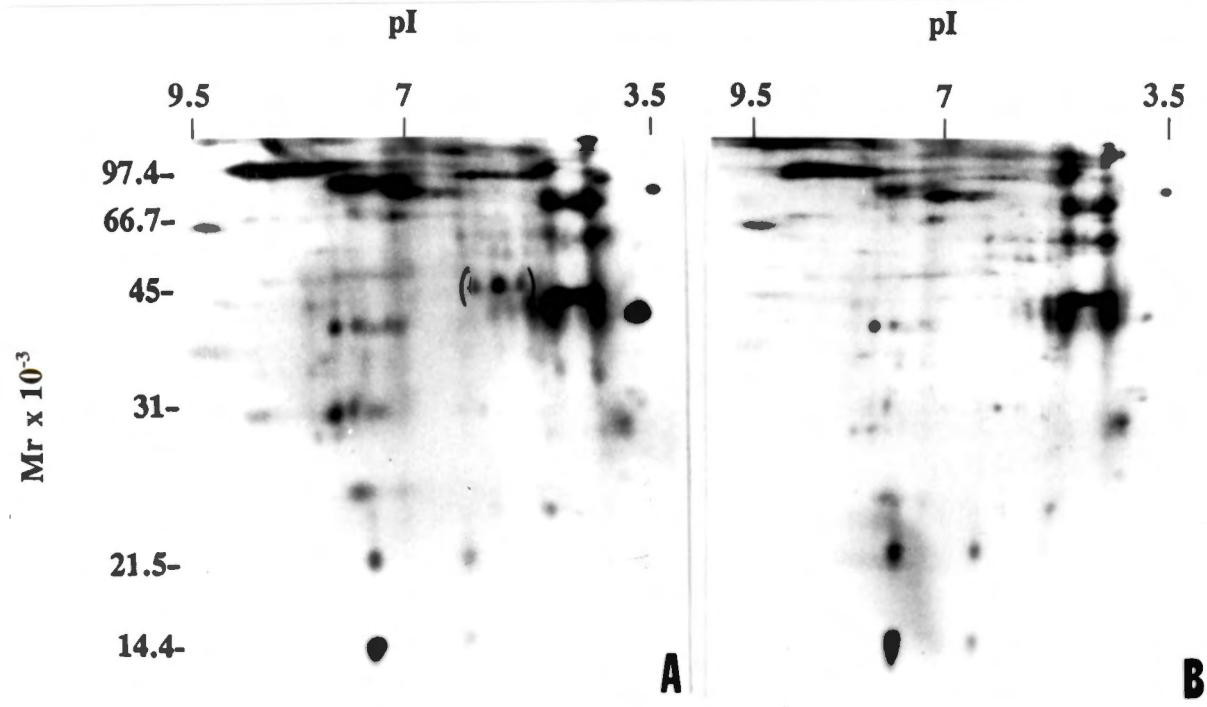
*Log transformed data expressed as mean percent incorporation (SE = 0.28) per μg DNA of three experiments performed in triplicate.

[a]Significantly different from oxytocin (OT); ($p<0.05$).

Two-Dimensional PAGE of synthesized proteins released by glandular epithelial cells co-cultured with stromal cells.

Pattern of proteins produced by co-cultured cells were similar regardless of treatment. Co-cultured cells (Figure 6A) produced four low molecular mass (14-22 KDa; pI 6.2-7) proteins, which were also present on fluorograms obtained from glands cultured alone (Figure 6B). Differences were present in the array of proteins produced by co-cultured cells and gland cells cultured alone. Differences include the presence of a group of acidic (45 KDa, pI 5.5 - 6.8) proteins which were present on fluorograms obtained from co-cultured cells and absent from gland cell preparations.

Figure 6. Fluorograms of 2D SDS PAGE gels of ^{35}S-methionine labeled proteins synthesized and released by uterine co-cultured glandular epithelial and stromal cells (A) and glandular epithelial cells cultured in the absence of stromal cells (B). One hundred thousand cpm were loaded on each gel and fluorograms were exposed to dried gels for 14 days. Different protein patterns were observed between co-cultured and non co-cultured cells. Fluorograms from co-cultured cells indicated the presence of a group of acidic proteins (45 KDa, pI 5.5-6.8) (brackets) which were absent on fluorograms prepared from glandular cells cultured alone.


pI
9.5
7
3.5
pI
9.5
7
3.5
97.4-
66.7-
45-
31-
21.5-
14.4-
Mr x 10-3
A
B

CHAPTER 4

DISCUSSION

In the present study, bovine glandular epithelial cells were grown on millicell inserts which allowed them to establish polar organization. Polarized cells contain distinct apical/basal domains and tight junctions which allows them to express normal phenotypic expression (Rodriguez-Boulan and Nelson 1989). Electrical resistance occurs in polarized cells as a result of the presence of functional tight junctions (Cereijido et al., 1978). Uterine cells in the present study, established polarity as indicated by cross-membrane resistance measurements. These resistance measurements are in agreement with measurements reported by Glasser et al., (1988).

Recently in a study by Cherny and Findlay (1990) isolated ovine endometrial cells and stromal cells were grown on matrigel coated millicell inserts. They demonstrated that PGF and PGE were secreted asymmetrically with basal release being larger than apical release. An asymmetrical release of PGF was also demonstrated by mouse uterine epithelial cells where a 3.5 fold greater basal release over apical release occurred (Jacobs et al., 1990). In early studies (Bazer and Thatcher, 1977) PGF secretion was believed to be secreted mainly toward the uterine vasculature and transported to the ovary by a counter-current mechanism, thus supporting the above study by Cherny

and Findlay (1990). In another study, bilateral perfusion devises were used to determine which direction (luminal or myometrial) endometrial PG release favored. Results demonstrated PGF secretion favored the luminal direction in ovine (Lacroix and Kann, 1983) and bovine (Gross et al., 1988c). In the present study, prostaglandin secretion was determined in apical secretions because basolateral secretions would contain contributions from both gland and stromal cells.

