5-2008

Phospholipase A_2 Expression During the Estrous Cycle and Early Pregnancy

Gregory Joseph Ochs
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Gregory Joseph Ochs entitled "Phospholipase A_2 Expression During the Estrous Cycle and Early Pregnancy." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

James D. Godkin, Major Professor

We have read this thesis and recommend its acceptance:

Patricia K. Tithof, Jun Lin

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting here within a thesis written by Gregory Joseph Ochs Jr. entitled “Phospholipase A_2 expression during the estrous cycle and early pregnancy” I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

James D. Godkin, Major Professor

We have read this thesis and
recommend its acceptance:

Patricia K. Tithof

Jun Lin

Acceptance for the Council:

Carolyn R. Hodges
Vice Provost and Dean of the
Graduate School

(Original signatures are on file with official student records)

PHOSPHOLIPASE A_2 EXPRESSION DURING THE ESTROUS CYCLE AND EARLY PREGNANCY

A Dissertation
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Gregory Joseph Ochs Jr.
May 2008

DEDICATION

The following work is dedicated to the support of my parents, the laughter provided by my friends and the patience of Jessica Satterfield. With out all of three of these it would not have been possible.

ACKNOWLEDGMENTS

I would like to thank many people for their help with the completion of this research. I appreciate Dr. Godkin's willingness to have both his door and his mind open to all questions that were presented. Mary Roberts deserves credit for her thankless job of overseeing my every move which was not always welcome, but always helpful. The work crews at Blount farm and JARTU were always reliable and eager to help with our projects. The clerical staff also deserves recognition for helping with the red tape surrounding the UT system. This project would still be ongoing if not for Dr. Edwards and her lab and Dr. Yuan's help with adjusting protocols and allowing me to use their equipment. I would especially like to thank all the graduate students for the support that only a person in a similar situation can provide.

ABSTRACT

Acute control of prostaglandin production is essential for normal estrous cyclicity and maintenance of early pregnancy. The rate limiting step for prostaglandin production is the activation of Phospholipase A_2. There are many phospholipase A_2s, but few have been investigated in reproductive studies. The objective of this study was to examine PLA_2 Groups IV and VI protein and mRNA expression in the uterine endometrium during the estrous cycle and early pregnancy in ewes. Ewes were monitored for estrous and uterine tissues were collected surgically on days 5 (n=3), 10(n=3) and 15(n=3) of the estrous cycle. Endometrium from pregnant animals were harvested on days 14(n=3), 15(n=1), 16(n=2), 17(n=1) or 20(n=2). Endometrial scrapings were collected in attempts to harvest luminal epithelial cells primarily and tissue samples were collected to harvest samples containing all cellular endometrial components. Samples were analyzed by western blot analysis and qRT-PCR to detect protein and mRNA expression of both PLA_2s Group IV and VI. Western blot results revealed

that protein expression of Group IVA was greatest on day ten of the estrous cycle but was not significantly different form days 5 and 15, possibly due to animal variation. Group IVA was significantly elevated on day 14 of pregnancy ($P<0.05$) and remained elevated until day 16 when it diminished and a 50kD band appeared. Group VIA analysis showed a cross-reactive 50kD band that showed no significant change. qRT-PCR analysis for Group IVA of scraping samples revealed similar findings showing not only an increase of Group IVA mRNA at pregnancy but also during day 10 of the estrous cycle ($P<0.0001$) but little difference was seen in tissue samples ($P<0.0001$). Group VIA was shown to have no mRNA difference in both tissue and scraping samples. These results suggest that PLA_2 expression is not the sole regulator of prostaglandin production, but it does play an integral role that is tissue and cell type specific in both the estrous cycle and early pregnancy.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Estrous Cycle and Early Pregnancy of the Ewe

Maintenance of pregnancy results from complex communication between the developing embryo and the maternal unit allowing for the maintenance of the corpus luteum and resultant progesterone production. Particular regulatory signals of interest at this time are prostaglandins. This thesis will attempt to provide a basic understanding of the differences in prostaglandin production between a cyclic animal and one that is in the early stages of pregnancy. The enzymatic cascade that generates prostaglandins will also be explored for possible points of local regulation.

Luteolysis and the Regulation of Estrous Cyclicity

Normal estrous cyclicity in ruminants results from the demise of the corpus luteum (CL), the source of progesterone (Goff, 2004; Schams and Berisha, 2004), termed functional luteolysis. Toward the end of the estrous cycle, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), the luteolytic signal in ruminants, is produced in a

pulsatile fashion by the uterine luminal epithelium of sheep and cattle (McCracken et al., 1972). Uterine oxytocin receptor concentrations increase toward the end of the cycle. Oxytocin of neurohypophysial and luteal origin bind oxytocin receptors (OTR) and initiate pulsatile $PGF_{2\alpha}$ secretion, which in turn, stimulates release of luteal oxytocin creating a positive feedback loop that results in a series of pulses of short duration which are effective in causing luteolysis. $PGF_{2\alpha}$ is delivered to the corpus luteum through a counter-current exchange system between the utero-ovarian vein and the ovarian artery (McCracken et al., 1972). Early in the estrous cycle oxytocin does not stimulate $PGF_{2\alpha}$ production, but after progesterone priming (Pate, 1988), oxytocin stimulates $PGF_{2\alpha}$ production under the influence of estrogen during an infertile cycle (Barcikowski et al., 1974). Uterine production of $PGF_{2\alpha}$ in response to oxytocin coincides with the cyclical variation of OTR concentration (Roberts et al., 1976). However, diminished OTR concentration does not fully explain luteal maintenance during early pregnancy. For example, OTR receptor number in pregnant ewes, though

decreased, is not limiting (Mann et al., 2001). Also lacking clarity is the regulation of the oxytocin receptor. It has been documented that the up-regulation of the estrogen receptor is followed by an up-regulation of the oxytocin receptor (Spencer et al., 1996). Oxytocin regulation by estradiol may be possible by interaction through the AP-1 and Sp-1 sites on the OTR gene promoter region (Bazer et al., 2003)since the promoter does not contain an estrogen response element (Ivell and Walther, 1999). But, other studies have documented up-regulation of the OTR preceding estrogen receptor up-regulation (Wathes and Hamon, 1993). In addition neurohypophyseal release of oxytocin is influenced by gonadal steroids (McCracken et al., 1996). Cumulatively, these results suggest a complex regulation of $PGF_{2\alpha}$ production involving estradiol, progesterone, oxytocin, and their respective receptors, as well as elements of the prostaglandin production pathway such as cyclooxygenases, terminal synthases and phospholipases, which will later be discussed in detail.

Deviation from Luteolysis During Early Pregnancy

In order to maintain a successful pregnancy, it is essential that progesterone levels be maintained. For ruminants to accomplish this, the normal cyclic progression into luteolysis must be averted (Spencer et al., 2004; Spencer et al., 2007). The developing conceptus signals its presence to the maternal unit and maintains luteal function. Upon receiving this signal, the maternal unit responds by providing an environment for the growth and development of the conceptus.

Pulsatile $PGF_{2\alpha}$ production is diminished in pregnant animals (Peterson et al., 1976) even though $PGF_{2\alpha}$ production is elevated above basal levels in some species (Zarco et al., 1988). Oxytocin stimulation of $PGF_{2\alpha}$ production in pregnant animals is diminished when compared to corresponding time periods in cyclic animals (McCracken, 1980). OTR concentrations are reduced in pregnant animals (McCracken, 1980; Sheldrick and Flint, 1985). The change in OTR regulation coincides with the production of a substance by the embryo between days 12 and 21 in the sheep that was originally referred to as ovine

trophoblast protein-1 (Godkin et al., 1982). More recently, this conceptus product was identified as a unique interferon (Imakawa et al., 1987) and has been designate interferon-τ (IFNτ)(Roberts et al., 1998). In all ruminant species investigated, IFNτ has been implicated as the conceptus product responsible for the maintenance of early pregnancy. It has been proposed that interferon-τ breaks down the positive feed back loop between oxytocin and uterine $PGF_{2\alpha}$ that initiates functional luteolysis (Flint et al., 1992). But, as alluded to earlier, OTR concentrations cannot completely explain this process (Mann et al., 2001; Wathes and Hamon, 1993).

Interferon-τ is a protein of 20-24kD produced by the extra embryonic trophectoderm of ruminants (Farin et al., 1989; Godkin et al., 1984). The sheep product has a mass of 20kD, while the bovine and caprine isoforms produce higher mass isoforms due to possible N-glycosylation sites (Baumbach et al., 1990). Interferon-τ acts by binding high and low affinity receptors on the endometrium known as IFNAR1 and IFNAR2 (Godkin et al., 1984; Kaluz et al., 1996). Interferon-τ binding is thought to initiate an atypical

JAK-STAT pathway resulting in the release of several interferon transcription factors that may directly or indirectly suppress uterine estrogen receptors (Bazer et al., 1997; Spencer et al., 2007), cytokines, and a myriad of enzymes (Hansen et al., 1999; Martal et al., 1997). It is speculated that the timing of interferon-τ expression is genetically programmed through stimulation and inhibition by transcription factors associated with blastocyst elongation and implantation. It has been demonstrated that the transcription factors c-fos, c-jun and Ets-2 stimulate interferon-τ production, in part, by acting at the AP-1 and Ets-2 sites, respectively, that have been identified in the promoter region of the interferon-τ gene (Adunyah et al., 1991; Ezashi et al., 1998; Imakawa et al., 1993; Yamaguchi H, 1999). The cessation of interferon-τ expression is not completely understood but negative regulatory domains have been identified on the promoter of the bovine interferon-τ gene (Yamaguchi H, 1999).

The corpus luteum of pregnancy is more resistant to luteolysis than the corpus luteum of the estrous cycle. Studies have shown that pregnant ewes require

a higher dose of luteolysins to induce luteolysis when compared to cyclic ewes (Inskeep et al., 1975; Nancarrow et al., 1982; Silvia and Niswender, 1986). One possibility for this action is the increase in PGE_2 production (Silvia et al., 1984), a reduction of ECE1 and increased catabolism of $PGF_{2\alpha}$(Costine et al., 2007). PGE_2 acts in an opposite manner to that of $PGF_{2\alpha}$ (Henderson and McNatty, 1975)and may counteract its effects in the pregnant animal (Magness et al., 1981).

Prostaglandin Production

Prostaglandins (PG) are key regulators of many reproductive processes, including those of estrous cyclicity, pregnancy and parturition (Poyser, 1995). Concerning ruminant reproduction, the primary focus has been on $PGF_{2\alpha}$ and PGE_2 because these are produced in greatest concentrations by the endometrial epithelium during the estrous cycle (Liggins CG et al., 1980) and pregnancy (Marcus, 1981).

A general overview of the prostaglandin production pathway, figure 1-1, yields the following events. First, membrane phospholipids are cleaved by

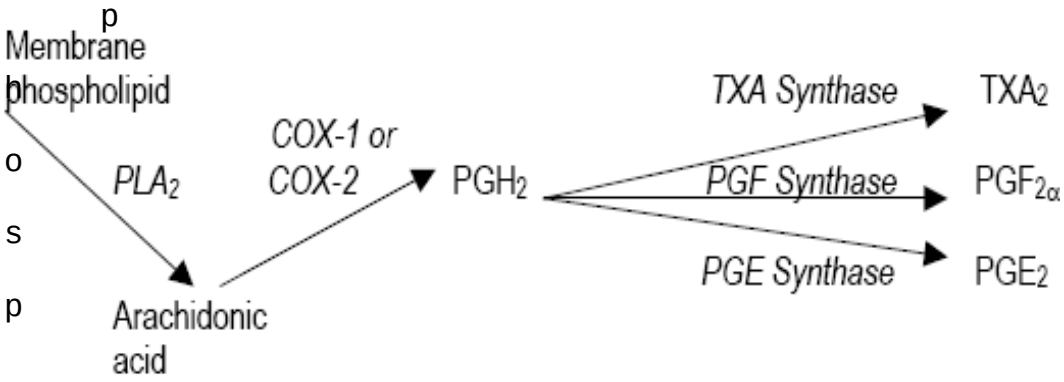

Figure 1-1. Schematic of prostaglandin production

phospholipase A_2 (PLA_2) at the *sn-2* position in the rate-limiting step of PG synthesis resulting in the release of arachidonic acid (AA) and a resultant lysophospholipid. AA is then acted upon by a cyclooxygenase (COX) in two distinct reactions. First, the enzyme performs a cyclooxygenase reaction in which two molecules of O_2 are added to AA to form PGG_2. PGG_2 then undergoes a peroxidase reaction where it is reduced by two electrons to form PGH_2. The resultant bioactive PGH_2 can then be converted into specific PGs by different PG synthases. This pathway has many points of complex regulation and not all are completely understood. Better understanding of these regulatory factors and enzymes may result in the development of better control of estrous, increased fertility and novel concepts for contraception.

Cyclooxygenases

There are two isoforms of cyclooxygenase, COX-1 and COX-2, which execute the committed step in the biosynthesis of prostaglandins. The two enzymes have similar amino acid sequences (60-65% homology) and have similar function and activities, with the predominant structural difference in the membrane binding domain (Kujubu et al., 1991). Both cyclooxygenases are homodimers (Luong et al., 1996) with each monomer having 3 structural domains: epidermal growth factor domain, membrane binding domain, and the catalytic domain. The epidermal growth factor domain is found at the N terminus and is essential for proper folding. The membrane binding domain, found adjacent to the epidermal growth factor domain, is formed by hydrophobic and aromatic residues that stick out of the helices forming a hydrophobic patch that can associate with a hydrophobic side of the membrane bi-layer. The globular catalytic domain is found at the C terminus and contains a hydrophobic channel through the center with the upper half containing the cyclooxygenase active site (Picot et

al., 1994). The globular catalytic domain also contains many KDEL-like sequences that direct the cyclooxygenases to the endoplasmic reticulum and the nuclear envelope, with COX-2 being more readily associated with the nuclear envelope (Song and Smith, 1996).

There is no real difference in the overall location of COX1 and COX2 besides the slightly higher concentration of COX2 around the nuclear envelope and they operate at similar rates. Each monomer has a hydrophobic channel that begins at the membrane binding domain and allows arachidonic acid, which can diffuse freely within cells, and O_2 to enter the enzyme directly from the lipid bilayer (Luong et al., 1996; Picot et al., 1994) from which it has been freed by PLA_2s.

