REGULATION OF KININS DURING EARLY PREGNANCY AND THE ESTROUS CYCLE OF THE GILT

By

MELANIE JANE ALLEN

Bachelor of Science

Oklahoma State University

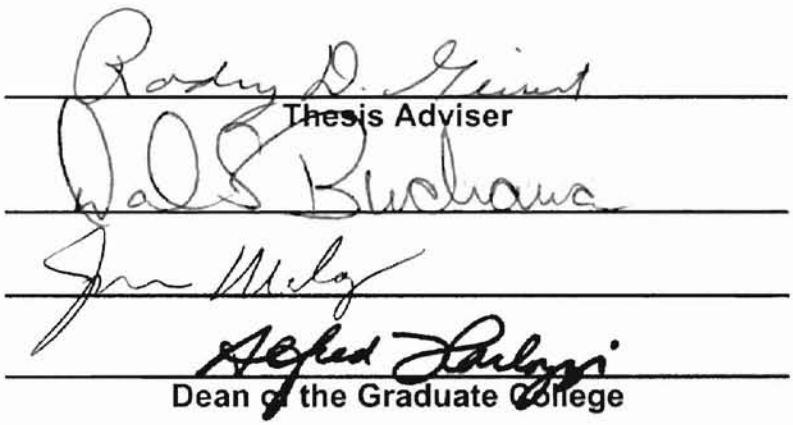
Stillwater, OK

1997

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
In partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2000

REGULATION OF KININS DURING EARLY PREGNANCY AND THE ESTROUS CYCLE OF THE GILT

Thesis Approved:



Thesis Adviser

Dean of the Graduate College

ACKNOWLEDGMENTS

I would first like to thank my family for their support, encouragement and counseling during my academic career here at Oklahoma State University. To my husband, Joe, thank you for keeping my head above water, increasing my awareness, and never loosing faith. To my mother and father, Don and Patricia, your support, encouragement and prayers through the hardest of times helped me accomplish the tasks before me and build the foundation of who I am today. To the rest of my family, your faith and unwavering support encouraged me to do my best.

To Dr. Rodney Geisert, thank you for the opportunity to work with you in the pursuit of my Masters degree. The diversity in training and the increased awareness of new technological advances made it challenging yet intriguing to study under you. Thank you for your persistence, patients, and encouragement and for sharing your knowledge of research and teaching.

To Dr. Malayer and Dr. Buchanan. Thank you for your assistance and willingness to answer my questions and concerns. Thank you for your support.

My last thanks go out to the staff and graduate students here in the college of Animal Science. Thank you for your support and encouragement.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Humans, primates, rodents and domestic farm animal species undergo similar events during the first few days of embryonic development. Following hatching from the zona pellucida the domestic farm species undergo a period of prolonged noninvasive implantation, which differs from that of the human, primate and rodent. Conceptus development in the pig is unique from that of other domestic animal species. In the pig, the conceptuses undergo a rapid transformation from spherical (10 mm) to tubular (15-40 mm) and finally, filamentous (150-200 mm in length) morphology between days 11 and 12 of pregnancy (Perry & Rowlands, 1962; Anderson, 1978). Attachment of the conceptus to the uterine surface epithelium begins on day 12 through 18 of pregnancy. This implantation period is the most critical period for early embryonic loss.

Production of estrogen by the developing conceptuses on day 12 of pregnancy provides the maternal recognition signal to maintain CL function throughout the 114 days of gestation in the pig (Bazer & Thatcher, 1977). The release of estrogen by the conceptuses alters secretion of uterine proteins and

prostaglandins, stimulates uterine blood-flow and changes uterine cell morphology necessary for establishment of pregnancy (Geisert et al., 1982b).

Implantation by the pig conceptuses is non-invasive, thus forming the diffuse, epitheliochorial type of placentation (Keys & King, 1990). Secretion of numerous protease inhibitors from the porcine endometrium prevents invasion into the endometrium by the normally protelytic conceptus (Fazlebas et al., 1983).

The following review of literature will discuss the events associated with early embryogenesis, factors affecting uterine receptivity, and the participation of the embryo itself in the establishment of a successful pregnancy in the pig. The possible role of the Kallikrein-Kininogen-Kinin system in embryo implantation and establishment of pregnancy will also be reviewed.

CHAPTER II

LITERATURE REVIEW

Embryonic Mortality

Much effort has been devoted to discover the time period and cause of embryonic loss since Corners' 1923 conclusion that the number of conceptuses was almost always less than the number of corpura lutea present on the ovaries. A minimum of two embryos per uterine horn must be present before day 16 of gestation for pregnancy to be established in the pig (Dziuk, 1968). Estimates of embryonic mortality in swine range from 20 to 46 percent with the majority of embryonic loss occurring between day 10 and 20 of gestation (Pope, 1994). Although the amount of uterine space available per conceptus is related to later fetal loss in the pig, it is not considered a cause of embryonic mortality normally observed before day 30 of pregnancy (Webel and Dzuik, 1974). Pope et al., (1982) concluded that during early conceptus growth, the differential development of embryos gave the more advanced developing embryos the preferential chance for survival over the less-developed embryos. Studies have concluded that it is not the lack of smaller conceptus' ability to develop and survive in the uterus, but its inability to compete with its more advanced littermates that causes early embryonic loss (Wilde et al., 1988). It is believed

that although uterine capacity is not the major cause of mortality before day 30 it limits the number of conceptuses that will survive after day 30 of gestation. Experiments to test this theory have included superovulation, superinduction (transferring additional embryos into the uterine lumen) and unilateral-hysterectomy-ovariectomy (UHO) (Christenson et al., 1987). Litter size resulting from superovulated or superinduced sows have similar number of fetuses compared to the control groups (Dzuik, 1968; Huang et al., 1987). UHO females have similar CL number as control females with no difference in the number of embryos when measured at day 30 of gestation (Huang et al., 1987). However, when compared on day 86 of gestation, the single horn of UHO females contained about half the number of fetuses as control pigs (Christenson et al., 1987). These results suggest that uterine capacity is a major factor regulating embryonic mortality during later gestation in the pig.

Peri-Implantation Conceptus Development

The pig is well known for being one of the few species that the prei-implantation conceptus undergoes rapid elongation within the uterine lumen (Anderson, 1978; Geisert et al., 1982b; Pope, 1994). Just prior to trophoblastic elongation, during the spherical stage of conceptus development, several key steroidogenic enzymes become available for the conversion of lumenal steroids precursors by the conceptus to synthesize estradiol (Gadsby et al., 1980; Fischer et al., 1985; Modschein et al., 1985; Pusateri et al., 1990; Conley et al., 1994; Ko et al., 1994; Yelich et al., 1997). Although we don't know much about the mechanism(s) controlling this event, we do know that cytochromes P450 17α-

hydroxylase and aromatase, two key enzymes responsible for high levels of conceptus estrogen, are present and coincident with conceptus elongation. Expression of 17α-hydroxylase is limited to the trophectoderm (Conley et al., 1994; Ko et al., 1994) and is responsible for the conversion of progesterone to 17α hydroxyprogesterone. Aromatase, localized in the inner cell mass layer (hypoblast) of the trophectoderm (Conley et al., 1994, Ko et al., 1994), is the enzyme essential for the conversion of testosterone to estradiol. Gene expression of these two key enzymes is notably increased during the development of 4 to 7 mm spherical conceptuses (Yelich et al., 1997). Immediately following elongation of the trophoblast, gene expression for both of these enzymes decreases dramatically (Conley et al., 1994; Ko et al., 1994; Green et al., 1995).

The increase in conceptus estradiol production is first detected on day 10 of pregnancy with an enhanced elevation in synthesis on days 11-12 (Perry et al., 1973). The first increase of estrogen is associated with the expansion of the spherical conceptus, which occurs through mitotic increase of trophoectodermal cells (Fischer, 1985). The conceptus continues to expand until it reaches the 10 mm spherical morphology at about day 11 of pregnancy. The sharp increase of estrogen production noted on approximately day 11 to 12 of gestation provides the signal for maternal recognition of pregnancy in the pig (Bazer and Thatcher, 1977). In 1986, Pope proposed that this early, surge release of conceptus estrogen on day 11 to 12 of pregnancy sets the stage for equidistant spacing of

the developing conceptuses, an important mechanism to embryonic survival in polytocous species such as the pig.

The 10 mm spherical stage in conceptus development is critical because this is the stage proposed to be associated with cellular remodeling for trophoblast elongation in the pig (Geisert et al., 1982b, Gupta et al., 1996). Yelich et al. (1997) indicated the appearance of the mesoderm within the embryoblast was temporally associated with initial expression of the brachyury gene in the conceptuses. The brachyury gene encodes for a transcription factor that is necessary for mesodermal differentiation (Herrmann et al., 1990). Brachyury gene expression parallels expression of P450 17α- hydroxylase and aromatase, suggesting that brachyury may be involved in regulating steroidogenic events in the conceptus. Once conceptus diameter reaches 10 mm, the conceptus rapidly undergoes a transition to a tubular (12-30mm) and finally a thin filamentous form measuring 100 mm in length (Geisert et al., 1982a). In 1987, Morgan and coworkers reported that conceptus elongation was partially regulated by developmental maturity of individual conceptuses. These researchers observed that the 7 to 8 mm conceptuses did not appear to elongate at the same time as their 10 mm littermates. Once the conceptus reaches a diameter of 10 mm it begins to take on an ovoid morphology with the trophectoderm cells having a cuboidal shape (Anderson, 1978; Geisert et al., 1982b).

The transformation from tubular to filamentous morphology is a rapidly occurring event lasting less than 2 to 4 hours in duration (Geisert et al., 1982b).

During this morphological transformation, the trophectoderm and endodermal cells undergo an alteration in shape and ultrastructure (Geisert et al., 1982b; Mattson et al., 1990). During rapid elongation, the actin cytoskeleton, consisting of filamentous actin (f-actin), mediates organizational events within the trophectoderm (Mattson et al., 1990). The period of rapid trophoblastic elongation does not occur through cellular hyperplasia but through remodeling of the trophoblast and endoderm layers of the conceptus (Geisert et al., 1982b; Pusateri et al., 1990). By day 15 of gestation the filamentous conceptus becomes twisted and coiled as it makes contact along the apical uterine luminal epithelial surface (Keys and King, 1990). The initial adhesion of the conceptus to the surface epithelium on day 12 of gestation may be essential to early trophoblastic elongation followed by a more permanent continuous adhesive attachment of the placenta throughout gestation (King et al., 1982).

The luteolysin that is released from the uterine epithelium into the peripheral circulation of the cyclic gilt induce the corpora lutea to lysis is $PGF_{2\alpha}$ (Bazer and Thatcher, 1977). In the pregnant animal, conceptus estrogen release causes a diversion in $PGF_{2\alpha}$ secretion from endocrine to an exocrine direction resulting in the majority of prostaglandins being secreted into the uterine lumen rather than the uterine vascular system. Thus, the uterine luteolysin $PGF_{2\alpha}$ is sequestered in the lumen and does not reach the CL to stimulate luteolysis.

Factors Involved in Conceptus Development

Trophoblast elongation is primarily controlled by maturational development of individual conceptuses within a litter (Morgan et al., 1987), however uterine secretions play a large part in its growth and survival. Retinol, which is transported by retinol binding protein (RBP), is a primary growth promoting and cellular remodeling factor. Retinol (Vitamin A) is important because it can function to induce or alter extracellular matrix proteins (De Luca, 1991) and cellular adhesion molecules (Agura et al., 1992). Retinol has been detected in uterine endometrium during the period of conceptus elongation in the pig (Schweigert et al., 1999). Retinol bound to RBP is transported into the uterine lumen where it can further metabolize it into the biologically active form of retinoic acid. Retinoic acid is a cellular morphogen that may regulate trophoblastic morphology during elongation through changes in the presence of retinoic acid receptors (RAR) within the conceptus (Harney et al., 1990). There are many isoforms of the retinoic acid receptor, RARα, RARβ, and RARγ. Yelich et al., (1997) indicated that gene expression for these isoforms in conceptus tissues changed during early conceptus development. RARα gene expression increased linearly during early conceptus development until it plateaued at the 9 to10 mm spherical conceptus stage while RARβ gene expression was detected in diminutive amounts across all stages of conceptus development. RARγ gene expression, although evident, was not affected by developmental stage (Yelich et al., 1997). Retinol binding protein (RBP) transports retinol from the plasma into the uterine lumen where it can be utilized by the conceptus. Retinol binding

protein mRNA can be detected not only in the uterine tissue layers but also the conceptus (Harney et al., 1994). Retinol binding protein gene expression was significantly affected by the stage of conceptus development (Yelich et al., 1997). RBP mRNA levels increase with conceptus size, increasing in the 2-8 mm spherical stage, decreasing in the tubular stage and then increasing during the filamentous stage, between days 11 and 12 of gestation (Harney et. al., 1994, Yelich et al., 1997). This pattern of RBP gene expression is proposed to protect the early developing conceptus from lethal concentrations of retinol within the uterine lumen (Harney et al.,1990; Trout et al, 1991) in addition to directing retinol to existing target cells (Trout et al., 1991). Another function of RBP proposed by Vallet et al., (1996) is protection of the uterine and conceptus tissues from the lipid oxidizing activity of uteroferrin. All of the roles of RBP mentioned above suggest a model for retinol, RAR and RBP possible involvement in the trophoblastic elongation and conceptus development process (Geisert and Yelich, 1997).

Uteroferrin, an iron transport protein, was one of the first and most extensively studied progesterone stimulated uterine proteins investigated in the pig (Roberts et al., 1993a). This glycoprotein serves to transport iron to the fetus throughout pregnancy in addition to its proposed role as a haematopoietic stem cell growth factor during early conceptus development (Bazer et al., 1991; Michel et al., 1992). Uteroferrin increases in uterine secretions at the time of maternal recognition in the pig (Vallet et al., 1996). Uteroferrin is capable of catalyzing lipid peroxidation in the presence of ascorbic acid, which is found in the uterine

environment (Vallet, 1995). Vallet and coworkers (1996) noted that the increase of acid phosphatase activity (uteroferrin) on day 13 of the estrous cycle and pregnancy was highly correlated with the presence of retinol binding protein and transferrin, both known inhibitors of acid phosphatase activity. Transferrin, an iron containing protein found in plasma, does not catalyze lipid peroxidation.

Transforming growth factors (TGF's) can also play a role in porcine conceptus development. TGFβ's 1, 2, and 3 have been identified in porcine conceptus tissues and the intensity of appearance in relation to conceptus development suggests that TGFβ's play important roles during this crucial period of conceptus growth (Gupta et al., 1998a). The role of each TGFβ isoform is not well understood but this family of cytokines is involved in a multitude of functions associated with early pregnancy such as cellular proliferation, cellular differentiation, extracellular matrix protein synthesis, tissue repair, intergrin modification, immunoSupplression, and angiogenesis (Gupta et al., 1998b). The increase in TGFβ's gene expression and TGFβ receptors in uterine tissues along with the detection of bioactive TGFβ's in the uterine lumen on days 12, 13 and 14 of pregnancy suggest a potential role of TGFβs in the interactions between the uterus and conceptus during early pregnancy. In contrast to Gupta's findings Yelich et al., (1997) did not detect gene expression of TGF-β2 in conceptus tissues whereas TGF-β3 gene expression increased throughout early conceptus development, remaining high throughout trophoblastic elongation.

Another family of growth factors which have been shown to regulate cell proliferation, cell differentiation, matrix formation and cell movement are acidic

and basic fibroblast growth factors (aFGF and bFGF). These two growth factors appear to play different roles during early porcine conceptus development and both are present in both the conceptus and uterus during early pregnancy (Gupta et al., 1997).

Two other growth factors expressed in the uterus at the time of trophoblastic elongation are epidermal growth factor (EGF) and transforming growth factor α (TGFα) (Brigstock et al., 1990; Kim et al., 1995). TGFα serves to increase fluid acquisition and subsequently blastocoel expansion in the early developing mouse conceptus (Dardick and Schultz, 1991). This could also be a suggested role for TGFα in porcine conceptus development. Both TGFα and EGF have been localized in the surface and glandular epithelium of the endometrium (Kennedy et al, 1994; Kim et al., 1995). Diehl et al, (1994) found a substantial increase of EGF in uterine secretions on day 12 of pregnancy. The fact that both EGF and TGFα can bind the EGF receptor (Prigent and Lemoine, 1992) and receptor expression can be found in both endometrial and conceptus tissues (Kennedy et al., 1994), suggests that uterine secretion of EGF and TGFα could have a regulatory role in conceptus and uterine growth and differentiation.

The conceptus production of cytokines and growth factors may play important roles in maternal reactions and adjustments to facilitate a proper environment for advanced embryo development (Mathialagan et al., 1992). Interleukin -1(IL-1β) is a cytokine produced by the porcine conceptus that may play a temporary role in maternal recognition of pregnancy (Tuo et al., 1996). Conceptus IL-1β could also play a communicative role between trophoblast and

uterus during the time of establishment of pregnancy through its influence on conceptus remodeling and stimulation of prostaglandin E release (Geisert and Yelich, 1997). Porcine conceptus tissues also express cytokine genes such as interferon-γ (INF-γ) (Lefevre et al., 1990) and interferon-α (INF-α) (Cross and Roberts, 1989) prior to implantation. In ruminant species interferon-τ exerts and antiluteolytic effect for the maintenance of CL function (Bazer et al., 1992). The pig does not express interferon-τ and the interferon's produced by the conceptuses do not have any antiluteolytic affect (Lefevre et al., 1990). Interferons produced by the porcine conceptuses may play more of an immunological protective role.

Another estrogen-controlled participant in conceptus differentiation and remodeling is plasmin. Plasminogen activator, a serine protease, produced by the conceptus, cleaves plasminogen to yield plasmin (Christman et al., 1977). Plasmin plays important roles in cellular remodeling (associated with embryo development) and degradation of fibrin. Bode et al., (1979) first identified the role of plasminogen activator in midgestation mouse embryos. Midgestation is the time of mouse embryogenesis that the embryo undergoes rapid growth. In the pig, plasminogen is present in the greatest amounts on day 12 of pregnancy (Fazleabas et al., 1983). To regulate plasmin conceptus estrogen stimulates the release of plasmin/trypsin inhibitor from the endometrium as a defense against its normally proteolytic functions thus blocking the invasive nature of the porcine conceptuses (Fazlebas et al., 1982).

Porcine Conceptus Estrogen Synthesis

In the pig, conceptus estrogen synthesis is vital to the establishment of pregnancy. Conceptus estrogens' role in the establishment and maintenance of pregnancy involves, and increase in uterine blood flow, stimulation of endometrial secretions, maintenance of the CL, initiation of placental attachment, and participation in conceptus elongation (Geisert et al., 1990). Synthesis of estrogen by the porcine conceptus is the signal to the uterus and ovary to undergo the necessary adjustments to establish and maintain early pregnancy. The estrogen synthesized by the conceptus is the result of its metabolism of androgens and progesterone to estrogen (Fischer et al., 1985). The conceptus does not continually synthesize and release estrogens. The earliest detection of conceptus-produced estrogen is at the 5 mm spherical stage of development, which is about day 11 of gestation (Geisert et al., 1982a). This is the stage temporally associated with mesodermal development and trophoblastic growth. There is a surge release of estrogen as the porcine conceptuses elongate through the uterine horns. A second surge of conceptus estrogen synthesis is detected around day 16 of gestation (Geisert et al., 1982b). This is the critical time for allantoic membrane development from the hindgut and fluid accumulation within the allantois to attach the chorion to the entire uterine lumen. Changes in conceptus estrogen production are parallel with the cellular content of P450 17α - hydroxylase and aromatase discussed earlier in this chapter. In response to conceptus estrogen synthesis, many endometrial substances are released to maintain pregnancy. These substances will be described in detail in

a later section. One would conclude that the larger amounts of conceptus estrogen could be detrimental to the embryo as well as the maternal system if released into the vascular system in a free, active form. However, the presence of progesterone stimulated endometrial sulfotransferase activity, for example sulfoconjugated estrone, estradiol, and estriol allows the sow to regulate the activity of estrogen (Geisert et al., 1982a; Meyers et al., 1983; Stone et al., 1985). Another suggested mechanism for conceptus and maternal protection in the presence of large amounts of estrogen is the ability of the conceptus to synthesize estrogen 2 and 4 hydroxylase, which converts estrogen to catechol estrogens (Chakraborty et al., 1989). Mondschein et al., 1985, noted an initial peak in catechol estrogen synthetic activity on day 12 and 13 of pregnancy in the pig and Stice et al., (1987) concluded that the presence of these catechol estrogens plays a large role in the control of uterine blood flow, which increases at the time of placental attachment in the pig.