Oxytocin had no significant effect on PGF and PGE release by gland cells cultured in the presence of stromal cells. However, in a previous study, oxytocin caused a significant increase in PGF and PGE release by cells cultured on rat tail collagen coated plastic (Smith and Godkin, unpublished results). The increase in PGF production by oxytocin has been demonstrated <u>in vivo</u>. Injections of oxytocin during the late luteal phase of the cycle will increase PGF production and luteolysis (Roberts and McCracken, 1976; Fairclough et al., 1984). In another study administration of oxytocin on days 14,15 and 16 of the cycle caused an increase in PGF levels and no extension of the cycle (Kotwica et al., 1988).

Oxytocin binding studies demonstrate that receptor concentrations begin to rise on day 15 of the cycle and then decline on day 3 after estrous in cattle (Jenner et al., 1991). Therefore, when cells were isolated in the present

study, oxytocin receptor levels were on the rise since uteri were utilized on days 15-17 after estrous. A reason for the lack of response by co-cultured gland cells may be due to stromal cell secretions. These secretions could contain factors which may interfere with oxytocin receptor production. Another reason may be due to the fact that gland cells in the previous study were cultured on rat-tail collagen. While in the present study cells were grown on matrigel and expressed polarity. The expression of polarity may allow oxytocin to stimulate basal secretion of PGF.

The addition of rbTP-1 and brIFN reduced secretion of PGF by co-cultured uterine cells. This is consistent with results from a study utilizing isolated bovine uterine cells grown on rat tail collagen coated plastic, where rbTP-1 and brIFN significantly reduced PGF secretion (Smith and Godkin, unpublished results). The reduction in PGF release by bTP-1 also occurs in endometrial explants obtained from cyclic cows (Helmer et al., 1989; Johnson, unpublished results). The present results support conclusions by Salamonsen et al., (1988), that oTP-1 reduces PGF release by isolated ovine endometrial cells.

Gross et al., (1988b) suggested that a putative inhibitor is present in bovine endometrium and may act to inactivate cycloxygenase. If this were true, then PGF and PGE secretion would be diminished. In contrast, Helmer et al., 1989 reported that bTP-1 increased PGE secretion by

explants but Johnson (unpublished results) observed no effect of bTP-1 on explant PGE production. In addition, ovine uterine epithelial cells were reported to diminish PGE production by uterine cells in response to oTP-1. In our laboratory, it was observed that bTP-1 did not diminish PGE production by stromal cells and reduced PGE production numerically but not significantly by epithelial cells (Smith and Godkin, unpublished results).

In the present study, rbTP-1 lowered PGE levels but not significantly. Bovine rIFN significantly reduced PGE levels by co-cultured glandular epithelial cells. A significant decrease by brIFN but not rbTP-1, could be due to the fact that brIFN is newer and has not been stored frozen for more than a year. Therefore, brIFN may have greater activity than rbTP-1. To determine if rbTP-1 and brIFN could inhibit oxytocin stimulation of glandular secretion of prostaglandins, cells were incubated in the presence of rbTP-1 or brIFN prior to the addition of oxytocin. Present results have shown that rbTP-1 and brIFN were unable to inhibit oxytocin stimulation of PGF release.

PGE secretion by co-cultured cells was not significantly reduced by rbTP-1. In contrast, brIFN caused a significant decrease in PGE release by cells treated with oxytocin. However, decreased amounts of oxytocin stimulated PGF and PGE release were detected in the presence of rbTP-1 and brIFN. Therefore, suggesting that rbTP-1 and brIFN were

functioning to diminish oxytocin stimulated prostaglandin synthesis. Stromal cells as demonstrated in this study, caused gland cells to secrete significantly greater levels of PGF and PGE than gland cells cultured in the absence of stromal cells. The increase in prostaglandin levels may be due to the diffusion of prostaglandins produced by stromal cells. This event is unlikely for PGF since stromal cell production of PGF is minimal (Smith and Godkin, unpublished results).

The effects of bTP-1 on uterine protein secretion is not very clear. Incorporation of radiolabeled amino acids into proteins by bovine endometrial explants were reduced in the presence of bTP-1 and bCSP (Helmer et al., 1989; Johnson, unpublished results). In addition, no change in secretion of radiolabeled proteins were detected between pregnant and nonpregnant cows or explants treated with bCSP (Bartol et al., 1985; Geisert et al., 1988; Gross et al., 1988a). In the present study, rbTP-1, brIFN and oxytocin or the combined treatments had no effect on percent incorporation of radiolabeled amino acids into proteins by co-cultured cells. The only significant difference in percent incorporation of radiolabeled amino acids into protein by gland cells was due to the presence of stromal cells. Physical contact between glandular epithelial and stromal cells may be necessary for bTP-1 to cause a reduction in protein secretion as was evident in the

aforementioned explant studies.

In a preliminary experiment, glandular cells were isolated and seeded on millicell inserts while being co-cultured with stromal cells. As a result of this combination, glandular cells grew very slowly, failed to reach confluency and eventually died. Stromal cells grew at normal rates to confluency in the presence of glandular cells. These results made it necessary to culture glandular cells to confluency in the absence of stromal cells. When confluent glandular cells on millicell inserts were placed in wells containing stromal cells no deleterious effects were observed. Thus, it was apparent that stromal cells released some inhibitory factor that interfered with glandular cell growth.