Despite the multitude of similarities between the isoforms, there are discernable differences that, though not completely understood, allow the two enzymes to function in different physiological capacities. It has been documented that COX -2 releases PGH_2 more efficiently than COX-1 at concentrations of substrate less than 2.5uM

(Shitashige et al., 1998; Swinney et al., 1997). The active site of COX-2 for the cyclooxygenase reaction is about 20% larger than that of COX-1 caused in part by a change in the position of helix D at the membrane binding domain to produce a larger opening allowing more substrate flexibility for the COX-2 active site (Luong et al., 1996; Picot et al., 1994). This increased flexibility may aid in substrate recognition and explain some of the subtle kinetic differences between the isoforms.

The isoforms are also known to have varying expressions of mRNA and protein during the estrous cycle and early pregnancy in many species. COX-1 is expressed constitutively in the endometrial epithelium and stromal cells through out the estrous cycle and pregnancy in sheep (Charpigny et al., 1997) whereas it is not expressed at detectable levels in the bovine in either tissue (Arosh et al., 2002). This constitutive expression is not seen with COX-2, which is inducible. COX-2 mRNA and protein fluctuate greatly during the estrous cycle and pregnancy in both sheep and cattle. In cattle, COX-2 mRNA and protein are expressed at a higher level starting at day 13 and continuing through

the estrous cycle (Arosh et al., 2002). Similarly in ewes, it appears that COX-2 mRNA and protein are expressed at basal levels until they are elevated around days 10-12 and remain elevated until they decrease between days 14 and 16 of the estrous cycle (Charpigny et al., 1997; Kim S et al., 2003).

In pregnant ewes COX-2 mRNA expression increases between days 10 and 12, but remains elevated through day 16 and declines slowly thereafter (Kim S et al., 2003). Despite elevated levels of COX-2 mRNA a decrease in COX-2 protein has been document past day 17 of pregnancy (Charpigny et al., 1997). In the bovine COX-2 protein and mRNA expression remain elevated to day 24 of pregnancy (Emond et al., 2004). This time of increased expression coincides with the production of interferon-τ by the conceptus (Bartol et al., 1985; Godkin et al., 1982).

COX-2 expression is up-regulated in the endometrium at the times of luteolysis during the estrous cycle, pre-implantation, and implantation during early pregnancy. The fluctuating expression of the cyclooxygenases at various time points may

illustrate multiple roles played by eicosanoids during luteolysis and implantation.

Despite the fact that the cyclooxygenases are the committed step of prostaglandin synthesis and are differentially expressed in diverse tissues throughout the estrous cycle and pregnancy, they do not independently determine which PGs are synthesized. They appear to simply be necessary machinery for PG production but have no bearing on the biosynthetic fate of their released bioactive PGH_2.

Functional Coupling in Prostaglandin Production

The fate of the released PGH_2 is decided by the final action of the terminal synthase that then acts on the PGH_2. There are several reports of terminal synthases coupling with both COX-1 and COX-2 (Liou et al., 2000; Murakami et al., 2000; Nakashima et al., 2003; Ueno et al., 2001). Specifically, Murakami et al. have shown prostaglandin E synthase is coupled with COX-1 and COX-2 enzymes forming two distinct pathways in rats. Each pathway appeared to be differentially regulated according to AA availability (Murakami et al., 2000). Also suggested is that

prostaglandin F synthase(PGFS) is coupled with the COX-2 in HEK293 cells but this observation still needs clarification concerning the many different types of PGFS (Nakashima et al., 2003). As well as terminal synthases, COX-1 and 2 have been shown to couple with PLA_2s (Ueno et al., 2001). These findings suggest compartmentalization of PLA_2-COX-Synthase within the membrane resulting in production of specific prostaglandins.

Prostaglandin Synthases

PGH_2 is subject to conversion into several different prostaglandins through the action of specific terminal prostaglandin synthases. In respect to luteolysis and recognition of pregnancy, PGE_2 and $PGF_{2\alpha}$ are the most readily produced prostaglandins of the ruminant uterus and therefore are of the greatest interest to the current studies (Liggins et al., 1980; Marcus, 1981).

$PGF_{2\alpha}$ can be produced by several prostaglandin F synthases and is known to affect physiological processes in many tissue types, including luteolysis in ruminants (McCracken et al., 1972). There are

three distinct pathways for $PGF_{2\alpha}$ production. PGD_2 11-keto reductase has been shown to convert PGD_2 to $PGF_{2\alpha}$ in the presence of NADPH in rabbit liver (Reingold et al., 1981; Wong, 1981) and rat lung (Watanabe et al., 1981). PGE_2 can also be transformed to $PGF_{2\alpha}$ by PGE_2 9-keto reductase activity as characterized in the chicken heart (Lee et al., 1975) and pre-ovulatory follicles of the ewe (Murdoch and Farris, 1988). The final common pathway for the formation of $PGF_{2\alpha}$ is known to be through the action of PGH_2 9,11-endoperoxide reductase. This enzymatic activity is the primary pathway for $PGF_{2\alpha}$ biosynthesis in the bovine lung (Watanabe et al., 1985), and sheep vesicular glands (Hamberg and Samuelsson, 1967). In addition, other studies have shown PGH2 to be the primary precursor for $PGF_{2\alpha}$ biosynthesis in the bovine uterus (Madore et al., 2003). However, in the bovine uterus, $PGF_{2\alpha}$ is thought to be produced by a novel PGFS (Madore et al., 2003) as postulated by Urade and colleagues (Urade et al., 1995). Madore et al. showed that, despite the ability of endometrial cells to produce PGE_2 and PGD_2, the primary source of $PGF_{2\alpha}$ comes

from the reduction of PGH_2. The reduction most likely results from the action of the aldoketoreductase 1B5 enzyme (AKR1B5) localized in the luminal and glandular epithelium. An identifying factor of this enzyme is the lack of 11-ketoreductase activity that allows most other PGF synthases to transform PGD_2 into $PGF_{2\alpha}$ (Madore et al., 2003). In addition to PGFS activity, AKR1B5 was shown to exhibit 20α-hydroxysteroid dehydrogenase activity that is involved in the breakdown of progesterone. This secondary activity may reduce progesterone's inhibitory activities that are seen through diestrous since AKR1B5 is shown to be up-regulated by high levels of progesterone (Madore et al., 2003). The up-regulation of AKR1B5 in the uterus begins at day 12 of the cycle and remains elevated through day 19 approaching luteolysis in cattle (Madore et al., 2003). Interestingly, Arosh et al., 2004 found that AKR1B5 is down regulated in both the CL and the uterus during the maternal recognition of pregnancy. This contradicts reports that $PGF_{2\alpha}$ levels are elevated from basal levels in some species (Zarco et al., 1988)suggesting a possible alternate method of regulation during pregnancy.

PGE_2, produced by prostaglandin E synthases (PGES), has many biological activities depending on the cell type and species. PGE synthases have been detected in both the microsomal fraction (mPGES) (Hamberg and Samuelsson, 1973) and the cytosolic fraction (cPGES) (Ogorochi et al., 1987) of cells. Microsomal PGES is generally recognized as the predominant producer of uterine PGE_2 and it is found on the perinuclear membrane co-localized with COX-2 (Murakami et al., 2000). In the cow uterus overall mPGES has been documented to be unvarying during the cycle and during the recognition of pregnancy (Arosh et al., 2004)despite a documented increase in the production of PGE_2 versus $PGF_{2\alpha}$ (Asselin et al., 1997). Conversely, PGES mRNA and protein levels increase in decidual cells and in epithelial cells at the sites of implantation in rats (Ni et al., 2002). Combined, this evidence suggests a specific role for increased PGE_2 production in implantation and decidualization. Further support of PGE_2 as a promoter of pregnancy is seen in the documented increase of mPGES in the CL (Arosh et al., 2004)that supports increased PGE_2

production which may help prevent luteolysis (Pratt et al., 1977).

Phospholipase A_2s

Phospholipase A_2s (PLA_2) are a broad family of acylhydrolases that act on the *sn*-2 position of membrane phospholipids resulting in the release of AA and the production of lysophospholipids. Arachidonic acid can act as a second messenger (Hallak et al., 1994)and is a precursor for eicosanoids and several other chemical mediators. Also, lysophospholipids can form an array of important inflammatory mediators in many different cell types (Graler and Goetzl, 2002). An example is the metabolization into platelet activating factor (PAF) (Jackson et al., 1998). Despite the ability to perform the previously described reaction, assortments of PLA_2s have multiple enzymatic capabilities. Due to subtle differences between enzymes, PLA_2s have been divided into 16 groups with many subgroups to organize over 60 different enzymes identified as PLA_2 isoforms. These groups divide the enzymes according to specific substrate specificity and amino acid sequence and are then

further split into sub-groups depending on homology and splice variants (Six and Dennis, 2000). This would seem to be straight forward, but several enzymes were named before the group designation began, resulting in several names often describing the same enzyme. For simplicity the enzyme will be referred to by the corresponding group and sub-group designation in the remainder of the paper.

For the purposes of this study, only a few PLA_2 groups were analyzed. Specifically groups IVA and VIA are of interest in the present study. This does not rule out the activity of other PLA_2s in reproductive physiology, but the aforementioned have been implied to have function in prostaglandin synthesis and/or are known to localize in the perinuclear area in close proximity to the other enzymes involved in prostaglandin biosynthesis.

As more evidence emerges suggesting functional coupling of PLA2-COX-Terminal Synthase, PLA_2s are likely candidates to explore as regulators of prostaglandin production since they catalyze the rate limiting step in prostaglandin production. Also, if specific PLA_2s act solely in certain prostaglandin

pathways, they can be regulated in target tissues with minimal impact on other cascades involving AA release. Elucidation of such pathways will likely yield new therapeutic options.

PLA_2 Group IVA

Group IVA is the best characterized PLA_2 of interest in our current studies. Its influence has been shown in multiple tissues with several physiological functions that all involve the release of AA and the production of a resultant lysophospholipid. The pathway for activation is controlled by multiple phosphorylation sites that can be activated by multiple pathways. It has been suggested, in some cell types, to be activated directly through G-protein interaction (Burch et al., 1986; Jelsema et al., 1987) although more recent research still suggests a link with the PLC pathway (Graf et al., 1999; Lee J and Silvia, 1994),or p38 mitogen- activated protein kinase (Waterman et al., 1996), and extracellular-signal-regulated kinase (Hazan et al., 1997) in human neutrophils in a PKC dependant pathway.

Group IVA, in its inactive state, is found in the cytoplasm but has been shown to be translocated to perinuclear membranes by vimentin fibrils in response to an influx of Ca++ at concentrations consistent with those found in physiological responses (Channon and Leslie, 1990; Nakatani et al., 2000; Schievella et al., 1995). Translocation, along with phosphorylation (Qiu et al., 1993), results in the active form of the enzyme that is able to process membrane phospholipids. Several phosphorylation sites have been identified, including Ser-437, Ser-454, Ser-505, and Ser-727 (de Carvalho et al., 1996), but no distinct pathway has been divulged for activation (de Carvalho et al., 1996; Qiu et al., 1993). Also demonstrated in fibroblast 3T3 cultures, phosphorylation of Group IVA must precede the calcium influx for full activity (Schalkwijk et al., 1996). It is presently unclear if the timing of phosphorylation is more imperative for the activity of the vimentin head or for PLA_2 Group IVA activity. Once phosphorylated, Nakatani et al., 2000 showed translocation occurs through the direction of vimentin

fibrils attached to the C2 domain of PLA_2 Group IVA at the vimentin head. Vimentin is believed to assist in proper localization of Group IVA with specific membrane associated compartments (Nakatani et al., 2000).

Group IVA activity in reproductive tissues has been studied in many species including the mouse, sheep, human, and bovine. Gene deletion (knock-out) studies in mice have associated Group IVA activity with some prostaglandin regulated functions of reproduction including implantation and parturition (Bonventre et al., 1997; Song et al., 2002; Uozumi et al., 1997). These Group IVA deficient mice were characterized as having decreased litter size with improper implantation and complications with natural induction of labor (Bonventre et al., 1997; Song et al., 2002; Uozumi et al., 1997). Although these studies clearly illustrate Group IVA's importance in regulation of reproductive processes, they do not delineate Group IVA's physiological roles during estrous cyclicity and pregnancy. Also when compared with COX-2 knock-out studies, it becomes evident that other PLA_2s are active in AA release allowing for

reproduction, though at reduced efficiency, whereas COX-2 deficiency in mice is often fatal and surviving females are sterile (Langenbach et al., 1999).

Few studies have investigated Group IVAs role in ruminant reproduction. Studies in the ewe demonstrate that Group IVA expression of mRNA and protein during a simulated estrous cycle remain constant during days 11-14 of the cycle and maintain the ability to respond to stimulation (Graf et al., 1999). These data are further supported *in vivo* by the demonstration that samples at multiple time points during the estrous cycle maintain constant Group IVA activity (Tamby et al., 1993). These studies were performed on endometrial tissue samples that may not contain a high enough concentration of luminal endometrial epithelium, the origin of most prostaglandin production in the uterus(McCracken et al., 1972).

PLA_2 activity may be regulated by oxytocin and interferon-τ since these signals regulate maternal recognition of pregnancy and luteolysis. Oxytocin stimulates $PGF_{2\alpha}$ production which is dependent upon PLA_2 activity; however, oxytocin stimulation of PLA_2 does not induce an increase in Group IVA mRNA,

protein, or activity in the ewe (Burns et al., 2000; Graf et al., 1999) suggesting that other PLA2 enzymes may be involved in maternal recognition of pregnancy and luteolysis in ruminants(Tithof et al., 2007). Indirect mechanisms have been suggested in the sheep (Graf et al., 1999; Lee J and Silvia, 1994) and cattle (Burns et al., 1997). A proposed mechanism involves oxytocin induced increases in Ca^{++} by activation of phospholipase C pathway resulting in activation of MAPK/PKC activity leading to full activation of Group IVA through the Ca^{++} and phosphorylation activities described earlier(Burns et al., 1997; Graf et al., 1999; Lee and Silvia, 1994). Unlike oxytocin, interferon-τ appears to directly affect Group IVA synthesis and activity. Pregnant sheep exhibited a steady decrease in Group IVA activity after day 12 (Asselin et al., 1997; Burns et al., 1997; Graf et al., 1999; Lee and Silvia, 1994). Similarly, interferon-τ was shown in bovine endometrial epithelial cells to decrease Group IVA mRNA and protein and subsequently decrease $PGF_{2\alpha}$ production after 12 hours even when stimulated by a phorbol ester *in vitro* (Binelli et al., 2000). Unfortunately,

interferon-τ's mechanism for action in either species is not known and could be through cytosolic and/or nuclear actions. Not to be ruled out, as noted by Bonney et al. 1987, are possible activities of other PLA_2s and species differences in luteolysis and maternal recognition of pregnancy that have yet to be classified.