Uterine Secretions During the Estrous Cycle and Early Pregnancy

Many uterine secretions play an important role in conceptus development and maternal preparation of the endometrium for embryo implantation and survival. The majority of these secretions are stimulated by the coordinated efforts of estrogen and progesterone. Prolonged ovarian progesterone stimulation initiates the secretion of endometrial proteins by the pregnant uterus (Knight et al., 1974). Prolonged CL progesterone release down-regulates the progesterone receptor (PR) on the uterine surface and glandular epithelium,

while PR is maintained in the uterine stroma and myometrium (Geisert et al., 1994). Down-regulation of epithelial PR is suggested to be the critical period when conceptus factors involved in growth and placentation must be activated (Geisert et al., 1994). One of those growth factors critical to conceptus growth would be keratinocyte growth factor (KGF). Keratinocyte growth factor/fibroblast growth factor-7 (KGF/FGF-7) has been identified in abundance in porcine endometrium between days 12 and 15 of the estrous cycle and pregnancy (Ka et al., 2000). KGF is an established paracrine mediator of hormone-regulated epithelial growth and differentiation. KGF, is produced by cells of mesynchymal origin that includes the uterus (Pekonen et al., 1993), functions to induce epithelial growth as a stromal - derived paracrine regulator of epithelial proliferation (Koji et al., 1994). It could also play a protective role by blocking apoptosis and preventing oxidative damage to the conceptus (Ka et al., 2000).

Two additional uterine factors associated with this time of development is the increased release of calcium (Ca^{++}) and prostaglandin E (PGE) (Geisert et al., 1982a). The increase of Ca^{++} and PGE in addition to conceptus $PGF_{2\alpha}$ suggests that conceptus estrogens may function to initiate endometrial secretion of histotroph into the uterine lumen in the efforts to provide nutrients for the developing conceptus (Geisert et al., 1982a).

During the time of conceptus elongation many haematopoietic cytokines can be detected in uterine flushings but one of the most notable cytokines, secreted by the pig uterus, involved in cellular differentiation and growth, is leukemia inhibitory factor (LIF) (Angeon et al., 1994). LIF plays a vital role in

mouse embryo development (Stewart, 1994). Evidence of increased endometrial gene expression and increased levels of LIF in the uterine lumen on days 11to 12 of pregnancy and days 7 and 13 of the estrous cycle (Anegon et al., 1994) which is in co-ordinance with increased conceptus gene expression of LIF receptors associated with the conceptus (Yelich et al., 1997a) suggests that LIF may play a role in embryo development. LIF also binds to the receptor for colony-stimulating factor 1 (CSF-1) (Bazan, 1991). Gene expression for CSF-1 increased in endometrial and conceptus tissue on day 10 of pregnancy, but greatest gene expression is not detected until after day 30 of pregnancy, suggesting a primary role for CSF-1 in placental and fetal growth following implantation (Tuo et al., 1995).

It is well known that relaxin is released from the luteal cells of the CL in the pig (Sherwood, 1994). Most recently relaxin mRNA has been identified in the uterine epithelium during the time of conceptus placental attachment in the pig (Knox, 1994). Bazer et al., (1981) suggests that relaxin plays a stimulatory role in growth of the uterus for accommodating the expanding conceptus.

Another growth factor that has been completely characterized in uterine flushings of the pig (Simmen et al., 1993) is insulin like growth factor–1 (IGF-I). IGF-I is a mediator of cellular growth and differentiation and has demonstrated measurable biological actions in the uterus and placenta (Simmen et al., 1993). It is believed that uterine secreted IGF-I plays more of an autocrine directed role in uterine development because the uterus has an abundance of IGF-I receptors (Simmen et al., 1992b) where in contrast, the conceptus expresses the receptor

in very low levels (Chastant et al., 1994). The maximal concentrations of IGFs in the porcine uterine lumen occur during the period of conceptus elongation (day 11 to 12), which would suggest vital roles of these growth factors in early development of the conceptus (Simmen et al., 1989; Ko et al. 1994). IGF-I can bind to IGF-II receptor with some affinity and therefore could exert a biological effect. Chastant et al., (1994) identified trophectodermal conceptus expression of IGF-II receptor. The identification of IGF-II receptor on the conceptus and the proposed binding of IGF-I to this receptor suggests IGF-II's possible role in conceptus growth and differentiation. Lewis et al., (1992) set out to determine whether the embryo actually responds to insulin as a possible embryonic growth factor during the period of conceptus elongation. They concluded that the pig conceptuses respond to physiological levels of insulin in similar ways as other species. Lewis also concluded that because there is the possibility of cross-reaction between the insulin receptor and IGF–I, IGF–I could potentially play a role as an embryonic growth factor.

Connective tissue growth factor (CTGF) is another growth factor whose essential role is in proliferation of connective tissue and production of ECM (Ball et al., 1998). The target cells for CTGF are fibroblasts and smooth muscle cells where CTGF acts to induce mitosis, chemotaxis, and construction of extracellular matrix (ECM) (Brigstock et al., 1997). With the identification of CTGF in the trophectoderm and inner cell mass of the perimpantation mouse embryo as well as localization in both luminal and glandular uterine epithelial cells and myometrium, Surveyor et al., (1998) suggested a role for CTGF in growth,

cellular migration, cellular adhesion, and extracellualr matrix production in both the uterus and the embryo. The identification of multiple low mass forms of CTGF in both pregnant and nonpregnant pigs along with its ability to interact with basic fibroblast growth factors present on days 10-14 of pregnancy, suggest that CTGF plays an important role at this stage of development of porcine pregnancy (Ball et al., 1998).

Placental Attachment

The last decade of study into regulation of reproductive function in the pig has identified many factors regulating and promoting placental attachment. MUC-1, a heavily glycosylated integral transmembrane glycoprotein is one of the major factors exhibiting its affects on the timing of placental attachment and has been quantified in a number of species (Pemberton et al., 1992). Mouse and human research has associated the loss of this glycoprotein with the time of implantation (Braga and Gendler, 1993; Alpin et al., 1994; Surveyor et al., 1995). Investigators purpose that the molecular structure of MUC-1 yields itself to being an antiadhesive molecule (Hilkens et al., 1992). Previous studies have identified gene expression of MUC-1 in the periimplantation uterus but at the time of implantation MUC-1 gene expression levels decrease to basal levels (1993; Braga et al., 1993; Surveyor et al., 1995). This down regulation of MUC-1 seemed to parallel down regulation of progesterone receptors (Surveyor et al., 1995). With this in mind Bowen et al., (1996) set out to quantify the gene expression of MUC-1 in the pig. Bowen's studies revealed that MUC-1 is present

on apical uterine epithelium on day 0 but absent by day 10 in both cyclic and pregnant gilts. This down-regulation of MUC-1 in response to progesterone-regulated events could serve to promote the transition of the uterus from a prereceptive to a receptive state by exposing adhesion molecules necessary for successful implantation (Bowen et al., 1996).

Many adhesion molecules have been characterized during placental attachment in mammalian species, such as the rodent and humans. Among those characterized are proteoglycans, lectins, cadherins, extracellular matrix components (ECM), and integrins (Cross et al., 1994). Integrins are a family of cell surface glycoproteins that are involved in cell-to-cell and cell-to-ECM interactions (Cross et al., 1994). Integrins function as transmembrane heterodimers comprising α and β subunits that are closely associated with cytoskeletal and cell signaling proteins (Clark et al., 1995). These subunits determine the function of each individual integrin and there individual binding affinity (Bowen et al., 1996). There are 16 α and 9 β subunits of integrins known at this time. The β_1 subunit can form heterodimers with 12 of the 16 α subunits. The pig demonstrates spatial and temporal expression of, α_4, α_5 , β_1 ,and possibly α_1 subunits, suggesting control of their expression within the uterine epithelium by reproductive steroids (Bowen et al., 1996). Subunits α_4, α_5, α_v, β_1 and β_3 are also expressed on trophectoderm during the peri-implantation period (Bowen et al., 1996). The integrin subunits highly expressed during the peri-implantation period are members of the fibronectin and vitronectin families of receptors and could be candidates for stabilizing attachment in the pig (Bowen et al., 1997).

Fibronectin gene expression has been detected in conceptus and uterine tissues throughout pregnancy (Tuo and Bazer, 1996). Work in the human, has identified integrin subunits $\alpha_v\beta_3$ and $\alpha_4\beta_1$ in uterine epithelium at the time of maternal recognition of pregnancy and implantation (Lessey et al., 1994). The uterine epithelium of the pig also expresses heterodimers $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_1$ during the time of implantation (Bowen et al., 1996). The expression of fibronectin associated integrin subunits during the time of implantation infers a major role of integrins in the establishment of epitheliocorial type placentation in the pig.

Embryo implantation depends on a multitude of contributing factors. At the time of implantation, three families of proteases are involved in the matrix degradation: the cysteine, serine and matrix metalloproteinases (Salamonsen, 1999). In the pig endometrium, the cysteine proteases that have been identified are cathepsins B, D, E, (Roberts et al., 1976) and L (Geisert et al., 1997). Cathepsins work to cleave the N-terminal peptides of collagen that contain the covalent cross-links that occur in and between molecules (Salamonsen, 1999). Cathepsins have been characterized as regulators of invasive implantation in rats (Elangovan and Moulton, 1980). Progesterone induces cathepsin L activity in the pig uterus (Geisert et al., 1997). Cathepsins have a high affinity for collagen and elastin (Salamonsen, 1999). Cathepsins affinity for collagen and elastin in cordinance with the fact that elastase activity and collagen remodeling play an important role in uterine growth and expansion during early pregnancy (Renegar, 1982), suggests a possible role for cathepsins in embryo attachment. An additional member of the cystine protease family is interleukin 1β-converting

enzyme. This enzyme releases active interleukin 1β from its precursor allowing it to regulate the transcription of matrix metalloproteinases (Salamonsen, 1999). A couple of the serine proteases extensively investigated are urokinase-type plasminogen activator, plasmin, and kallikrein (Salamonsen, 1999). These enzymes exert their action on matrix degradation. The third class of these protease's is the metalloproteinases (MMP's). MMP's fall into four categories: the collagenases, gelatinses, stromelysisns and membrane type.

Despite having an epitheliochorial type of placentation the porcine trophoblast will invade extracellular matrix when transplanted to ectopic sites (Samuel and Perry, 1972). Trophoblast production of proteolytic enzymes facilitates this non-uterine associated invasion (Fazleabas et al., 1983). Therefore, the lack of uterine disruption of conceptuses within the uterine lumen must occur through production of uterine protease inhibitors (Samuel and Perry, 1972; Fischer et al., 1985). Uterine expression of the antileukoproteinase gene, also known as secretory leukocyte protease inhibitor (SLPI) is regulated by conceptus estrogen secretion (Badinga et al., 1994). SLPI as an inhibitor of neutrophil elastase and cathepsin G would provide protection against epithelial cell destruction (Thompson et al., 1986). In pregnant gilts, SLPI is located specifically in glandular and luminal epithelial cells (Reed et al., 1996). Reed et al., (1998) suggests that TGFα plays a possible role in up-regulation of SLPI gene expression. Another protease inhibitor secreted by porcine surface epithelium of the endometrium at the time of trophoblastic elongation is uterine plasma/trypsin inhibitor (UTPI) (Badinga et al., 1999). SLPI and UTPI exhibit

potential growth factor activity in addition to their protease inhibition during early pregnancy suggesting an autocrine function for these two classic protease inhibitors (Badinga et al., 1999).

Recently, a new serine protease inhibitor has been detected in porcine endometrium during the estrous cycle and early pregnancy known as the inter-α-trypsin inhibitor (IαI) family (Geisert et al., 1995; Diederich et al., 1997). This family consists of four heavy chains and the light chain known as bikunin. These inhibitors consist of a combination of two heavy chains IαIH1, IαIH2 and bikunin, or IαIH3 and bikunin or IαIH2 and bikunin (Salier et al., 1996). Bikunin contains two kunitz-type protease inhibitor domains giving it the ability to inhibit serines such as trypsin, cathepsin G, elastase and plasmin (Hochstrasser et al., 1981). Although a clear biological role for inter-α-trypsin inhibitors is not established, Diedrich et al, (1997) suggests that, the detectable gene and protein expression of bikunin on days 12 and 18 of pregnancy indicates bikunin could assist with regulation of endometrial invasion of the porcine trophoblast. The IαI family act as acute phase proteins from the liver during trauma (Gonzalez-Ramon, 1995). Acute phase proteins are involved with inflammatory processes as indicated by its regulation by interleukin-6, an important inflammatory mediator (Sarafan et al., 1995). Therefore, a possible function of these acute phase proteins would be their role in prevention of tissue degradation during the period of embryo attachment and implantation. IαI heavy chains contain a von Willebrand type A domain that is known as a target for adhesion molecules like integrins, collagen, proteoglycans and heparin (Salier et al., 1996). The preceding research along

with the latest research by Bost et al., (1998) suggest an important role in extracellular matrix stabilization.

Many theories have evolved as to how conceptus estrogen initiates the biological effects associated with its elongation and implantation. Geisert et al., (1998) provided some possible insight into its mechanism with the discovery of inter-α-trypsin inhibitor heavy chain 4 (IαIH4), which is produced by the porcine endometrium. Initially these researchers identified a 30 kDa glycoprotein (GP30), which was homologous to the C-terminal region of a larger 120 kDa pig plasma glycoprotein, IαIH4 (Geisert et al., 1995). IαIH4 was most highly expressed in the porcine endometrium at the time of conceptus attachment. Heavy chain 4 in the pig is unique compared to the other Iαl heavy chains discussed earlier because it does not possess protease inhibitory ability and it lacks the consensus sequence to bind bikunin. Inter-α-trypsin inhibitor H4 is however a substrate for the plasma serine protease, kallikrein (Nishimura et al., 1995). In the pig, plasma kallikrein could cleave IαIH4 to release several fragments including GP30 (Geisert et al., 1995). The cleavage of IαIH4 by kallikrein could induce local alterations in receptivity of the uterus to the expanding conceptus that allows it to have contact with integrins, developing a firm attachment to the uterine epithelium (Bowen et al., 1997).

Kallikrein – Kininogen – Kinin System

In addition to the previously mentioned activity of kallikrein, the increased serine protease activity detected during conceptus elongation suggests the

presence of the Kallikrein-Kininogen-Kinin (K-K-K) system within the porcine uterus (Vonnahme et al., 1999). The K-K-K system consists of several members that include kallikrein, kininogens, kinins, kallikrein binding proteins, and kininases (Bhoola et al., 1992). In this system, tissue or plasma kallikrein cleaves either high or low molecular weight kininogens (HMWK and LMWK) releasing kinins, mainly the vasoactive peptide, bradykinin (Boohla et al., 1992). A number of studies have quantified the K-K-K system in reproductive processes such as ovulation (Gao et al., 1992), implantation (Valdes et al., 1996; Corthorn et al., 1997), menses (Clements and Mukhtar, 1994) and parturition (Brann et al., 1995). Figure 2.1 describes the two different pathways resulting in bradykinin release from kallikrein and kininogen.

Bradykinin is a vasoactive peptide that plays major roles in calcium release, blood flow, uterine contractions, decreased membrane permeability, and release of prostaglandin's (Cooper et al., 1985; Bhoola et al., 1992). The K-K-K system has been previously implicated in the process of embryo implantation and parturition in the rat (Valdes et al., 1993; Brann et al., 1995; Corthorn et al., 1997). The presence of the K-K-K system at the time of implantation suggests a major steroid regulated role in the induction of prostaglandin and histamine cascade known to be involved in increasing endometrial receptivity of the rodent conceptus.

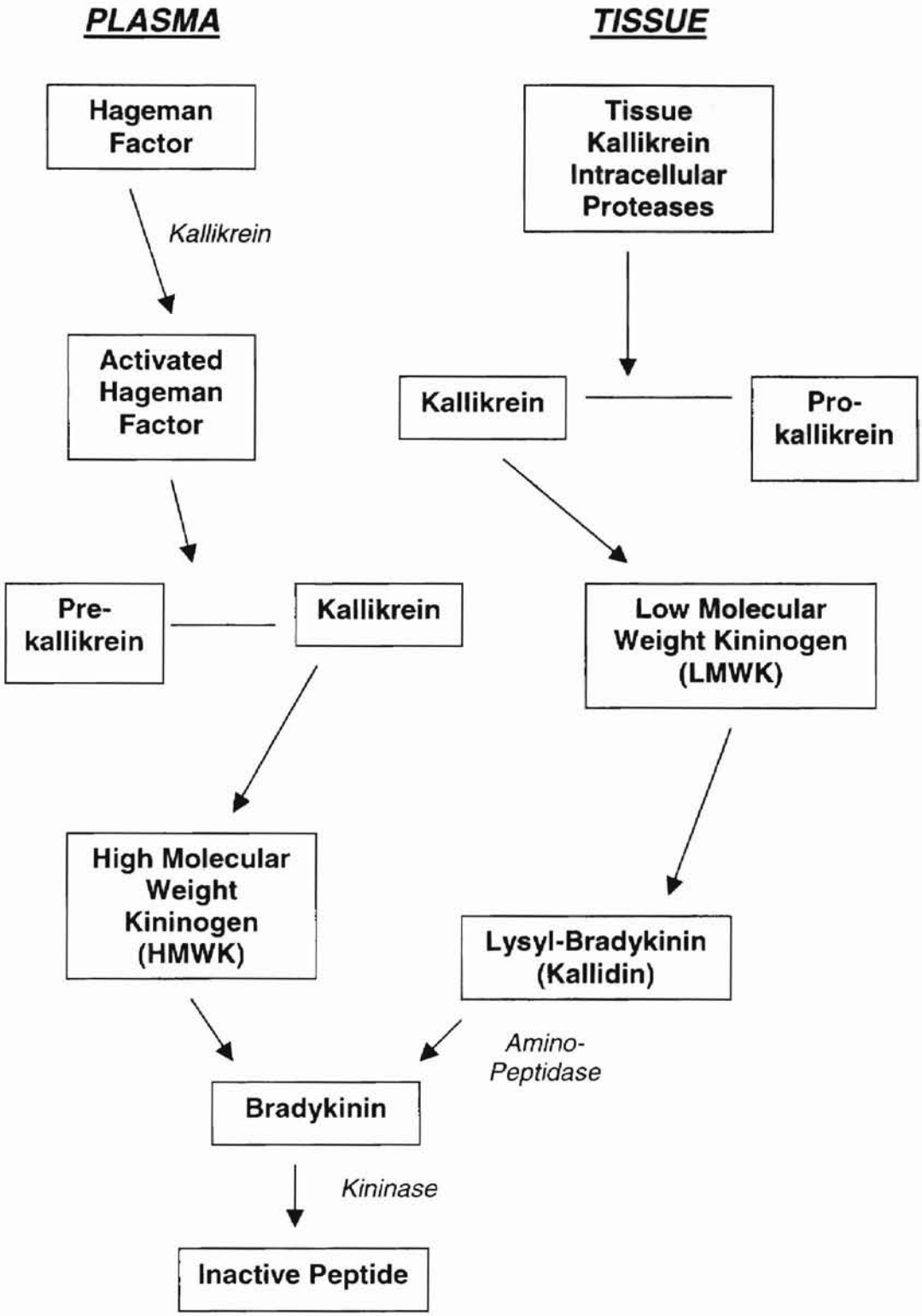


Figure 2.1 Plasma and tissue kallikrein proteolytic pathways involved in the generation and destruction of bradykinin. Taken from Rusiniak and Back, (1995)

Kallikrein

Kallikrein is a serine protease that is identified by its site of synthesis or detection: plasma and tissue (glandular) kallikrein, each having a different function. Plasma kallikrein cleaves HMWK releasing the nonapeptide, bradykinin, whereas tissue kallikrein acts on LMWK to release lysl-bradykinin or kallidin, which is later converted to bradykinin by amino-peptidase (Bhoola et al., 1992; Margolius, 1996). Plasma kallikrein is derived from a single gene in the liver (Bhoola et al., 1992), but tissue kallikrein is a multiple gene family (Bhoola et al., 1992; Rusiniak and Back, 1995; Margolius, 1996). In addition to its role in the K-K-K system, kallikrein also converts pro-renin (Sealy et al., 1978) and plasminogen to plasmin (Mandle and Kaplan, 1977). Kallikrein content in the kidney, uterus, ovary, and anterior pituitary of the rat can be increased by estrogen (Clements et al. 1986; Powers, 1986;Chen et al., 1992; Clements et al. 1997; Corthorn et al. 1997). Another known action of kallikrein is its cleavage of IGF-BP's. Seminal plasma kallikrein, otherwise referred to as prostate-specific antigen, is an IGF-BP protease that cleaves IGF-BP3 (Cohen et al., 1992).

