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Temporal expression of protein mediators during PGF2alpha-induced luteolysis in sheep

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TEMPORAL EXPRESSION OF PROTEIN MEDIATORS DURING PGF2α-INDUCED LUTEOLYSIS IN SHEEP

BY

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ABSTRACT

TEMPORAL EXPRESSION OF PROTEIN MEDIATORS DURING PGF2α-INDUCED LUTEOLYSIS IN SHEEP

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To study dynamic in vivo biochemical changes within the corpus luteum (CL) during luteolysis, a physiological model mimicking the onset of natural luteolysis was established, in which sheep received sequential systemic pulses of PGF2α (20ug/min/1hr) at mid-luteal phase of the estrous cycle. We previously demonstrated that after one pulse of PGF2α, the protein levels of extracellular matrix (ECM) regulators, tissue inhibitors of metalloproteinases (TIMPs) -1 and -2 decreased dramatically within one hour of PGF2α infusion, while matrix metalloproteinase (MMP)-2 activity increased 4 hrs post infusion. Protein levels of steroidogenic acute regulatory (StAR) protein decreased (40%) at 8 hrs, which paralleled the decline in peripheral plasma progesterone (P), before recovering by 16 and 24 hrs. COX-1 was unchanged, while COX-2 displayed a dramatic increase (~300%) at 16 and 24 hrs compared to all other time points. In the present study, we examined the protein expression patterns of TIMP-1, TIMP-2, MMP-2, MMP-9, COX-1, COX-2, and StAR following two systemic pulses of PGF2α (20ug/min/1hr) given 16 hrs apart at mid-luteal phase. Corpora lutea were removed surgically 1 hr before and 1, 8, 16, and 24 hrs after the second pulse of PGF2α (n=4 sheep/time point). Peripheral blood

for monitoring plasma progesterone (P) was collected hourly before and after each pulse of PGF2α. The TIMP-1 protein showed a sharp decline (~50%; $p<0.05$) one hour post-infusion before returning to control values by 8 hrs and continuing to rise above control levels by 24 hrs (30%; $p<0.05$). The TIMP-2 protein also declined sharply 1 hr and remained low throughout the sampling period. Active MMP-2 increased 1 hr following infusion (~20%; $p<0.05$), while COX-1, and COX-2, and StAR protein levels showed no change compared to controls. In summary, the early and dramatic decrease in TIMP-1 and TIMP-2 proteins, accompanied by an increase in MMP-2 activity, indicate an extension and amplification by the second pulse of PGF2α on regulators of the ECM within the CL. These findings confirm a critical role for regulators of the ECM in mediating both structural and steroidogenic changes during physiological PGF2α-induced luteolysis in sheep.

CHAPTER I

TEMPORAL EXPRESSION OF PROTEIN MEDIATORS DURING PGF2α-INDUCED LUTEOLYSIS IN SHEEP: A LITERATURE REVIEW

Introduction

The goal of all organisms is to pass their genetic information to subsequent generations. To accomplish this, mammals reproduce sexually. It can be said that all facets of an organism's life, ranging from growth, development, nutritional health, and physiological functions, can be attributed to facilitating reproduction. The ability of mammals to reproduce is dependent on several key features of sexual reproduction. There must be a component contributed by a male, the sperm, and from the female, the ovum. The research that is covered under the present review is limited to female reproduction, specifically the ovarian follicle and a gland called the corpus luteum (CL), whose functions are essential for pregnancy. It has been reported and widely accepted that both of these reproductive structures, the follicle and CL, are influenced by eicosanoid hormones, the prostaglandins (PGs) (Espey 1980; McCracken et al. 1981).

Since the focus of this review is mainly on the role of PGs in luteolysis, it is important to first introduce the background information on the sheep reproductive cycle to provide context for the sequence of events that culminate with the process of luteolysis, or the structural and functional demise of the CL. Mammals display an estrous cycle, excluding primates. Sheep and cows display a polycyclic estrous cycle, which means that if pregnancy does not occur after ovulation, luteolysis occurs to

allow a new cycle to start. However, sometimes there are periods of anestrous, or times when there are breaks in the cycle (e.g. sheep) (Garverick and Smith 1993). The estrous cycle is broken into two dominant phases, the follicular phase and the luteal phase. The follicular phase encompasses the growth and development of the follicle and culminates with ovulation, and is characterized by the predominance of the hormone estradiol. The luteal phase entails the luteinization, maturation, and regression of the CL, and this period is characterized by progesterone production (Niswender et al. 1994). The estrous cycle as a whole is controlled by various components of the reproductive system, ranging from the hypothalamus to the anterior pituitary to the reproductive tract. The reproductive tract is composed of the ovary and duct system, spanning from the external genitalia, vagina, cervix, uterus, and oviducts to the ovary.

To understand how the estrous cycle is controlled by the reproductive system it is important to grasp the structural makeup of the reproductive tract. The two separate, highly vascularized, bipartite uterine horns of the ewe and cow are composed of three tissue layers. The outermost layer is called the myometrium and consists of a longitudinal muscle layer between two circular layers of muscle. The middle layer is called the endometrium. This layer is composed of glandular tissue that builds and regresses over the course of an estrous cycle (Niswender and Nett 1994). The endometrium is the layer that the fetus implants if fertilization does occur. And finally, the innermost layer is the epithelium, which lines the lumen of the uterus.

The duct system continues to the two ovaries, the site of most interest for this study. The ovary is a very dynamic organ that is constantly undergoing stages of remodeling. The ovary is composed of a central medulla that contains the nerve, blood, and lymph supply (Espey and Lipner 1994). The next layer is the peripheral cortex, which contains the developing ova in follicles until maturation and subsequent ovulation. The tunica albuginea, a thin layer of dense connective tissue surrounds the peripheral cortex and basement membranes. The serous/germinal epithelium sits atop the tunica albuginea which is periodically broken to permit the release of the oocyte during ovulation (Espey and Lipner 1994).