Specific bovine uterine proteins were described by Bartol et al., (1985). Comparisons of these proteins produced by endometrial explants obtained from pregnant and non-pregnant cattle revealed no differences in the array of proteins released. In the present study, low molecular weight acidic and basic proteins (14 - 22 KDa; pI 6.2 - 7.5) were present on fluorograms obtained from cocultured and isolated glandular epithelial cells. These proteins have similar electrophoretic mobility to proteins enhanced by pregnancy, bCSP and rbTP-1. Geisert et al., (1988) reported an enhanced secretion of two low molecular mass basic proteins (14 - 16 KDa; pI 6.8 - 7.2) by endometrial explants

obtained from pregnant cows. Endometrial explants cultured in the presence of bCSP also induced secretion of two low molecular weight basic proteins (10 KDa; 13 KDa; pI 7.8 - 8.4) (Gross et al, 1988a). Analysis of 1D-PAGE demonstrated bCSP, but not bTP-1 stimulates endometrial synthesis of a 15 KDa protein (Helmer et al., 1989). In another study, two molecular weight classes of proteins (12 KDa; pI 7.0; 28 KDa, pI 5.0) were induced by endometrial explants in response to increasing doses of rbTP-1 (Rueda et al., 1991). In the present study, no differences in amount or pattern of proteins between treated and untreated cell types were observed.

Coculturing stromal cells with glandular cells had no apparent qualitative affect on protein synthesis. Some protein patterns present on fluorograms obtained from co-cultured cells were different than protein patterns from isolated gland cells but this was apparently the result of contributions from stromal cells. A group of proteins (45 KDa; pI 5.8 - 6.7) were present on fluorograms from co-cultured cells that were absent on fluorograms from gland cells cultured alone. However, this protein was present on fluorograms from isolated stromal cells but absent on fluorograms from isolated glandular cells (Smith and Godkin, unpublished results).

The effect of rbTP-1 on co-cultured glandular epithelial cell prostaglandin and protein metabolism was

similar to gland cells cultured in the absence of stromal cells. However, the presence of stromal cells altered PGF metabolism and decreased percent incorporation of radiolabeled amino acids into proteins. Thus, stromal cells may act to alter the biochemical activity of glandular epithelial cells.

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PART 4: EFFECTS OF RECOMBINANT BOVINE TROPHOBLAST PROTEIN-1 ON THE REGULATION OF GENE EXPRESSION BY ISOLATED BOVINE UTERINE GLANDULAR EPITHELIAL AND STROMAL CELLS

CHAPTER 1

INTRODUCTION

Maintenance of early pregnancy is dependent on progesterone secretion by the corpus luteum. In the nonpregnant cow, progesterone levels decline toward the end of the estrous cycle as a result of uterine prostaglandin F-2α (PGF) secretion. During early pregnancy the conceptus must attenuate PGF secretion, thus protecting the corpus luteum from the luteolytic actions of PGF (see Thatcher et al., 1984).

The conceptus rescues the CL from regression through the antiluteolytic effect of bovine trophoblast protein-1 (bTP-1). This protein is secreted by the trophoblast cells of the developing conceptus between days 16 - 24 (Bartol et al., 1985). Infusion of bTP-1 into the uterine lumen of cyclic cattle between days 14 - 17 extended the lifespan of the corpus luteum (Helmer et al., 1989a). In vitro, bTP-1 reduced PGF synthesis by uterine endometrial explants (Helmer et al., 1989b; Johnson, unpublished results).

The mechanism of how endometrial prostaglandin synthesis during pregnancy is modulated is unknown. Earlier studies have reported the presence of an endometrial intracellular factor induced by pregnancy, which inhibits enzymes involved in prostaglandin synthesis. This inhibitor was reported to be present in placental tissue (Shemesh et al., 1984) and endometrial tissue from cyclic cattle

(Wlodover et al., 1976). Basu and Kidahl (1987) found the presence of an endogenous inhibitor of prostaglandin synthesis to be increased during early pregnancy when compared to nonpregnant cows. Gross et al.,(1988) characterized some physicochemical properties of the putative intracellular endometrial prostaglandin inhibitor. Prostaglandin synthesis by cotyledonary microsomes was slightly inhibited in response to intracellular preparations of endometrium from cyclic cows. Microsomes treated with intracellular preparations from pregnant cows greatly reduced prostaglandin synthesis. It was determined that the inhibitor(s) may be proteinaceous but it has yet to be isolated or defined.

In another study by Helmer et al., (1989b) cotyledonary microsomes from parturient cattle were treated with intracellular preparations of endometrial explants which had been treated with bovine conceptus secretory proteins (bCSP) or bTP-1. Results indicated that both bCSP and bTP-1 reduced PGF secretion and induce synthesis of an intracellular inhibitor of PGF production.

In sheep, Salamonsen et al., (1988) demonstrated that oTP-1 reduced the release of PGF by isolated endometrial cells and suggested that this reduction may occur by regulation of prostaglandin synthase (PGS) enzyme. Prostaglandin synthase has two functions. It is the first enzyme in the pathway from arachidonic acid to prostanoids,

and plays a central role in prostanoid synthesis (Needleman et al., 1986). The two enzymatic activities include a bis-dioxygenase (cyclooxygenase) which catalyzes the formation of prostaglandin G and a hydroperoxidase which reduces prostaglandin G to prostaglandin H from which prostaglandin of the E and F series are derived.

Salamonsen and Findlay (1990) demonstrated by immunocytochemical localization of PGS that differential regulation occurs in the two major cell types of the ovine uterus. Early in the cycle (day 4) staining was mainly located in stromal cells, with little staining in glandular cells. In tissues obtained from day 14 to 16, PGS immunocytochemical location was mainly in outer luminal epithelium and glandular epithelium. Staining was also located in stromal cells in the intercaruncular regions. However, no differences in immunocytochemical staining was present between pregnant and nonpregnant ewes on day 15.

Recently, Salamonsen et al., (1991) defined the role of ovarian steroids in the regulation of mRNA expression for PGS in whole endometrial tissue. Results indicated no significant differences in the concentration of mRNA for PGS on days 4 to 14 of the estrous cycle or between tissue obtained from day 15 pregnant or nonpregnant cows. Although a significant reduction in PGS mRNA concentration was found in ovariectomized ewes treated with estrogen.