Kininogen

Kininogen can be of two forms based on molecular weight, high (HMWK) or low (LMWK) (Bhoola et al., 1992; Cabinin et al., 1993). The gene for kininogen is divided into three regions: the heavy chain, which is common to both HMWK and LMWK, the bradykinin moiety, and the light chains, which are unique to each kininogen (Schmaier, 1997). Each domain has its own unique bioactivity;

bioactivities such as calcium binding, cell binding, and site-specific binding, i.e. bradykinin site serving as binding site for kallikrein (Schmaier, 1997). In addition to light chain sequence differences, HMWK and LMWK differ in their bradykinin moiety domain (Bhoola et al., 1992; Schmaier, 1997).

The kininogen gene contains eleven exons. The first nine exons are identical between HMWK and LMWK. Exon ten encodes for the bradykinin sequence common to both HMWK and LMWK and the 3' end of HMWK (which encodes the HMW-kininogen-specific gene). Exon 11 contains the sequence specific for LMWK.

Kininogen has been detected in regions of the body affecting reproductive function such as the placenta (Hermann et al., 1996), adrenal (Wang et al., 1996), and rat uterus (Brann et al., 1995).

Kinins

The four known mammalian kinins are kalladin, bradykinin, T-kinin and Met-T-kinin (Bhoola et al., 1992). T-kinin and Met-T-kinin identification has been limited to the plasma of rats (Bhoola et al., 1992). Kallidin, otherwise known as lysl-bradykinin, is the result of tissue kallikrein action on LMW kininogen. Kallidin is readily converted to bradykinin by aminopeptidases (Bhoola et al., Rusiniak and Back, 1995; Margolius, 1996). Bradykinin is a nonapeptide released from HMW kininogen by plasma kallikrein (Bhoola et al., 1992). Bradykinin is identified by its eight amino acid sequence, which is common to all of the mammalian kinins: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (Bhoola et al., 1992).

Kinins are rapidly degraded by kininases and have a half-life of less than ten seconds in the blood (McCarthy et al., 1965; Rusiniak and Back, 1995; Margolius, 1996). Kinases are peptidases, such as aminopeptidases and carboxypeptidases that hydrolyze kinins and terminate their biological activity (Bhoola et al.,1992).

In recent years researchers have studied and proposed theories on the biological affects kinins have on a variety of different tissues. Kinins mediate a wide range of physiological actions such as, vasodilatation, stimulation of GnRH release, increased sperm motility, increased vascular permeability, smooth muscle contractions, and increased blood flow (Schill and Haberland, 1974; Busse and Fleming, 1996; Mombouli et al., 1996; Regoli et al., 1997; Andre et al., 1998; Shi et al., 1998). On a cellular level bradykinin stimulates; calcium increase, cell division, cGMP increase, phospholipase A_2 activation, release norepinephrine, histamine, activation of platelet-activator factor leukotrienes, and prostaglandins (Bhoola et al., 1992). Kinins exert their action by adhering to membrane receptors. Kinins bind to their receptor inducing smooth muscle tone, the release of hormones, neurotransmitters, autocoids, and the activation of ion transport in epithelia and endothelia (Regoli et al., 1993). Kinins can also induce effects indirectly by the release of agents, such as nitric oxide, platelet activating factor, arachidonic acid metabolites, tumor necrosis factor, interleukin-1, histamine, acetylcholine, noradrenaline, and neuropeptides (Regoli et al., 1993).

Bradykinin Receptors

Kinins act through specific receptors that belong to two major catagories; β_1 , and β_2. The bradykinin β_1 receptor (BK_1r) mediates the rapid acute responses such as smooth muscle contraction or relaxation in addition to slower effects such as collagen synthesis, which appear to modulate interleukins (1980; Regoli et al., 1993). Bradykinin β_2 receptor mediates the majority of physiological effects of kinins. The bradykinin β_2 receptor (BK_2r), in the rat, has a protein sequence of 366 amino acids with a molecular mass of ~42 kDa (Margolius, 1996). Bradykinin and lysl-bradykinin have similar affinities for BK_2r (Bhoola et al., 1992). BK_2r has been shown to be a G-protein coupled, rhodopsin type receptor consisting of seven hydrophobic membrane domains connected by extracellular and intracellular loops (Regoli et al., 1993). This would suggest that kinins can exert their effects through both a paracrine and an autocrine pathway (Regoli et al., 1996). Kinin receptors utilize a variety of mechanisms (i.e. mediators or second messengers) to exert their effects. Kinins can bind to their receptors that act as mediators stimulating the release of substances such as nitric oxide, prostaglandins, platelet activating factor, noradrenaline and a number of other substances (Regoli et al., 1993). Kinins can also bind to their receptors exerting secondary messenger affects such as the regulation of activities of cyclases and induce changes in intracellular concentrations of cyclic nucelotides such as cAMP and cGMP (Regoli et al., 1993).

Bradykinin β_2 receptor can be found in the uterus of a number of different species, such as the rat (Yaqoob and Snell, 1994; Murone et al., 1999),

sheep (Murone et al., 1996), human (Shams et al., 1996), canine (Figueroa et al., 1997), the guinea-pig (Murone et al., 1996), and the rabbit (Damas et al., 1995). The receptor can also be found in a number of different reproductive tissues, like the human adrenal gland (Wang et al., 1996), human umbilical artery (Abbas et al., 1998), guinea-pig brain (Murone et al., 1996), and human decidua cells (Rehbock et al., 1997).

Bradykinin stimulates uterine contractions through the induction of smooth muscle contraction, mediated by the BK_2r (Bhoola et al., 1992; Damas et al., 1995). To further understand the mediation of BK_2r Murone and coworkers (1999) localized this receptor in the endometrial and myometrial layers of the rat uterus. In addition to their localization studies researchers also examined the effects of estrogen and progesterone on the gene expression of BK_2r. Murone and coworkers discovered that during diestrus BK_2r were localized to both the circular and longitudinal smooth muscle layers of the myometrium, the endometrial stroma, the glandular epithelium, and the layer subjacent to the luminal epithelium. During early proestrus BK_2r gene expression in both myometrium and endometrium was at its lowest. In contrast, during late proestrus myometrial BK_2r protein and mRNA levels were highest. This increase of BK_2r protein and mRNA levels, when estrogen levels are at their highest, suggests that estrogen regulates myometrial BK_2r gene expression. Further conclusions of this study suggest that both myometrial and endometrial BK_2r gene expression are regulated during the estrous cycle. These changes may be due to regulation by estrogen and progesterone, as myometrial receptor levels

are at their highest when estradiol levels peak, both myometrial and endometrial receptor levels are at their lowest when progesterone levels peak. Previous studies by Murone and coworkers (1996) identified similar gene expression of BK_2r in the ovine uterus and detected down-regulation of BK_2r in the myometrium during pregnancy. This information, in coordination with the knowledge that in the rat elevated levels of uterine bradykinin on day 22 of rat gestation play a role in uterine contraction during parturition (Brann et al., 1995), suggests that BK_2r down-regulation could play a vital role in prohibiting preterm uterine contractions.

Studies into the localization and characterization of BK_2r have established potential roles for bradykinin in the different regions of the uterus. Scientists have well established a role for bradykinin in myometrial function (Bhoola et al., 1990; Damas et al., 1995). Unfortunately, bradykinins' role in the endometrium is not as easy to determine. Endo and coworkers (1991) suggest a role as a growth factor because of its role in mobilizing intracellular calcium and induction of DNA synthesis in what would be normally quiescent endometrial stromal cells. Another suggested role would be in maintaining uterine electrolyte environment through its ability to enhance sodium absorption (Matthews et al., 1993) and changes in increasing arachidonic acid release, stimulating prostaglandin synthesis from endometrial stromal cells and glands (Bonney et al., 1993). In addition to these roles, Shams and coworkers (1996) also suggested that bradykinin acting though BK_2r increases vascular permeability and vasodilation associated with implantation.

Statement of Problem

Conceptus derived estrogen, the signal for maternal recognition of pregnancy in the pig (Bazer and Thatcher, 1977) begins a cascade of events in the uterus that have yet to be completely understood. We do know that there are events in the uterus, which need to be in complete concert with the conceptus for successful embryonic development and placental attachment to occur.

Identification of kallikrein enzymatic activity in the porcine uterine lumen in co-ordinance with kallikrein and kininogen gene expression in the uterus suggests a functional role for the kallikrein-kininogen-kinin system in the porcine uterus (Vonnahme et al., 1999). Kinins mediate physiological events such as prostaglandin and histamine release, increase in vascular permeability, and increase in blood flow, all essential components of conceptus development and placental attachment in the pig.

The presence of kinins in the porcine uterine lumen needs to be evaluated. In addition, gene expression of the bradykinin β_2 receptor throughout the estrous cycle and early pregnancy should also be investigated to establish a pathway mediating kinin induced events possibly involved in conceptus elongation and placental attachment.

CHAPTER III

DETECTION OF KININS IN THE PORCINE UTERUS DURING THE ESTROUS CYCLE AND EARLY PREGNANCY

Introduction

Porcine conceptus development is unique among the farm animal species because they undergo a rapid morphological transformation from a spherical shape to a long filamentous thread in a matter of just a few hours on approximately Day 12 of gestation (Geisert et al., 1982b). During this process, the conceptus releases estrogen, which acts as the signal for maternal recognition of pregnancy in the pig (Bazer & Thatcher, 1977). Conceptus estrogen stimulates the release of various proteins (Roberts et al., 1993), prostaglandins (Bazer and Thatcher, 1977), alters uterine cellular morphology (Geisert et al., 1982b; Keys and King, 1990), and increases uterine blood-flow (Ford et al., 1982). Numerous studies have thoroughly described the influence of conceptus estrogen on uterine secretory activity but the underlying factors activated by estrogen in the uterus that influence conceptus development and survival have yet to be fully elucidated.

Conceptus implantation is not an accurate description of placental formation as the trophoblast in the pig forms a non-invasive, epitheliochorial

placenta (Keys and King, 1990). However when placed outside of the uterine environment, porcine conceptuses produce several proteolytic enzymes that allow them to invade into ectopic tissues (Samuel and Perry, 1972) suggesting that uterine protease inhibitors play a significant role in formation of the diffuse, epitheliolchorial placentation in the pig.

Recently our laboratory has isolated and characterized a glycoprotein that is homologous to inter-α-trypsin inhibitor heavy chain 4 (IαIH4) (Geisert et al., 1998). IαIH4 is unique, compared to the other IαIH family members as it does not have a binding site to bikunin and is cleaved by the serine protease, kallikrein (Nishimura et al., 1995). In addition to its interaction with IαIH4, kallikrein could also interact with its normal substrate kininogen to release kinins (Bhoola et al., 1992) within the porcine uterine lumen. Kinins are vasoactive peptides that are normally involved with inflammatory associated effects such as tissue prostaglandin synthesis and release, increased blood flow, histamine release and induction of smooth muscle contractions (see Bhoola et al., 1992). The detection of kallikrein enzymatic activity in the porcine uterine lumen and endometrial gene expression, as well as identification of endometrial LMW kininogen protein and gene expression (Vonnahme et al., 1999) indicates the possible presence of an active kallikrein-kininogen-kinin system in the porcine uterus.

The objective of the current investigation was to determine the changes of bradykinin content in the porcine uterine lumen and alteration of endometrial bradykinin receptor gene expression during the estrous cycle and early pregnancy. Although bradykinin can effect tissues through two specific

receptors, β_1 and β_2; bradykinin β_2 receptor mediates the majority of physiological effects of kinins (Bhoola et al., 1992). Therefore, we evaluated the presence and alteration of endometrial bradykinin β_2 receptor gene expression during the estrous cycle and early pregnancy.

Materials and Methods

Evaluation of Uterine Lumenal Content of Bradykinin and Endometrial Bradykinin β_2 Receptor Gene Expression of Cyclic and Pregnant Gilts

Cyclic, crossbred gilts of similar age (8-12 months) were checked twice daily of estrus behavior by contact with an intact boar. Onset of estrus was considered day 0 of the estrous cycle. Gilts assigned to be mated were bred naturally with fertile boars at the onset of estrus and 12 hours later.

Cyclic (n=20) and pregnant (n=12) gilts were surgically hysterectomized through midventral laparotomy on days 0, 5, 12, 15, and 18 of the estrous cycle and days 12, 15, and 18 of pregnancy as previously described by Gries et al, (1989). The initial induction of anesthesia was through a 2.5 cc i.m. administration of a cocktail consisting of 2.5 cc Rompum (zylazime; 100mg/ml) (Miles, Inc., Shawnee Mission, KS), 2.5 cc Vetamine (ketamine HCL; 100mg/ml) (Mallickrodt Veterinary, Mundelein, IL) in 500 mg of Telazol (tiletamine HCL and zolazepam HCL) (Fort Dodge, Syracuse, NE). Anesthesia was maintained with a closed-circuit system of halothane (Halocarbon Laboratories, Riveredge, NJ) and oxygen (1.0 liter/min). After exposure by midvental laparotomy, the uterine horns

and ovaries were surgically removed. The incision site was closed using routine surgical procedures, and gilts were treated i.m. with penicillin (20,000 IU/kg BW).

Uterine flushings (UTF) and endometrium were collected immediately following removal of the uterus. The two horns were isolated and one horn was flushed with 20 ml of phosphate buffered saline (PBS; pH 7.4) while the second horn was flushed with 20 ml of PBS containing 200 µl of an enzyme cocktail inhibitor solution consisting of; EDTA (132.35 mg/100 ml; Sigma, St. Louis, MO), 1, 10 phenanthroline (90 mg/100 ml; Sigma, St. Louis, MO), chicken-egg-albumin trypsin inhibitor (7.3 mg/100 ml; Boehringer Mannheim, Mannheim, Germany), hexadimethrine bromide (30.5 mg/100 ml; Sigma, St. Louis MO), and aprotinin (2.0 mg/100ml; Boehringer Mannheim, Mannheim, Germany) to prevent the rapid proteolytic cleavage of bradykinin. Uterine flushings were examined to confirm pregnancy in mated gilts. Conceptuses collected on Day 12 of pregnancy were characterized as to stage of development (spherical, tubular, or filamentous) and then were immediately snap frozen in liquid nitrogen. Uterine flushings were placed on ice until centrifugation (3000 x g, 20 min; 4°C) at the laboratory, and stored at -80°C. After flushing, the uterine horn was cut along its anti-mesometrial border and endometrium was exposed for removal with sterile scissors. Endometrium was collected, snap frozen in liquid nitrogen and stored at -80°C until processed for extraction of RNA.

Bradykinin Radioimmunoassay

Content of bradykinin in uterine flushings was quantified by RIA following manufacturers recommendations for the Bradykinin RIA Kit (Peninsula Labs, Belmont, CA). Aliquots of two individual samples of different bradykinin concentration diluted with RIA buffer (25, 50, 75,100 µl) displaced the I^{125}-labeled bradykinin from antiserum to produce a binding curve parallel to the standard curve (Figure 3.1). Addition of 64, 128, 256, and 512 pg bradykinin to a flushing sample resulted in a recovery of 61, 138, 226, and 508 pg (Figure 3.2). The sensitivity of the assay was 2 pg/ml. Uterine flushing samples (100 µl) were assayed in duplicate in a single assay. The intra-assay coefficient of variation was 19.18%.

RNA Extraction

TRIzol reagent (Gibco/Life Sciences, Gaithersburg, MD) was utilized to extract total RNA from endometrium. A total of 0.5 grams of endometrium was homogenized in 5 ml of TRIzol reagent using a Virtishear homogenizer (Virtis Co. Inc., Gardiner, NY). One ml of chloroform was added and the samples were centrifuged for 30 min at 3500 x g (4°C). The aqueous layer was removed and placed into a new tube. RNA was precipitated by the addition of 2.5 ml of isopropanol at room temperature. After centrifugation (3500 x g; 4°C) for 10 min, the supernatant was poured off. RNA pellets were washed with 5 ml of 75% ethanol and centrifuged (5 min, 3500 x g; 4°C). RNA was rehydrated with 10mM Tris 1mM EDTA (pH 7.4) and stored at -80°C until further analysis. Total RNA

was quantified spectrophotometrically by absorbance at 260nm. RNA purity was determined from calculation of 260/280 ratios.

Complementary DNA Preparation

Total RNA was reverse transcribed to cDNA in a Perkin Elmer Cetus (Norwalk, CT) DNA Thermal Cycler Model 480. Total volume of the reactions was 20 µl containing 200 U of Moloney murine leukemia virus reverse transcriptase-Rnase H (M-MLV-RT), 1.0 µg of oligo$(dT)_{15}$ primer, 0.5 mM each of dATP, dCTP, dGTP, dTTP, 50mM Tris-HCL (pH 8.3), 75 mM KCL, 3 mM MgCl, 10mM dithiothreitol, 20 U of Rnasin, and 2.0 µg total RNA brought to volume with DEPC water. The sample preparation was incubated at 22°C for 15 min, 42°C for 30 min, and terminated by heating to 95°C. The samples were then cooled to 4°C and placed in the –20°C freezer for long-term storage. Quality and quantity of endometrial cDNA was checked by evaluating PCR expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as previously described (Yelich et al., 1997). M-MLV-RT, reaction buffer, Rnasin, and oligo$(dT)_{15}$ primer were obtained from Promega Corporation (Madison, WI).

Bradykinin β_2 Receptor Primer, Optimization, and Sequencing

Primers to bradykinin β_2 receptor cDNA were designed to regions of homology between human (Hess et al., 1992) and mouse bradykinin receptor (BK-2) mRNA (Yokoyama et al., 1994). The sequence of human bradykinin β_2 receptor mRNA (bp 668-1269) coinciding to homologous region of the mouse

was utilized to construct the 5' TCTACAGCTTGGTGATCTGGGG and 3' GTTTGTGAATCTGGCGTTCCAC primers (Hess et al., 1992). PCR reactions were carried out in 25 µl volumes covered with 30 µl of mineral oil. To optimize PCR conditions, pooled porcine endometrial cDNA from various days of the estrous cycle and pregnancy were amplified with 0.6 U of Taq DNA polymerase and its supplied $MgCl_2$ –free buffer (Promega, Madison, WI) and a 3X2X3 factorial (see table 3.1) combination of primer (50, 150, or 250 nM), deoxynucleotide triphosphates (dNTPs 50 or 100 µM) and $MgCl_2$ (1.25, 2.5, or 3.75 mM). All samples were maintained on ice until loaded directly into the heat block of the thermocycler, which was allowed to heat to 95°C before loading. The first cycle used a denaturation temperature or 95°C for 2 min, annealing temperature of 55°C for 1 min, and an extension at 72°C for 2 min. The subsequent 29 cycles utilized a denaturation temperature of 95°C for 1 min, annealing at 55°C for 1 min, and a 1 min extension at 72°C. The terminal cycle ran a 9 min extension at 72°C and cooled to 4°C for completion of the PCR. The resulting PCR product was resolved in a 3% agarose gel run at 90 volts for 1.5 h, followed by a 30 min staining in ethidium bromide (1.0mg/ml). The gel was destained in distilled water for 15 min. The optimal conditions chosen for endometrial bradykinin β_2 receptor gene amplification were 3.75 mM $MgCl_2$, 100 µM dNTPs, and 150 nM primer (see Figure 3.3). To verify PCR product as bradykinin β_2 receptor, pooled cDNA was amplified with the previously described optimal conditions, run on a 2% agarose gel, stained with ethidium bromide, and bands were cut from the gel with a razor blade. The PCR product was extracted

using Qiaquick (Qiagen, Santa Clara, CA), and sequenced by the Recombinant DNA/Protein Research Facility at Oklahoma State University. The resulting 625 base pair PCR product (Figure 3.4) was determined to be 90% homologous to Bos taurus bradykinin β_2 receptor (Fahrenkrug et al., 2000) and 85% homologous to Homo sapien bradykinin β_2 receptor mRNA (Hess et al., 1992) .