PLA_2 Group VI

Group VI PLA_2 was first purified in $P388D_1$ macrophage cells although calcium-independent PLA_2 activity had been previously described in multiple species and cell types (Ackermann et al., 1994). Unfortunately, little is known about the multiple enzymes and isoforms that are classified within Group VI. The gap in knowledge is considerably large with regards to Group VI's roles in reproductive processes. Group VIA is a 85-88kDa protein comprised of 8 N-terminal ankyrin repeats and a conserved lipase motif (Tang et al., 1997); but, there are multiple splice variants, see figure 1.2, resulting in many active and inactive gene products that arise from the same gene in humans, rats (Forsell et al., 1999; Larsson et al.,

1998) and most likely some other species. Of these splice variants, 40kDa and 50kDa variants have been identified (Ackermann and Dennis, 1995; Brant and Caruso, 2005; Brant et al., 2006; Manguikian and Barbour, 2004). The separate variants have split Group VIA into 5 different splice variants of known structure as described by Winstead and colleagues (Winstead et al., 2000). Group VIA-1 is an active phospholipase comprised of 752 amino acids containing

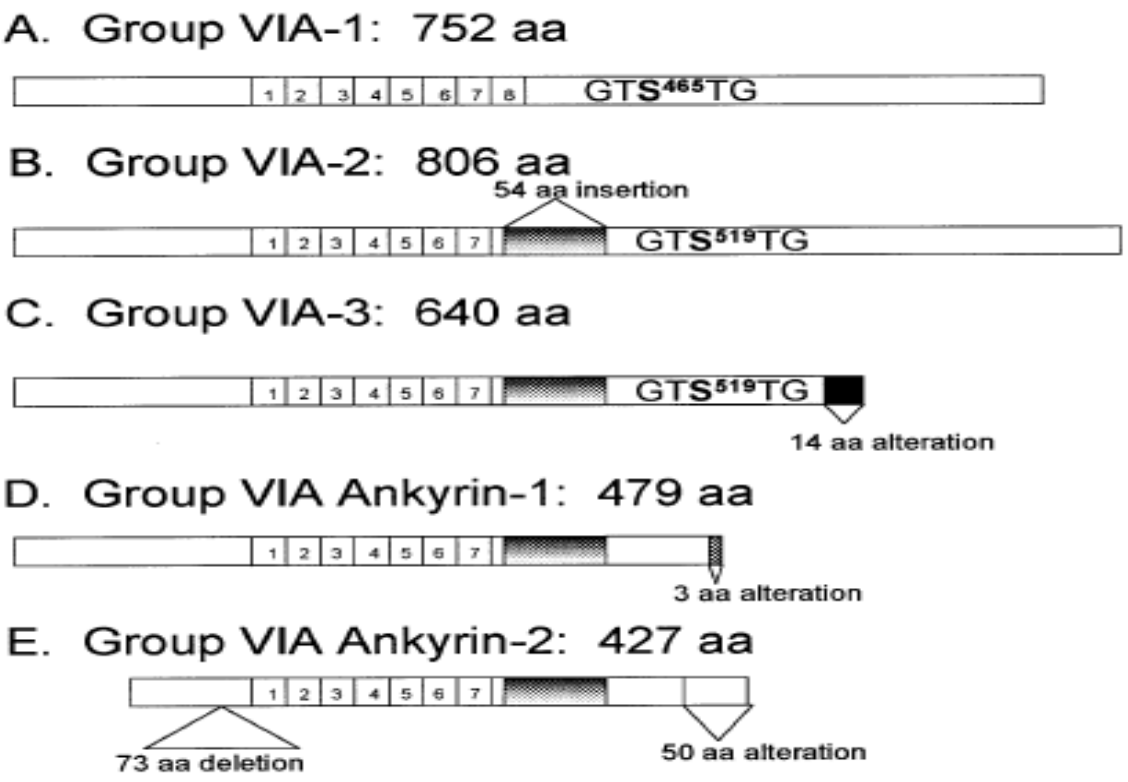

Figure 1-2. Schematic of Group VI Isoforms from Winstead et al., 2000.

eight ankyrin repeats and the conserved GTSTG lipase motif. Group VIA-2, 806 amino acids, is similar to 1 except, in place of the eighth ankyrin repeat, it contains a 54 amino acid insertion derived from exon 9 of the human gene which is believed to determine its localization (Forsell et al., 1999). Group VIA-3 is very similar to VIA-2 but at only 640 amino acids it has a shortened C-terminus and it is not yet known if this isoform is active. Also produced are Ankyrin-1 and Ankyrin-2. These gene products result in inactive enzymes because they lack the lipase motif despite containing structure similar to Group VIA-2 and are dominant negative inhibitors(Forsell et al., 1999; Larsson et al., 1998; Winstead et al., 2000).

Although the molecular details of Group VI activity are still largely undefined, the structure helps to explain some trends that have been identified in past experiments. In early studies, Group VI was found to form a tetramer of 270-350kDa that was catalytically active (Ackermann et al., 1994; Tang et al., 1997). The formation of an oligomeric structure is most likely due to the protein to protein interactions common between ankyrin repeats. It has

been documented that ankyrin repeats of group VI are necessary in some capacity for standard activity to be achieved (Tang et al., 1997). Structural interaction between splice variants is also believed to be a point of regulation for group VI. Ankyrin-1 and 2 are believed to bind catalytically competent Group VI PLA_2 enzymes, in turn playing an inhibitory role by preventing the formation of catalytically active tetramers (Forsell et al., 1999; Manguikian and Barbour, 2004; Tang et al., 1997). In some cases ankyrin repeats also coordinate localization to phospholipid membranes. There is mixed evidence for such sub cellular localization for Group VI. There are multiple reports describing both the cytosolic (Forsell et al., 1999; Saavedra et al., 2006; Tang et al., 1997) and membrane (Akiba et al., 1999; Forsell et al., 1999; Manguikian and Barbour, 2004) localization and activity of these enzymes. These results require more experimentation, and suggest that the localization is not only species, but also cell type specific.

Several other important regulatory aspects of Group VI have also been described. Despite being

active in the absence of Ca^{2+} *in vitro*, Group VI activity has been shown to be intricately regulated by calcium stores. It is known that calmodulin can be functionally coupled to Group VI in an inhibitory manner (Wolf and Gross, 1996). But, this inhibition can be lifted through localized calcium store depletion and the actions of calcium/calmodulin-dependent protein kinase type II in pancreatic islet β-cells (Wang et al., 2005). Also, different kinases are involved in the activity of Group VI. PKCα is imperative to the membrane localization and activity in $P388D_1$ cells (Akiba et al., 1999; Akiba et al., 2002)whereas PKCε phosphorylation is implicated in Group VIA activity in ventricular myocytes (Steer et al., 2002). A final kinase implicated is p38 MAPK which affects Group VI activity in a dose-dependant manner in smooth muscle cells (Brant and Caruso, 2005; Yellaturu and Rao, 2003). Many of these activities are dependant upon association with ATP (Ackermann et al., 1994; Steer et al., 2002; Wolf and Gross, 1996). It has since been shown that ATP acts to protect Group VI from degradation in oxidation studies (Song et al., 2006).

Group VI PLA2 has just as many proposed physiological roles as points of regulation. Group VI has been demonstrated to be a house keeping enzyme involved in phospholipid remodeling in many cell types (Baburina and Jackowski, 1999; Barbour et al., 1999; Herbert and Walker, 2006; Perez et al., 2004; Saavedra et al., 2006). But this is not true for all cell types (Ma et al., 2001; Ramanadham et al., 1999)and is not the only function for Group VIA. Group VIA acts in a signaling fashion promoting the production of eicosanoids (Akiba et al., 1998; Brant and Caruso, 2005), lysozyme secretion (Balboa et al., 2003), cell division (Herbert and Walker, 2006; Manguikian and Barbour, 2004), differentiation (Birbes et al., 2000)and apoptosis (Balboa et al., 2003; Perez et al., 2004). These activities can be stimulated by exogenous sources (Akiba et al., 1999; Yellaturu and Rao, 2003) or are initiated by the physiological intricacies of Group VI splice variant regulation that varies between cell types and species. Such variation makes it difficult to extrapolate results from previous experiments as universal knowledge. But, the previous descriptions provide insight to some

possibilities for activity and regulation within reproductive tissues and processes.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Fisher Scientific, Pittsburgh, PA unless otherwise noted. Mature cross-bred ewes were provided by the University of Tennessee, Knoxville Experiment Station. Protease Inhibitor Complete EDTA free tablets were purchased from Roche Diagnostic Corporation, Indianapolis, IN. diethylpyrocarbonate (DEPC) was bought from Sigma-Aldrich, St. Louis, MO. Correlate EIA kits were purchased from Assay Designs Inc., Ann Arbor, MI. BCA Protein Assay Kit, IgG Anti-rabbit HRP conjugate, Super Signal West Femto Maximum Sensitivity Substrate, CL-Xposure X-ray film and PVDF membranes were procured from Pierce Biotechnology, Rockford, IL. MagicMark XP molecular weight markers were purchased from Initrogen, Carlsbad, CA. High Range Pre-stained SDS-PAGE Standards and iScript Select were bought from Bio-Rad, Hercules, CA. GIVA specific monoclonal antibody (SC-454) was obtained from Santa Cruz Biotechnology, Santa Cruz. GIV antibody (MAB166A) was procured from Chemicon International Inc., Temecula, CA. IgG Anti-mouse ECL Peroxidase was acquired from

GE Healthcare Bio-Sciences Corp., Piscataway, NJ. iPLA2 antibody (Group VIA) was obtained from Upstate Ltd., Hampshire, United Kingdom. RNAlater, RLT buffer, RNeasy Mini Kit, and RNease-Free DNase set were procured from Qiagen Inc., Valencia, CA. Power SYBR was purchased from Applied Biosystems, Foster City, CA. All primers were ordered from Integrated DNA Technologies, Coralville, IA. Clonetech Nucleotrap Kit was obtained from Takara Bio Company, Otsu, Japan.

In Vivo Collection of Luminal Epithelium

Mature cross-bred ewes were monitored for standing heat twice daily with vasectomized rams. Upon observation of estrus, sheep were scheduled for hysterectomy on day 5 (n=3), 10 (n=3), or 15 (n=3) of the cycle with estrus being day 0 or bred by intact rams and scheduled for hysterectomy on days 14 (n=3), 15 (n=1), 16 (n=2), 17 (n=1) or 20 (n=2) of pregnancy. Day of the cycle was further confirmed by macroscopic evaluation of the reproductive tract (Ireland et al., 1980; Miyamoto et al., 2000). Pregnancy was verified by the presence of an embryo. Excess blood was

removed from the uterus after hysterectomy and it was placed immediately on ice. Horns were separated and opened vertically revealing the luminal epithelium. Tissue samples were removed containing both epithelium and stromal layers from each horn with scissors. Remaining Luminal epithelium was scraped from each horn with a scalpel. These samples were weighed and placed in 3 volumes (w/v) of Western lysis buffer (154 mM NaCl, 1 mM KH_2PO_4, and 5.6 mM Na_2HPO_4, 1% NP-40, 0.1% SDS, and 0.5% deoxycholate, pH 7.4 freshly supplemented with 1mM DTT, and 2.4% (v/v) protease inhibitor solution composed of 4 Complete EDTA Free Tablets dissolved in 5mL of water) on ice. Samples were immediately homogenized with a Tissumizer SDT-1370 (Tekmar, Cincinnati, OH) for thirty seconds on ice. Samples were sonicated with a Sonic Dismembrator 100 (Fisher Scientific, Pittsburgh, PA) for 3 pulses of 10 seconds on ice, aliquoted, and stored at -80^{o}C until further use.

***In Vivo* RNA Collection**

As the luminal epithelium scrape and tissue samples were removed from each horn for Western blot

analysis samples were also obtained, weighed and placed in 1mL of RNAlater overnight at 4^0C. The next day 1mL of ice cold DEPC treated PBS was added and the mixture was centrifuged at 5000 x g for 5 minutes at 4^oC. The liquid was then drained and tissue was stored at -80^oC until the RNA could be extracted.