The present study was designed to examine if rbTP-1

altered expression of mRNA encoding PGS in isolated bovine uterine glandular epithelial and stromal cells.

CHAPTER 2

MATERIAL AND METHODS

Materials

Cell culture medium, Hank's balanced salt solution (HBSS), HBSS without (Ca^{+2}) and (Mg^{+2}), Dulbeccos modified Eagle's medium (low glucose) (DMEM), DMEM methionine free, Minimal essential media (MEM), fetal calf serum (FCS) (charcoal treated; 5 g/100 ml), amino acids and basal medium Eagle vitamin solution were purchased from Gibco Laboratories, Grand Island, NY. Insulin was obtained from Eli Lilly and Co. Indianapolis, IN. Enzymes, collagenase A and dispase were purchased from Boehringer Mannheim, Indianapolis, IN. Corning 100 mm/tissue culture dishes were obtained from Corning Glass Works, Corning, NY. Rat tail type I collagen, bovine serum albumin (BSA), oxytocin and glucose were obtained from Sigma Chemical Co., St. Louis, MO. Recombinant bovine trophoblast protein-1 (rbTP-1), specific activity (1.5×10^7 U/mg) (GBK-1/VSV) was gift from R. M. Roberts. RNA isolation supplies, Guanidium isothiocynate, Tris-base, ethylenediaminetetraacetic acid (EDTA), N-laural sarcosine, diethylpyrocarbonate (DEPC) and electrophoresis buffers (molecular biology grade) were purchased from Sigma Chemical Co., St. Louis, MO. Cesium trifluoroacetate (CsTFA) was obtained from Pharmacia, Uppsala, Sweden. Agarose (low electroendosmosis) was

purchased from Mallinckrodt GenAR, Paris, Ky. Random prime labeling kits and formamide was purchased from United States Biochemical, Cleveland, OH. Molecular weight standards for RNA were obtained from IBI, New Haven, CT. Radionucleotide ^{32}P-dCTP (3,000 ci/mmole) was obtained from ICN biochemical, Irvine, CA. Nytran, nylon membrane (positively charged) was purchased from Schleicher and Schuell, Keene, NH. The PGS probe (1.6 Kb cDNA linear fragment) was obtained from Oxford Biomedical Research Inc. Oxford, MI.

Animals and collection of uterus

In this study, two cell types were isolated from the bovine uterus. These include glandular epithelial and stromal cells. Since, actions of bTP-1 critical to pregnancy maintenance are believed to occur between days 15 and 17, endometrium was obtained from cows between days 15 and 17 of the cycle (estrus=0). Uteri from 4 cows were utilized in this study. The estrous cycles of cows were synchronized with a single I.M. injection of Lutalyse (25 mg; Upjohn Co., Kalamazoo, MI). Cows were observed twice daily for estrous behavior. Reproductive tracts were collected at slaughter within 40 minutes of death, placed on ice and transported to the laboratory where they were trimmed, the exterior washed in water, then ethanol and placed in a laminar flow hood for dissection.

Isolation and culture of glandular epithelial and stromal cells.

Endometrial strips obtained from above were placed into a 150 x 25 mm petri dish, containing sterile HBSS without Ca^{+2} and Mg^{+2} and minced into small fragments (2-4 mm^2) using two scalpel blades. Minced material was placed into a sterile 500 ml Erylenmyer flask containing 0.1% collagenase and 0.5 U/ml dispase in 250 ml of HBSS without Ca^{+2} and Mg^{+2}. The Erylenmyer flask containing the digestion media was placed in a 37° C shaking water bath for approximately 2 hours, during which the tissue was checked periodically to determine the progress of digestion. After digestion, HBSS without Ca^{+2} and Mg^{+2} was added to digesta and transferred to a 150 x 25 mm petri dish. Undigested material and cellular debris was removed manually and the remainder was passed through a 200 *ím* sterile nylon mesh (Spectrum Medical Industries, Inc., Los Angeles, CA) through which allows small glandular fragments isolated cells and small clumps of cells passed pass through. Fragments were isolated by slow speed centrifugation (30 x g). Dispersion of glandular fragments through a sterile pipette produced smaller fragments. Stromal cells were obtained by high speed centrifugation (500 x g) of the supernatant from glandular cell washings.

Isolated cell types were seeded onto 0.01% rat tail collagen coated 100 mm culture dishes, following

resuspension in DMEM supplemented with 10% heat inactivated FCS, 4x glucose, 2x ABAM, 1x insulin, 1x basal medium Eagle vitamin solution and 1x non-essential amino acids. Cells were incubated at 38° C in a humidified atmosphere of 5% CO_2 : 95% air. Following 48 hours of incubation glandular cells were treated with 0.05% trypsin/ 0.04% EDTA to remove unwanted stromal cells. Media was changed every 48 hours until confluent. Once confluent, cells were washed 3x in HBSS prior to treatment. Treatments were prepared in MEM (1x) and contained the following: 1) control (no treatment) 2) BSA (30 ng/ml) 3) rbTP-1 (30 ng/ml). In the presence of treatment, cells were incubated for 36 hours.

RNA isolation

Using a cell scraper, isolated uterine glandular and stromal cells were removed from culture dishes in the presence of 4 M- guanidinium thiocyanate containing 0.1M Tris Cl (pH 7.5) and 1 % β-mercaptoethanol. Cells were further solubilized by repeated aspiration through a narrow gauge needle followed by csTFA isopycnic ultracentrifugation (117,000 x g, 24 h, 20° C, Beckman sw401TI rotor).