Endometrial cDNA (2 μg) was amplified using the optimal conditions previously described to determine bradykinin β_2 receptor gene expression in the uterus during the estrous cycle (Days 0, 5, 12, 15, and 18) and early pregnancy (Days 12, 15, and 18). The PCR products were resolved on a 2% agarose gel at 95 V for 1 h followed staining with ethidium bromide. Agarose gel was exposed to ultraviolet light and photographed with an MP4 Polaroid Camera System (Fotodyne, Inc., Hartland, WI). Molecular standards were obtained from Boehringer Mannheim (Indianapolis, IN). To quantitate the PCR synthesis procedure for comparable amounts of starting cDNA in samples, glyceraldehyde-3-phosphate dehydrogenase primers (Yelich et al., 1997) were utilized. The products were run in a 3% agarose gel, stained with ethidium bromide and bands were observed.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Bradykinin receptor endometrial gene expression was quantified by using the one-step RT-PCR reaction following manufacturers recommendations for TaqMan® Gold RT-PCR kit (P/N N808-0233) (PE Applied Biosystems, Foster City, Ca). The TaqMan® Gold RT-PCR kit is designed to reverse transcribe and

amplify target RNA that results in alterations of fluorescence from the bradykinin receptor specific probe permitting a quantitative measure of gene expression. The Taq Man probe contains a 5' reporter dye (FAM) and a 3' quencher dye (TAMRA). Cleavage of the probe by the endogenous 5' nuclease activity of AmpliTaq Gold DNA polymerase results in increased fluorescence of the reporter dye. The total reaction volume of 50 µl contained, TaqMan Buffer A, 5.5 mM $MgCl_2$, 300 µM deoxyATP, 300 µM deoxyCTP, 300 µM deoxyGTP, 600 µM deoxyUTP, 200 nM bradykinin receptor forward primer (bp 427-446), GCCTCCTACGTGGCCTACAG, 200 nM bradykinin receptor reverse primer (bp 475-493) AGTGCTTGCCCACGATCAC, 100 nM fluorescent labeled bradykinin receptor probe (bp 448-469) AACAGCTGCCTCAACCCGCTGG, all designed from the porcine bradykinin β_2 receptor mRNA sequence (Figure 3.4), 0.025 U/µl AmpliTaq Gold DNA polymerase, 0.25 U/µl MultiScribe reverse transcriptase, 1.25 U/µl recombinant Moloney murine leukemia virus (MuLV) reverse transcriptase, 0.4 U/µl Rnase Inhibitor and 100 ng of total RNA brought to volume with Rnase-free water. The PCR amplification was carried out in the ABI PRISM® 7700 sequence detection system (Applied Biosystems, Foster City, CA). Thermal cycling conditions were 50°C for 2 min, 95°C for 10 min followed by repetitive cycles of 95°C for 15 sec and 60°C for 1 min. Ribosomal 18S RNA control kit (43108993E, Applied Biosystems, Foster City, CA) was run as a control for RNA loading.

Following RT-PCR, quantitation of gene amplification was made by setting the threshold on the FAM layer in the geometric region of the plot after examining

the semi-log view of the amplification plot (Figure 3.5). Relative quantitation of bradykinin β_2 receptor gene expression was evaluated using the comparative C_T (cycle threshold) method as described in the manufacture's bulletin (Applied Biosystems, Foster City, CA). The ΔC_T value is determined by subtracting the bradykinin β_2 receptor C_T of each sample from its ribosomal 18S C_1 value. Calculation of $\Delta\Delta C_T$ involves using the highest sample ΔC_T value as an arbitrary constant to subtract from all other ΔC_T sample values. Fold changes in gene expression of bradykinin receptor is determined by evaluating the expression, $2^{-\Delta\Delta Ct}$. A validation of the quantitative PCR was performed with 5, 1, 0.2, 0.04, 0.008, 0.0016 ng RNA from a selected sample of RNA using both bradykinin β_2 receptor and 18S primers within the same PCR well. Expression of bradykinin β_2 receptor and 18 S RNA was parallel across the dilutions of RNA (Figure 3.6). Endometrium from an additional group of animals was collected on day 10 of the estrous cycle and early pregnancy for quantitative analysis of bradykinin β_2 receptor gene expression.

Statistical Analysis

Data were analyzed by ordinary least-squares analysis of variance using the General Linear Models Procedure of SAS (SAS, 1990). The statistical model used to analyze UTF bradykinin concentration and endometrial gene expression of bradykinin β_2 receptor during the estrous cycle included day (0, 5, 10, 12, 15, and 18). Model for analysis of reproductive status effects included the effects of Day, (0, 5, 10, 12, 15, and 18), reproductive status (estrous cycle and pregnant),

and day x reproductive status. Data were log transformed for analysis of bradykinin concentrations of UTF.

Results

Bradykinin Radioimmunoassay

A Day effect ($P<0.01$) for total bradykinin content in UTF during the estrous cycle was detected (Figure 3.7). During the estrous cycle, bradykinin in the UTF is greatest on Day 0 (estrus) followed by a 5-fold decrease on Day 5. A moderate 2-fold increase in bradykinin occurred between Day 12 and 18 of the estrous cycle. Pregnancy affected ($P<0.001$) bradykinin concentrations in UTF. There was no day x reproductive status interaction ($P>0.10$). Pregnant UTF bradykinin concentration was 5-fold greater than UTF from cyclic gilts on Day 12 with a 8 to 10-fold increase in bradykinin content detected on days 15 and 18 respectively (Figure 3.7).

Semi-Quantitative Analyses of Bradykinin β_2 Receptor Gene Expression

PCR amplification of endometrial G3PDH mRNA expression was similar across the days of the estrous cycle and early pregnancy (Figure 3.8 a and b). Endometrial gene expression of bradykinin β_2 receptor was detected on Day 0, 5, 12, 15, and 18 of the estrous cycle and Day 12, 15 and 18 of early pregnancy (Figure 3.9 a and b). Although this particular PCR technique is only a semi-quantitative measure of gene expression, endometrial gene expression of bradykinin β_2 receptor was decreased on Day 5 compared to Day 0 (estrus),

followed by a gradual increase of gene expression from Day 12 to 18 of the estrous cycle. Endometrial gene expression of bradykinin β_2 receptor appeared to be higher throughout early gestation in the pig. similar to expression observed on the same days of the estrous cycle.

Quantitative RT-PCR of Bradykinin β_2 Receptor with the ABI Prism System

Relative quantitation of bradykinin β_2 receptor gene expression was evaluated using the comparative C_T (cycle threshold) method. Endometrial gene expression of bradykinin β_2 receptor was detected on Day 0, 5, 10, 12, 15, and 18 of the estrous cycle and early pregnancy. Fold changes in gene expression of bradykinin β_2 receptor were determined by evaluating the expression, $2^{-\Delta\Delta Ct}$ (see Table 3.2). There was no effect of reproductive status on endometrial gene expression of bradykinin β_2 receptor ($P > .19$) between Days 12 to 18. There was a significant effect during the estrous cycle on bradykinin β_2 receptor gene expression ($P < .0001$). Bradykinin β_2 receptor gene expression was highest on Days 0, 15, and 18 of the estrous cycle. Gene expression decreased almost 6-fold on Days 5 and 10 of the estrous cycle (Figure 3.10a). There was a tendency for day x reproductive status interaction to effect receptor gene expression ($P < .06$) for comparison of Day 10, 12, 15 and 18, endometrial gene expression of bradykinin β_2 receptor between cyclic and pregnant gilts (Figure 3.10b). Bradykinin β_2 receptor gene expression was 3-fold greater in the endometrium of pregnant gilts on Day 10 and 12 of pregnancy compared to the estrous cycle.

However, bradykinin β_2 receptor gene expression was similar between cyclic and pregnant gilts on Day 15 and 18.

Discussion

Porcine conceptuses undergo a rapid morphological transformation from a 10 mm sphere to a thin filamentous shape on Day 12 of gestation (Anderson, 1978; Geisert et al., 1982b). Developing conceptuses produce estrogens that alter uterine cellular morphology (Geisert et al., 1982b), vascular permeability (Keys and King, 1990), uterine blood flow (Ford et al., 1982), secretion of endometrial derived proteins (Roberts et al., 1993), and prostaglandin release into the uterine lumen (Bazer and Thatcher, 1977).

Porcine conceptus attachment to the uterine surface epithelium begins on Day 12 and continues to Day 18 of pregnancy. This implantation process is non-invasive, therefore forming the diffuse epitheliochorial type of placentation of the pig (Keys and King, 1990). Many of the events associated with conceptus development and implantation in the mouse and rat are associated with uterine activation by estrogen and uterine changes that mirror the inflammatory process (Clements et al., 1997). Although porcine conceptuses do not implant into the uterine tissue in stark contrast to rodents, conceptus estrogen release is involved with placental attachment to the uterine epithelium and a inflammatory reaction also occurs during placental attachment in the pig (Keys et al., 1986; Keys and King, 1988).

Recent identification of kallikrein in the porcine uterine lumen during early gestation (Vonnahme et al., 1999) in coordination with the presence of IαIH4 suggested a possible role for kallikrein in placental attachment in the pig (Geisert et al., 1998). Inter-α-trypsin inhibitor heavy chain 4 has been identified in the porcine uterus during the estrous cycle and early gestation (Geisert et al., 1998). Heavy chain 4 in the pig is unique compared to the other Iαl heavy chains because it does not possess protease inhibitory activity due to the lack of a binding site for the serine protease inhibitor, bikunin and is cleaved by the serine protease, kallikrein (Nishimura et al., 1995). The release of endometrial derived kallikreins into the uterine environment (Vonnahme et al., 1999) were suggested to play a potential role for attachment of the placenta to the uterine surface through the possible alteration in IαIH4 during conceptus elongation and estrogen release. Presence of kallikrein in the porcine uterus suggested that the kallikrein-kininogen-kinin (K-K-K) system may be active during the period of placentation and could be associated with the increase in uterine blood flow, prostaglandin release and changes in uterine tone observed during early pregnancy in the pig (see Ford, 1989). The substrate for kallikrein, LMW-kininogen is present in the uterine lumen of the pig throughout the estrous cycle and early pregnancy in the gilt (Vonnahme and Geisert, unpublished results). Uterine tissue kallikrein could cleave LMW-kininogen releasing the vasoactive nonapeptide bradykinin. The presence of bradykinin in the uterine flushings supports our proposal for kallikrein stimulated release of bradykinin from LMW-kininogen during the estrous cycle and early pregnancy in the pig. The greatest

uterine content of bradykinin during the estrous cycle was detected at estrus. These data would suggest that estrogen could play a regulatory role in endometrial release of bradykinin in the pig. In cyclic animals, bradykinin initially decreased from Day 0 to Day 5 then increased moderately to Day 18, suggesting that progesterone may have a suppressive effect on bradykinin release during the early diestrous period. Kallikrein, the key regulatory enzyme in the liberation of bradykinin from LMW-kininogen, activity increases slightly in porcine UTF after Day 10 of the estrous cycle (Vonnahme et al., 1999). The increase in kallikrein enzyme activity on Day 12 and 15 of the estrous cycle and pregnancy are not significantly different (Vonnahme et al., 1999), however, there is a 5 to 10-fold increase in bradykinin content in the uterine lumen of pregnant compared to cyclic gilts. Although, the association of kallikrein enzyme activation and conceptus estrogen release during early pregnancy (Vonnahme et al., 1999) supports a role participation of the conceptus in the release of bradykinin into the uterine lumen, the release of bradykinin may not occur through the uterine tissue kallikrein. Kallikrein belongs to a multigene family of serine proteases, which consists of approximately 15 related genes in the rat (Gauthier et al., 1992) and three in human (Carbini et al., 1993). The kallikrein family has diverse substrate specificity's and variable sensitivities to inhibitors such as aprotinin (Gauthier et al., 1992), however the genes have extensive homology to each other and between species (Clements, 1997). It is possible that the porcine conceptus produces a kallikrein specific for LMW-kininogen that is different from the uterine kallikrein detected during the estrous cycle. Vonnahme et al., (1999) reported

gene expression for kallikrein by early porcine conceptuses. It is possible that uterine kallikrein enzyme activation is involved with changes in the extracellular matrix of the uterine surface necessary during trophoblast attachment, through cleavage of IαIH4, and is also involved with the removal of insulin-like growth factor binding proteins observed during Day 10 to 12 of the estrous cycle and pregnancy (Lee et al., 1998; Geisert et al., 1999). Conceptus synthesis and activation of kallikrein specific for LMW-kininogen would provide a local control of bradykinin release. The release of bradykinin in the uterine lumen during early pregnancy is consistent with the known effects of bradykinin in prostaglandin synthesis, smooth muscle contraction, increased blood flow, and altered vascular permeability (Bhoola et al., 1992), which are all observed changes in the porcine uterus during early pregnancy (see Geisert et al., 1990).

Further supporting evidence for a role of bradykinin in the establishment of early pregnancy is the identification of endometrial gene expression for bradykinin β_2 receptor during the estrous cycle and early pregnancy in the pig. Endometrial gene expression of bradykinin β_2 receptor changed throughout the estrous cycle with an increase in gene expression occurring after Day 10 of the estrous cycle and early pregnancy. Higher levels of bradykinin β_2 receptor gene expression on Day 0 of the estrous cycle would further support that estrogen may stimulate bradykinin β_2 receptor gene expression in the endometrium as an increase in expression is also detected on Day 12 of pregnancy when conceptus estrogen occurs for maternal recognition of pregnancy. It is clear that bradykinin β_2 receptor gene expression increases close to the period of conceptus

trophoblast elongation and attachment to the uterine surface. Corthorn and Valdes (1994) previously suggested that kallikrein-kininogen-kinin system plays an important role for implantation in the rat. The increased gene expression of bradykinin β_2 receptor and subsequent increase of kallikrein and bradykinin during the time of implantation in the present study suggests that the kallikrein-kininogen-kinin system may play an important part in the establishment of pregnancy in the pig.

Figure 3.1 RIA validation using two representative samples measured by dilutions (0, 25, 50, and 75 µl) with RIA buffer.

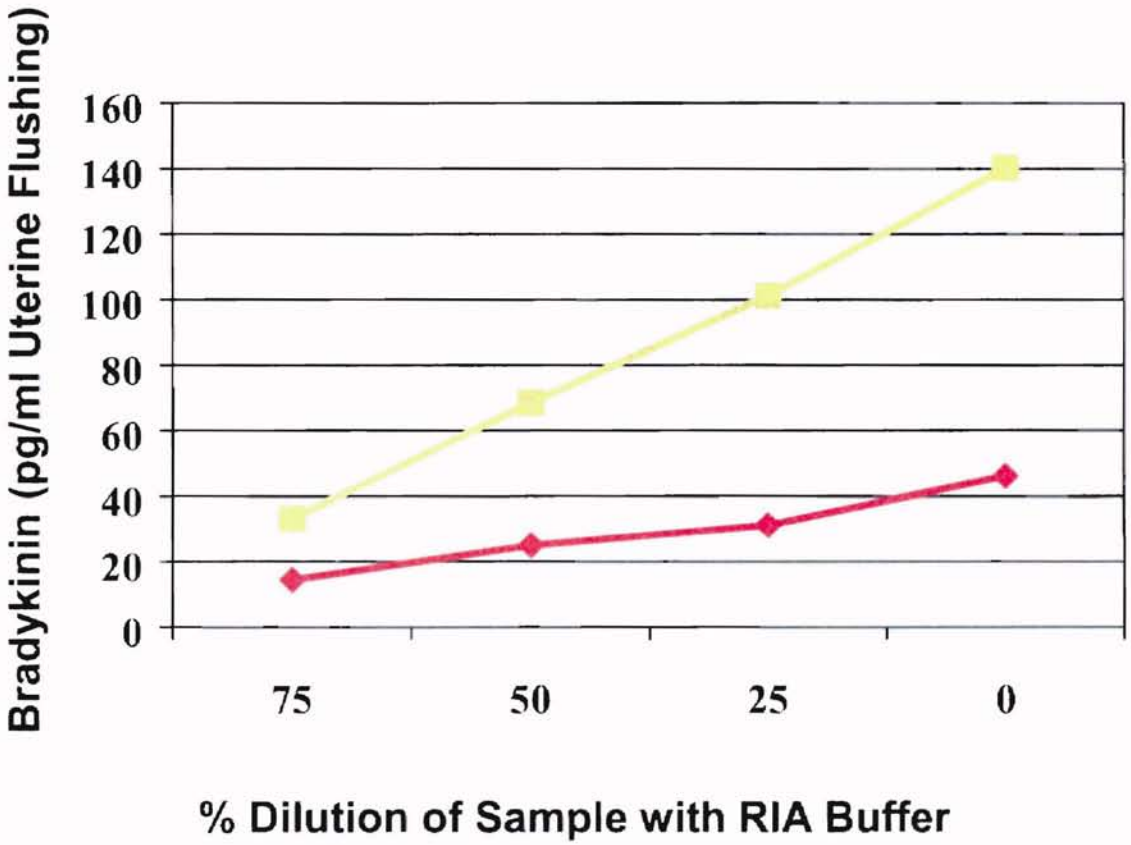

Bradykinin (pg/ml Uterine Flushing)
160
140
120
100
80
60
40
20
0
75
50
25
0
% Dilution of Sample with RIA Buffer

Figure 3.2 Recovery of mass measured in uterine flushings. Yellow line indicates the amount of mass recovered; green line indicates the calculated mass to be recovered.

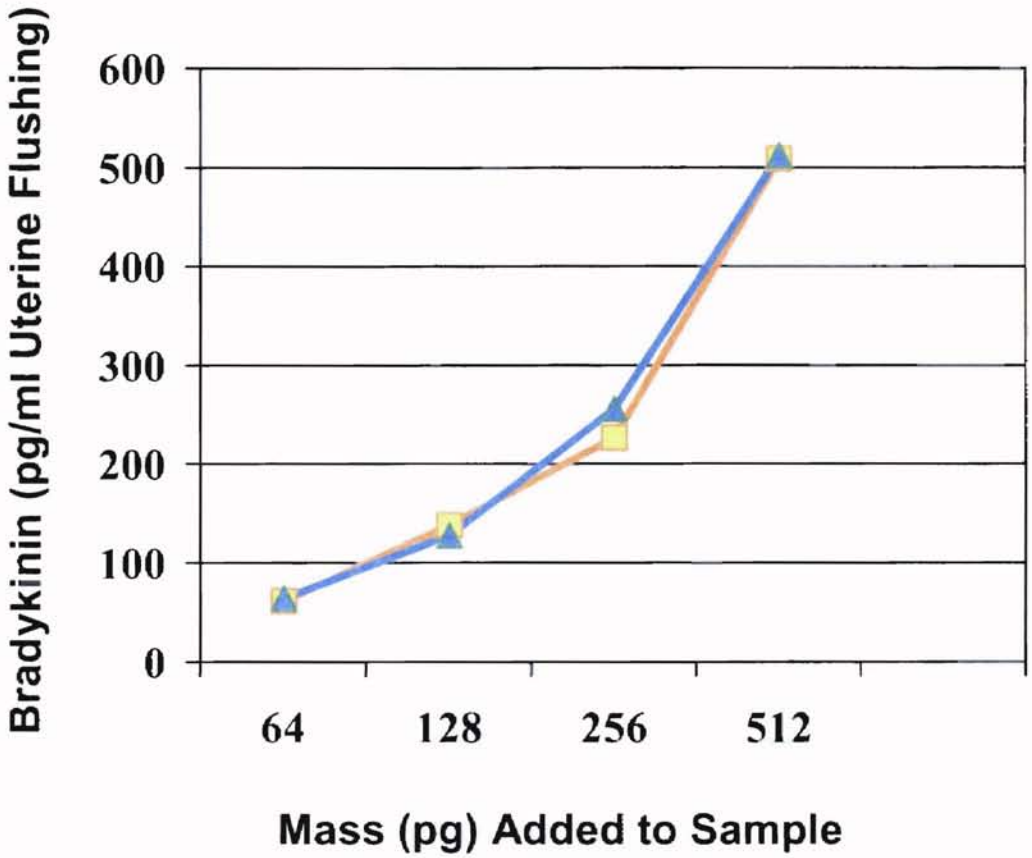

Bradykinin (pg/ml Uterine Flushing)
600
500
400
300
200
100
0
64
128
256
512
Mass (pg) Added to Sample

Table 3.1 Conditions for PCR optimization depicting changes in primer, dNTP's, and $MgCl_2$.