Prostaglandins regulate a variety of proteins that mediate events associated with follicular development, ovulation, luteotropism, and luteolysis. In the present study, the focus is on how PGF2α influences various protein mediators during the process of luteolysis. These mediators include matrix metalloproteinases (MMPs), which are enzymes responsible for degrading the extracellular matrix (ECM), and their endogenous inhibitors, the inhibitors of metalloproteinases (TIMPs). The balance between MMPs and TIMPs influences the rate of proteolysis and the type of ECM degradation that occurs, which are thought to ultimately lead to structural regression of the CL. Another mediator of interest is the steroidogenic acute regulatory protein (StAR). StAR is responsible for cholesterol transport allowing for steroid synthesis, thus providing insight into the functional aspects of the corpus luteum. Also important in this study are the cyclooxygenase (COX) enzymes. COX enzymes regulate the synthesis of PGs, fueling the auto-amplification of luteolysis

through PG production within the CL. Therefore, the present literature review is intended to discuss the aspects of ovarian function that relate PGF2α and luteolysis.

Prostaglandins

Collectively eicosanoids encompass an entire group of compounds that include PGs, thromboxanes (TXs), leukotrienes, lipoxins (LPXs), and hydroxyeicosanoic acids (HETEs) (McCracken 2005). Prostaglandins are hormones derived from the essential 20 carbon fatty acids. The predominant 20 carbon precursor is arachidonic acid (AA) (Poyser 1973). AA is readily liberated from membrane phospholipids, which are found in all cell membranes. Prostaglandins are very biologically active and are metabolized readily by most tissues in the body, particularly the lung (Samuelsson 1964). As a result of their rapid metabolism, prostaglandins are synthesized in many tissues to serve in a variety of biological processes, particularly at sites of inflammation. For the purpose of the present review, I will focus mainly on PGs, particularly PGF2α.

Prostaglandin (PG) Biosynthesis

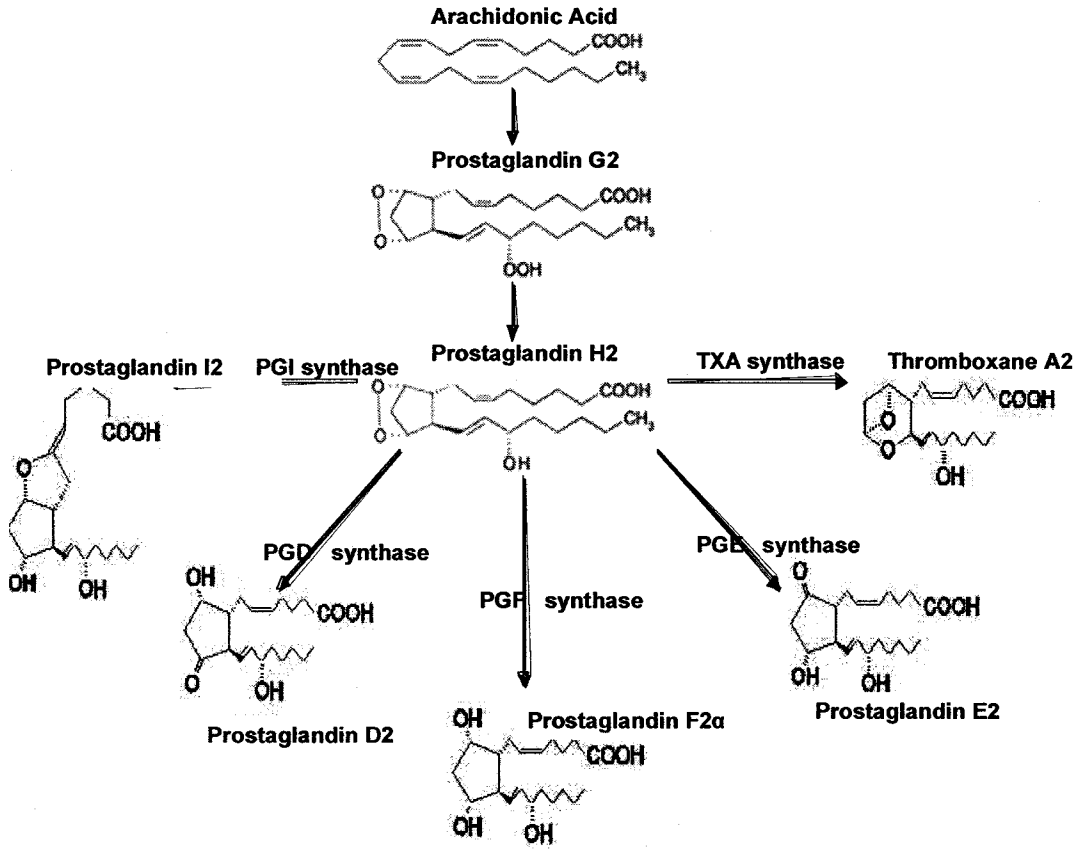
PG synthesis begins with the liberation of AA from plasma membrane phospholipids by two phospholipases, A_2 and C. These enzymes are responsible for providing AA, the preferred precursor to the 3-step cyclooxygenase pathway that leads to PG synthesis (Kunze and Vogt 1971; Lands and Samuelsson 1968). There are three forms of the PG endoperoxide H synthases responsible for AA metabolism: cyclooxygenase 1 (COX-1), which is the constitutively expressed isoform;

cyclooxygenase 2 (COX-2), which is the induced isoform; and cyclooxygenase 3 (COX-3), a relatively new isoform with little known function. These COX enzymes convert AA into prostaglandin G_2 (PGG_2) and subsequently to prostaglandin H_2 ($PGH_{2)}$ (Vane and Botting 1995). PGH_2 then undergoes an isomerization by prostaglandin F synthase to yield the product PGF2α (Smith et al. 1996).

Cyclooxygenase Enzyme Function

Cyclooxygenase (COX) enzymes are membrane bound enzymes (Smith and Dewitt 1996) capable of both peroxidase and cyclooxygenase activities (Smith et al. 1996). The cyclooxygenase activity enables the addition of two oxygen molecules to AA through an oxidation reaction to produce PGG_2, and the peroxidase activity follows, which adds two electrons through a reduction reaction to produce PGH_2 (Smith and Dewitt 1996; Smith et al. 1996). **Figure 1** depicts how PGH_2 is converted into a number of PGs and related products by their specific synthases.