Western Blot

The total protein concentration was determined using BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) as described by the manufacturers protocol. Absorbance was measured at 595nM with a Multiscan RC 96-well plate reader (Lab System Multiscan RC; Thermo Lab System, Franklin, MA). 10ug of total protein from each sample was loaded onto a 10% (w/v) SDS polyacrylamide gel and electrophoresed at 125 V for 1.5 hours. Also added to the gel was one lane each of chemiluminescent MagicMark XP molecular weight markers (Initrogen, Carlsbad, CA) and High Range Pre-stained SDS-PAGE Standards (Bio-Rad, Hercules, CA) in order to calculate molecular weight, confirm transfer, and monitor migration during electrophoresis. Protein contents were transferred to

PVDF membranes (Pierce Biotechnology, Rockford, IL) using the Bio-Rad Transblot SD (Bio-Rad, Hercules, CA) semi dry transfer system at 1.5 mAmps per cm^2 of membrane for 30 minutes. Before transfer, the PVDF membrane and a transfer pad were soaked in 60mM tris base, 40mM CAPS, and 0.37 M methanol for 30 minutes while another transfer pad and the gel was soaked in 60mM tris base, 40mM CAPS, and 0.1% SDS. After transfer the gels were stained with 0.1% Coomassie Blue stain to verify transfer. The membranes were immediately blocked for 1 hour at room temperature by shaking in 5% non-fat dry milk (NFDM) in TBST (10 mM Tris, 0.05% Tween, and 154mM NaCl). The blocked membrane was placed in 1mL of primary antibody diluted with 5% NFDM in TBST to a concentration of 1ug/mL for Group IVA specific monoclonal antibody, 2ug/mL for Group IV antibody, and incubated overnight at 4^oC. The Group VI antibody was diluted to 1:500. The Group IVA and IV antibodies recognize different peptides. This allows for group IVA to only react with Group IVA and its various breakdown products where as the Group IV antibody can also recognize other Group IV PLA2s such as Group IVC. After incubation, membranes were washed

4 times in 200mL of TBST for 5 minutes. 1mL corresponding secondary antibody diluted by 5% NFDM in TBST was applied at a dilution of 1:5000 for IgG Anti-mouse ECL Peroxidase or 1:1000 for IgG Anti-rabbit HRP conjugate (Pierce Biotechnology, Rockford, IL) and incubated for 2 hours at room temperature. Membranes were then washed in 200mL of TBST 3 times for 20 minutes each. Super Signal West Femto Maximum Sensitivity Substrate was used according to manufacturer's instructions. Protein bands were visualized by a 30 second exposure to CL-Xposure X-Ray film. Band intensity was analyzed with the FluoroChem 5500 (Alpha Innotech, San Leandro, CA) for densitometry values.

Both Group IV antibodies are mouse monoclonal antibodies and recognize a sequence in the first 216 amino acids. Exactly which sequences are recognized is not disclosed by either manufacturer. The primary antibody from santa cruz biochemical claims to be specific to Group IVA. Chemicon's Group IV antibody recognizes Group IVA and other Group IV isoforms. Both antibodies will recognize group IVA and may also cross react with breakdown products or novel PLA_2s yet

to be classified. Similarly the Group VIA antibody recognizes a peptide that to date is specific to Group VIA. The antibody will recognize Group VIA and some cleavage products but an uncharacterized PLA_2 is not outside of the realm of possibilities.

RNA Extraction

Tissue and scraping samples treated with RNAlater were thawed, weighed and homogenized for two 20 second bursts with a Tissumizer SDT-1370 in 600 uL of RLT buffer per 20mg of sample. Samples were then centrifuged at 10,000 x g for 5 minutes and the supernatant collected for RNA extraction. RNeasy Mini Kit spin columns were used for extraction and DNA contamination was removed with an on column RNease-Free DNase Kit treatment. 200uL of supernatant was added to each spin column and manufacturer's instructions were followed with the addition of a 5 minute incubation after the addition of RPE buffer. RNA was eluted in 50uL of RNase free water. RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Life Sciences and Chemical Analysis, Foster City, CA). Final RNA concentration

was determined using a ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE) and samples were stored at -80^{o}C.

Reverse Transcription

Reverse transcription of RNA samples was performed using iScript Select following the manufacturer's instructions using the supplied oligo d(T) primer. 500ng and 175ng of total RNA were added to each reaction for scrape and tissue samples, respectively. After reverse transcription the cDNA samples were stored at -20^{o}C until use in real time PCR.

Semi-quantitative Realtime PCR (qRT-PCR)

cDNA was diluted with DEPC water to 1:5, 1:25, and 1:125 for each sample. 2uL of each dilution was plated in duplicate wells along with forward and reverse primers (500nM) and 12.5uL of Power SYBR and DEPC water resulting in a 25uL reaction volume. Also, a control sample of the previous dilutions was plated for quantitative analysis (sheep 121). Analysis was performed under universal conditions with an ABI 7000

Sequence Detection System (Applied Biosystems, Foster City, CA). Ribosomal protein L19 was used as a reference gene with primer sequences of ACCCCAATGAGACCAATGAAAT (F) and CAGCCCATCTTTGATCAGCTT (R) (Chen et al., 2006). Phospholipase Group IVA was analyzed using the following primer sequences GGAAACCATAAAGGAGAACATGAAGAA (F) and GCACATCACGTGTAGAATGCAA (R). These were designed using Primer Express software (Applied Biosystems, Foster City, CA) to choose primer pairs that span across exons of the bovine sequence (NM_001075864) described by ensemble. Partial sheep sequences, produced by our lab, were examined with Primer Express software for phospholipase Group VIA primer generation. Comparison with the bovine sequence for GVIA variant-2 (XM_615452.3) was used to determine possible splice sites. Phospholipase Group VIA was evaluated using as CGCACGTGGGACTGCAT (F) and GGTGGCAACTGGGAACAATACT (R) as primer sequences. Clontech Nucleotrap Kit (Takara Bio Company, Otsu, Japan) was used to purify PCR products for subsequent sequencing at University of Tennessee, Knoxville Molecular Biology Resources Facility in order to validate primer specificity. A

melting curve was also generated following PCR analysis in order to detect any primer dimmers or false products in the reactions.

Statistical Analysis

All statistical analysis was performed using SAS 9.1 software (SAS Institute, Cary, NC). Densitometry values obtained from Western blot data were analyzed with the following model, $y = \mu + \text{treatment} + \text{sheep(treatment)} + \varepsilon$, with each antibody being analyzed separately. Pairwise contrasts were performed between each treatment to determine differences. Analysis of qRT-PCR data for primer efficiency was performed, as described by Yuan et al., using a simple linear regression, $Ct = \beta_o + \beta_{con}X_{lcon} + \varepsilon$, to determine data quality and primer efficiency (Yuan et al., 2006). PROC GLM was used to construct a multiple regression model, $Ct=\beta_o+\beta_{con}X_{icon}+\beta_{gene}X_{igene}+\beta_{treat}X_{itreat}+\beta con_{treat}X_{icon}X_{itreat}+\beta_{congene}X_{icon}X_{igene}+\beta_{genetreat}X_{igene}X_{itreat}+\beta_{congenetreat}X_{icon}X_{itreat}X_{igene}+\varepsilon$ also described by Yuan et al., in order to estimate $\Delta\Delta Ct$ number which was the ΔCt for RPL19 minus ΔCt for the target gene between our target and control samples

(Yuan et al., 2006). A general linear model, $y = \mu +$ treatment + sheep(treatment) + ε, was used to analyze differences of the means between the $\Delta\Delta$Ct numbers of each treatment. qRT-PCR data are presented as fold change ($2^{-\Delta\Delta Ct}$)and significance for all experiments was determined as $P < 0.05$.

RESULTS

Western blot analysis during the estrous cycle

Western blot analysis of proteins prepared from luminal endometrial epithelial scrapings collected from animals over the estrous cycle revealed (Figure 3-1) a protein that migrated to a position coinciding with 108kD. This protein was visualized with both group IV and Group IVA specific antibodies leading to the conclusion that this protein is PLA_2 Group IVA. Statistical analysis of densitometry values (Figure 3-2) revealed that the greatest Group IVA expression during the estrous cycle occurred at day 10 of the cycle before becoming diminished again at day 15. However, days 5, 10, and 15 were not seen to be statistically different. Also seen were 45 and 50kD bands that do not correspond to any previously reported group IV or IVA cross reactive proteins.

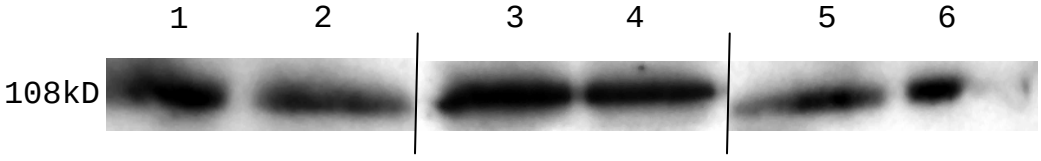

Figure 3-1. Representative expression of PLA_2 Group IVA during the estrous cycle. Lanes 1&2 Day 5 (n=3). Lanes 3&4 Day 10 (n=3). Lanes 5&6 Day 15 (n=3).

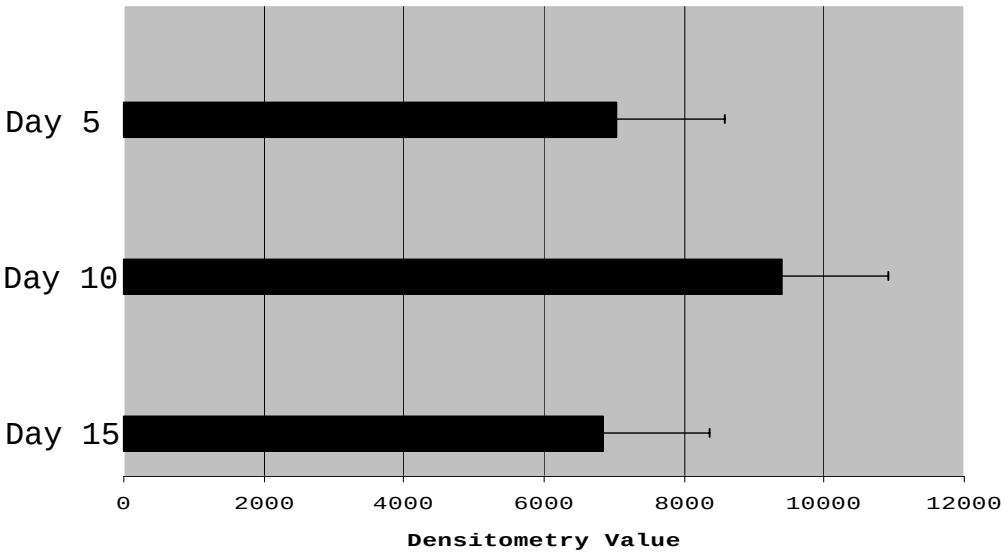

Figure 3-2. Estimated densitometry values for PLA_2 Group IVA blot (Day 5=6831.33, Day 10=9289.67, and Day 15=7041.33).

Western blot analysis of pregnant ewes

Western blot analysis of proteins prepared from luminal endometrial epithelial scrapings of pregnant sheep between days 14 and 20 of pregnancy also revealed (Figure 3-3) a protein that migrated to a position of 108kD. This protein was identified as Group IVA by using both a group IV non-specific and a Group IVA specific antibody as described previously. Protein expression, as determined by densitometry (Figure 3-5), was shown to be highest at days 14 and 15 of pregnancy. Pair wise comparison revealed that Group IVA expression decreased following day 15 of pregnancy ($P<0.04$).

Also of interest is the appearance of cross reactive proteins against both the Group IV and Group IVA antibodies between days 15 and 17 (Figure 3-4) of pregnancy that migrates to positions of 68kD and 38kD. No Group IV cross reactive proteins have been documented at this position. The 68kD protein exhibits greatest expression on day 16 of pregnancy where as the 32kD protein remains relatively consistent from day 14 to 17. There are also 45kD and 50kD proteins that are cross reactive with the group IV and Group IVA antibodies that reveal no defined pattern and at this time can only be considered as possible breakdown products until further study.

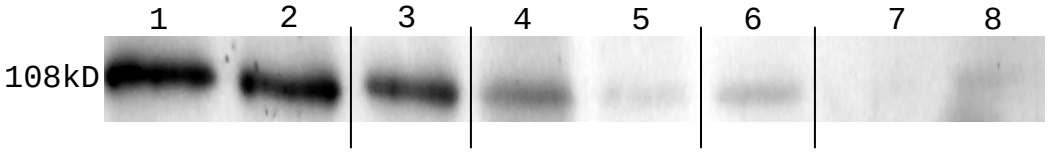

Figure 3-3. Expression of PLA_2 Group IVA during pregnancy. Lanes 1&2: Day 14 of pregnancy. Lane 3: Day 15 of pregnancy. Lane 4&5: Day 16 of pregnancy. Lane 6: Day 17 of pregnancy. Lanes 7&8: Day 20 of pregnancy

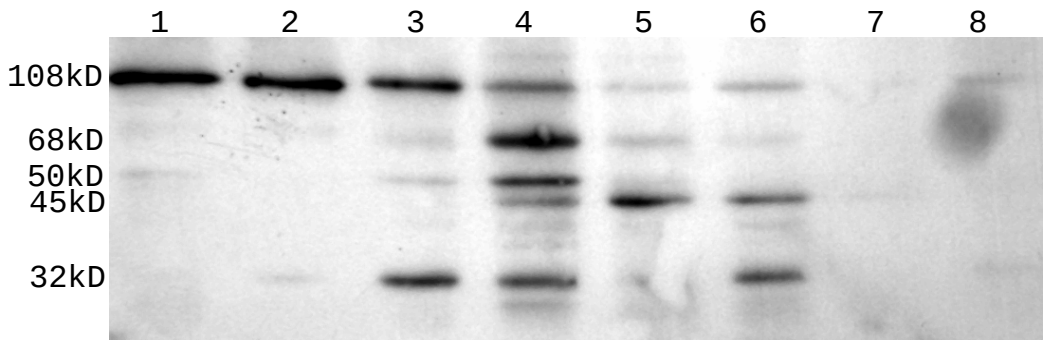

Figure 3-4. Expression of PLA_2 Group IV during pregnancy. Lanes 1&2: Day 14 of pregnancy. Lane 3: Day 15 of pregnancy. Lane 4&5: Day 16 of pregnancy. Lane 6: Day 17 of pregnancy. Lanes 7&8: Day 20 of pregnancy.