Lane	Primer (5μM stock)	DNTPs (10mM stock)	10 x MgCl
1	0.5 μl	0.5 μl	1.25 μl
2	0.5	0.5	2.50
3	0.5	0.5	3.75
4	0.5	1.0	1.25
5	0.5	1.0	2.50
6	0.5	1.0	3.75
7	1.5	0.5	1.25
8	1.5	0.5	2.50
9	1.5	0.5	3.75
10	1.5	1.0	1.25
.11	1.5	1.0	2.50
12	1.5	1.0	3.75
13	2.5	0.5	1.25
14	2.5	0.5	2.50
15	2.5	0.5	3.75
16	2.5	1.0	1.25
17	2.5	1.0	2.50
18	2.5	1.0	3.75

Figure 3.3 Photograph of an ethidium bromide stained 3% gel with PCR products from bradykinin β_2 receptor primer optimization. Conditions utilized for amplification of bradykinin β_2 receptor in lane 12 (see Table 3.1) were selected for analysis of gene expression in porcine endometrium. The arrow indicates the proper band size (625 bp). L represents the molecular ladder lane.

Lanes

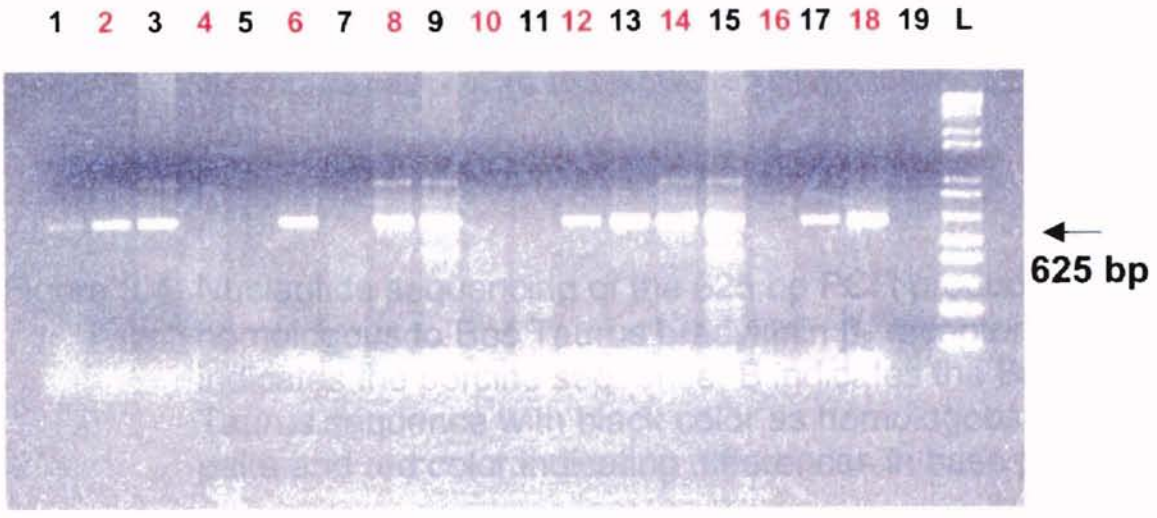


Figure 3.4 Nucleotide sequencing of the 625 bp PCR product is 90% homologous to Bos Taurus bradykinin β_2 receptor. P indicates the porcine sequence; B indicates the Bos Taurus sequence with black color as homologous base pairs and red color indicating differences in base pairs.

```
P1:     TCTACAGCTTGGTGATCTGGGGCTGCACGCTGCTCTTGAG
B176:   TCTACAGCCTGGTGATCTGGGGCTGCGCGCTGCTTCTGAG

P41:    CTCGCCCATGCTGGCCTTCCGGACCATGCAGCACGAGTAC
B216:   CTCGCCCATGCTGGCCTTCCGCACCATGCAG___GAGTAC

P81:    ACGGCCGAGGGCCACAACGTCACCGCCTGCATCATCAAGT
B256:   AACGCCGAGGGCCACAACGTCACCGCCTGCGTCATCAATT

P121:   ACCCCTCGCGCAAGCTGGGTGGTGTTCACCAACATCCTCC
B296:   ACCCATCCCACA_GCTGGGAGGTCTTCACCAACATCCTCC

P161:   TCAACTCCGTGGGCTTCCTGCTGCCCCTGAGCATCATCAC
B336:   TGAACTCTGTGGGCTTCCTGCTGCCCCTGAGCGTCATCAC

P201:   CTACTGCACCGTGCAGATCCTGCAGGTGCTGCGCAACAAC
B376:   CTTCTGCACCGTGCAGATCATGCAGGTGCTGCGTAACAAC

P241:   GAGATGCAGAAGTTCAAGGAGATCCAGACGGAGAGGAAGG
B416:   GAGATGCAGAAGTTCAAGGAGATCCAGACTGAGAGGAAGG

P281:   CCACGGTGCTGGTCCTGGCCGTGCTGCTGCTGTTCGTCGT
B456:   CCACGCTGCTGGTCCTGGCCGTCCTGCTGCTGTTCGTGGT

P321:   CTGCTGGCTGCCCTTCCAGATCAGCACCTTCCTGGACACG
B496:   CTGCTGGCTGCCCTTCCAGATCAGCACCTTCCTGGACACG
```

P361: **CTGCTGCGCCTCGACGTCCTCTCGGGCTGCTGGGACGAGC**

B536: **CTGCTGCGCCTCCACGTCCTCTCGGGCTGCTGGGACGAGT**

P401: **ACGTGGTCGACGTCCTCACGCAGATCGCCTCCTACGTGGC**

B576: **ACGTGATCGACATCTTCACACAGATCGCGTCCTTTGTGGC**

P441: **CTACAGCAACAGCTGCCTCAACCCGCTGGTGTACGTGATC**

B616: **TTACAGCAACAGCTGCCTCAACCCCCTGGTGTACGTGATC**

P481: **GTGGGCAAGCACTTCCGCAAGAAGTCGCGGGAGGTGTACT**

B656: **GTGGGCAAGCGCTTCCGCAAGAAGTCGCAGGAGGTGTACG**

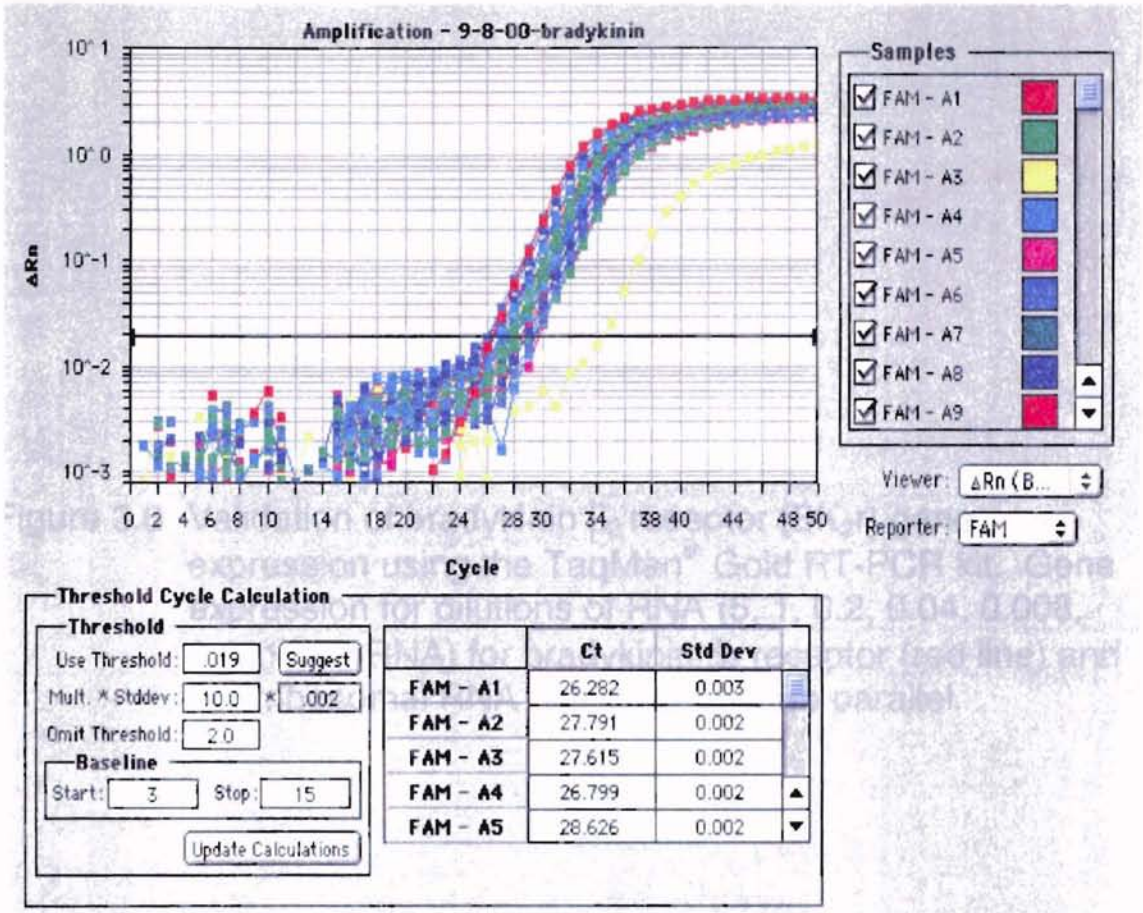
P521: **GGCGGCTGTGCGGGAAAGTGGGCTGCGGGCCC___GAGCC**

B696: **CGCGGCTGTGCCGGCCAGGGGGCTGCGGGTCCGCGGAGCC**

P558: **CAGCCAGACGGAGAATTCCCTGGGC**

B736: **CAGCCAGACGGAGAACTCCGTGGGC**

Figure 3.5 Quantitation of bradykinin β_2 gene amplification, made by setting the threshold on the FAM layer in the geometric region of the plot after examination of the semi-log view of the amplification plot.



	Ct	Std Dev
FAM - A1	26.282	0.003
FAM - A2	27.791	0.002
FAM - A3	27.615	0.002
FAM - A4	26.799	0.002
FAM - A5	28.626	0.002

Figure 3.6 Validation of bradykinin β_2 receptor (BK_2r) gene expression using the TaqMan® Gold RT-PCR kit. Gene expression for dilutions of RNA (5, 1, 0.2, 0.04, 0.008, 0.0016 ng RNA) for bradykinin β_2 receptor (red line) and 18S ribosomal RNA (yellow line) were parallel.

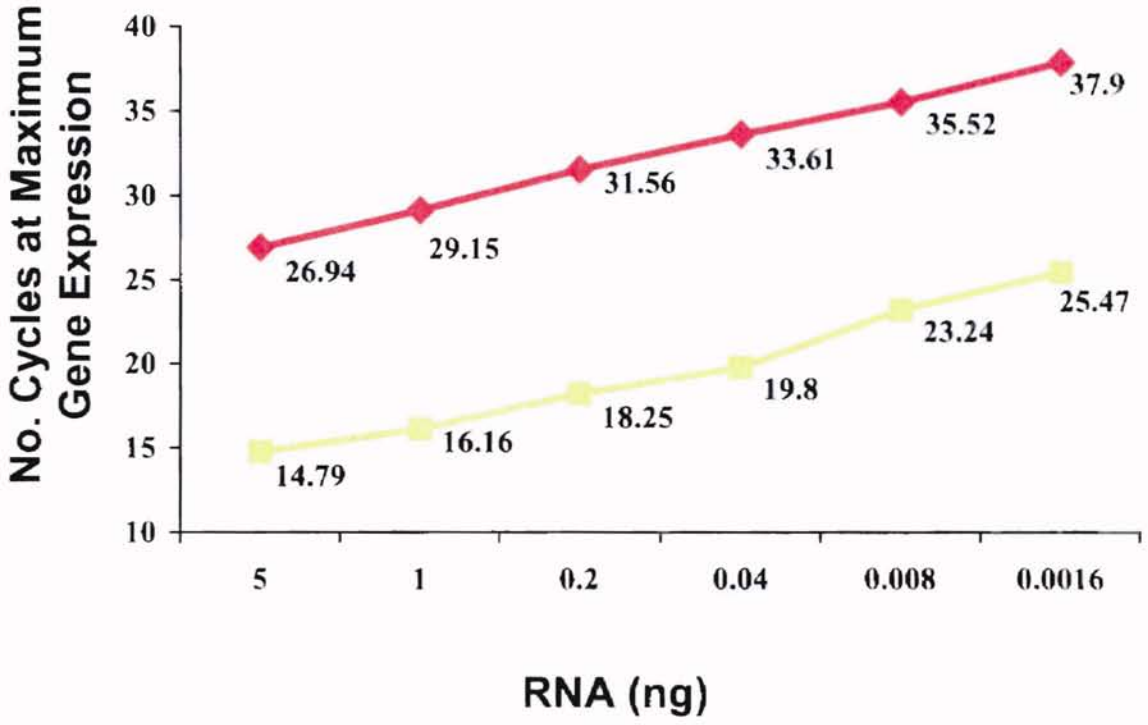

No. Cycles at Maximum Gene Expression
40
35
30
25
20
15
10
26.94
29.15
31.56
33.61
35.52
37.9
14.79
16.16
18.25
19.8
23.24
25.47
5
1
0.2
0.04
0.008
0.0016
RNA (ng)

Figure 3.7 Bradykinin content in uterine flushings from cyclic (blue bar) and pregnant (red bar) gilts. An effect of Day was detected ($P < .01$) in addition to an effect of reproductive status ($P < .0001$). There was no day x reproductive status interaction ($P > .10$). Data were log transformed for statistical analysis.

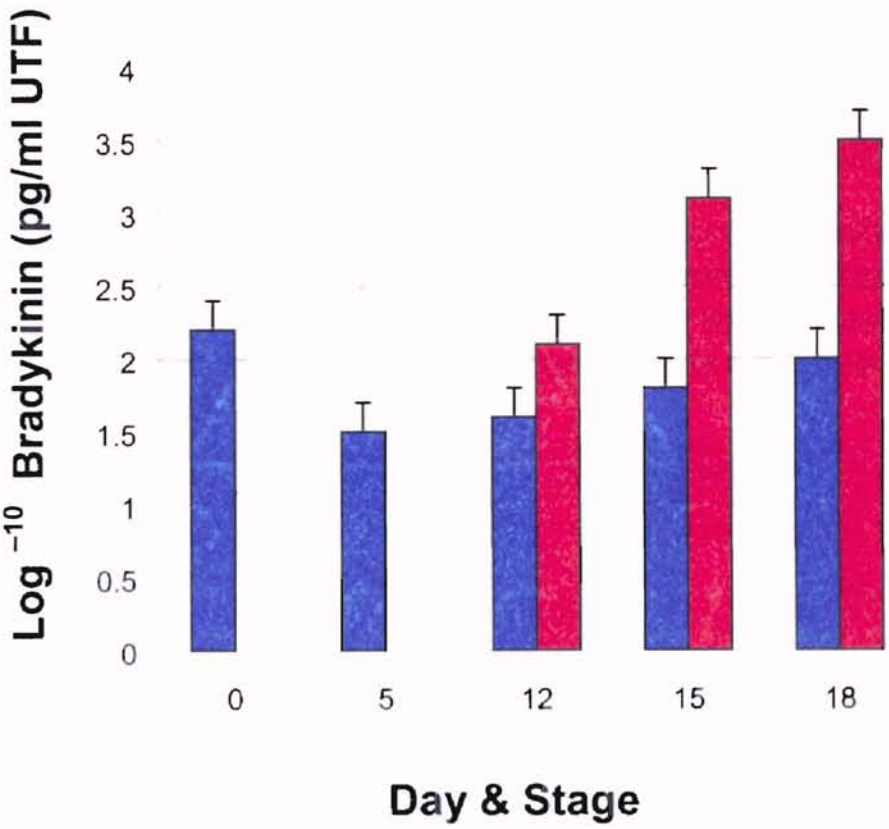
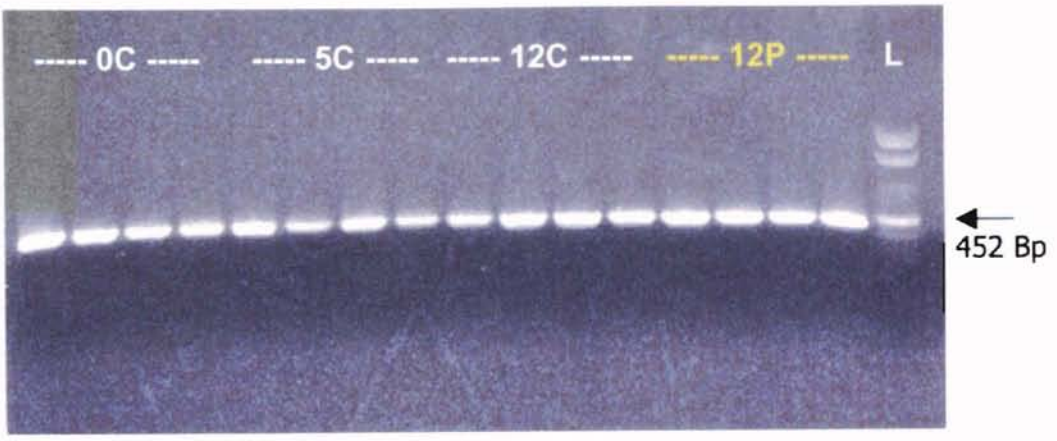

Log $^{-10}$ Bradykinin (pg/ml UTF)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
5
12
15
18
Day & Stage

Figure 3.8 Gene expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in endometrium from the estrous cycle and pregnancy used to quantitate the PCR synthesis procedure for comparable amounts of starting cDNA in samples.

A



B

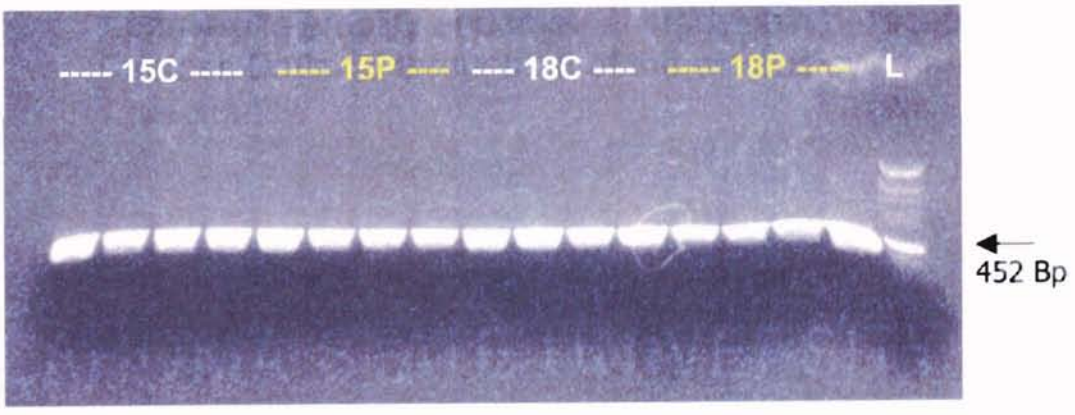
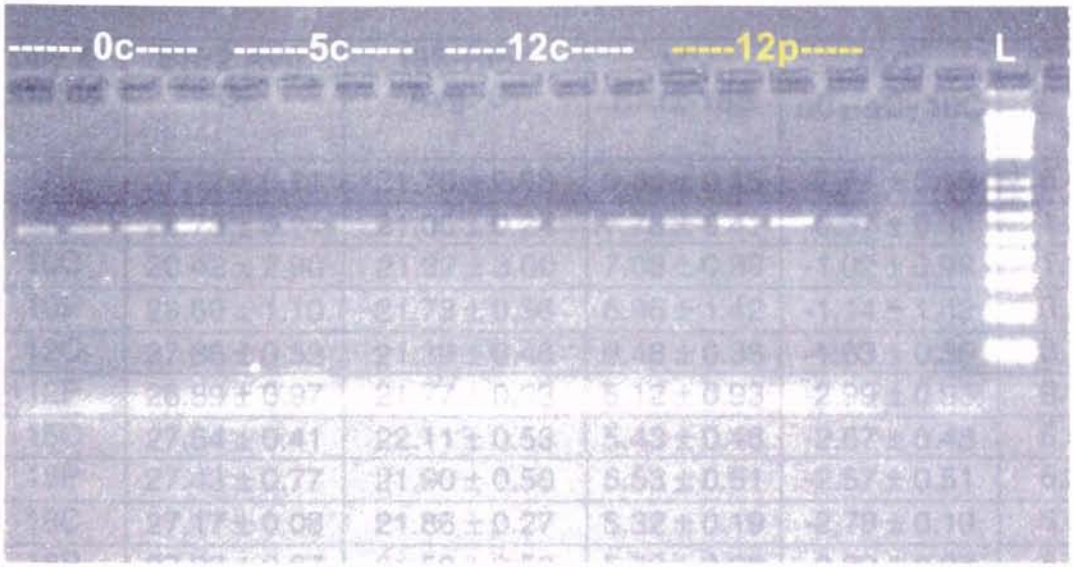


Figure 3.9 a. Representative PCR using bradykinin receptor primers to amplify endometrial bradykinin β_2 receptor gene expression across Days 0 through 12 of the estrous cycle (C) and pregnancy (P).
b. Representative PCR using bradykinin receptor primers to amplify endometrial bradykinin β_2 receptor across Days 15 through 18 of the estrous cycle and pregnancy.