FIGURE 1: Arachidonic Acid Cascade



Adapted from (McCracken 2005).

There are two well known and highly documented isoforms of the COX enzyme, COX-1 and COX-2 (Herschman 1994). Recently a third isoform was identified. While this isoform is not very well characterized, it is localized predominantly in the brain (Botting 2000; Hersh et al. 2005). Therefore, I will focus on COX-1 and COX-2 and their relevance to ovarian function. Both COX-1 and COX-2 cDNA share up to 60% amino acid sequence homology (Vane et al. 1998). This homology extends beyond the bounds of evolution, and can remain up to 90% homologous between species (Kulkarni et al. 2000). **Table 1** depicts some of the general and distinguishing characteristics of COX-1 and COX-2.

Table 1. Comparisons of COX-1 and COX-2 Characteristics

Physiology	• constitutive form • produces PGs that regulate normal kidney, stomach function and vasculature homeostasis	• inducible form • induced during periods of inflammation, produces PGs involved in the inflammatory response; may have roles in regulating mitogenesis and cell growth
Amino Acids	• 599 aa	• 604 aa
Molecular Weight	• ~70 kDa	• ~72 kDa
Regulation and Expression	• gene is 22 Kb, 11 exons • gene is located on chromosome 9 • mRNA transcript is 2.8 to 3.0 Kb • mRNA transcript is not degraded quickly • promoter gene has low inducibility • not inhibited by glucocorticoids	• gene is 8.3 Kb, 10 exons • gene is located on chromosome 10 • mRNA transcript is 4.0 to 4.5 Kb • mRNA transcript is degraded quickly • promoter region contains many transcriptional factors which can be upregulated by proinflammatory cytokines • inhibited by glucocorticoids
Phase of Inflammation	• chronic inflammation phase	• acute inflammation phase

Adapted from (Kulkarni et al. 2000).

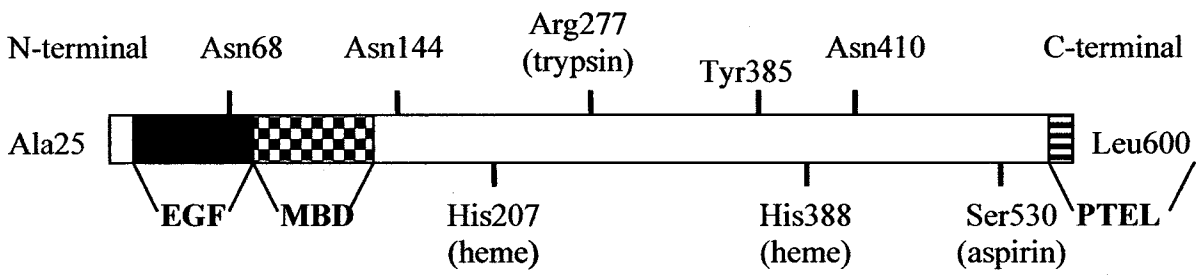
The COX enzymes are integral membrane proteins that undergo post-translational modifications (e.g. glycosylation and homodimerization), however, the COX enzymes are slightly different in size and in the composition of their active sites (Kulkarni et al. 2000; Luong et al. 1996). As mentioned in **Table 1**, the COX enzymes differ in length by 5 amino acids. COX-1 is 599 amino acids and has a relative molecular mass of ~70 kDa, while COX-2 is 604 amino acids and has a relative molecular mass of ~72 kDa (Kulkarni et al. 2000). COX-1 is constitutively

expressed and is responsible for regulating renal water and sodium metabolism, stomach acid, and vascular homeostasis (Smith and Dewitt 1996; Smith et al. 1996; Vane et al. 1998). COX-2 is the inducible form of the gene and is upregulated in the early inflammation response. Upregulation of COX-2 is initiated by factors such as growth factors and cytokines during times of acute inflammatory responses, as seen in disease states (e.g. arthritis, colon cancer, and Alzheimer's diease) (Vane et al. 1998) and also during ovulation and luteolysis (Espey 1980; McCracken et al. 1972).

COX structure

COX was first purified in 1976 (Hemler and Lands 1976; Miyamoto et al. 1976), and first cloned in 1988 (DeWitt and Smith 1988; Merlie et al. 1988). In 1994, X-ray crystallography revealed the structure of ovine COX-1 (Picot et al. 1994), as depicted in **Figure 2**. The three-dimensional structure of the integral membrane protein, COX-1, is a bifunctional enzyme comprised of three independent folding units: an epidermal growth factor domain (EGF), a membrane-binding motif (MBD) and an enzymatic domain. Two adjacent active sites were found for its heme-dependent peroxidase and cyclooxygenase activities (Picot et al. 1994). The cyclooxygenase active site is created by a hydrophobic channel that is the site of non-steroidal anti-inflammatory drug binding (aspirin or COX inhibitors) (Vane and Botting 1995). The conformation of the membrane-binding motif suggests that the enzyme integrates into only one leaflet of the lipid bilayer and is thus a monotopic membrane protein (Picot and Garavito 1994).

FIGURE 2. Ovine COX-1 protein



Adapted from (Smith and Dewitt 1996)

The EGF domain ranges from residue 34 to 72, contains two β-sheets and three intra-domain disulfide bonds. The disulfide bond that connects cysteines 37 to 159 is responsible for attaching the EGF domain to the enzymatic domain, or active site (Picot et al. 1994).

The MBD is composed of four α-helices and ranges from residues 73-116; this is the portion of the protein that inserts into the organelle membranes (Picot and Garavito 1994; Picot et al. 1994). Some organelles, in particular, are the endoplasmic reticulum (ER) and the nuclear envelope (NE). Immunocytofluorescence reveals that COX-1 is localized in lipid bodies as well as the ER and NE of murine 3T3 cells, and human (HUVEC) and bovine (BAEC) endothelial cells (Dvorak et al. 1994). COX-2 is predominantly localized in the NE over the ER, and has been seen within the nucleus (Vane et al. 1998). Although the patterns of localization of COX-1 and COX-2 are somewhat similar, it is believed that COX-1 is active primarily in the ER while COX-2 is active in the NE as well as the ER (Morita et al. 1995).