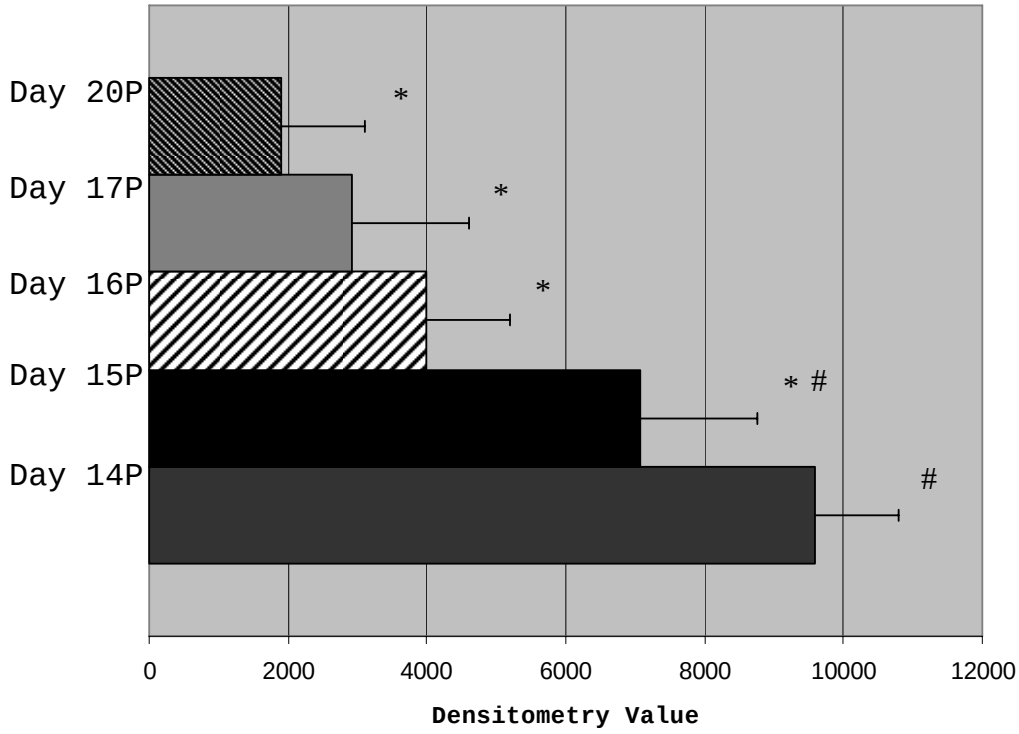

Figure 3-5. Estimated densitometry values for PLA_2 Group IV blot (Day 14P=9589.5, Day 15P=7065.00, Day 16P=3987.5, Day 17P=2917, and Day 20P=1906.5).

Western blot analysis of pregnancy versus non-pregnancy

Western blot analysis of pregnant and non-pregnant samples with both Group IV and Group IVA antibodies reveals similar results as seen in the pregnancy and estrous cycle blots described earlier. But, from this blot it is determined that group IVA (Figure 3-6) is expressed at its highest level on days 14 and 15 of pregnancy before decreasing as described previously. Day 14 of pregnancy was shown to be significantly higher than day 15 of the estrous cycle (P<0. 05). This blot once again demonstrated not only the band corresponding to Group IVA, but also the unidentified 68, 32, 50 and 45kD bands that were described earlier.

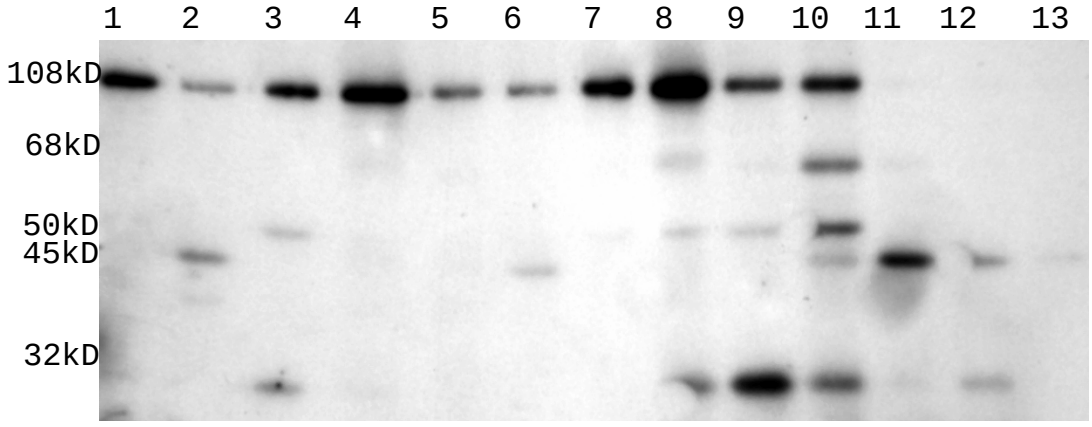

Figure 3-6. Expression of PLA_2 Group IV. Lanes 1&2: Day 5. Lanes 3&4: Day 10. Lanes 5&6: Day 15. Lanes 7&8: Day 14 of pregnancy. Lane 9: Day 15 of pregnancy. Lane 10&11: Day 16 of pregnancy. Lane 12: Day 17 of pregnancy. Lane 13: Day 20 of pregnancy.

qRT-PCR Analysis of endometrial epithelium

All samples were analyzed by qRT-PCR in order to demonstrate the fluctuations of phospholipase A_2 mRNA during the estrous cycle and early pregnancy. Analysis of Group IVA expression by luminal endometrial epithelial scrapings (Figure 3-7) revealed greatly elevated expression ($P<0.0001$) at day 10 of the estrous cycle (11.59), followed by day 14 of pregnancy (2.197). That level was reduced during days 5 and 15 of the estrous cycle (0.744,0.613) and days 15 and 16 of pregnancy (0.613,1.013). Expression

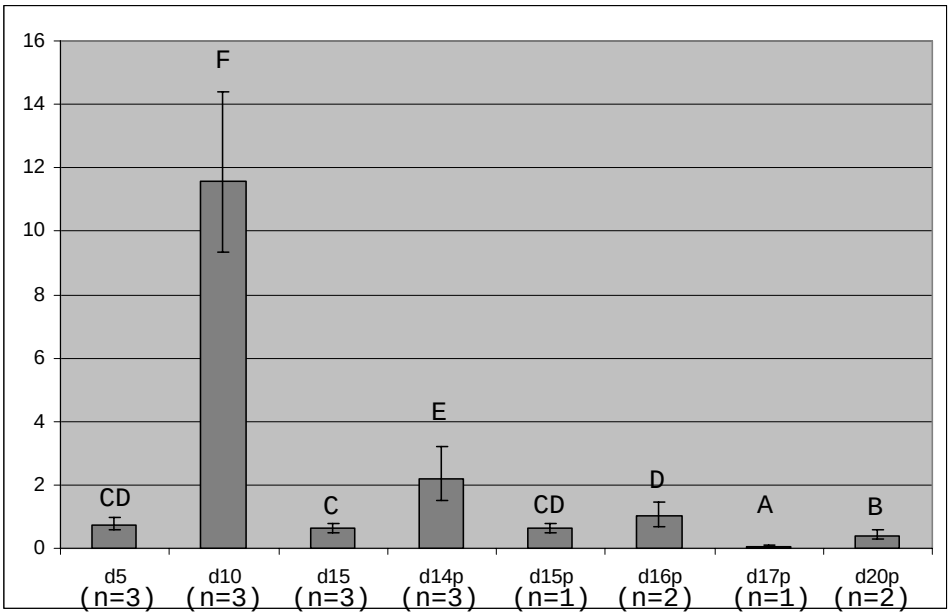

Figure 3-7. Analysis of qRT-PCR results for Group IVA scrape mRNA expression reported as $2^{-\Delta\Delta Ct}$. Error bars represent the 95% confidence intervals with letter grouping representing significance.

was greatly decreased after day 16 of pregnancy with the lowest expression occurring at day 17 of pregnancy (0.051).

Group IVA mRNA expression in tissue samples (Figure 3-8) did not show differences between most treatments ($P<0.001$). Similar to the results seen from endometrial scrapings, the tissue samples showed days 5 and 15 of the estrous cycle (0.273,0.339) once again had decreased expression with day 10 exhibiting a significant increase. Unlike the scraping samples, all pregnant samples expressed Group IVA mRNA at increased levels that were comparable to expression on day 10 of the estrous cycle with day 14 of pregnancy (1.917) exhibiting the highest expression.

Group VIA expression in scraping samples (Figure 3-9) revealed elevated mRNA expression at days 5 and 10 of the estrous cycle (0.487,0.792) and decreased expression on day 15 of the estrous cycle($P<0.0002$). The highest mRNA expression levels were observed on day 10 and the lowest were observed on day 15 of the estrous cycle (0.115). Expression from all pregnant time points for Group VIA was diminished and did not significantly differ from one another.

Tissue samples (Figure 3-10) showed virtually no significant difference in Group VIA expression ($P<0.0062$). Day 15 of the estrous cycle (0.656) and days 14 and 20 of pregnancy (0.673,1.232) were seen to be the highest in Group VIA mRNA expression with the remaining being slightly decreased. The day 20 value may be an artifact due to decreased replications of this treatment. Day 20 tissue RNA extraction only yielded one viable sample.

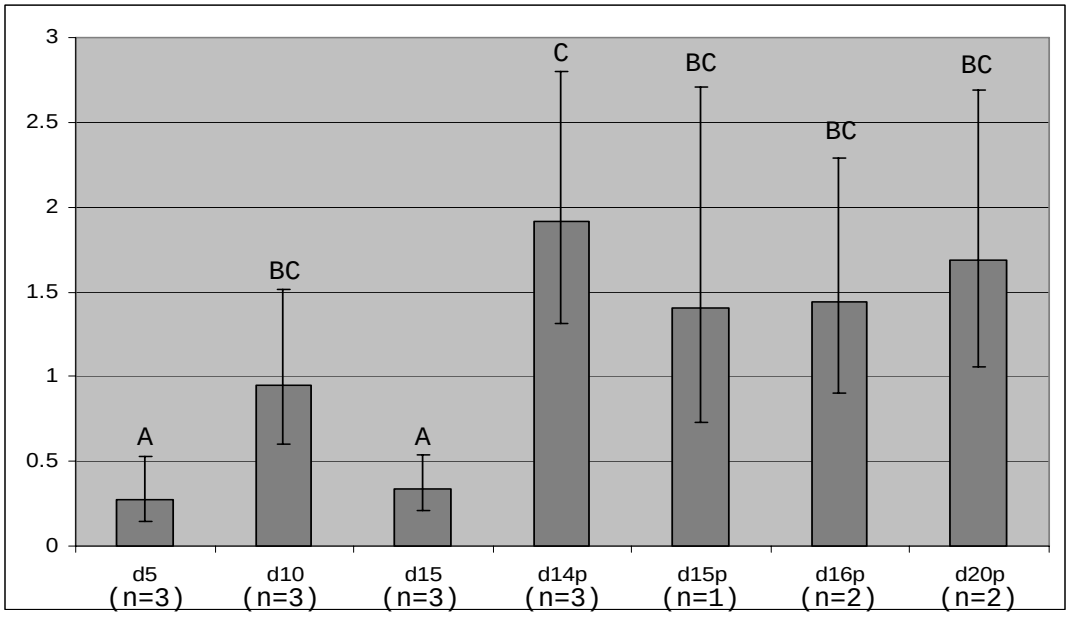

Figure 3-8. Analysis of qRT-PCR results for Group IVA tissue mRNA expression reported as $2^{-\Delta\Delta Ct}$. Error bars represent the 95% confidence intervals with letter grouping representing significance.

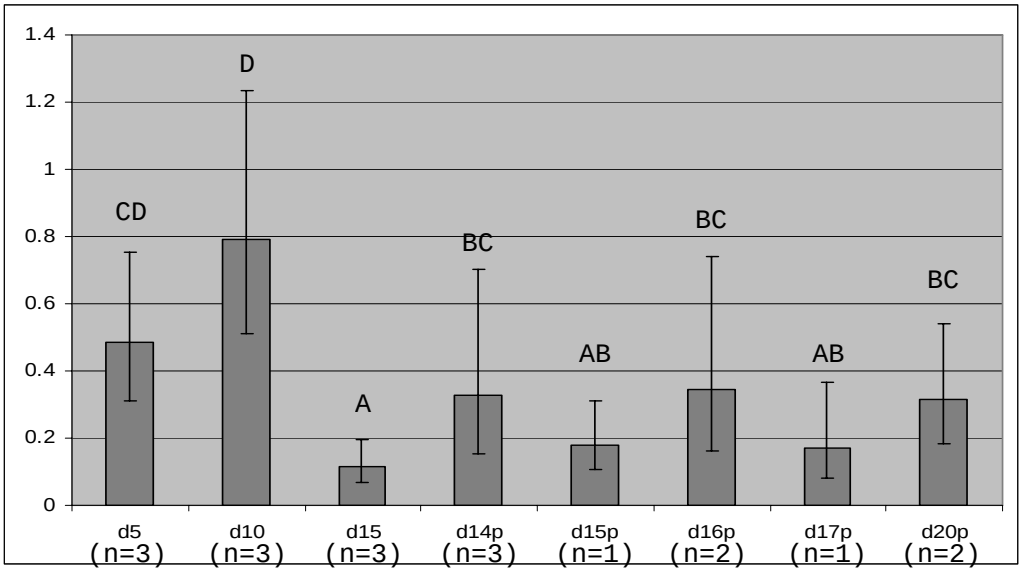

Figure 3-9. Analysis of qRT-PCR results for Group VIA scrape mRNA expression reported as $2^{-\Delta\Delta Ct}$. Error bars represent the 95% confidence intervals with letter grouping representing significance.

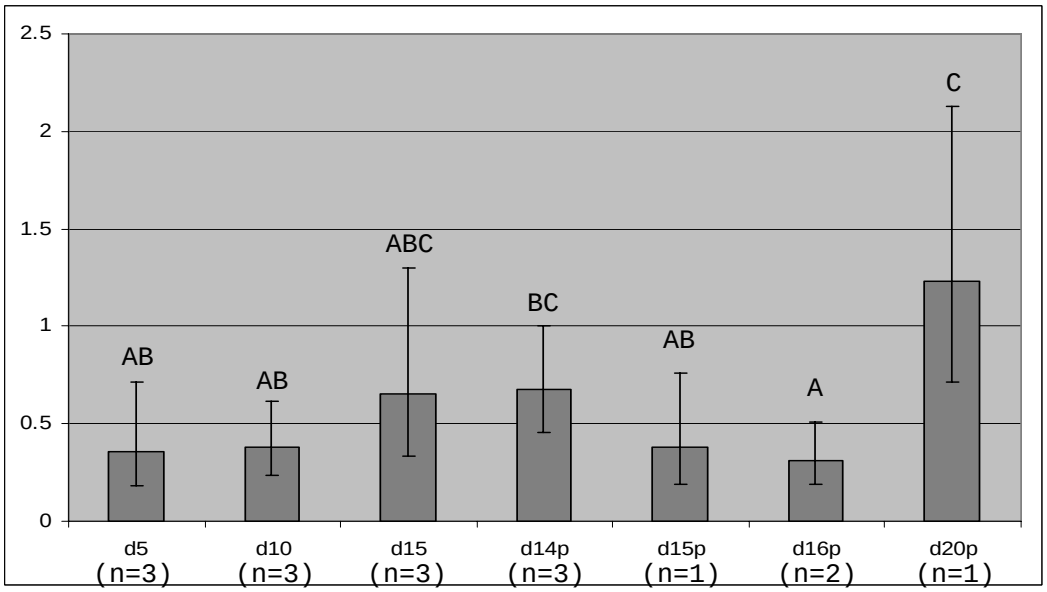

Figure 3-10. Analysis of qRT-PCR results for Group VIA tissue mRNA expression reported as $2^{-\Delta\Delta Ct}$. Error bars represent the 95% confidence intervals with letter grouping representing significance.