A



B

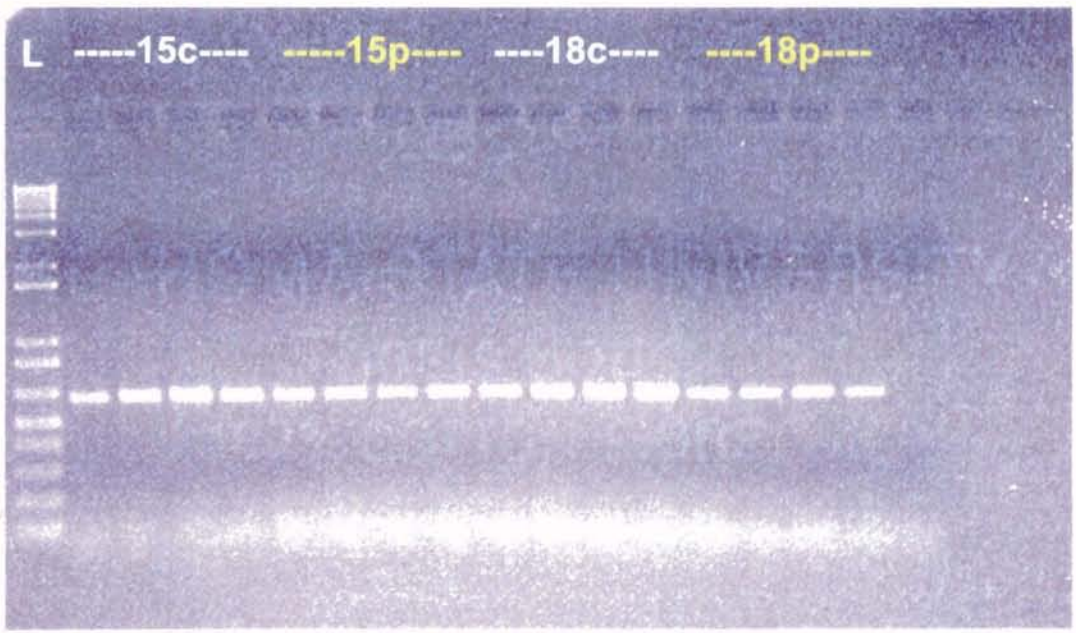


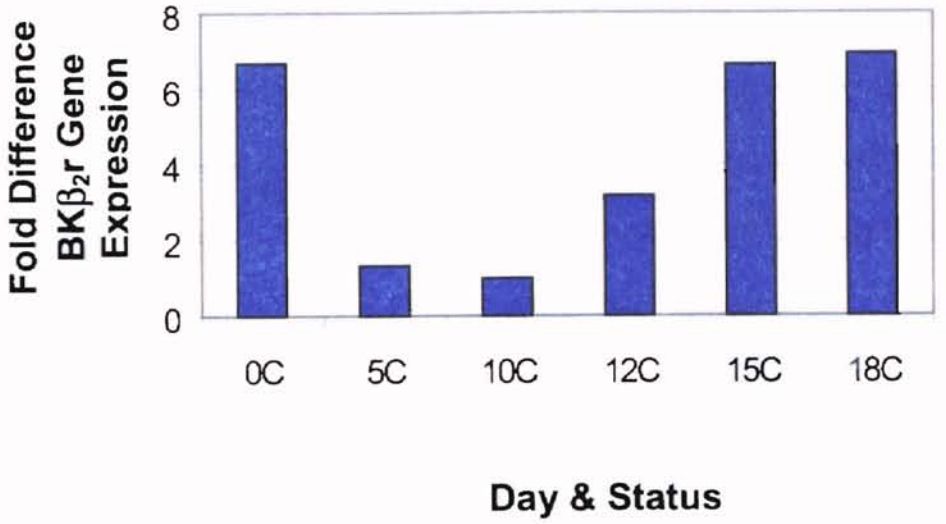
Table 3.2 Relative Quantitation Using the Comparative C_T Method for Bradykinin β_2 Receptor (BKr2)

Day & Status	BKr2 Avg. C_T	18S-Ribo Avg. C_T	ΔC_T BKr2-18S	$\Delta\Delta C_T$ ΔC_T-ΔC_T 10C	BKr2 $2^{-\Delta\Delta Ct}$
0C	27.12 ± 0.71	21.76 ± 0.58	5.36 ± 0.15	-2.74 ± 0.15	6.67
5C	28.78 ± 0.24	21.05 ± 0.24	7.73 ± 0.41	-0.37 ± 0.33	1.32
10C	29.42 ± 2.90	21.32 ± 3.00	7.08 ± 0.99	-1.02 ± 0.99	1.00
10P	28.69 ± 1.10	21.73 ± 0.36	6.96 ± 1.42	-1.14 ± 1.42	3.11
12C	27.86 ± 0.53	21.39 ± 0.46	6.48 ± 0.38	-1.63 ± 0.38	3.17
12P	26.89 ± 0.97	21.77 ± 0.23	5.12 ± 0.93	-2.99 ± 0.93	9.18
15C	27.54 ± 0.41	22.11 ± 0.53	5.43 ± 0.48	-2.67 ± 0.48	6.62
15P	27.43 ± 0.77	21.90 ± 0.50	5.53 ± 0.51	-2.57 ± 0.51	6.21
18C	27.17 ± 0.08	21.86 ± 0.27	5.32 ± 0.19	-2.78 ± 0.19	6.92
18P	27.22 ± 0.67	21.50 ± 0.52	5.72 ± 0.28	-2.39 ± 0.28	5.30

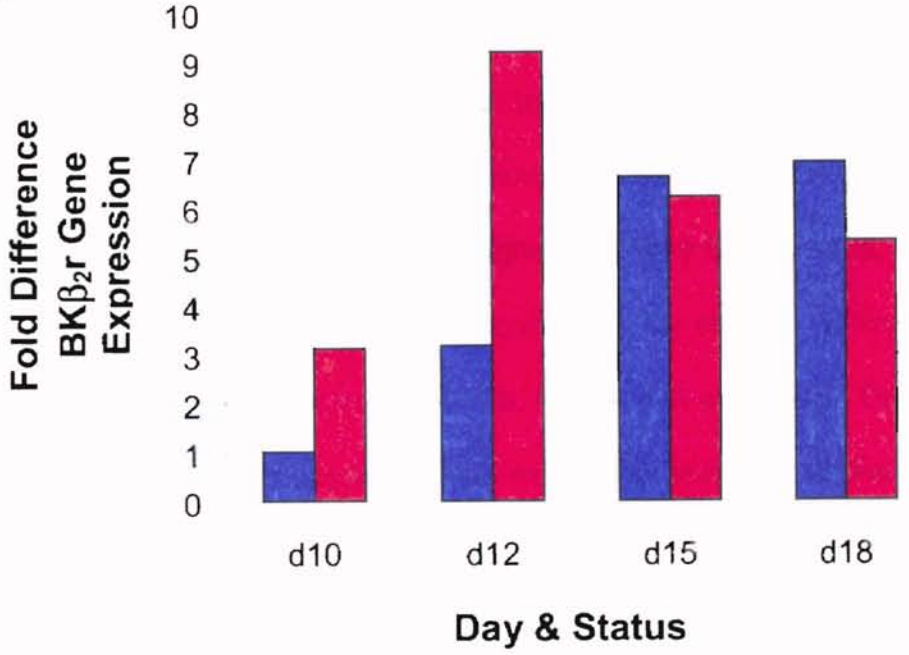
Figure 3.10 Relative quantitation of bradykinin β_2 receptor gene expression across days of the estrous cycle and early pregnancy in relation to 10C.

a. Blue bars indicate cyclic animals. An effect of Day was detected ($p < .0001$).
b. Red bars indicate pregnant animals and blue bars indicated cyclic animals. A tendency for day x reproductive status interaction to effect receptor gene expression ($p < .06$). There was no effect of reproductive status on endometrial gene expression ($p > .10$).

A


Fold Difference BKβ2r Gene Expression
8
6
4
2
0
0C
5C
10C
12C
15C
18C
Day & Status

B


Fold Difference BKβ2r Gene Expression
10
9
8
7
6
5
4
3
2
1
0
d10
d12
d15
d18
Day & Status

CHAPTER IV

GENERAL DISCUSSION

Domestic animal species are different from the rodent, human and primate in that they must undergo a prolonged period of implantation for successful establishment of pregnancy. The pig is well known for being one of the few species that the peri-implantation conceptus undergoes rapid elongation within the uterine lumen (Anderson, 1978; Geisert et al., 1982a; Pope, 1994). Conceptuses undergo a rapid morphological transformation from spherical (10 mm) to tubular (15-40 mm) and finally, filamentous (150-200 mm in length) morphology between days 11 and 12 of pregnancy (Perry & Rowlands, 1962; Anderson, 1978). Conceptus elongation occurs uniquely enough without an increase in cell number (Geisert et al., 1982b; Pusateri et al., 1990). During this transformation porcine conceptuses release estrogens which act as the maternal recognition signal for pregnancy (Thatcher and Bazer, 1977), in addition to influencing protein secretions (Roberts et al., 1993) redirecting prostaglandin release into the uterine lumen (Bazer and Thatcher, 1977), increasing uterine blood-flow (Ford et al., 1982), and altering uterine cellular morphology (Geisert et al., 1982b; Keys and King, 1990). A second surge of estrogen can be observed

on day 15 of gestation. This second surge is essential for maintaining prolonged corporal lutea function.

Implantation by the pig conceptuses is non-invasive, thus forming the diffuse, epitheliochorial type of placentation (Keys & King, 1990). In-vivo the conceptuses are observed to be non-invasive but when placed outside of the uterine lumen the conceptuses will aggressively invade into the surrounding tissue (Samuel and Perry, 1972). In light of this observation, one would deduce the porcine endometrium must produce proteolytic inhibitors to prevent invasive implantation.

Attachment of the conceptus to the uterine surface epithelium begins on day 12 through 18 of pregnancy. The conceptus trophoblast is secured by the uterine epithelial glycocalyx present on the microvilli (Keys and King, 1990). The timing at which this occurs is largely controlled by the presence of the glycoprotein, MUC-1 (Bowen et al., 1996, 1997). Mouse and human research has associated the loss of this glycoprotein with the time of implantation (Braga and Gendler, 1993; Alpin et al., 1994; Surveyor et al., 1995). When MUC-1 is removed from the uterine surface epithelium, extracellular matrix proteins readily bind to uterine surface integrins, promoting placental attachment (Hynes, 1992). Integrins are a family of cell surface glycoproteins that are involved in cell-to-cell and cell-to-ECM interactions (Cross et al., 1994). The pig demonstrates spatial and temporal expression of integrin subunits, α_4, α_5, β_1 ,and possibly α_1, suggesting control of their expression within the uterine epithelium by reproductive steroids (Bowen et al., 1996). Integrin subunits are also expressed

on the trophectoderm during the peri-implantation period (Bowen et al., 1996). The integrin subunits highly expressed during the peri-implantation period are members of the fibronectin and vitronectin families of receptors and could be candidates for stabilizing attachment in the pig (Bowen et al., 1996). The inter-α-trypsin inhibitor (IαI) family members, serine protease inhibitors, are also recognized as extracellular matrix stabilizers (Bost et al., 1998). This family consists of four heavy chains and the light chain known as bikunin (Salier et al., 1996). IαI heavy chains contain a von Willibrand domain which is known to bind adhesion molecules such as collagen, integrins, heparin and proteoglycans (Salier et al., 1992), which suggests a possible involvement in placental attachment. IαI heavy chain 4 (IαIH4), recently identified in the porcine uterus (Geisert et al., 1998), supports the hypothesis that IαI heavy chains could play a role in trophoblastic attachment. Although a clear biological role for inter-α-trypsin inhibitors is not established, Diedrich et al, (1997) suggests that, the detectable gene and protein expression of bikunin on days 12 and 18 of pregnancy indicates bikunin could assist with regulation of endometrial invasion of the porcine trophoblast. Heavy chain 4 (IαIH4) in the pig is unique compared to the other IαI heavy chains because it does not possess protease inhibitory ability because it lacks the consensus sequence to bind bikunin. Inter-α-trypsin Inhibitor H4 is however a substrate for the plasma serine protease, kallikrein (Nishimura et al., 1995). In the pig, plasma kallikrein could cleave IαIH4 to release several fragments including GP30 (Geisert et al., 1995). The cleavage of IαIH4 by kallikrein could induce local alterations in receptivity of the uterus to the

expanding conceptus that allows it to contact with integrins, developing a firm attachment to the uterine epithelium (Bowen et al., 1997).

Kallikrein can also bind to its natural substrate, low molecular weight kininogen (LMWK), releasing the vasoactive, nonapeptide bradykinin (Bhoola et al., 1992). Vonnahme et al., (1999) detected kallikrein activity in the uterine lumen and kallikrein endometrial gene expression during the estrous cycle and early pregnancy in the pig. In addition, Vonnahme and coworkers discovered LMW kininogen in the uterine lumen and LMW kininogen gene expression in the porcine endometrium.

The present investigation examined bradykinin concentrations in the porcine uterine lumen and endometrial gene expression of bradykinin β_2 receptor across the days of the estrous cycle and early gestation. This study detected varying concentrations of bradykinin in uterine flushings (UTF) examined from different stages of the estrous cycle and early pregnancy. In cyclic animals, bradykinin initially decreased from Day 0 to Day 5 then increased to Day 18. Kallikrein activity also increases in porcine UTF during this time (Vonnahme et al., 1999). Kallikrein, the key regulatory enzyme in the liberation of bradykinin from LMW-kininogen, activity increases slightly in porcine UTF after Day 10 of the estrous cycle (Vonnahme et al., 1999). The increase in kallikrein enzyme activity on Day 12 and 15 of the estrous cycle and pregnancy are not significantly different (Vonnahme et al., 1999), however, there is a increase in bradykinin content in the uterine lumen of pregnant compared to cyclic gilts. Although, the association of kallikrein enzyme activation and conceptus estrogen release

during early pregnancy (Vonnahme et al., 1999) supports a role participation of the conceptus in the release of bradykinin into the uterine lumen, the release of bradykinin may not occur through the uterine tissue kallikrein. Kallikrein belongs to a multigene family of serine proteases, which consists of approximately 15 related genes in the rat (Gauthier et al., 1992) and three in human (Carbini et al., 1993). The kallikrein family has diverse substrate specificity's and variable sensitivities to inhibitors such as aprotinin (Gauthier et al., 1992), however the genes have extensive homology to each other and between species (Clements, 1997). It is possible that the porcine conceptus produces a kallikrein specific for LMW-kininogen that is different from the uterine kallikrein detected during the estrous cycle. Vonnahme et al., (1999) reported gene expression for kallikrein by early porcine conceptuses. It is possible that uterine kallikrein enzyme activation is involved with changes in the extracellular matrix of the uterine surface necessary during trophoblast attachment, through cleavage of IαIH4, and is also involved with the removal of insulin-like growth factor binding proteins observed during Day 10 to 12 of the estrous cycle and pregnancy (Lee et al., 1998; Geisert et al., 1999).

Kallikrein levels have been shown to be regulated by ovarian hormones such as progesterone and estrogen (Brann et al., 1995). Valdes et al., (1996) noted an increase in kallikrein activity, and subsequent kinin release, associated with increased estrogen, in the rat uterus. Progesterone seems to have a negative and estrogen a positive affect on kallkrein production. When observing the estrous cycle of the pig by Day 12-15, progesterone levels are declining due

to the regression of the corpora lutea and follicles are growing, therefore releasing increased levels of estrogen. These events could be influencing factors affecting the increased kallkrein activity and subsequent bradykinin concentrations in cyclic UTF's.

Significantly, higher and increasing levels of bradykinin can be detected in pregnant UTF's. Again, this correlates with the findings of Vonnahme et al., 1999. Steroid influence would seem to be the controlling factor at this stage as well but in this case, the source of estrogen would be the conceptuses themselves. Synthesis of estrogen by the porcine conceptus is the signal to the uterus and ovary to undergo the necessary adjustments to establish and maintain early pregnancy (Thatcher and Bazer, 1977). Previous investigations have identified estrogen mediated events such as influencing protein secretions (Roberts et al., 1993) prostaglandin release (Bazer and Thatcher, 1977), increasing uterine blood-flow (Ford et al., 1982), and altering uterine cellular morphology (Geisert et al., 1982b; Keys and King, 1990). Increased concentrations of bradykinin paralleled with the conceptus production of estrogen could explain some of the unexplained uterine dynamics associated with conceptus estrogen release. Increased uterine blood flow could be indirectly regulated by estrogen through bradykinin due to its physiological ability to increase increased blood flow (Shi et al., 1998). Increased blood flow was detected in the pregnant uterus by Ford and Christenson (1979). Another biological action of bradykinin is the stimulation of phospholipase A_2 which in turn stimulates prostaglandin release (Bhoola et al., 1992), a factor associated with

early pregnancy in the pig. In addition to the previous mediation, bradykinins could also play an important role in conceptus orientation in the uterus through its ability to stimulate smooth muscle contractions. Of course, without its receptor, bradykinin β_2, bradykinin would not be able to exert any of its affects on uterine responses. Kinins exert their action by adhering to membrane receptors. Bradykinin β_2 receptor mediates the majority of physiological effects of kinins.

The present research detected endometrial gene expression of bradykinin β_2 receptor throughout the estrous cycle and early pregnancy of the pig. Endometrial gene expression of bradykinin β_2 receptor in the cyclic animal was similar to the concentrations of bradykinin found in the uterine flushings of those same animals, exhibiting a significant decrease in expression on Day 5 of the cycle then increasing till Day 18. These results suggest a possible regulating role for steroid hormones in the gene expression of bradykinin β_2 receptor. Increased gene expression of bradykinin β_2 receptor in pregnant endometrium suggesting again their mediation of biological events stimulated by bradykinins.

There is an interesting similarity between events stimulated by conceptus estrogen in the pig and inflammatory events occurring during tissue damage (Clements et al., 1997). Events such as histamine increased vascular permeability, vasodilation, edema and a number of other things. It is a well-known fact that the K-K-K system is associated with inflammatory responses (Bhoola et al., 1992). The immunological biological affects of bradykinins that are released from endometrial sources could be acting in co-ordinance with the conceptus to stimulate these effects. Research performed by Mathialagan et al.,

(1992) suggests that IL-6 is secreted from the porcine conceptuses between Days 13 to 17 of gestation. IL-6 upregulates Iαl heavy chains 1 through 3. Therefore another responsibility of bradykinin mediated events would be to stimulate IL-6 release from the porcine conceptus to upregulate Iαl members, supporting conceptus attachment.

Further investigation into the regulation of bradykinins and their receptors need to be performed. One means of investigation would be the observation of hormone supplemented ovarectomized gilts, measuring kallikrein, kininogen, bradykinin and bradykinin receptor gene expression in the uterus. Bradykinin affects also need to be elucidated by localizing and characterizing bradykinin β_2 receptor in the uterus.

The present data and its relation to the previously explained events taking place in the porcine uterus at the time of conceptus elongation and attachment supports this laboratories hypothesis that the K-K-K system is active in the porcine uterus and may play an important role in conceptus elongation and attachment.