As mentioned above the MBD region contains four α-helices. This is important because the fourth α-helix is associated with the heme-dependent active site of the enzyme (Vane et al. 1998). This active site facilitates both cyclooxygenase and peroxidase reactions (Luong et al. 1996; Picot et al. 1994). These reactions take place in a hydrophobic channel in the COX enzyme that is stereochemically accommodating for membrane associated AA. The α-helices compose this hydrophobic channel that allows COX to interact with AA within a lipid bilayer (Picot et al. 1994). The C-terminus of the protein contains an ER targeting and retention signal (Pro-Thr-Glu-Leu) (Smith and Dewitt 1996; Smith et al. 1996). Although the COX-1 and COX-2 amino acid structures are highly conserved evolutionarily (Luong et al. 1996), there are some major differences in their regulation and expression.

COX Regulation

The differences in COX-1 and COX-2 transcripts lie on different genes (Smith and Dewitt 1996; Smith et al. 1996). The 3' untranslated region of COX-2 contains several Shaw-Kamen sequences, which are amino acid sequences commonly associated with mRNA degradation (Jouzeau et al. 1997). These 18 amino acid inserts may account for the relatively short half-life of COX-2 mRNA and protein (Smith et al. 1996).

Recent research has shown that protein kinase C (PKC) directly regulates COX-2 transcription in large luteal cells (LLCs) through several upstream leucine zipper transcription factors (Wu and Wiltbank 2001a; Wu and Wiltbank 2001c).

These upstream transcription factors bind to an E-box (-CACGTG-) that lies only 50 base pairs upstream from the COX-2 transcript (Wu and Wiltbank 2001c). In addition, when LLCs are treated *in vitro* (Tsai and Wiltbank 1997) and *in vivo* (Wu and Wiltbank 2001a) with a protein kinase C activator, ionomycin (calcium ionophore), COX-2 mRNA concentrations increase significantly.

There is debate over the belief that nitric oxide (NO) influences COX expression and is a primary regulator of COX activity. Binding kinetics show NO to be a weak ligand for COX-1, leading researchers to believe that no direct interactions exist between NO and COX-1 expression, at least at physiological concentrations (Tsai and Wiltbank 1997). Although COX-1 does not seem to be a primary target of NO, evidence suggests that endogenous NO or endogenous NO donors may be a regulator of COX-2 activity (Mollace et al. 2005; Salvemini et al. 1994). It is probable that there are many regulators of COX expression, however, many of them have yet to be characterized.

COX Expression

As mentioned previously COX-1 is constitutively expressed, whereas COX-2 is inducible. In sheep, COX-1 is expressed constitutively throughout the estrous cycle and pregnancy mainly in the myometrium of the uterus, whereas COX-2 is expressed mainly in the endometrium and is expressed in the highest concentrations on days 12-15 of the estrous cycle (Charpigny et al. 1997). Results of *in vitro* experiments show that COX-2 mRNA expression increases within 4 hours after ovine luteal cells are treated with 100 nM PGF2α (Tsai and Wiltbank 1997; Tsai and

Wiltbank 2001), and remains high for up to 12 hours following treatment. Tsai and Wiltbank also demonstrated that LLCs contain 1000 times more COX-2 mRNA transcripts than small luteal cells (SLCs). These studies propose a positive feedback loop for PGF2α upregulating intraluteal COX expression, which suggests that COX-2 may play an integral role in luteolysis. These findings, coupled with those that indicate NO may regulate COX expression, suggest a possible synergistic relationship between PGF2α, NO, COX during luteolysis.

PGF2α Mechanism of Action

PGF2α receptors belong to the family of 7-transmembrane heterotrimeric G protein-coupled receptors (Abramovitz et al. 1994; Anderson et al. 2001). Binding of PGF2α leads to activation of the PGF2α receptor via phosphorylation. Receptor activation induces a cascade of events leading to the activation of various cellular components including phospholipase C (PLC). PLC is a membrane bound enzyme responsible for catalyzing the formation of inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (Bourdage et al. 1984) from the membrane component phosphatidylinositol (PI). IP3 initiates a release of intracellular Ca^{++} from the smooth ER (Berridge and Irvine 1984; Davis et al. 1988) and also opens calcium channels on the plasma membrane causing an influx of extracellular Ca^{++} (Kuno and Gardner 1987). These effects are thought to be limited to LLCs, and not SLCs (Alila et al. 1989). DAG acts in concert with excess Ca^{++} activate another membrane bound, calcium-dependent enzyme called protein kinase C (PKC) (Niswender et al. 2000).

The activation of PKC through binding of PGF2α to its receptor leads to an acute inhibition of progesterone synthesis 8 hours following treatment (Beal et al. 1980; Wiltbank et al. 1991). This pathway is believed to affect LLCs through decreasing cholesterol availability (Behrman et al. 1971) and ECM stability, and even apoptosis (Niswender et al. 2000). PKC has been linked to the stimulation of PGF2α production via an auto-amplification feedback loop (Shemesh et al. 1989; Wiltbank and Ottobre 2003), PKC affects gene expression and/or the modification of various cellular proteins that play a role in PGF2α production (e.g. COX enzymes/PLA2)(Shemesh et al. 1989). PKC's effects are not limited to PGF2α production. Examples of genes/proteins affected by PKC are steroidogenic acute regulatory protein (StAR), which is responsible for progesterone production (Manna and Stocco 2005), and ECM mediators such as tissue inhibitors of metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs) (Smith et al. 1997).