DISCUSSION

Mouse knock-out studies have identified both cyclooxygenases and PLA_2 Group IVA enzymes to be essential to many reproductive processes. Langenbach and colleagues demonstrated that COX-1 knockouts developed normally and were relatively healthy. Reproduction appeared to be normal in both male and female COX-1 deficient mice with normal litter size and development. Only the initiation of labor in homozygous COX-1 matings appeared to be problematic in some animals (Langenbach et al., 1995). Later studies found that labor could be initiated by administering $PGF_{2\alpha}$ suggesting a COX-1 specific pathway for $PGF_{2\alpha}$ production at the time of parturition (Sugimoto et al., 1997). COX-2 deficient mice exhibited marked differences when compared with normal mice or COX-1 deficient mice. COX-2 deficient mice showed decreased survivability with only 60% of pups surviving to weaning and only 75% of those survived a year (Morham et al., 1995). The surviving females were infertile (Morham et al., 1995). Lim and associates took an in depth look at the causes of

infertility in COX-2 deficient mice. The most likely cause of infertility was due to failed ovulation and severely hampered oocyte development. Outside of unsuccessful ovulation and oocyte maturation, the transfer of normal blasocysts into COX-2 deficient dams revealed severely restricted implantation and an inhibited decidualization reaction (Lim et al., 1997). Bonventre and associates demonstrated that PLA_2 Group IVA knockouts have similarities with both COX-1&2 knockouts. Like COX-1, Group IVA deficient mice were seen to develop normally with a typical lifespan. Unlike COX-1 knockouts, Group IVA deficient mice had decreased reproductive efficiency with decreased litter size and improper spacing of implantation sites (Bonventre et al., 1997; Song et al., 2002). The pups at parturition, though anatomically normal, were born dead due to problems with the initiation of labor (Bonventre et al., 1997) similar to results seen in COX-1 studies. Such results implicate Group IVA's involvement in reproduction though it is not clear what roles it plays in the processes of decidualization, implantation, and parturition. When both cyclooxygenase and Group IVA knockout results are

compared it is of interest that the COX-2 knockout resulted in sterility on many levels where as Group IVA knockout significantly reduced fertility but did not completely inhibit the reproductive process. Such results implicate that, though efficiency may be reduced, alternate PLA_2s may be responsible for, or act in redundancy with PLA2 GIVA in initiating AA release allowing for reproductive processes to continue.

Group IVA protein expression in endometrium samples was demonstrated to be consistent across the estrous cycle with similar levels of activity at different time points (Graf et al., 1999). These findings are consistent with statistical results from western blot analysis (Figures 3-1 and 3-2) which showed no difference in protein expression between endometrial epithelium samples from day 5, day 10 and day 15 of the estrous cycle despite being numerically increased at day 10 of the cycle. Group IVA mRNA expression in endometrium samples (Figure 3-8), containing stroma, glands, and blood vessels, was seen to have only a slight increase at day 10 of the estrous cycle of approximately 25% which is similar to previously seen results in endometrial samples (Graf

et al., 1999). This is puzzling due to the change in prostaglandin production during this time. Specifically, $PGF_{2\alpha}$ production increases as the estrous cycle progresses. PLA_2, the rate limiting enzyme in prostaglandin production, would be expected to alter its expression and/or activity. Conversely, Group IVA protein expression in endometrium samples appeared to remain relatively constant and shows no response to oxytocin stimulation despite the ability of broad spectrum PLA_2 inhibitors to block $PGF_{2\alpha}$ production (Lee and Silvia, 1994). Such results suggest an alternative regulation of the prostaglandin production pathway that may act through different tissues, a down stream enzyme in the cascade, another PLA_2 whose activity is yet to be characterized, or regulation of enzyme activation.

Interestingly, a distinct increase, by 16 fold, of Group IVA mRNA was seen in luminal epithelial scraping samples at day 10 of the cycle (Figure 3-7). This level then returned to a level similar to that seen on day 5 of the cycle by day 15. These results contradicted those previously seen (Graf et al., 1999) in endometrial samples. This discrepancy between

sample types seen both in COX-2 (Charpigny et al., 1997; Kim et al., 2003)and Group IVA expression identifies the key role that the epithelial cell layer plays in prostaglandin production and uterine regulation. Western blot analysis of endometrial epithelium also showed an increase at day 10 of the estrous cycle (Figures 3-1, 3-2, & 3-6). Unfortunately the increase is not significant when densitometry values were analyzed. With the development of internal controls, such as β-actin, allowing for normalization, gel to gel comparison and increased number of samples, the increased protein expression at day 10 of the cycle may become significant due to greater statistical power. The development of internal controls would be necessary since there are no pure forms for each of our target proteins currently available for standard curve generation in order to normalize the data obtained from densitometry analysis. These results are supported by the qRT-PCR findings in endometrial epithelium (Figure 3-7). Also, the naked eye detects a difference especially when viewing the western blot of our most representative samples (Figure 3-6).

These results follow more closely the physiological production of prostaglandin and reported production of prostaglandins specifically by the luminal endometrial epithelium (McCracken et al., 1972). Though the insight into Group IVA protein and mRNA expression is valuable it does not completely explain the regulation of prostaglandin production since there are multiple regulatory events controlling maximal activity, such as calcium influx and phosphorylation, yet to be characterized in this model.

PLA_2 expression during early pregnancy exhibits dramatic changes (Figures 3-3, 3-4 & 3-5). At day 14 of pregnancy, the time of early interferon-τ production by the trophoblast in sheep, group IVA protein was seen to be elevated when compared with day 15 of the estrous cycle (Figure 3-6). Group IVA mRNA was also increased 3 fold in luminal epithelial scrapings at this time (Figure 3-7). Conversely, interferon-τ was shown *in vitro* to suppress group IVA expression and decrease $PGF_{2\alpha}$ production in BEND cells stimulated by phorbol esters. Our results support in vivo prostaglandin production which increases during early pregnancy in sheep (Silvia et al., 1984). In addition,

the promoter region of the Group IVA gene contains multiple interferon response elements (Wu et al., 1994) which could result in its up regulation *in vivo*. Tamby and colleagues observed that PLA_2 activity decreased beginning at day 12 of pregnancy in the ewe (Tamby et al., 1993). Our results do not show a significant decrease in Group IVA protein or mRNA expression until day 16 of pregnancy in luminal epithelial scrapings (Figures 3-3 & 3-7). The discrepancy between the day of decrease in expression and activity is due to animal, sample, or methodology differences. Endometrium samples were observed to have no variation in Group IVA mRNA expression (Figure 3-8) between day 15 of the estrous cycle and all days of pregnancy tested. Also, an intact enzyme does not denote activity due to the involvement of further processing and cofactors resulting in maximal activity as discussed earlier. Interestingly, the decrease in Group IVA protein and mRNA expression on day 16 of pregnancy in our study coincides with maximal interferon-τ production by the embryo (Bartol et al., 1985; Godkin et al., 1982). Further study is needed

to determine if interferon-τ directly or indirectly stimulates these changes.

Interestingly, there was an appearance of 68 and 32kD Group IVA cross reactive proteins during days 15-17 of pregnancy with maximum expression on day 16 of pregnancy (Figures 3-4 & 3-6). The proteins were recognized by both the Group IV and Group IVA specific antibodies. These findings have not been reported in *in vivo* samples before, but similar findings have been demonstrated in studies of Group IVA cleavage by caspases(Adam-Klages et al., 1998; Luschen et al., 1998). Early reports demonstrated that caspase-3, 4, and 8 possessed the ability to cleave PLA_2 group IVA to enzymatically inactive 68/70kD and 32kD fragments (Adam-Klages et al., 1998; Luschen et al., 1998). Later studies found the 68/70kD fragment had greater membrane affinity than the intact enzyme resulting in dual competitive inhibition for membrane/substrate binding and phosphorylation (Atsumi et al., 2000). Caspase activity has been shown to be increased in the presence of interferon-α(Boccellino et al., 2004). Collectively this may suggest an interferon mediated mechanistic shift away from inflammation, similar to

that noted during apoptosis, allowing differentiation and successful implantation without initiation of an inflammatory response. Another point of interest is the timing of possible caspase cleavage of Group IVA. This appears to occur at or after day 16 of pregnancy. At this time the uterus is preparing for implantation and decidualization in sheep (Spencer et al., 2004). This is a time of cellular reorganization in which caspases have been implied in other tissues to play an essential role in the cascade signaling for terminal differentiation (Okuyama et al., 2004) resulting in the restructuring of cellular membranes and function.

A final result of interest is the lack of change in Group VIA mRNA with in both endometrium and endometrial epithelial samples (Figures 3-9 & 3-10). Previous studies had shown a sharp decrease in $PGF_{2\alpha}$ production when Group VIA was inhibited by bromoenol lactone in endometrial cell cultures under the stimulation of oxytocin (Tithof et al., 2007). Such results implicate changes in Group VIA expression and/or activity as a key factor in $PGF_{2\alpha}$ production at the time of luteolysis. Our mRNA and western blot results did not support such findings *in vivo*.

Western Blots (not pictured) were seen to be inconclusive yielding a faint unidentified 50kD band that was constitutively expressed. A similar band was recognized as a caspase-3 cleavage product by Lauber and associates but it's activity was never described (Lauber et al., 2003). Such results may be due to the lack of sheep specific antibodies. The qRT-PCR results displayed minimal expression of Group VIA when compared with Group IVA, with little change between day of the cycle or early pregnancy in both endometrial and endometrial epithelium samples.

A preliminary result sparking curiosity was that a previous preparation of samples abandoned by our lab involving multiple high speed centrifugations after sonication, yielded a 28kD band that appeared to increased during the estrous cycle and become diminished in early pregnancy. Though further research is needed for verification, this could be the result of Group VIA cleavage by caspase. Group VIA can be cleaved by caspase-3 resulting in a 28kD fragment that increases fatty acid release in apoptotic cells (Atsumi et al., 2000; Lauber et al., 2003). If this were the case, the rapidly changing

endometrial cells may undergo a change resulting in an increase in localized caspase-3 activity that would splice the small amounts of Group VIA into active 28kD enzymes resulting in increased activity. Zhoa and colleagues have identified a unique pathway in ovarian cancer cells that does not induce apoptosis in which Caspase-3 cleaved Group VIA produces lysophosphatidic acid which in turn initiates the activation of Group IVA causing an increase in AA production (Zhao et al., 2006). Increased release of AA by cleaved Group VIA and activated Group IVA may result in elevated prostaglandin production that is seen in response to oxytocin. Further investigation of protein expression and activity is needed using PLA_2 activity assays and western blot analysis to verify this theory.

These findings further implicate the role of PLA_2s in complex regulations that maintain proper uterine function during the estrous cycle and early pregnancy. Though PLA_2's expression is not the sole regulator of prostaglandin production, PLA_2s do play an integral role that is tissue and cell type specific. Their roles appear to be intertwined with the processes of the estrous cycle and early pregnancy both as

substrates and enzymes within multiple cascades that allow for their activity in divergent processes. Future studies should explore the expression of other PLA_2s, such as secretory PLA_2s, in these tissues as well as the activity of all studied isoforms to further elucidate the relevance of the previous findings.

REFERENCES

Ackermann, E. J., and E. A. Dennis. 1995. Mammalian calcium-independent phospholipase a2. Biochim Biophys Acta 1259: 125-136.

Ackermann, E. J., E. S. Kempner, and E. A. Dennis. 1994. Ca(2+)-independent cytosolic phospholipase a2 from macrophage-like p388d1 cells. Isolation and characterization. J Biol Chem 269: 9227-9233.

Adam-Klages, S. et al. 1998. Caspase-mediated inhibition of human cytosolic phospholipase a2 during apoptosis. J Immunol 161: 5687-5694.

Adunyah, S., T. Unlap, F. Wagner, and A. Kraft. 1991. Regulation of c-jun expression and dap-1 enhancer activity by granulocyte-macrophage colony stimulating factor. The Journal of Biological Chemistry 266: 5670-5675.

Akiba, S., M. Hayama, and T. Sato. 1998. Inhibition of ca2+-independent phospholipase a2 by bromoenol lactone attenuates prostaglandin generation induced by interleukin-1 beta and dibutyryl camp in rat mesangial cells. FEBS Lett 437: 225-228.

Akiba, S., S. Mizunaga, K. Kume, M. Hayama, and T. Sato. 1999. Involvement of group vi ca2+-independent phospholipase a2 in protein kinase c-dependent arachidonic acid liberation in zymosan-stimulated macrophage-like p388d1 cells. J Biol Chem 274: 19906-19912.

Akiba, S. et al. 2002. Protein kinase calpha-dependent increase in ca2+-independent phospholipase a2 in membranes and arachidonic acid liberation in zymosan-stimulated macrophage-like p388d1 cells. Biochem Pharmacol 63: 1969-1977.

Arosh, J. A. et al. 2004. Effect of interferon-tau on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: Evidence of polycrine actions of prostaglandin e2. Endocrinology 145: 5280-5293.

Arosh, J. A., J. Parent, P. Chapdelaine, J. Sirois, and M. A. Fortier. 2002. Expression of cyclooxygenases 1 and 2 and prostaglandin e synthase in bovine endometrial tissue during the estrous cycle. Biol Reprod 67: 161-169.

Asselin, E., D. Lacroix, and M. A. Fortier. 1997. Ifn-tau increases pge2 production and cox-2 gene expression in the bovine endometrium in vitro. Mol Cell Endocrinol 132: 117-126.