APPENDIX

ABI- Quantiative RT-PCR Reaction Conditions

Reagent	Total Volume In Master Mix (µl)	Conc. Of Reagent in Solution
10X Taq Man Buffer A	210	
25 mM MgCl	462	5.5 mM
10mM dATP	63	300 µM
10mM dGTP	63	300 µM
10mM dCTP	63	300 µM
20mM dUTP	63	600 µM
10 µM Ribosomal Forward Primer	10.5	200 nM
10 µM Ribosomal Reverse Primer	10.5	200 nM
40 µM Ribosomal RNA Probe	2.63	50 nM
10 µM Bradykinin Forward Primer	42	200 nM
10 µM Bradykinin Reverse Primer	42	200 nM
5 µM Bradykinin Probe	2.1	100 nM
Amplitaq Gold DNA Polymerase	10.5	.25 U / µl
Multiscribe Reverse Trans.	10.5	.25 U / µl
RNAse Inhibitor	42	.4 U / µl

100 ng of RNA was added to each reaction tube.

The Master Mix (MM) was separated in equal amounts into 46 tubes (reactions). Tubes 1-40 were tubes measuring sample gene expression. Tubes 41-46 were tubes containing increasing amounts of RNA.

DEPC water was used to bring the reactions to volume.

LITERATURE CITED

Abbas F, Clayton JK, Marshall KM, and Senior J. Characterization of kinin receptors on the human isolated umbilical artery. J Endocrinol 1998; 156: 386-394.

Agura ED, Howard M and Collins SJ. Identification and sequence analysis of the promoter for the leukocyte integrin β-subunit (CD18): A retinoic acid-inducible gene. Blood 1992; 79:602-609.

Alpin JD. Adhesion molecules in implantation. Rev Reprod 1997; 2:84-93.

Anderson LL. Growth, protein content and distribution of early pig embryos. Anatomy Rec 1978; 190:142-154.

Andre M, Rahimi S, Schollmeyer P, Pavenstadt H. Bradykinin stimulates the intracellular calcium activity in human mesothelial cells. Nephrology Dialysis Transplantation 1998; 13:2047-2052.

Angeon I, Cuturi MC, Godard A, Moreau M, Terqui M, Martinat-Botte F, and Soulillou JP. Presence of inhibitory factor and interleukin 6 in porcine uterine secretions prior to conceptus attachment. Cytokine 1994; 6:493-499.

Badinga L, Michel FJ, and Simmen RCM. Uterine-associated serine protease inhibitors stimulate deoxyribonucleic acid synthesis in porcine endometrial glandular epithelial cells of pregnancy. Biol Reprod1999; 61: 380-387.

Badinga L, Michel FJ, Fields MJ, Sharp DC, and Simmen RCM. Pregnancy associated endometrial expression of antileukoproteinase gene is correlated with epitheliochorial placentation. Mol Reprod Dev 1994; 38: 357-363.

Badinga L, Michel FJ, and Simmen RCM. Uterine associated serine protease inhibitors stimulate deoxyribonucleic acid synthesis in porcine endometrial glandular epithelial cells of pregnancy. Bio Reprod 1999; 61: 380-387.

Ball DK, Surveyor GA, Diehl JR, Steffen CL, Uzumcu M, Mirando MA, Brigstock DR. Characterization of 16- to 20- kilodalton (kDA) connective tissue

growth factors (CTGFs) and demonstration of proteolytic activity for 38-kDa CTGF in pig uterine luminal flushings. Biol Reprod 1998; 59:828-835.

Bazan JF. Neuropoietic cytokines in the hematopoietic fold. Neuron 1999; 7:197-208.

Bazer FW, Goldstein MH, and Barron DH. Water and electrolyte transport by the pig chorioallantois. 1981; In: Fertilization and Embryonic Development In Vivo., Eds. L Mastroianni, Jr and JD Biggers Plenum Plublishing Corp, New York: pp 299-321.

Bazer FW, Worthington-White D, Fliss MF, and Gross S. Uteroferrin: A progesterone-induced hematopoietic growth factor of uterine origin. Experimental Hematology 1991; 19:910-915.

Bazer FW. Mediators of maternal recognition of pregnancy in mammals. Proc Soc Exp Biol Med 1992 ; 199 (4):373-384.

Bazer FW and Thatcher WW. Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin F2 alpha by the uterine endometrium. Prostaglandins 1977; 14:397-401.

Bhoola KD, Figueroa CD, and Worthy K. Bioregulation of kinins: Kallikreins, kininogens, and kinases. Pharmacological Reviews1992; 44 (1):1-80.

Bode VC, and Dziadek MA. Plasminogen activator secretion during mouse embryogenesis. Dev Biol 1979; 73:272-289.

Bonney RC, Beesley JS, Rahman C, Franks S. Arachidonic acid release and inositol lipid metabolism in response to bradykinin and related peptides in human indometrial cells in vitro. Prostaglandins Leukotrienes Essential Fatty Acids 1993; 48:253-260.

Bost F, Diarra-Mehrpour M, Martin J-P. Inter-α-trypsin inhibitor proteoglycan family: A group of proteins binding and stabilizing the extracellular matrix. European Journal of Biochemistry 1998; 252:339-346.

Bowen JA, Bazer FW, and Burghardt RC. Spatial and temporal analyses of integrin and muc-1 expression in porcine uterine epithelium and trophectoderm in vivo. Biol Reprod 1996; 55:1098-1106.

Bowen JA, Bazer FW, Burghardt RC. Spatial and temporal analyses of integrin and muc-1 expression in porcine uterine epithelium and trophectoderm in vitro. Biol Reprod1997; 56:409-415.

Bowen JA, Bazer FW, Burghardt RC. Spatial and temporal analyses of integrin and muc-1 expression in porcine uterine epithelium and trophectoderm in vivo. Biol Reprod 1996; 55:1098-1106.

Braga VMM and Gendler SJ. Modulation of muc-1 expression in the mouse uterus during the estrus cycle, early pregnancy and placentation, J Cell Sci 1993; 105: 397-405.

Braga VMM and Gendler SJ. Modulation of muc-1 expression in the mouse uterus during the estrus cycle, early pregnancy and placentation. J Cell Sci 1993; 105:397-405.

Brann DW, Greenbaum L, Mahesh VB, and Gao X. Changes in kininogens and kallikrein in the plasma brain, and uterus during pregnancy in the rat. Endocrinology 1995; 136 (1):46-51.

Brigstock DR, Heap RB, Baker PJ, and Browtn KD. Purification and characterization of heparin-binding growth factors from porcine uterus. Biochem Journal 1990; 266:273-282.

Brigstock DR, Steffen CL, Kim GY, Vegunta RK, Diehl JR, Harding PA. Purification and characterization of novel heparin-binding growth factors in uterine secretory fluids: Identification as heparin-regulated 10000-Mr forms of connective tissue growth factor. J Biological Chemistry 1997; 272:20275-20282.

Brown TL, Moulton BC, Witte DP, Swertfeger DK, and Harmony JAK. Apolipoprotein j/clusterin expression defines distinct stages of blastocyst implantation in the mouse uterus. Biol Reprod 1996; 55: 740-747.

Busse R and Fleming I. Molecular responses of endothelial tissue to kinins. Diabetes 1996; 45 (Suppl. 1): S8-S13.

Carbinin LA, Scicli AG, and Carretero OA. The molecular biology of the kallikrein-kinin system: III. The human kallikrein gene family and kallikrein substrate. J Hypertension 1993; 11: 893-898.

Carnahan KG, Prince BC, Mirando MA. Exogenous oxytocin stimulates uterine secretion of prostaglandin F2α in cyclic and early pregnant swine. Biol Reprod 1996; 55:838-843.

Chakraborty C, Dey SK, and Davis DL. Pattern of tissue distribution of catechol estrogen forming activity by pig conceptuses during the peri-implantation period. J Anim Sci 1989; 67:991-998.

Chastant S, Monget P, and Terqui M. Localization and quantification of insulin-like growth factor – I (IGF– I) and IGF– II / Mannose-6-phosphate receptors in pig embryos during early pregnancy. Biol Reprod1994; 51: 588-596.

Chen LM, Chung P, Chao S, Chao L, and Chao J. Differential regulation of kininogen gene expression by estrogen and progesterone in vivo., Biochimica et Biophysica Acta 1992; 1131:145-151.

Christenson RK, Leymaster KA, Young LD. Justification of unilateral hysterectomy-ovariectomy as a model to evaluate uterine capacity in swine. J Anim Science 1987; 65:738-744.

Christman JK, Silverstein SC and Acs G. Plasminogen activators. 1977. In: Proteinases in Mammalian Cells and Tissues. Ed AJ Barrett. Elsevier/North-Holland. New York. pp 91-149.

Clark RA, Brugge JS. Integrins and signal transduction pathways, the road taken. Science 1995; 268:233-239.

Clements J, Mukhtar A, Ehrlich A, and Yap B. Glandular kallikrein gene expression in the human uterus. Braz J Med Biol Res 1994; 27:1855-1863.

Clements J, Mukhtar A. Tissue kallikrein and the bradykinin β_2 receptor are expressed in endometrial and prostate cancers. Immunopharmacology 1997; 36:217-220.

Clements J, Mukhtar A, Yan S, and Holland A. Kallikreins and kinins in inflammatory like events in the reproductive tract. Pharm Res1997; 35 (6): 537-539.

Clements JA, Fuller PJ, McNally M, Nikolaidis I, and Funder JW. Estrogen regulation of kallikrein gene expression in rat anterior pituitary. Endocrinology 1986; 119 (1):268-272.

Cohen P, Graves HCB, Peehl DM, Damarei M, Guidice LC, Rosenfeld RG. Prostate specific antigen PSA is an IGF binding protein-3 (IGFBP-3) protease found in seminal plasma. J Clin Endocrinol Metab 1992; 75:1046-1053.

Conley AJ, Christenson LK, Ford SP, and Christenson RK. Immunocytochemical localization of cytochromes P450 17α- hydroxylase and aromatase in embryonic cell layers of elongating porcine blastocysts. Endocrinology 1994; 135:2248-2254.

Conley AJ, Christenson RK, Ford SP, Geisert RD, and Mason JI. Steroidogenic enzyme expression in porcine conceptuses during and after elongation. Endocrinology 1992; 131: 896-902.

Cooper CL, Shaffer JE, Malik KU. Mechanism of action of angiotensin and bradykinin on prostaglandin synthesis and vascular tone in isolated rat kidney: effect of Ca^{++} antagonists and calmodulin inhibitors. Circ Res, 1985; 56:97-108.

Corthorn J, and Valdes G. Variations in uterine kallikrein during cycle and early pregnancy in the rat. Biol Reprod 1994; 50:1261-1264.

Corthorn J, Figueroa C, and Valdes G. Estrogen and luminal stimulation of rat uterine kallikrein. Biol Reprod 1997; 56:1432-1438.

Cross JC, and Roberts RM. Porcine conceptuses secrete an interferon during the preattachment period of early pregnancy. Biol Reprod1989; 40:1109-1118.

Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: Key pieces of the development puzzle. Science 1994; 266:1508-1518.

Damas J, Bourbon V, Souza PJ-C. The myostimulating effect of tissue kallikrein on rat uterus. Arch Pharmacol 1995; 351:535-541.

Dardick A, and Schultz RM. Blastocoel expansion in the preimplantation mouse embryo: Stimulatory effect of TGF-α and EGF. Development 1991; 113:919-930.

De Luca LM. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. FASEB Journal 1991; 5:2924-2933.

Diederich JJ, Yelich JV, and Geisert RD. Gene and protein expression of the inter-trypsin inhibitor, bikunin, in the porcine conceptus and endometrium. J Anim Sci 1997; 75 (Suppl 1):83.

Diehl JR, Henricks DM, and Gray SL. EGF and IGF-1 in the uterine and oviductal fluids of pregnant and nonpregnant pigs from day 10 to day 18. Biol Reprod 1994; 50 (Suppl 1):122.

Dziuk PJ. Effect of number of embryos and uterine space on embryo survival in the pig. J Anim Sci 1968; 27:673-676.

Edgerton LA, Kaminski MA, Silvia WJ. Changes in uterine secretion of prostaglandin $F_{2\alpha}$ in response to oxytocin during the estrous cycle, early pregnancy, and estrogen-induced pseudopregnancy in swine. Biol Reprod 1996; 55:657-662.

Elangovan S, and Moulton BC. Blastocyst implantation in the rat and the immunohistochemical distribution and rate of synthesis of uterine lysosomal cathepsin D. Biol Reprod 1980; 23:663-668.

Endo T, Fulane H, Kanaya M, Mizunuma M, Fujii M, Yamamoto H, Tanaka S, Hashimoto M. Bombesin and bradykinin increase inositol phosphates and cytosolic free Ca^{2+}, and stimulate DNA synthesis in human endometrial stromal cell. J Endocrinol 1991; 131:313-318.

Fazleabas AT, Geisert RD, Bazer FW and Roberts RM. Relationship between release of plasminogen activator and estrogen by blastocysts and secretion of plasmin inhibitor by uterine endometrium in the pregnant pig. Biol Reprod 1993; 29:225-238.

Figueroa CD, Ulises N, Valdes G, Corthorn J, Muller-Esterl W. Localization of the bradykinin β_2 receptor in uterus, bladder and Madin-Darby canine kidney cells. Immunopharmacology, 1997; 36:127-133.

Fischer HE, Bazer FW, and Fields MJ. Steroid metabolism by endometrial and conceptus tissues during early pregnancy and pseudopregnancy in gilts. J Reprod Fert 1985; 75:69-78.

Ford SP, Christenson RK, Ford JJ. Uterine blood flow and uterine arterial, venous and lumenal concentrations of oestrogens on days 11, 13, and 15 after oestrus in pregnant and non-pregnant sows. J Reprod Fert 1982; 64:185-190.

Frankenkrug SC, Freking BA, Rexroad CE III, Leymaster KA, Kappes SM, and Smith TPL. Gen Bank, Ref. gb | AF207860.1 | AF207862S2.

Gadsby JE, Heap RB and Burton RD. Oestrogen synthesis by blastocyst and early embryonic tissue of various species. J Reprod Fert 1980; 60:409-417.

Gao X, Greenbaum LM, Mahesh VB, and Brann DW. Characterization of the kinin system in the ovary during ovulation in the rat. Biol Reprod 1992; 47: 945-951.

Gauthier F, Moreau T, Gutman N, EL Moujahed A, Brillard-Bourdet M, Functional diversity of proteinases encoded by genes of the rat tissue kallikrein family, Recent Progress on Kinins 1992; 42-50.

Geisert RD, and Yelich JV. Regulation of conceptus development and attachment in pigs. J Reprod Fert 1997; 52 (Suppl):133-149.

Geisert RD, Blair RM, Pratt T, Zavy MT. Characterization and proteolytic activity of a cathepsin l-like polypeptide in endometrium and uterine flushings of cycling, pregnant and steroid-treated ovariectomized gilts. Reprod Fert Dev 1997; 9:395-402.

Geisert RD, Brookbank JW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: II Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biol Reprod 1982b; 27:941-955.

Geisert RD, Dixon MJ, Pratt T, Schmitt RAM, Lessley BA, and McCann JP. Isolation and characterization of a 30-kDa endometrial glycoprotein synthesized during the estrous cycle and early pregnancy of the pig. Biol Reprod 1995; 53:1038-1050.

Geisert RD, Pratt TN, Bazer FW, Mayes JS, Watson GH. Immunocytochemical localization and changes in endometrial progestin receptor protein during the porcine oestrous cycle and early pregnancy. Reprod Fert Dev 1994; 6:749-760.

Geisert RD, Renegar RH, Thatcher WW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: I Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. Biol Reprod 1982a; 27:925-939.

Geisert RD, Yelich JV, Pratt T, and Pomp D. Expression of an inter-α-trypsin inhibitor heavy chain-like protein in the pig endometrium during the oestrous cycle and early pregnancy. J Reprod Fert 1998; 114: 35-43.

Geisert RD. Zavy MT, Moffatt RJ, Blair RM, and Yellin T. Embryonic steroids and the establishment of pregnancy in pigs. J Reprod Fert 1990; 40 (Suppl):293-305.

Gonzales-Ramon N, Alava MA, Sarsa JA, Pineiro M, Escartin A, Garcia-Gil A, Lampreave F, Pineir A. The major acute phase serum protein in pigs is homologous to human plasma kallikrein sensitive PK-120. FEBS Letters 1995; 371:227-230.

Green ML, Simmen RCM and Simmen FA. Developmental regulation of steroidogenic enzyme gene expression in the peri-implantation porcine conceptus: a paracrine role for insulin-like growth factor-1. Endocrinology 1995; 136:3961-3970.

Gries LK, Geisert RD, Zavy MT, Garrett JE, Morgan GL. Uterine secretory alterations coincident with embryonic mortality in the gilt after exogenous estrogen administration. J Anim Sci 1989; 67:276-284.

Gupta A, Bazer FW, and Jaeger LA. Differential expression of beta transforming growth factors (TGFβ1,2,and 3) and their receptors (type I and type II) in peri-implantation porcine conceptuses. Biol Reprod 1996; 55:796-802.

Gupta A, Bazer FW, Jaeger LA. Immunolocalization of acidic and basic fibroblast growth factors in porcine uterine and conceptus tissues. Biol Reprod 1997; 56:1527-1536.

Gupta A, Dekaney CM, Bazer FW, Mardrigal MM, Jaeger LA. Beta transforming growth factors (TGFβ) at the porcine conceptus-maternal interface Part II: Uterine TGFβ bioactivity and expression of immunoreactive TGFβ's (1,2,and 3) and their receptors (type I and type II). Biol Reprod 1998; 59:911-917.

Gupta A, Ing NH, Bazer FW, Bustamante LS, and Jaeger LA. Beta transforming growth factors (TGFβ) at the procine conceptus-maternal interface Part I: Expression of TGFβ1, TGFβ2, and TGFβ3 messenger ribonucleic acids. Biol Reprod 1998; 59:905-910.

Hansen PJ, Bazer FW, Roberts RM. Appearance of beta-hexosaminidase and other lysosomal-like enzymes in the uterine lumen of gilts, ewes and mares in response to progesterone and oestrogens. J Reprod Fert 1985; 73:411-424.

Harney JP, Mirando MA, Smith LC, and Bazer FW. Retinol-binding protein: A major secretory product of the pig conceptus. Biol Reprod 1990; 42:523-532.

Harney JP, Smith LC, Simmen RCM, Fliss AE, and Bazer FW. Retinol-binding protein: Immunolocalization of protein and abundance of messenger ribonucleic acid in conceptus and maternal tissues during pregnancy in pigs. Biol Reprod1994; 50:1126-1135.

Hermann A, Buchinger P, Somlev B, Rehbock J. High and low molecular weight kininogen and plasma prekallikrein/plasma kallikrein in villous capillaries of human term placenta. Placenta 1996; 17:223-230.

Hermann BG, Labiet S, Poustka A, King TR, and Lehrach H. Cloning of the T gene required in mesoderm formation in the mouse. Nature 1990; 343:617-622.

Hess JF, Borkowski JA, Young GS, Strader CD, and Ransom RW. Cloning and pharmacological characterization of human bradykinin (BK-2) receptor. Biochem Biophys Res Commun 1992; 184 (1):260-268.

Hikens J, Ligtenberg MJL, Vos HL, Litvinov SV. Cell membrane associated mucins and their adhesion modulating property. Trends Biochem Sci 1992; 17:359-363.

Hochstrasser K, Schonberger OL, Rossmanith I, and Wachter E. Kunitz-type proteinase inhibitors derived by limited proteolysis of the inter-trypsin inhibitor. Hoppe-Seyler's Zeitscrift fur Physiologische Chemie 1981; 362:1357-1362.

Hofig A, Michel FJ, Simmen FA, Simmen RCM. Constitutive expression of uterine receptors for insulin-like growth factor–I during the peri-implantation period in the pig. Biol Reprod 1991; 45:533-539.

Huang Y-T, Johnson RK, and Eckardt GR. Effect of unilateral hysterectomy and ovariectomy on puberty, uterine size and embryo development in swine. J Anim Sci 1987; 65:1298-1305.

Hynes RO. Integrins: Versatility, modulation, and signaling in cell adhesion. Cell 1992; 69:11-25.