Follicular Development

The follicular phase describes the growth and development of ovarian follicles. This is a process that is continuous throughout the estrous cycle, but most developing follicles never reach full matu23rity. Most follicles (99.9%) that are selected and recruited for development regress through a natural process called atresia (Fortune et al. 1991; Peters et al. 1975; Quirk et al. 1986). For many years the development of the follicle has been reported to be influenced by PGs. The PGs are produced by follicular tissues in considerable quantities throughout development (Erickson et al. 1977; Triebwasser et al. 1978), due to PG synthesis stimulated by

gonadotropins (Evans et al. 1983). This is predominantly characterized by the steroid hormone, 17β-estradiol (E2), which is being produced at higher quantities than the rest of the cycle. The developing follicles are producing E to bathe the maturing oocytes, eventually leading to a surge of LH and eventual ovulation (Fortune 1994; Fortune and Armstrong 1978). Before delving into the follicle's role in steroidogenesis, it would be pertinent to first review follicular structure and function.

Follicle Development and Structure

The multiplication of oogonia (primordial germ cells) and the development of primordial follicles occurs during embryonic development in female mammals (Peters et al. 1975). It was believed that by the time gestation is complete, the ovary will contain the most oocytes that it will ever possess, and from this point on, the number of oocytes will decrease through the above mentioned process of atresia. However, there is now evidence that mammals possess the ability to produce germ cells and follicular renewal postnatally (Bukovsky et al. 2004; Johnson et al. 2004). Although several follicles are recruited each cycle, most will never reach maturity and ovulate (Quirk et al. 1986).

Follicles have various stages of development according to their morphological characteristics. The first stage of development begins with primordial follicles, which describes the immature oocyte being surrounded by a single layer of squamous (pregranulosa) follicle cells (Baca and Zamboni 1967; Sirois and Fortune 1988). Primordial follicles then develop into primary follicles with the addition of a basement membrane surrounded by a single layer of cuboidal cells (Bjersing 1982;

Fortune et al. 2000; Stegner et al. 1976). If the follicle is still being recruited, it then develops into a secondary follicle, which is characterized by the addition of a thick translucent layer around the oocyte, called the zona pellucida, and is accompanied by the growth of multilaminar granulosa cells that are 2-6 layers thick (Bjersing 1982). As development continues, the number of theca and granulosa cells increases, which leads to the formation of a tertiary, or antral follicle due to the accumulating fluid in the antrum. This follicular fluid contains various proteins, carbohydrates, steroids, and polysaccharides that bathe the developing ovum through maturation (McNatty 1978; McNatty and Baird 1978). The final stage of follicular development is characterized by a decrease in systemic progesterone (P) concentrations, due to the regression of the CL from the previous estrous cycle, and an increase in estradiol (E2) concentration which is secreted by developing follicles (McNatty et al. 1979). This last stage is when the follicle is referred to as a dominant, or Graffian follicle.

Follicular Function

Follicular function is characterized by the development of the theca layer, and subsequent production of E (McNatty et al. 1984; McNatty et al. 1979). Both granulosa and theca cells are involved in the process of steroid production, but for most species, each is incapable of metabolizing cholesterol into 17β-estradiol due to lack of appropriate enzymes. The cooperation of the granulosa and theca cells in steroid production is described as the "two-cell theory" (Falck and Hillarp 1959; Short 1962). The complete conversion of cholesterol > progestogens > androgens >

estrogens (Payne and Hales 2004) possible is only through a combination of enzymes found in both the granulosa and theca cells.

Steroid production begins in the theca interna cells with their ability to produce and secrete androgens in response to an LH stimulus (McNatty et al. 1979). The theca interna cells possess a large concentration of 17α-hydroxylase activity required for androgen synthesis, however, they lack enzymes required to metabolize androgens any further (Bjersing 1968). Instead, thecal androgens (androstenedione and testosterone) cross the basement membrane to the granulosa cells, where they are converted to estradiol by the aromatase enzymes (Fortune and Armstrong 1978). Aromatase activity increases in granulosa cells in response to follicle stimulating hormone (FSH), and thus increasing E producion (Fortune and Hilbert 1986). As follicular development continues, there is an increase of E concentration in the follicular fluid, which increases granulosa cell sensitivity to FSH and luteinizing hormone (LH) (Richards et al. 1987). Estradiol also crosses the basement membrane where it is transported through the blood stream to various target tissues, including the brain.

Endocrine Regulation of Follicle Development

The hypothalamus and the anterior pituitary play a large role in regulating the growth and development of follicles. There are two groupings of nerve bodies, called nuclei, which are involved in regulating the estrous cycle. These nuclei are known as the tonic center (ventromedial and arcuate nucleus) and the surge center (preoptic and

suprachiasmatic nuclei, and anterior hypothalamus). Both of these brain centers are responsible for producing the decapeptide, gonadatropin releasing hormone (GnRH). GnRH is released from the nerve endings of these nuclei and into the capillary network called the hypothalamo-hypophyseal portal system (Niswender and Nett 1994). It arrives at the anterior pituitary where it stimulates the release of FSH and LH in a pulsatile manner (Garverick and Smith 1993; Turzillo et al. 1995). Since 1940, it has been accepted that both FSH and LH are required for follicular development (Fevold 1941), which are required for stimulating E production (Goodman et al. 1981).

In ruminants, tonic GnRH secretions are responsible for the waves of follicular growth that result mainly in atresia, or death of follicles (Noel et al. 1993). As follicular development continues, small quantities of a glycoprotein hormone called inhibin are produced. Inhibin is responsible for inhibiting the release of FSH (Ying 1988), which prevents the further recruitment of follicles, but leaves secondary and tertiary follicles unaffected. At this time, it appears that the follicles switch from their dependency on FSH to LH, as seen by a decrease in FSH receptors and an increase in LH receptors on granulosa cells (Carson et al. 1979; Niswender et al. 1985a).

Under conditions of low progesterone, E feedback reaches a threshold causing the pulse generator in the hypothalamus to increase in amplitude and frequency (Baird and McNeilly 1981). The surge center of the pituitary responds with a release of LH, which ultimately causes ovulation.