Following centrifugation, the RNA pellet was resuspended in 0.01% DEPC treated 30 mM sodium citrate and precipitated by the addition of 3M sodium acetate pH 5.2 and ice cold 100% ethanol. Precipitated RNA was pelleted by spinning for 30 minutes (14,000 x g) at 4° C. The RNA pellet was resuspended in 1X MOPS (containing 50mM sodium

acetate and 5mM EDTA) and was quantitated by absorbance at 260 nm.

Northern and slot blot analysis

A sample of RNA was electrophoresed through 2.2 M formaldehyde / 1.2% agarose gel and stained with 0.2 μg/ml ethidium bromide to determine if the RNA was intact. Northern blotting was carried out as described by Maniatis et al., (1982). Briefly, 20 μg of total RNA was denatured in 2.2 M formaldehyde / 1.2 % agarose gel and electrophoresed. Following electrophoresis RNA was transferred to nylon membranes by capillary blotting in 10x SSPE (1x SSPE = 150 mM NaCl, 10 mM $Na_2H_2PO_4$, 1 mM EDTA) and covalently linked to nylon membranes by UV crosslinking (Hoeffer, UV Crosslinker). The PGS probe was randomly primed with ^{32}P dCTP to a specific activity of 10^8 - 10^9 cpm/μg. Blots were hybridized with the ^{32}P-labeled probe in 50% Denhardts solution, 5x SSPE, 0.1% SDS and 100 μg/ml denatured salmon sperm DNA at 4° C for 12-20 hours. After hybridization, membranes were washed three times in 2x SSPE, 0.1% SDS at 42° C and two times with 0.1x SSPE, 0.1% SDS at 65° C. Autoradiography was performed using Kodak X-Omat XAR film at -90° C for 6 to 24 hours.

Slot blots were performed using a Minifold slot blot apparatus (Schleicher and Schuell). Ten μg of RNA from isolated gland cells and 20 μg of stromal RNA, were applied to Nytran membranes, immobilized, hybridized and detected as

described above. Membranes containing the PGS probe were subjected to boiling for three minutes in sterile, deionized water and checked for the completeness of probe removal by exposing the membrane to Kodak X-omat XAR film. Signal intensity was determined with a computer-integrated laser densitometer (Ultrascan XL, LKB) and the accompanying analysis software (Gelscan XL, version 2, Pharmacia LKB Biotechnology). Relative expression of rat β-actin was used to correct for total RNA loading.

Statistics

The densitometric estimation of mean glandular cell PGS mRNA, corrected for loading were analyzed by analysis of variance followed by Duncans multiple range F test. Isolated stromal cell PGS mRNA was not subjected to analysis of variance since their densitometric values were below detection level.

CHAPTER 3

RESULTS

Northern blot analysis

Hybridization of the ^{32}P- labeled PGS probe to isolated uterine glandular epithelial and stromal cells detected a mRNA of ~2.8 Kb when compared to the standard RNA ladder. This is consistent with the size of PGS mRNA as reported by Salamonsen et al., (1991). Stromal cell mRNA endcoding for PGS was present but at a barely detectable level (Figure 1).

Slot blot analysis

The effect of rbTP-1 on PGS expression by isolated glandular epithelial cells from day 17 of the estrous cycle showed no significant difference when compared to control (no treatment) and BSA treated cells. After correcting for total RNA loading by comparison with rat β-actin, average concentrations of mRNA for PGS in the three groups were: control (no treatment), 0.61 +/- .10 (n = 3) BSA, 0.82 +/- 0.10 (n = 3), rbTP-1, 0.51 +/- 0.088 (n = 4) (Figure 2A). Messenger RNA encoding PGS from isolated stromal cells was barely detectable regardless of treatment (Figure 2B).

Figure 1. Northern blot analysis of the expression of mRNA encoding PGS by isolated uterine glandular epithelial and stromal cells. Total RNA from isolated gland cells (10 μg/lane) hybridized with ^{32}P-labeled riboprobe for PGS and β-actin. Isolated stromal cell total RNA (20 μg/lane) hybridized with ^{32}P-labeled riboprobe for PGS and β-actin. However, detection of PGS expression by stromal cells was very low. Expression of PGS mRNA appeared to be independent of treatment.