Ka H, Spencer TE, Johnson GA, Bazer FW. Keratinocyte growth factor: Expression by endometrial epithelia of the porcine uterus. Biol Reprod 2000; 62:1772-1778.

Kaye PL. Preimplantation growth factor physiology. Rev of Reprod 1997; 2:121-127.

Kennedy TG, Brown KD, and Vaughan TJ. Expression of the genes for the epidermal growth factor receptor and its ligands in porcine oviduct and endometrium. Biol Reprod 1994; 50:751-756.

Keys JL, and King GJ. Microscopic examination of porcine conceptus-maternal interface between days 10 and 19 of pregnancy. Amer J Anat 1990; 188:221-238.

Keys JL, and King GJ. Morphological evidence for increased uterine vascular permeability at the time of embryonic attachment in the pig. Biol Reprod 1988; 39:473-487.

Kim GY, Besner GE, Steffen CL, McCarthy DW, Downing MT, Luquette MG, Abad MS, and Brigstock DR. Purification of heparin binding EGF-like growth factor from pig uterine flushings and its production by endometrial tissues. Biol Reprod 1995; 52:561-571.

King GJ, Atkinson BA, and Robertson A. Implantation and early placentation in domestic ungulates. J Reprod Fert 1982; 31 (Suppl):17-30.

Knight JW, Bazer FW, and Wallace HD. Effect of progesterone induced increase in uterine secretory activity on development of the porcine conceptus. J Anim Sci 1974; 39 :743-746.

Knox RV, Zhang Z, Day BN, and Anthony RV. Identification of relaxin gene expression and protein localization in the pig uterine endometrium during early pregnancy in the pig. Endocrinology 1994; 135:2517-2525.

Ko Y, Choi I, Green ML, Simmen FA, and Simmen RCM. Transient expression of the cytochrome P450 aromatase gene in elongating porcine blastocysts is correlated with uterine insulin-like growth factor levels during peri-implantation development. Mol Reprod and Dev 1994; 37:1-11.

Lefevre F, Martinat-Botte F, Guillomot M, Zouari K, Charley B, LaBonnardiere C. Interferon-gamma gene and protein are spontaneously expressed by the porcine trophectoderm early in gestation. European Journal of Immunology 1990; 20:2485-2490.

Lessey BA, Castelbaum AJ, Buck CA, Lei Y, Yowell CW, Sun J. Further characterization of endometrial integrins during the menstrual cycle and in pregnancy. Fertility and Sterility 1994; 62:497-506.

Lewis A, Kaye PL, Lising R, Cameron RDA. Stimulation of protein synthesis and expansion of the pig blastocysts by insulin in vitro. Reprod Fert and Dev 1992; 4:119-123.

Lindqvist A, Rouet P, Salier JP, Akerstrom B. The alpha 1-microglobulin/bikunin gene: Characterization in mouse and evolution. Gene 1999; 234:329-336.

Mandle R, Kaplan AP. Hageman factor substrates. J Biol Chem 1977; 252 :6097-6104.

Margolius HS. Molecular characteristics and cellular and tissue responses. Diabetes 1996; 45 (Suppl 1):S14-S19.

Mathialagan N, Bixby JA, and Roberts RM. Expression of interleukin-6 in porcine, ovine, and bovine preimplantation conceptuses. Mol Reprod and Dev 1992; 32:324-330.

Matthews CJ, McEwan GTA, Redfern CPF, Thomas EJ, Herst BH. Bradykinin stimulation of electrogenic ion transport in epithelial layers of cultured human endometrium. Eur J Physiol 1993; 422:401-403.

Mattson BA, Overstrom EW, and Albertini DF. Transitions in trophectoderm cellular shape and cyto-skeletal organization in the elongating pig blastocyst. Biol Reprod 1990; 42:195-205.

McCarthy DA, Potter DE, and Nicolaides ED. An in vivo estimation of the potencies and half-lives of synthetic bradykinin and kallidin. J Pharmacology and Experimental Therapeutics 1965; 149 :117-122.

Meyers SA, Lozon MM, Corombos JD, Saunders DE, Hunter K, Christensen C, and Brooks SC. Induction of porcine uterine estrogen sulfotransferase activity by progesterone. Biol Reprod 1983; 28:1119-1128.

Michel FJ, Fliss MF, Bazer FW, and Simmen RCM. Characterization and developmental expression of binding sites for the transplacental iron transport protein, uteroferrin, in fetal hematopoietic tissues. Biol Neonate 1992; 61:82-91.

Mirando MA, Becker WC, Whiteaker SS. Relationships among endometrial oxytocin receptors, oxytocin-stimulated phosphoinositide hydrolysis and prostaglandin $F_{2\alpha}$ secretion in vitro, and plasma concentrations of ovarian steroids before and during corpus luteum regression in cyclic heifers. Biol Reprod 1993; 48:874-882.

Mombouli JV, Bissiriou I, Agboton V, Vanhoutte PM. Endothelium-derived hyperpolarizing factor: A key mediator of the vasodilator action of bradykinin. Immunopharmacology 1996; 33:46-50.

Mondschein JS, Hersey RM, Dey SK, Davis DL and Weisz J. Catechol estrogen formation by pig blastocysts during the preimplantation period: Biochemical characterization of estrogen–2/4–hydroxylase and correlation with aromatase activity. Endocrinology 1985; 117:2339-2346.

Morgan GL, Geisert RD, Zavy MT, Shawley RV, and Fazleabas AT. Development of pig blastocysts in a uterine environment advanced by exogenous oestrogen. J Reprod Fert 1987; 80:15-131.

Murone C, Chai SY, Muller-Esterl W, Mendelsohn FAO, and Clements J. Localization of bradykinin β_2 receptors in the endometrium and myometrium of rat uterus and effects of estrogen and progesterone. Endocrinology 1999; 140:3372-3382.

Murone C, Perich RB, Schlawe I, Chai SY, Casley D, MacGregor DP, Muller-Esterl W, Mendelsohn FAO. Characterization and localization of bradykinin β_2 receptors in the guinea-pig using a radioiodinated HOE140 analogue. Eur J Pharm 1996; 306:237-247.

Murone C, Perich RB, Schlawe I, Muller-Esterl W, Mendelsohn FAO, Chai SY. Localization of bradykinin β_2 receptors in sheep uterus. Immunopharmacology 1996; 33:108-112.

Nie GY, Li Y, Wang J, Minoura H, Findlay JK, Salamonsen LA. Complex regulation of calcium-binding protein D9K (calbindin-D9K) in the mouse uterus during early pregnancy and at the site of embryo implantation. Biol Reprod 2000; 62:27-36.

Nishimura H, Kakizaki I, Muta T, Sasaki N, Pu PX, Yamashita T, and Nagasawa S. cDNA and deduced amino acid sequence of human PK-120, a plasma kallikrein-sensitive glycoprotein. FEBS Letters 1995; 357:207-211.

O'Neill LA, Geisert RD, Zavy MT, Morgan GL, Wettemann RP. Effect of estrogen inhibitors on conceptus estrogen synthesis and development in the gilt. Domestic Animal Endocrinology 1991; 8 :139-153.

Pekonen F, Nyman T, Rutanen EM. Differential expression of keratinocyte growth factor and its receptor in the human uterus. Mol Cell Endo 1993; 95:43-49.

Perry JS, and Rowlands IW. Early pregnancy in the pig. J Reprod Fert 1962; 4:175-188.

Perry JS, Heap RB, Amorosa EC. Steroid hormone production by pig blastocysts. Nature 1973; 245:45-47.

Pope WF. Embryonic mortality in swine. 1994; In: Embryonic Mortality in Domestic Species. Eds. RD Geisert and MT Zavy. CRC Press. Boca Raton. pp 53-78.

Pope WF, Lawyer MS, Butler WR, Foote RH, First NL, Effect of asynchronous superinduction on embryo survival and range of blastocyst development in swine. Biol Reprod 1986 35:133-137.

Pope WF, Maurer RR, and Stormshak F. Survival of porcine embryos after asynchronous transfer. Proc Soc Exp Biol Med 1982; 171:179-183.

Powers JC, Harper JW. Inhibitors of metalloproteases. 1986; In: Proteinase Inhibitors. Chapter 6. Barrett and Salvesen. Elsevier Science Publishers BV.

Prigent SA and Lemoine NR. The type I (EGF related) family of growth factor receptors and their ligands. Progress in Growth Factor Research 1992; 4:1-24.

Pusateri AE, Rothschild MF, Warner CM, and Ford SP. Changes in morphology, cell number, cell size and cellular estrogen content of individual littermate pig conceptuses on days 9 to 13 of gestation. J Anim Sci 1990; 68:3727-3735.

Reed KL, Badinga L, Davis KL, Chung TE, Simmen RCM. Porcine endometrial glandular epithelial cells in vitro: Transcriptional activities of the pregnancy-associated genes encoding antileukoproteinase and uteroferrin. Biol Reprod 1996; 55:469-477.

Reed KL, Blaeser LL, Dantzer V, Green ML, and Simmen RCM. Control of secretory leukocyte protease inhibitor gene expression in the porcine peri-implantation endometrium: A case of maternal-embryo communication. Biol Reprod 1998; 58:448-457.

Regoli D, Jukic F, Gobeil F, and Rhaleb NE. Receptors for bradykinin and related kinins: A critical analysis. Can J Physiol Pharmacol 1993; 71: 556-567.

Regoli D, Pheng LH, Allogho SN, Nguyen-Le XK, Gobeil F. Receptors for kinins: From classical pharmacology to molecular biology. Immunopharmacology 1996; 33:24-31.

Regoli D, Rizzi A, and Calo G. Pharmacology of the kallikrein-kinin system. Pharmacological Research 1997; 35 (6): 513-515.

Rehbock J, Chondromatidou A, Miska K, Buchinger P, Hermann A. Evidence for bradykinin β_2 receptors on cultured human decidua cells. Immunopharmacology 1997; 36:135-141.

Roberts JS, McCracken JA, Gavagan JE, Soloff MS. Oxytocin stimulated release of prostaglandin $F_{2\alpha}$ from ovine endometrium in vitro: Correlation with estrous cycle and oxytocin-receptor binding. Endocrinology 1976; 99:1107-1114.

Roberts RM, Bazer FW, Baldwin N, and Pollard WE. Progesterone induction of lysozyme and peptidase activities in the porcine uterus. Archives of Biochemistry and Biophysics 1976; 177:499-507.

Roberts RM, Xie S, and Trout WE. Embryo-uterine interactions in pigs during week 2 of pregnancy. J Reprod Fert 1993; 48 (Suppl):171-186.

Ross SA, Ahrens RA, de Luca LM. Retinoic acid enhances adhesiveness, laminin and integrin beta 1 synthesis, and retinoic acid receptor expression in F9 tetraocarcinoma cells. J Cell Physio 1994; 159 :263-273.

Rusiniak ME, Back N. Kallikrein-kininogen-kinin system. 1995; In: Molecular Biology and Biotechnology: A Comprehensive Desk Reference. Ed. RA Meyers VCH Publishers. Inc New York, NY.

Salamonsen LA. Role of proteases in implantation. Rev Reprod 1999; 4:11-22.

Salier J-P, Rouet P, Raguenez G, and Daveau M. The inter-α-inhibitor family: From structure to regulation. Biochemical Journal 1996;315:1-9.

Samuel CA, and Perry JS. The ultrastructure of pig trophoblast transplanted to an ectopic site in the uterine wall. J Anatomy 1972; 113 :139-149.

Sarafan N, Martin JP, Bourguignon J, Borghi H, Calle H, Sesboue H, Diarra-Mehrpour M. The human inter-α-inhibitor genes respond differently to interleukin-6 in HepG2 cells. Eur J Biochem 1995; 227:808-815.

SAS SAS User's Guide: Statistics (version 5 Ed) Cary, NC: Statistical Analysis System Institute Inc; 1985.

Schill WB and Haberland GL. Kinin-induced enhancement of sperm motility. Hoppe-Seyler's Z Physiol Chem 1974; 355 (Suppl):229-231.

Schmaier AH. Contact activation: A revision. Thrombosis and Haemostasis 1997; 78 :101-107.

Schweigert FJ, Bonitz K, Siegling C, and Buchholz I. Distribution of vitamin A, retinol binding protein, cellular retinoic acid-binding protein I, and retinoid X receptor β in the porcine uterus during early gestation. Biol Reprod 1999; 61:906-911.

Schweigert FJ, Bonitz K, Siegling C, and Buchholz I, Distribution of vitamin a, retinol-binding protein, cellular retinoic acid-binding protein I, and retinoid X receptor β in the porcine uterus during early gestation. Biol Reprod 1999; 61:906-911.

Sealy JE, Atlas SA, Laragh JH. Human urinary kallikrein converts inactive renin and is a possible physiological activator of renin. Nature 1978; 275 :144-145.

Shams M, Sis P, and Ahmed A. Localization of bradykinin type II receptor mRNA in human endometrium. Mol Hum Reprod 1996; 2 :35-39.

Sherwood OD. Relaxin. 1994; In: The Physiology of Reproduction. Eds. E Knobil and J Neill. Raven Press, New York, NY. pp 861-1009.

Shi B, Mahesh VB, Bhat GK, Ping L, Brann DW. Evidence for a role of bradykinin neurons in the control of gonadotropin-releasing hormone secretion. Neuroendocrinology 1998; 67:209-218.

Shiokawa S, Yoshimura Y, Nagamatsu S, Sawa H, Hanashi H, Koyama N, Katsumata Y, Nagai A, and Nakamura Y. Function of β1integrins of human decidual cells during implantation. Biol Reprod 1996; 54:745-752.

Simmen RA, Simmen RCM, Geisert RD, Martinat-Botte F, Bazer FW, and Terqui M, Differential expression, during the estrous cycle and pre- and postimplatation conceptus development, of messenger ribonucleic acids encoding components of the pig uterine insulin-like growth factor system, Endocrinology 1992; 130:1547-1556.

Simmen RCM, Ko Y and Simmen FA. Insulin-like growth factors and blastocyst development. Theriogeneology 1993; 39:163-175.

Simmen RCM, Simmen FA, Ko Y, and Bazer FW. Differential growth factor content of uterine luminal fluids from large white and prolific meishan pigs during the estrous cycle and early pregnancy. J Anim Sci 1989; 67:1538-1545.

Stice SL, Ford SP, Rosazza JP, Van Orden DE. Interaction of 4-hydroxylated estradiol and potential-sensitive Ca^{2+} channels in altering uterine blood flow during the estrous cycle and early pregnancy in gilts. Biol Reprod 1987; 36:369-375.

Stone BA, and Seamark RF. Steroid hormones in uterine washings and in plasma of gilts between days 9 and 15 after oestrus and between days 9 and 15 after coitus. J Reprod Fert 1985; 75:209-221.

Stroband HWJ, Taverne N, and Bogaard MVD. The pig blastocyst: Its ultrastructure and the uptake of protein macromolecules. Cell Tissue Research 1984; 235:347-356.

Surveyor GA, Gendler SJ, Pemberton GL, Das SK, Chakraborty I, Julian J, Pimental RA, Wegner CC, Dey SK, and Carson DD. Expression and steroid hormonal control of muc-1 in the mouse uterus. Endocrinology 1995; 136 (8):3639-3647.

Surveyor GA, Wilson AK, and Brigstock DR. Localization of connective tissue growth factor during the period of embryo implantation in the mouse. Biol Reprod 1998; 59:1207-1213.

Takano M, Yokoyama K, Yayama K, Okamoto H. Murine fibroblasts synthesize and secrete kininogen in response to cyclic-AMP, prostaglandin E_2 and tumor necrosis factor. Biochem Biophys Acta 1995; 1265:189-195.

Thompson RC, Ohlsson K. Isolation, properties, and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase. Proc Natl Acad Sci USA 1986; 83:6692-6696.

Trout WE, Hall JA, Stallings-Mann ML, Galvin JM, Anthony RV, and Roberts RM. Steroid regulation of the synthesis and secretion of retinol-binding protein by the uterus of the pig. Endocrinology 1992; 130 :2557-2564.

Trout WE, McDonnell JJ, Dramer KK, Baumbach GA, and Roberts RM. The retinol binding protein of the expanding pig blastocyst: Molecular cloning and expression in trophectoderm and embryonic disc. Mol Endo 1991; 5:1533-1540.

Tuo W, and Bazer FW. Expression of oncofetal fibronectin in porcine conceptuses and uterus throughout gestation. Reprod Fert Dev 1996; 8:1207-1213.

Tuo W, Harney JP and Bazer FW. Developmentally regulated expression of interleukin-1β by peri-implantation conceptuses in swine. J Reprod Immuno 1996; 31:185-198.

Tuo W, Harney JP, and Bazer FW. Colony-stimulating factor-1 in conceptus and uterine tissues in pigs. Biol Reprod 1995; 53:133-142.

Valdes G, Corthorn J, Scicli AG, Gaete V, Soto J, Ortiz ME, Foradori A, and Saed GM. Uterine kallikrein in the early pregnant rat. Biol Reprod 1993; 49:802-809.

Valdes G, Figueroa CD, Corthorn J. Temporospatial changes of kallikrein-like enzymes during the estrous cycle and pregnancy in the rat uterus. Biol Reprod 1996; 55:236-245.

Vallet JL, Christenson RK, McGuire WJ. Association between uteroferrin, retinol-binding protein, and transferrin within the uterine and conceptus compartments during pregnancy in swine. Biol Reprod 1996; 55:1172-1178.

Vallet JL. Uteroferrin induces lipid peroxidation in endometrial and conceptus microsomal membranes and is inhibited by apotransferrin, retinol binding protein, and the uteroferrin-associated proteins. Biol Reprod 1995; 53: 1436-1445.

Vonnahme KA, Malayer JR, Spivey HO, Ford SP, Clutter A, Geisert RD. Detection of kallikrein gene expression and enzymatic activity in porcine endometrium during the estrous cycle and early pregnancy. Biol Reprod 1999; 61:1235-1241.

Wang D-Z, Song Q, Chen L-M, Chao L, and Chao J. Expression and cellular localization of tissue kallikrein-kinin system in human adrenal gland. Ameri J Physiol 1996; 271 : F709-F716.

Webel SK, and Dziuk PJ. Effect of stage of gestation and uterine space on prenatal survival in the pig. J Anim Sci 1974; 38:960-966.

Wilde MH, Xie S, Day ML, Pope WF. Survival of small and large littermate blastocysts in swine after synchronous and asynchronous transfer procedures. Theriogeneology 1988; 30:1069-1074.

Yaqoob M, Snell CR. Purification and characterization of β_2 bradykinin receptor from rat uterus. J Neurochemistry 1994; 62:17-26.

Yelich JV, Pomp D, and Geisert RD. Detection of transcripts for retinoic acid receptors, retinol-binding protein, and transforming growth factors during rapid trophoblastic elongation in the porcine conceptus. Biol Reprod 1997; 57:286-294.

Yelich JV, Pomp D, Geisert RD. Ontogeny of elongation and gene expression in the early developing porcine conceptus. Biol Reprod 1997; 57:1256-1265.

Yokoyama S, Kimura Y, Taketo M, Black JA, Ransom BR, and Gigashida H. β_2 bradykinin receptors in NG108-15 cells: cDNA cloning and functional expression. Biochem Biophys Res Commun 1994; 200 :634-631

Zavy MT, Roberts RM, Bazer FW. Acid phosphatase and leucine aminopeptidase activity in the uterine flushings of non-pregnant and pregnant gilts. J Reprod Fert 1984; 72:503-507.

VITA

Melanie J. Allen

Candidate for the Degree of

Master of Science

Thesis: REGULATION OF KININS DURING EARLY PREGNANCY AND THE ESTROUS CYCLE OF THE GILT

Major Field: Animal Science

Biographical:

Personal Data: Born in Harrison, Arkansas, On May 20, 1971, the daughter of Don and Patricia Rose.

Education: Graduated from Jasper High School, Jasper, Arkansas in May 1989; received Bachelor of Science degree in Animal Science from Oklahoma State University, Stillwater, Oklahoma in August of 1997. Completed the requirements for the Master of Science degree with a major in Reproductive Physiology at Oklahoma State University in December of 2000.

Experience: Worked summers on a family owned swine operation. Employed by Oklahoma State University, Department of Animal Science as a graduate research assistant; Oklahoma State University, Department of Animal Science, 1998 to present.

Professional Memberships: Sigma Xi, Society for the Study of Reproduction, American Association for the Advancement of Science