PGs in Ovulation

The growth and maturation of the follicle is designed for the single purpose of ovulation. Ovulation is the event that releases the oocyte to the Fallopian tubes in an attempt to facilitate the process of fertilization and subsequent pregnancy. Ovulation has been compared to an inflammatory response involving various hormones and proteins, but most importantly PGs (Espey 1980). The process begins with luteinizing hormone (LH), which is responsible for initiating the cascade of events culminating in ovulation (Niswender et al. 1986). Some of the many components affected by this LH surge are the morphology, biochemical, and endocrine function of the follicle cells. PGs are thought to contribute to this inflammatory process through their capacity to intensify vascular leakage induced by mediators such as histamine and bradykinin (Vane 1976). In concert with the effects on the vasculature, PGs facilitate the destruction and remodeling of connective tissue elements (Espey 1980) by induction of proteolytic enzymes, thus pushing the inflammation to the chronic stages, eventually leading to ovulation.

Several mechanisms may explain in part how PGs, particularly PGF2α, generate large amounts of PGF2α, in an auto-amplification manner, to propagate the inflammatory nature of this process. PGs are leukotactic, in that once leukocytes arrive at the site of inflammation, they release additional PGs into the area of inflammation (Lewis 1977). Another mechanism of action involves the PKC pathway discussed earlier. Since PGs act through a pathway that involves PKC, PKC may directly target genes/enzymes that are involved in PG synthesis, thus up-

regulating their expression/activation leading to increased production of PGs (Tsai and Wiltbank 1997; Wiltbank and Ottobre 2003).

Luteinization

Once ovulation has occurred, the cells that comprised the follicle undergo a process known as luteinization. This process is regulated, in part, by prostaglandin E (PGE) and PGF2α, which act in conjunction to promote luteinization of granulosa and theca cells, and subsequent stimulation of P synthesis (McArdle 1990). Luteinization is caused by the preovulatory surge of LH, and it involves the transformation of theca and granulosa cells into luteal cells, leading to drastic increases in P production (Pescador et al. 1999; Smith et al. 1994b). Luteinizing hormone is the luteotrophic hormone in both sheep (McCracken et al. 1971) and cows (Hansel and Seifart 1967). The follicular fluid contains LH inhibitors, thus preventing luteinization until after ovulation. These two inhibitors are inhibin and LH receptor binding inhibitor (LHRBI) (Channing et al. 1980). Once the follicular fluid containing the inhibitors is gone, LH is capable of binding its receptors which causes an increase in adenosine 3',5'-monophosphate (Dimino et al. 1976; Niswender and Nett 1994). The increase in cAMP levels causes cells to acquire cytoplasmic projections, along with large numbers of smooth endoplasmic reticulum and mitochondria, and most importantly, the ability to produce and secrete progesterone (Niswender and Nett 1994). With these acquired characteristics, granulosa and theca cells transform into luteal cells. Simultaneous with the transformation of luteal cells, the developing corpus luteum undergoes a stage of extensive vascularization, or angiogenesis, as endothelial cells

invade the avascular granulosa compartment (Smith et al. 1994b), making the corpus luteum one of the most vascularized tissues in the body (Ford et al. 1982).

Corpus Luteum (CL)

The corpus luteum (CL) is a transient endocrine gland (Loeb 1923b) that forms from the follicle, the structure that houses the ovum. The CL is responsible for producing P, the steroid hormone that allows for implantation and maintenance of the fetus. The CL is essential for maintenance of pregnancy until the placenta develops and takes over P production, thus allowing the fetus to survive until parturition. Ultimately, proper functioning of the CL is essential for mammals to pass on their genetic characteristics through reproduction.

Structure of the CL

The structure of the CL is comprised of a variety of cell types including fibroblasts, endothelial cells, macrophages, and steroidogenic luteal cells (Farin et al. 1986). All of these cell types act in concert to regulate vascularization, and luteal cell function, particularly steroidogenesis (Devoto et al. 2002). For the present discussion, I am going to focus only on the steroidogenic luteal cells of the CL.

There are two functionally distinct types of steroidogenic luteal cells present in the CL, large luteal cells (LLCs), and small luteal cells (SLCs) (Rodgers et al. 1986; Ursely and Leymarie 1979). It is generally accepted that LLCs develop from granulosa and SLCs develop from theca cells during the process of luteinization (Alila and Hansel 1984; Farin et al. 1986). These cell populations are distinguishable

based on cell diameter, with cells <22 μm being SLCs and >26 μm being LLCs (O'Shea et al. 1989; Rodgers et al. 1986). Both theca and granulosa cells acquire the morphological and functional characteristics of small and large luteal cells respectively *in vitro* (Meidan et al. 1990). During the process of luteinization, LLCs develop increased numbers of ER and mitochondria, both of which are essential for biosynthesis of steroids (Murphy 2000; Niswender et al. 2000; Niswender and Nett 1994). Morphologically LLCs have a polyhedral shape with a centrally located nucleus and distinct nucleoli, and large numbers of mitochondria surrounding the nucleus. Conversely, SLCs have an angular-spindle shape with a cup-shaped nucleus and irregular shaped nuclei, and an accumulation of lipid droplets within their cytoplasm (Niswender et al. 1994).

Both LLCs and SLCs comprise between 30% (O'Shea et al. 1989) to 36% (Farin et al. 1986; O'Shea 1987) of the cell population in the mid-cycle CL. These cell populations are found in a ratio of 6:1 of SLC:LLC (O'Shea et al. 1989) with LLCs comprising 4% of the total cell population and 25% of the CL total volume, and SLCs comprising 19% of the cell population and 18% of the CL total volume (Farin et al. 1986; McCracken et al. 1999; O'Shea et al. 1989). However, these estimates may be influenced by the age of the CL. LLCs exit the cell cycle upon luteinization, thus rendering them incapable of replication, however, SLCs are thought to replicate as much as 5-times between days 4-16 of the ovine estrous cycle (Farin et al. 1986; Niswender et al. 2000).