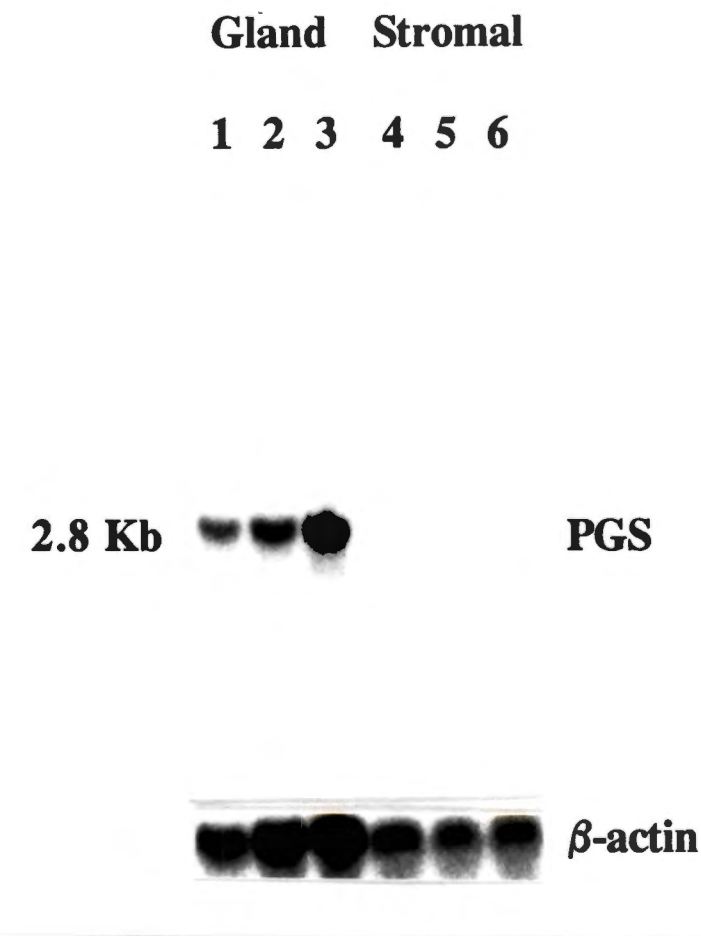
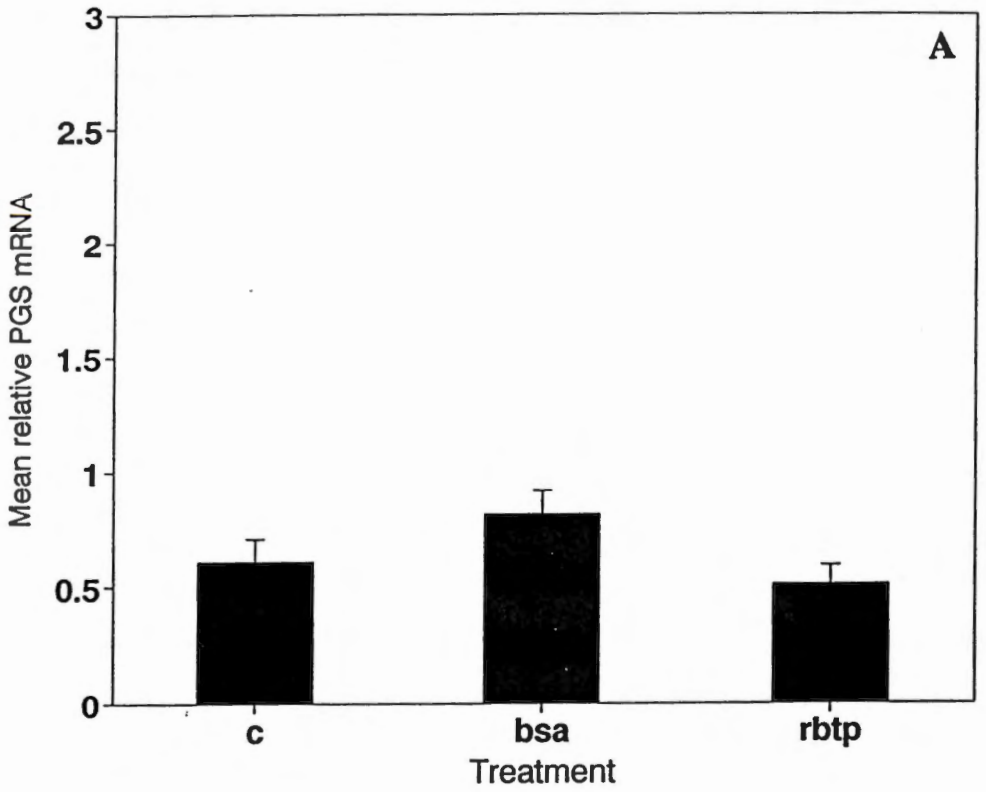
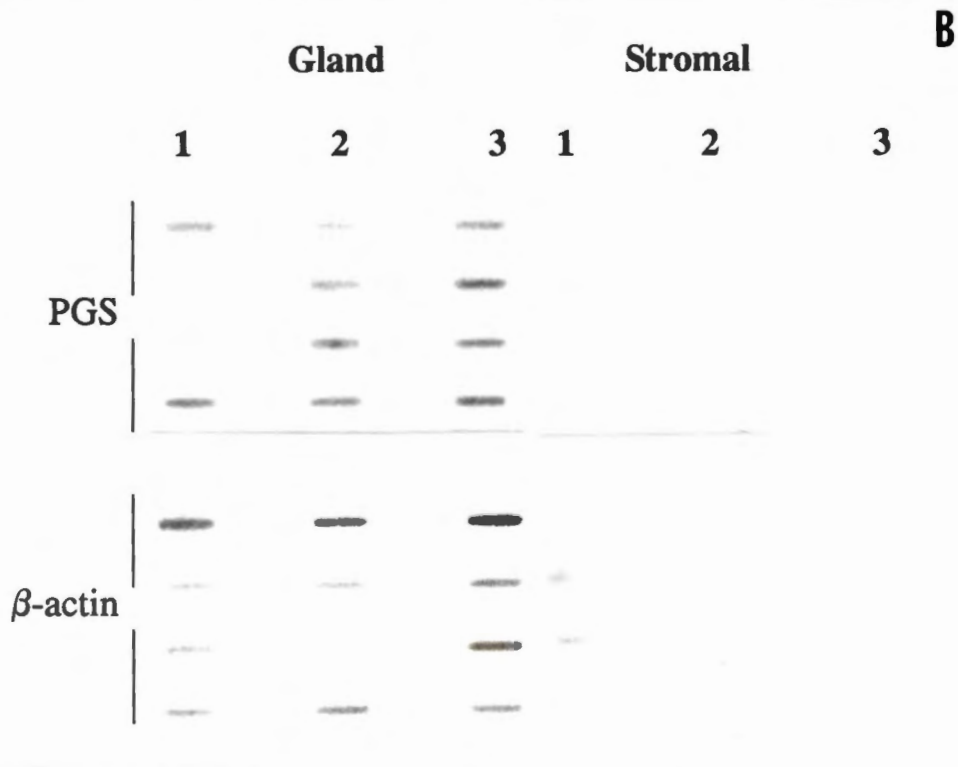

Gland
Stromal
1 2 3 4 5 6
2.8 Kb
PGS
β-actin

Figure 2. Mean relative mRNA for PGS in isolated bovine uterine glandular cells (A). Total RNA from isolated gland cells was subjected to slot-blot analysis and probed with ^{32}P-labeled riboprobe for PGS (B). Values are expressed as mean densitometric measurement after correction for loading as compared to β-actin. Note differences in expression of mRNA encoding for PGS were similar between treatments.