Atsumi, G. et al. 2000. Distinct roles of two intracellular phospholipase a2s in fatty acid release in the cell death pathway. Proteolytic fragment of type iva cytosolic phospholipase a2alpha inhibits stimulus-induced arachidonate release, whereas that of type vi ca2+-independent phospholipase a2 augments spontaneous fatty acid release. J Biol Chem 275: 18248-18258.

Baburina, I., and S. Jackowski. 1999. Cellular responses to excess phospholipid. J Biol Chem 274: 9400-9408.

Balboa, M. A., Y. SÃ¡ez, and J. Balsinde. 2003. Calcium-independent phospholipase a2 is required for lysozyme secretion in u937 promonocytes. J Immunol 170: 5276-5280.

Barbour, S. E., A. Kapur, and C. L. Deal. 1999. Regulation of phosphatidylcholine homeostasis by calcium-independent phospholipase a2. Biochim Biophys Acta 1439: 77-88.

Barcikowski, B., J. C. Carlson, L. Wilson, and J. A. McCracken. 1974. The effect of endogenous and exogenous estradiol-17beta on the release of prostaglandin f2alpha from the ovine uterus. Endocrinology 95: 1340-1349.

Bartol, F. F. et al. 1985. Characterization of proteins produced in vitro by periattachment bovine conceptuses. Biol Reprod 32: 681-693.

Baumbach, G. A., R. T. Duby, and J. D. Godkin. 1990. N-glycosylated and unglycosylated forms of caprine trophoblast protein-1 are secreted by preimplantation goat conceptuses. Biochem Biophys Res Commun 172: 16-21.

Bazer, F. W., J. Fleming, S. Safe, and T. E. Spencer. 2003. The ovine oxytocin receptor prometer/enhancer region is responsive to estrogen receptor alpha. Biology of Reproduction 68.

Bazer, F. W., T. E. Spencer, and T. L. Ott. 1997. Interferon tau: A novel pregnancy recognition signal. Am J Reprod Immunol 37: 412-420.

Binelli, M. et al. 2000. Interferon-tau modulates phorbol ester-induced production of prostaglandin and expression of cyclooxygenase-2 and phospholipase-a(2) from bovine endometrial cells. Biol Reprod 63: 417-424.

Birbes, H., E. Gothie, J. F. Pageaux, M. Lagarde, and C. Laugier. 2000. Hydrogen peroxide activation of ca(2+)-independent phospholipase a(2) in uterine stromal cells. Biochem Biophys Res Commun 276: 613-618.

Boccellino, M. et al. 2004. Apoptosis induced by interferon-alpha and antagonized by egf is regulated by caspase-3-mediated cleavage of gelsolin in human epidermoid cancer cells. J Cell Physiol 201: 71-83.
Bonventre, J. V. et al. 1997. Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase a2. Nature 390: 622-625.
Brant, K., and R. L. Caruso. 2005. Late-gestation rat myometrial cells express multiple isoforms of phospholipase a2 that mediate pcb 50-induced release of arachidonic acid with coincident prostaglandin production. Toxicol Sci 88: 222-230.
Brant, K., W. Guan, P. Tithof, and R. L. Caruso. 2006. Gestation age-related increase in 50-kda rat uterine calcium-independent phospholipase a2 expression influences uterine sensitivity to polychlorinated biphenyl stimulation. Biol Reprod 74: 874-880.
Burch, R. M., A. Luini, and J. Axelrod. 1986. Phospholipase a2 and phospholipase c are activated by distinct gtp-binding proteins in response to alpha 1-adrenergic stimulation in frtl5 thyroid cells. Proc Natl Acad Sci U S A 83: 7201-7205.
Burns, P. D., G. A. Graf, S. H. Hayes, and W. J. Silvia. 1997. Cellular mechanisms by which oxytocin stimulates uterine pgf2 alpha synthesis in bovine endometrium: Roles of phospholipases c and a2. Domest Anim Endocrinol 14: 181-191.
Burns, P. D., G. A. Graf, S. H. Hayes, and W. J. Silvia. 2000. Effect of oxytocin on expression of cytosolic phospholipase a2 mrna and protein in ovine endometrial tissue in vivo. Domest Anim Endocrinol 19: 237-246.
Channon, J. Y., and C. C. Leslie. 1990. A calcium-dependent mechanism for associating a soluble arachidonoyl-hydrolyzing phospholipase a2 with membrane in the macrophage cell line raw 264.7. J Biol Chem 265: 5409-5413.
Charpigny, G. et al. 1997. Expression of cyclooxygenase-1 and -2 in ovine endometrium during the estrous cycle and early pregnancy. Endocrinology 138: 2163-2171.
Chen, Y. et al. 2006. Effect of interferon-tau administration on endometrium of nonpregnant ewes: A comparison with pregnant ewes. Endocrinology 147: 2127-2137.
Costine, B. A., E. K. Inskeep, K. P. Blemings, J. A. Flores, and M. E. Wilson. 2007. Mechanisms of reduced luteal sensitivity to prostaglandin f2alpha during

maternal recognition of pregnancy in ewes. Domest Anim Endocrinol 32: 106-121.
de Carvalho, M. G. et al. 1996. Identification of phosphorylation sites of human 85-kda cytosolic phospholipase a2 expressed in insect cells and present in human monocytes. J Biol Chem 271: 6987-6997.
Emond, V. et al. 2004. Expression of cyclooxygenase-2 and granulocyte-macrophage colony-stimulating factor in the endometrial epithelium of the cow is up-regulated during early pregnancy and in response to intrauterine infusions of interferon-tau. Biol Reprod 70: 54-64.
Ezashi, T., A. D. Ealy, M. C. Ostrowski, and R. M. Roberts. 1998. Control of interferon-tau gene expression by ets-2. Proc Natl Acad Sci U S A 95: 7882-7887.
Farin, C. E., K. Imakawa, and R. M. Roberts. 1989. In situ localization of mrna for the interferon, ovine trophoblast protein-1, during early embryonic development of the sheep. Mol Endocrinol 3: 1099-1107.
Flint, A. P., H. J. Stewart, G. E. Lamming, and J. H. Payne. 1992. Role of the oxytocin receptor in the choice between cyclicity and gestation in ruminants. J Reprod Fertil Suppl 45: 53-58.
Forsell, P., B. Kennedy, and H. Claesseon. 1999. The human calcium-independent phospholipase a2 gene multiple enzymes with distinct properties from a single gene. European Journal of Biochemistry 262: 575-585.
Godkin, J. D., F. W. Bazer, J. Moffatt, F. Sessions, and R. M. Roberts. 1982. Purification and properties of a major, low molecular weight protein released by the trophoblast of sheep blastocysts at day 13-21. J Reprod Fertil 65: 141-150.
Godkin, J. D., F. W. Bazer, and R. M. Roberts. 1984. Ovine trophoblast protein 1, an early secreted blastocyst protein, binds specifically to uterine endometrium and affects protein synthesis. Endocrinology 114: 120-130.
Goff, A. K. 2004. Steroid hormone modulation of prostaglandin secretion in the ruminant endometrium during the estrous cycle. Biol Reprod 71: 11-16.
Graf, G. A., P. D. Burns, and W. J. Silvia. 1999. Expression of a cytosolic phospholipase a2 by ovine endometrium on days 11-14 of a simulated oestrous cycle. J Reprod Fertil 115: 357-363.
Graf, G. A., P. D. Burns, and W. J. Silvia. 1999. Expression of a cytosolic phospholipase a2 by ovine endometrium on days 11-14 of a simulated oestrous cycle. J Reprod Fertil 115: 357-363.

Graler, M. H., and E. J. Goetzl. 2002. Lysophospholipids and their g protein-coupled receptors in inflammation and immunity. Biochim Biophys Acta 1582: 168-174.
Hallak, H. et al. 1994. Covalent binding of arachidonate to g protein alpha subunits of human platelets. J Biol Chem 269: 4713-4716.
Hamberg, M., and B. Samuelsson. 1967. On the mechanism of the biosynthesis of prostaglandins e-1 and f-1-alpha. J Biol Chem 242: 5336-5343.
Hamberg, M., and B. Samuelsson. 1973. Detection and isolation of an endoperoxide intermediate in prostaglandin biosynthesis. Proc Natl Acad Sci U S A 70: 899-903.
Hansen, T. R. et al. 1999. Mechanism of action of interferon-tau in the uterus during early pregnancy. J Reprod Fertil Suppl 54: 329-339.
Hazan, I., R. Dana, Y. Granot, and R. Levy. 1997. Cytosolic phospholipase a2 and its mode of activation in human neutrophils by opsonized zymosan. Correlation between 42/44 kda mitogen-activated protein kinase, cytosolic phospholipase a2 and nadph oxidase. Biochem J 326 (Pt 3): 867-876.
Henderson, K. M., and K. P. McNatty. 1975. A biochemical hypothesis to explain the mechanism of luteal regression. Prostaglandins 9: 779-797.
Herbert, S. P., and J. H. Walker. 2006. Group via calcium-independent phospholipase a2 mediates endothelial cell s phase progression. J Biol Chem 281: 35709-35716.
Imakawa, K. et al. 1987. Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. Nature 330: 377-379.
Imakawa, K., S. D. Helmer, K. P. Nephew, C. S. Meka, and R. K. Christenson. 1993. A novel role for gm-csf: Enhancement of pregnancy specific interferon production, ovine trophoblast protein-1. Endocrinology 132: 1869-1871.
Inskeep, E. K., W. J. Smutny, R. L. Butcher, and J. E. Pexton. 1975. Effects of intrafollicular injections of prostaglandins in non-pregnant and pregnant ewes. J Anim Sci 41: 1098-1104.
Ireland JJ, Murphee RL, and C. PB. 1980. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. Journal of Dairy Science 63: 155-160.

Ivell, R., and N. Walther. 1999. The role of sex steroids in the oxytocin hormone system. Mol Cell Endocrinol 151: 95-101.
Jackson J et al. 1998. The role of platelet activation factor and other lipid mediators in inflammatory angiogenesis. Biochimica et Biophysica Acta 1392: 145-152.
Jelsema, C. 1987. Light activation of phospholipase a2 in rod outer segments of bovine retina and its modulation by gtp-binding proteins. Journal of Biological Chemistry 262: 163-168.
Kaluz, S., P. A. Fisher, M. Kaluzova, E. L. Sheldrick, and A. P. Flint. 1996. Structure of an ovine interferon receptor and its expression in endometrium. J Mol Endocrinol 17: 207-215.
Kim S, Choi Y, Spencer T, and B. F. 2003. Effects of the estrous cycle, pregnancy and interferon tau on expression of cyclooxygenase two in ovine endometrium. Journal of Reproductive Biology and Endocrinology 1.
Kujubu, D. A., B. S. Fletcher, B. C. Varnum, R. W. Lim, and H. R. Herschman. 1991. Tis10, a phorbol ester tumor promoter-inducible mrna from swiss 3t3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem 266: 12866-12872.
Langenbach, R., C. D. Loftin, C. Lee, and H. Tiano. 1999. Cyclooxygenase-deficient mice. A summary of their characteristics and susceptibilities to inflammation and carcinogenesis. Ann N Y Acad Sci 889: 52-61.
Langenbach, R. et al. 1995. Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. Cell 83: 483-492.
Larsson P, Claesson HE, and K. BP. 1998. Multiple splice variants of the human calcium-independent phospholipase a2 and their effect on enzyme activity. Journal of Biological Chemistry 273: 207-214.
Lauber, K. et al. 2003. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. Cell 113: 717-730.
Lee J, and Silvia. 1994. Cellular mechanisms mediating the stimulation of ovine endometrial secretion of prostaglandin $f_{2\alpha}$ in response to oxytocin: The role of phoslpholipase a_2. Journal of Endocrinology 141: 491-496.
Lee S, and L. L. 1975. Purification and regulatory properties of chicken heart prostaglandin e 9-

ketoreductase. Journal of Biological Chemistry 250: 4549-4555.
Liggins CG, Compos GA, Roberts CM, and S. SJ. 1980. Production rates of prostaglandin f, 6keto-pgf1a and thromboxane b2 by perfused human endometrium. Prostaglandins 19: 461-477.
Lim, H. et al. 1997. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 91: 197-208.
Liou J et al. 2000. Co-localization of prostacyclin synthase with prostaglandin h sythase-1 but not phorbol ester induced pghs-2 in cultured endothelial cells. Journal of Biological Chemistry 275: 15314-15320.
Luong C et al. 1996. Flexibility of the nsaid binding site in the structure of human cyclooxygenase-2. Nature Structural Biology 3: 927-933.
Luschen, S., S. Ussat, M. Kronke, and S. Adam-Klages. 1998. Cleavage of human cytosolic phospholipase a2 by caspase-1 (ice) and caspase-8 (flice). Biochem Biophys Res Commun 253: 92-98.
Ma, Z. M. et al. 2001. Studies of insulin secretory responses and of arachidonic acid incorporation into phospholipids of stably transfected insulinoma cells that overexpress group via phospholipase a(2) (ipla(2)beta) indicate a signaling rather than a housekeeping role for ipla(2)beta. Journal Of Biological Chemistry 276: 13198-13208.
Madore, E. et al. 2003. An aldose reductase with 20 alpha-hydroxysteroid dehydrogenase activity is most likely the enzyme responsible for the production of prostaglandin f2 alpha in the bovine endometrium. J Biol Chem 278: 11205-11212.
Magness, R. R. et al. 1981. Effect of chronic ipsilateral or contralateral intrauterine infusion of prostaglandin e2 (pge2) on luteal function of unilaterally ovariectomized ewes. Prostaglandins Med 6: 389-401.
Manguikian, A. D., and S. E. Barbour. 2004. Cell cycle dependence of group via calcium-independent phospholipase a2 activity. J Biol Chem 279: 52881-52892.
Mann, G. E., J. H. Payne, and G. E. Lamming. 2001. Hormonal regulation of oxytocin-induced prostaglandin f(2alpha) secretion by the bovine and ovine uterus in vivo. Domest Anim Endocrinol 21: 127-141.