The CL is one of the few adult tissues that undergoes regular periods of growth, development, and regression (Jablonka-Shariff et al. 1993). The growth and

development of the CL is very rapid. Following ovulation, the sheep CL weighs about 30-40 mg, and by day 12 of the ovine estrous cycle the CL can weigh as much as 750 mg, a 20-fold increase in tissue mass after only 12 days. At this rate, the doubling time for luteal tissue mass is about 60-70 hours (Reynolds et al. 1994). During this time it is essential that the tissue undergoes a time of rapid vascularization to maintain nutrient supply and waste removal. The endothelial cells present in the CL, which make up 30-40% of cell population (Devoto et al. 2002), help to facilitate this period of angiogenesis (Grazul-Bilska et al. 1997; Jablonka-Shariff et al. 1997). This rate of tissue growth is comparable to that of tumor growth (Reynolds et al. 1994), suggesting that there is, to an extent, an amount of flexibility within the CL to allow for rapid growth and expansion of the tissue (Redmer et al. 1988).

Function of the CL

The main purpose of the CL is the production of P, a steroid hormone that facilitates pregnancy through the preparation of uterine tissue, thus allowing embryo implantation (Channing et al. 1980; Moeljono et al. 1977; Niswender et al. 1994). After implantation of the fertilized ovum into the uterine wall, P production is still required by the CL until the fetal placenta matures enough to produce P on its own (Casida LE 1945). In fact, the CL remains functional throughout most of pregnancy (Hansel and Seifart 1967; Silvia and Niswender 1984).

The regulation of progesterone production differs between LLCs and SLCs. When both LLCs and SLCs are separated and treated *in vitro* with LH, LLC production of P is unaffected by LH, whereas SLC production of P increases by as

much as 20 times (Fitz et al. 1982). This indicates that LH stimulates P production by SLCs, but not LLCs. However, LLCs are the main source of P in the CL by producing 80% of the circulating P levels (O'Shea 1987).

Progesterone Biosynthesis

As mentioned above, the majority of luteal P is produced by LLCs (80%), nearly 20 times the amount synthesized by SLCs (Niswender et al. 1985b). LH stimulates P synthesis in SLCs by regulating genes necessary for P synthesis (Devoto et al. 2001). The upregulation of the steroid biosynthesis machinery leads to a 40-fold increase in P production from SLCs (Fitz et al. 1982) due to the fact that SLCs have over 10 times the number of LH receptors compared to LLCs (Glass et al. 1984). LLCs rate of P synthesis is not affected by LH. However, when over-stimulated by LH, SLCs have the ability to down-regulate LH receptors, suggesting that there is a defined biological regulation of P biosynthesis (Niswender and Nett 1994). As the young CL matures, serum concentrations of P can increase by as much as 25 times.

LH regulates P synthesis by affecting several key mediators. LH binds to a heterotrimeric G-coupled protein that leads to the activation of adenylate cyclase (Davis et al. 1989). This leads to an increase in cAMP concentrations, and subsequent activation of protein kinase A (PKA). PKA phosphorylates cellular proteins to elicit various biological responses such as gene transcription, protein synthesis, and protein activation (Niswender and Nett 1994).

One protein that is affected by PKA, in particular, is steroidogenic acute regulatory protein (StAR). PKA increases the amount of phosphorylated StAR, thus increasing its activity (Arakane et al. 1997; Stocco et al. 2001; Sugawara et al. 1997). StAR facilitates steroid synthesis by importing cholesterol across the mitochondrial membranes for metabolism into pregnenolone, a building block for all other steroid hormones (Stocco 2001). Specifically, StAR imports cholesterol from the outer to the inner mitochondrial membrane, where cholesterol is converted to pregnenolone by cytochrome P_{450} side-chain cleavage enzyme (Devoto et al. 2001; Stocco 2001), hence making cholesterol import into the mitochondria the rate limiting step of all steroid production (Stocco 2001; Wiltbank et al. 1993).

Steroidogenic Acute Regulatory (StAR) Protein

StAR was first isolated and cloned in MA-10 mouse leydig cells (Clark et al. 1994). The ~30 kDa StAR phosphoprotein is cleaved from larger ~32- and ~37 kDa precursors (Stocco and Sodeman 1991), which takes place in the mitochondria of steroidogenic cells. The 37- and 32 kDa precursors are the active forms of the protein that transport cholesterol from the outer to the inner mitochondrial membrane before they are processed to the 30 kDa protein (Stocco and Sodeman 1991). Once in the inner mitochondrial membrane, the 30 kDa protein loses contact sites and is no longer active in cholesterol transport (Pescador et al. 1996), however, it may remain associated with the inner membrane for a relatively long time.

Although StAR is paramount to cholesterol transport, there is evidence to suggest that StAR works in concert with several other molecules to facilitate this

process. StAR may function as a triad with peripheral-type benzodiazepine receptor (PBR) and endozepine (Niswender 2002). Previous research linked PBR to cholesterol translocation in adrenocortical cells (Krueger and Papadopoulos 1990; Papadopoulos et al. 1990) and steroidogenesis in mouse leydig cells (Krueger and Papadopoulos 1990; Papadopoulos et al. 1990). Using fluorescent energy transfer, recent research shows that PBR may contribute to StAR's ability to transport cholesterol across the mitochondrial membrane (West et al. 2001).

StAR is associated with steroidogenic tissues of many species, including the rat (Epstein and Orme-Johnson 1991), rabbit (Townson et al. 1996), human (Devoto et al. 2001), bovine (Pescador et al. 1996), and sheep (Juengel et al. 1995). The StAR cDNA sequence remains highly conserved across evolution, with the bovine and human sequences sharing 84% homology (Sugawara et al. 1997). Bovine StAR is transcribed as 3 kb and 1.8 kb transcripts, which differ only in their 3'polyadenylation sites. Both transcripts are highly expressed in the CL at mid to late cycle but are at low levels early in the cycle (Hartung et al. 1995). The human StAR gene is located on p11.2 of chromosome 8, with the 37 kDa form of StAR containing 285 amino acids (Sugawara et al. 1997).

The StAR protein (**Figure 3**) contains three primary structural components, the StAR related lipid transfer (START) domain, the central portion, and the mitochondrial matrix targeting sequence (MMTS).