A
Mean relative PGS mRNA
3
2.5
2
1.5
1
0.5
0
c
bsa
rbtp
Treatment


B
Gland
Stromal
1
2
3
1
2
3
PGS
β-actin

CHAPTER 4

DISCUSSION

The data presented here demonstrate that expression of PGS mRNA in the bovine endometrium during the late luteal phase of the cycle is a function of glandular epithelial cells but not stromal cells. These results correspond with immunocytochemical localization of PGS in sheep on days 14 through 16 where staining was most intense in luminal epithelial and glandular epithelial cells (Salamonsen and Findlay, 1990). The above studies support the contention that uterine epithelial is the major site of prostaglandin synthesis at the time of luteal regression.

In the present study, rbTP-1 had no effect on PGS expression, although it did cause a decreased in PGF released by isolated gland cells (Smith and Godkin, 1992). Similarly isolated endometrial glandular cells prepared from day 14 nonpregnant sheep treated with oTP-1 secreted significantly less PGF and PGE than those cultured in the absence of oTP-1. These isolated cells displayed no difference in the intensity of PGS immunostaining between cells cultured in the presence or absence of oTP-1, thus supporting the present observations. Further evidence is displayed in tissue obtained from day 15 pregnant and nonpregnant ewes that PGS location was essentially the same. Together, the above data suggest that the attenuation of prostaglandin synthesis by trophoblast IFN are not mediated

through alteration of PGS enzyme production. It is possible that trophoblast protein -1 affects PGS turnover rate which was not analyzed in this study.

Epidermal growth factor (Yokota et al., 1986), platelet derived growth factor (Lin et al., 1989) and interleukin I (Raz et al., 1988) have been demonstrated to alter PGS activity. Raw et al., (1988) has demonstrated by immunostaining that PGS expression and PGF release from ovine explant tissue was under progesterone control. PGS expression of mRNA in ewes treated with estradiol-17β was suppressed while ewes treated with progesterone overcame this suppression. During the estrous cycle PGS mRNA expression showed no significant variation between days. In endometrium from pregnant day 15 ewes, no significant difference in mRNA for PGS were evident when compared to day 14 or 15 of the cycle. It has been suggested (Salamonsen et al., 1990;1991) that estrogen may be responsible for the increased turnover rate of mRNA for PGS.

Studies by Ohta, (1985) utilizing steroid treated rats demonstrated that detection of PGS by immunocytochemistry was dependent on progesterone but not estrogen. Conversely, in women during the secretory phase of the menstrual cycle, estradiol enhanced PGS activity in isolated epithelial but not stromal cells (Schatz et at., 1987). Although we did not test the effects of estrogen on PGS mRNA expression, the present study concurs that PGS mRNA expression occurs in

isolated glandular cells while stromal cells express barely detectable levels of PGS mRNA. It would be of interest to evaluate the effects of estrogen and progesterone on isolated glandular and stromal cells.

The inability of rbTP-1 to alter PGS expression in glandular and stromal cells suggests that bTP-1 may possibly be acting via another mechanism. It has been demonstrated that an inhibitor of prostaglandin secretion exists in the endometrium from pregnant cows (Basu and Kindahl, 1987; Gross et al., 1988) and is induced by bTP-1 and bCSP (Helmer et al., 1989b). The inhibitor may function to block the conversion of arachidonic acid to PGF and PGE (Gross et al., 1988b). Bovine TP-1 decreased PGF secretion in bovine endometrial explants from day 17 cyclic animals and induced the putative intracellular inhibitor (Helmer et al., 1989b). Other enzymes may be involved in the regulation of the inhibitor since bTP-1 reduced PGF secretion and increased PGE secretion in endometrial explants (Helmer et al., 1989).

Ovine TP-1 and oxytocin are both potential regulators of prostaglandin synthesis. Receptors for oTP-1 and oxytocin have been located on glandular cells but not stromal cells (McCracken et al., 1984; Godkin et al., 1984a; Salamonsen et al., 1989). In the presence of oxytocin, endometrium from cyclic ewes, exposed to oTP-1 on days 12 through 14 demonstrate a significant reduction in inositol phospholipid metabolism (Mirando et al., 1990 a,b) and PGF secretion

(Vallet et al., 1989; Mirando et al., 1991) suggesting the absence of functional oxytocin receptors in ewes treated with oTP-1. In vivo studies utilizing pregnant animals demonstrated that oxytocin receptors are low in ewes (Flint et al., 1986; McCracken et al., 1984) and cows (Jenner et al., 1991). Infusion of oTP-1 into the uterus of cyclic ewes between Days 11 and 15 decreased the number of oxytocin receptors and the affinity of the receptor for oxytocin (Mirando et al., 1991). Thus, suggesting oTP-1 may regulate prostaglandin synthesis and secretion by altering the action of oxytocin.

In summary, rbTP-1 had no effect on PGS mRNA expression by isolated endometrial gland cells. Expression of PGS is barely detectable in stromal cells and is not responsive to rbTP-1. Therefore, this study has demonstrated that rbTP-1 may not directly regulate prostaglandin metabolism at the level of PGS.

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PART 4: General Conclusion

CONCLUSION

A major cause of reproductive inefficiency and economic loss in livestock is due to embryonic mortality. It is estimated that a 45 - 65% calving rate follows a 90 - 95% fertility rate (Gordon et al., 1976; Spalding et al., 1975; Roche et al., 1978). The majority of embryonic loss occurs during early pregnancy. Pregnancy failure may result from embryonic and uterine asynchrony (Pope, 1988). Communication between the trophoblast cells of the developing conceptus and the maternal endometrium are essential in order to maintain pregnancy. The maintenance of luteal function during early pregnancy is mediated through the actions of a protein released by the trophoblast cells. The antiluteolytic signal in sheep and cattle is a trophoblast interferon (IFN) previously referred to as ovine (o) or bovine (b) trophoblast protein -1 (TP-1) and recently named IFN (τ) (see Roberts, 1992).