Marcus, G. J. 1981. Prostaglandin formation by the sheep embryo and endometrium as an indication of maternal recognition of pregnancy. Biol Reprod 25: 56-64.
Martal, J. et al. 1997. Recent developments and potentialities for reducing embryo mortality in ruminants: The role of ifn-tau and other cytokines in early pregnancy. Reprod Fertil Dev 9: 355-380.
McCracken, J. A. 1980. Hormone receptor control of prostaglandin f2 alpha secretion by the ovine uterus. Adv Prostaglandin Thromboxane Res 8: 1329-1344.
McCracken, J. A. et al. 1972. Prostaglandin f 2 identified as a luteolytic hormone in sheep. Nat New Biol 238: 129-134.
McCracken, J. A., E. E. Custer, J. A. Eldering, and A. G. Robinson. 1996. The central oxytocin pulse generator: A pacemaker for the ovarian cycle. Acta Neurobiol Exp (Wars) 56: 819-832.
Miyamoto Y, Skarzynski DJ, and O. K. 2000. Is tumor necrosis factor a trigger for the initiation of endometrial prostaglandin f_2 release at luteolysis in cattle? Biology of Reproduction 62: 1109-1115.
Morham, S. G. et al. 1995. Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. Cell 83: 473-482.
Murakami, M. et al. 2000. Regulation of prostaglandin e2 biosynthesis by inducible membrane-associated prostaglandin e2 synthase that acts in concert with cyclooxygenase-2. Journal of Biological Chemistry 275: 32783-32792.
Murdoch, W. J., and M. L. Farris. 1988. Prostaglandin e2-9-ketoreductase activity of preovulatory ovine follicles. J Anim Sci 66: 2924-2929.
Nakashima, K. et al. 2003. Coupling between cyclooxygenases and prostaglandin f2a synthase detection of an inducible, glutathione-activated, membrane-bound prostaglandin f2a-synthetic activity. Molecular and Cell Biology of Lipids 1633: 96-105.
Nakatani, Y., T. Tanioka, S. Sunaga, M. Murakami, and K. I. 2000. Identification of a cellular protein that functionally interacts with the c2 domain of cytosolic phospholipase a2. 275: 1161-1168.
Nancarrow, C. D., B. M. Evison, and P. J. Connell. 1982. Effect of embryos on luteolysis and termination of early pregnancy in sheep with cloprostenol. Biol Reprod 26: 263-269.

Ni, H., T. Sun, N. Z. Ding, X. H. Ma, and Z. M. Yang. 2002. Differential expression of microsomal prostaglandin e synthase at implantation sites and in decidual cells of mouse uterus. Biol Reprod 67: 351-358.

Ogorochi, T., M. Ujihara, and S. Narumiya. 1987. Purification and properties of prostaglandin h-e isomerase from the cytosol of human brain: Identification as anionic forms of glutathione s-transferase. J Neurochem 48: 900-909.

Okuyama, R. et al. 2004. High commitment of embryonic keratinocytes to terminal differentiation through a notch1-caspase 3 regulatory mechanism. Dev Cell 6: 551-562.

Perez, R., R. Melero, M. A. Balboa, and J. Balsinde. 2004. Role of group via calcium-independent phospholipase a2 in arachidonic acid release, phospholipid fatty acid incorporation, and apoptosis in u937 cells responding to hydrogen peroxide. J Biol Chem 279: 40385-40391.

Pate, J. L. 1988. Regulation of prostaglandin synthesis by progesterone in the bovine corpus luteum. Prostaglandins 36: 303-315.

Peterson, A. J., H. R. Tervit, R. J. Fairclough, P. G. Havik, and J. F. Smith. 1976. Jugular levels of 13, 14-dihydro-15-keto-prostaglandin f and progesterone around luteolysis and early pregnancy in the ewe. Prostaglandins 12: 551-558.

Picot, D., P. J. Loll, and R. M. Garavito. 1994. The x-ray crystal structure of the membrane protein prostaglandin h2 synthase-1. Nature 367: 243-249.

Poyser, N. L. 1995. The control of prostaglandin production by the endometrium in relation to luteolysis and menstruation. Prostaglandins Leukot Essent Fatty Acids 53: 147-195.

Pratt, B. R., R. L. Butcher, and E. K. Inskeep. 1977. Antiluteolytic effect of the conceptus and of pge2 in ewes. J Anim Sci 45: 784-791.

Qiu, Z. H., M. S. de Carvalho, and C. C. Leslie. 1993. Regulation of phospholipase a2 activation by phosphorylation in mouse peritoneal macrophages. J Biol Chem 268: 24506-24513.

Ramanadham, S., F. F. Hsu, A. Bohrer, Z. Ma, and J. Turk. 1999. Studies of the role of group vi phospholipase a2 in fatty acid incorporation, phospholipid remodeling, lysophosphatidylcholine generation, and secretagogue-induced arachidonic acid release in pancreatic islets and insulinoma cells. J Biol Chem 274: 13915-13927.

Reingold, D., A. Kawasaki, and N. P. 1981. A novel prostaglandin 11-keto reductase found in rabbit liver. Biochimica et Biophysica Acta 659: 179-188.
Roberts, J. S., J. A. McCracken, J. E. Gavagan, and M. S. Soloff. 1976. Oxytocin-stimulated release of prostaglandin f2alpha from ovine endometrium in vitro: Correlation with estrous cycle and oxytocin-receptor binding. Endocrinology 99: 1107-1114.
Roberts, R. M., L. Liu, Q. Guo, D. Leaman, and J. Bixby. 1998. The evolution of the type i interferons. J Interferon Cytokine Res 18: 805-816.
Saavedra, G., W. Zhang, B. Peterson, and B. S. Cummings. 2006. Differential roles for cytosolic and microsomal ca2+-independent phospholipase a2 in cell growth and maintenance of phospholipids. J Pharmacol Exp Ther 318: 1211-1219.
Schalkwijk, C. G. et al. 1996. Maximal epidermal growth-factor-induced cytosolic phospholipase a2 activation in vivo requires phosphorylation followed by an increased intracellular calcium concentration. Biochem J 313 (Pt 1).
Schams, D., and B. Berisha. 2004. Regulation of corpus luteum function in cattle--an overview. Reprod Domest Anim 39: 241-251.
Schievella, A. R., M. K. Regier, W. L. Smith, and L. L. Lin. 1995. Calcium-mediated translocation of cytosolic phospholipase a2 to the nuclear envelope and endoplasmic reticulum. J Biol Chem 270: 30749-30754.
Sheldrick, E. L., and A. P. Flint. 1985. Endocrine control of uterine oxytocin receptors in the ewe. J Endocrinol 106: 249-258.
Shitashige, M., I. Morita, and S. Murota. 1998. Different substrate utilization between prostaglandin endoperoxide h synthase-1 and -2 in nih3t3 fibroblasts. Biochim Biophys Acta 1389: 57-66.
Silvia, W. J., and G. D. Niswender. 1986. Maintenance of the corpus luteum of early pregnancy in the ewe. Iv. Changes in luteal sensitivity to prostaglandin f2 alpha throughout early pregnancy. J Anim Sci 63: 1201-1207.
Silvia, W. J., J. S. Ottobre, and E. K. Inskeep. 1984. Concentrations of prostaglandins e2, f2 alpha and 6-keto-prostaglandin f1 alpha in the utero-ovarian venous plasma of nonpregnant and early pregnant ewes. Biol Reprod 30: 936-944.

Six, D. A., and E. A. Dennis. 2000. The expanding superfamily of phospholipase a(2) enzymes: Classification and characterization. Biochim Biophys Acta 1488: 1-19.
Song, H., S. Bao, S. Ramanadham, and T. J. 2006. Effects of biological oxidants on the catalytic activity and structure of group via phospholipase a2. Biochemistry 45: 6392-6406.
Song, H., H. Lim, and P. B. 2002. Cytosolic phopholipase a2 alpha is deficiency is crucial for "on-time" embryo implantation that directs subsequent development. Development 129: 2879-2889.
Song, I., and W. L. Smith. 1996. C-terminal ser/pro-thr-glu-leu tetrapeptides of prostaglandin endoperoxide h synthases-1 and -2 target the enzymes to the endoplasmic reticulum. Arch Biochem Biophys 334: 67-72.
Spencer, T. E., R. C. Burghardt, G. A. Johnson, and F. W. Bazer. 2004. Conceptus signals for establishment and maintenance of pregnancy. Anim Reprod Sci 82-83.
Spencer, T. E., G. A. Johnson, F. W. Bazer, and R. C. Burghardt. 2004. Implantation mechanisms: Insights from the sheep. Reproduction 128: 657-668.
Spencer, T. E., G. A. Johnson, F. W. Bazer, R. C. Burghardt, and M. Palmarini. 2007. Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. Reprod Fertil Dev 19: 65-78.
Spencer, T. E. et al. 1996. Effects of interferon-tau and progesterone on oestrogen-stimulated expression of receptors for oestrogen, progesterone and oxytocin in the endometrium of ovariectomized ewes. Reprod Fertil Dev 8: 843-853.
Steer, S. A., K. C. Wirsig, M. H. Creer, D. A. Ford, and J. McHowat. 2002. Regulation of membrane-associated ipla2 activity by a novel pkc isoform in ventricular myocytes. Am J Physiol Cell Physiol 283: C1621-1626.
Sugimoto, Y. et al. 1997. Failure of parturition in mice lacking the prostaglandin f receptor. Science 277: 681-683.
Swinney, D. C., A. Y. Mak, J. Barnett, and C. S. Ramesha. 1997. Differential allosteric regulation of prostaglandin h synthase 1 and 2 by arachidonic acid. J Biol Chem 272: 12393-12398.
Tamby, J. P., G. Charpigny, P. Reinaud, and J. Martal. 1993. Phospholipase a2 activity in endometrium from

early pregnant and non-pregnant ewes. Prostaglandins 46: 407-415.
Tang, J. et al. 1997. A novel cytosolic calcium-independent phospholipase a2 contains eight ankyrin motifs. J Biol Chem 272: 8567-8575.
Tithof, P. K., M. P. Roberts, W. Guan, M. Elgayyar, and J. D. Godkin. 2007. Distinct phospholipase a2 enzymes regulate prostaglandin e2 and f2alpha production by bovine endometrial epithelial cells. Reproductive Biology and Endocrinology 5.
Ueno, N. et al. 2001. Coupling between cyclooxygenase, terminal prostanoid synthase, and phospholipase a2. J Biol Chem 276: 34918-34927.
Uozumi, N. et al. 1997. Role of cytosolic phospholipase a2 in allergic response and parturition. Nature 390: 618-622.
Urade, Y., K. Watanabe, and O. Hayaishi. 1995. Prostaglandin d, e, and f synthases. J Lipid Mediat Cell Signal 12: 257-273.
Wang, Z. et al. 2005. Group via phospholipase a2 forms a signaling complex with the calcium/calmodulin-dependent protein kinase iibeta expressed in pancreatic islet beta-cells. J Biol Chem 280: 6840-6849.
Watanabe, K., T. Shimizu, and H. O. 1981. Enzymatic conversion of prostaglandin d2 to f2 alpha in the rat lung. Biochemistry International 2: 603-610 %.
Watanabe, K., R. Yoshida, and O. H. Shimizu T. 1985. Enzymatic formation of prostaglandin f2, from prostaglandin h2 and d2. Journal of Biological Chemistry 260: 7035-7041.
Waterman, W. H., T. F. Molski, C. K. Huang, J. L. Adams, and R. I. Sha'afi. 1996. Tumour necrosis factor-alpha-induced phosphorylation and activation of cytosolic phospholipase a2 are abrogated by an inhibitor of the p38 mitogen-activated protein kinase cascade in human neutrophils. Biochem J 319 (Pt 1): 17-20.
Wathes, D. C., and M. Hamon. 1993. Localization of oestradiol, progesterone and oxytocin receptors in the uterus during the oestrous cycle and early pregnancy of the ewe. J Endocrinol 138: 479-492.
Winstead, M. V., J. Balsinde, and D. Ea. 2000. Calcium-independent phospholipase a2: Structure and function. Biochemica et Biophysica Acta 1488: 28-39.
Wolf, M. J., and R. W. Gross. 1996. Expression, purification, and kinetic characterization of a

recombinant 80-kda intracellular calcium-independent phospholipase a2. J Biol Chem 271: 30879-30885.
Wong, P. 1981. Purification and partial characterization of prostaglandin d2 11-keto reductase in rabbit liver. Biochimica at Biophysica Acta 659: 169-178.
Wu, T., T. Ikezono, C. W. Angus, and J. H. Shelhamer. 1994. Characterization of the promoter for the human 85 kda cytosolic phospholipase a2 gene. Nucleic Acids Res 22: 5093-5098.
Yamaguchi H, I. Y., Taylor A, Katsumura M, Miyazawa T, Takahashi E, Imakawa K. 1999. Effects of pma and transcription factors on ovine interferon-tau transactivation in various cell lines. Endocrine Journal 46: 383-388.
Yellaturu, C. R., and G. N. Rao. 2003. A requirement for calcium-independent phospholipase a2 in thrombin-induced arachidonic acid release and growth in vascular smooth muscle cells. J Biol Chem 278: 43831-43837.
Yuan, J. S., A. Reed, F. Chen, and C. N. Stewart, Jr. 2006. Statistical analysis of real-time pcr data. BMC Bioinformatics 7: 85.
Zarco, L., G. H. Stabenfeldt, S. Basu, G. E. Bradford, and H. Kindahl. 1988. Modification of prostaglandin f-2 alpha synthesis and release in the ewe during the initial establishment of pregnancy. J Reprod Fertil 83: 527-536.
Zhao, X. et al. 2006. Caspase-3-dependent activation of calcium-independent phospholipase a2 enhances cell migration in non-apoptotic ovarian cancer cells. Journal of Biological Chemistry 281: 29357-29368.

VITA

Gregory J. Ochs Jr. was born in West Monroe, Louisiana, on January 9, 1980, and spent his childhood living around the southeast. He graduated from the University School of Jackson in 1998 and attended Mississippi State University. After receiving a bachelor’s degree in Animal Science, Greg spent 3 years traveling and working at a ski resort in Wyoming and as a fishing guide in Alaska. He then returned to school in 2004 at the University of Tennessee and completed a Master of Science degree in Animal Science in 2007.