In this study, isolated bovine endometrial glandular epithelial and stromal cells were used to determine: 1) if the effects of recombinant bovine trophoblast protein-1 (rbTP-1), bovine recombinant interferon (brIFN) and oxytocin altered prostaglandin and protein secretion by the isolated cell types, 2) if the effects of coculture of glandular epithelial and stromal cells alter secretion of prostaglandins and proteins, 3) if rbTP-1 regulated gene

expression of PGS in the isolated cell types. A reduction in prostaglandin release or alteration in PGS expression by the isolated cell types in response to rbTP-1 may provide information regarding the mechanism of action of the trophoblast interferon.

Results from the present study indicate that isolated gland cells are the major prostaglandin releasing cells when compared to stromal cells. Addition of rbTP-1 and brIFN caused a significant decrease in PGF release by isolated gland cells and cocultured cells. However, rbTP-1 and brIFN did not effect PGE release by isolated gland cells or stromal cells. A reduction in PGE was observed in cocultured gland cells when treated with rbTP-1 which supports studies utilizing endometrial explants (Helmer et al., 1989). However, in another study, PGE release by endometrial explants treated with bTP-1 was unaltered (Johnson, unpublished results). Stromal cell PGE release was also unaltered in response to rbTP-1.

In a preliminary dose response study, rbTP-1 reduced PGE release by gland cells which is inconsistent with results stated above. A reason for this inconsistency may be due to DNA analysis. In the dose response study, DNA was diluted prior to analysis. In the remainder of the study, DNA was not diluted and read directly in the culture plate. Direct readings of DNA are more consistent than readings from diluted DNA.

A reduction in PGF by rbTP-1 and the lack of effect on PGE release by both cell types provides evidence that trophoblast IFN may function to protect the corpus luteum from the luteolytic effects of PGF.

An interesting result was that stromal cells secrete very low levels of PGF and express low levels of PGS mRNA. Recombinant bTP-1 was unable to alter the expression of PGS by both cell types. Therefore, suggesting that rbTP-1 does not act at the level of PGS. It is possible that rbTP-1 acts by altering the regulation of oxytocin receptor formation. Jenner et al., (1991) has demonstrated that oxytocin receptor are low during early pregnancy. A time which bTP-1 is being secreted from the trophoblast cells. In cows, demise of the corpus luteum is due to the pulsatile secretion of PGF (Thatcher et al., 1986). In the present study, rbTP-1 was unable to reduce oxytocin stimulated release of PGF release by gland cells, however, oxytocin stimulated release of PGE was significantly reduced in the presence of rbTP-1. This significant reduction was unexpected since rbTP-1 had no significant effect on PGE release by isolated gland cells and cocultured cells. Perhaps rbTP-1 is acting at a level after the formation of PGH which is the precursor for PGF and PGE.

Addition of oxytocin caused a significant decrease in PGE release by stromal cells. The combination of rbTP-1, brIFN and oxytocin caused stromal cells to release

significantly greater levels of PGE when compared to stromal cells treated with oxytocin alone. The decrease in PGE release by stromal cells in the presence of oxytocin is difficult to interpret because of the variable results between animals. More animals are needed in order to determine oxytocin effects on PGE release by stromal cells.

Oxytocin addition to isolated gland cells increased PGE secretion whereas PGE secretion by cocultured gland cells was unaffected. It is possible that stromal cells produce products which inhibit the stimulatory effects of oxytocin on glandular PGE production. Alternatively it is plausible that oxytocin affected the directionality of PGE secretion by cocultured cells.

In cattle, attachment of the placenta is not complete until the third week of pregnancy (Amoroso 1952; King et al., 1979). During this time, endometrial secretions are believed to provide a proper environment for the growth, development and eventually implantation of the embryo. The effects of rbTP-1 on uterine protein secretion was similar in isolated gland cells and cocultured cells. Incorporation of radiolabeled amino acids into proteins by isolated gland and stromal cells and cocultured cells were unaffected by rbTP-1, brIFN and oxytocin. The only significant difference in percent incorporation of radiolabeled amino acid into proteins by gland cells was due to the presence of stromal cells in which a decrease in protein secretion was observed.

The array of proteins released by isolated gland cells grown on rat tail collagen coated plastic were similar to proteins secreted by gland cells grown on Matrigel coated millicell inserts. Twenty proteins appeared to be glandular cell specific while four proteins are stromal cell specific. A protein (MW 45 KDa; pI 5.5 - 6.8) released by isolated stromal cells was also present on fluorograms obtained from cocultured glandular cells suggesting that this protein may have been a result of stromal cell origin or a transport protein.

Results from the present study conclude that rbTP-1 does not affect protein release by the isolated cell types. Perhaps these cell types need to be in physical contact in order for rbTP-1 to elicit a response. The main findings of this project was that rbTP-1 does alter prostaglandin release by bovine uterine cells but may not mediate this effect through regulation of PGS mRNA. Therefore, this study has provided <u>in vitro</u> information regarding the interactions of the trophoblast and maternal endometrium during maternal recognition of pregnancy.

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VITA

Scott Edward Smith was born in Jackson, Michigan on March 19, 1963. He attended Vandercook Lake High School and graduated in June 1981. In September 1981, he entered Jackson Community College where he received an Associates degree in Arts and Science in June 1983. He then transferred to The University of Tennessee at Martin where he received his Bachelor of Science degree in animal Science in June 1986.

In September 1986, he was admitted as a graduate student in the Department of Animal Science at The University of Tennessee at Knoxville. Scott received his Master of Science degree in December 1988. He was accepted into a Doctorate program in January 1989 at UTK and was awarded a Doctor of Philosophy degree with a major in Animal Science in May 1993.