Figure 3. Structural Domains of StAR

START: StAR related lipid transfer domain

MMTS: Mitochondrial Matrix Targeting Sequence

The START domains of StAR are ~210 amino acid lipid binding domains that have been implicated in intracellular lipid transport, lipid metabolism, and cell signaling events (Soccio and Breslow 2003). The MMTS is located on the N-terminus of the protein and is responsible for directing StAR to the mitochondria. Upon entering the mitochondria, cleavage of the 37 kDa form involves the removal of a 25 amino acid mitochondrial targeting sequence to generate the 30 kDa inactive form (Sugawara et al. 1997), which may be catalyzed by metal-dependent matrix proteases (Epstein and Orme-Johnson 1991). StAR concentrations are directly correlated to steroidogenesis rates and seem to be influenced by cAMP levels (Kohen et al. 2003), which may be regulated by PGF2α via the PKC and PKA pathways (Wiltbank et al. 1993; Wiltbank et al. 1991)

Luteolysis

If the ovum is not fertilized during the functional luteal phase of the estrous cycle, then the CL will regress (die) to allow a new cycle to commence, providing another chance for an ovum to be fertilized. The process of CL regression is known

as luteolysis, the focus of my study. Luteolysis in estrous mammals is strongly tied with PGF2α and involves the actions of many protein mediators. In turn, these mediators participate in, (1) functional demise through the loss of P synthesis, and (2) structural demise which involves tissue degradation due in part to ECM remodeling.

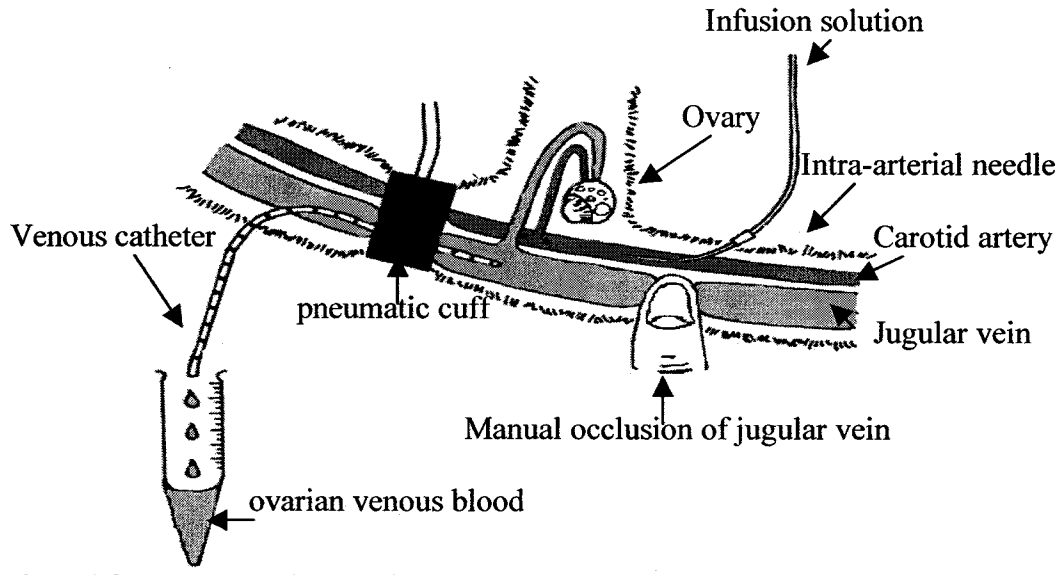
Role of the Uterus in Luteolysis

The role of the uterus in CL regression was first reported by Leo Loeb (Loeb 1923b; Loeb 1927) who demonstrated in guinea pigs that hysterectomy caused an extended lifespan of the CL. Similar findings were then reported in cyclic sheep and cows (Wiltbank and Casida 1956), rats (Bradbury 1937), and rabbits (Asdell and Hammond 1933). However, this only remains true for estrous cycling mammals and not primates (Beavis et al. 1969; Burford and Diddle 1936). With the persistence of the CL after hysterectomy, this suggested that the uterus possessed a component that facilitates luteolysis.

PGF2α Identified As the Mammalian Luteolysin

PGF2α is found in abundant quantities in the uterus and may play a role as a uterine luteolysin (Pharris and Wyngarden 1969). This information led to an ovarian autotransplant sheep model first established at the Worcester Foundation (McCracken et al. 1999). This model, originally established using the adrenal gland, involved removal of the ovary from the abdomen and subsequent attachment to the carotid artery and jugular vein of the neck as seen in **Figure 4.**

FIGURE 4. Ovarian Autotransplant: Diagram of the technique for intra-arterial infusion of autotransplanted ovary in sheep and periodic collection of ovarian venous blood. With inflation of pneumatic cuff above carotid arterial pressure, carotid arterial blood containing an infusate supplies ovary.



Adapted from (McCracken et al. 1969).

The transplanted ovary was then infused with PGF2α through the arterial supply, and the results mimicked that of natural luteolysis. However, when PGF2α was infused systemically, there was no effect on CL function (McCracken et al. 1972; McCracken et al. 1970). The negative results were explained in part by the dilution effect and the rapid metabolism of PGF2α by the lungs (Ferreira and Vane 1967). These negative results gave support to the belief that PGF2α must be the luteolytic hormone that was periodically released by the uterus, acting on the adjacent ovary to induce CL regression (McCracken et al. 1999). Furthermore, investigations using radiolabeled PGF2α suggest that diffusion of uterine PGF2α across blood vessel walls, in a counter-current exchange system, is sufficient to initiate luteolysis in sheep (McCracken et al. 1984)

Luteolytic Effects of PGF2α

As mentioned earlier, PGF2α acts through a 7-transmembrane heterotrimeric G-coupled protein receptor to initiate biological responses. PGF2α initiates the decline of luteal function through several mechanisms. The first is the rate of cholesterol transport across the mitochondrial membrane, a crucial component to steroid synthesis. Cellular transport of cholesterol involves several molecular mediators, including the interactions of sterol carrier protein-2 (SCP-2) with the cytoskeleton, and the before mentioned StAR. PGF2α decreases expression of SCP-2 (McLean et al. 1995) and drastically reduces expression of tubulin, a major component of microtubule fibers in sheep (Murdoch 1996). Evidence also suggests that StAR mRNA (Juengel et al. 1995) and protein (Pescador et al. 1996) is down-regulated following PGF2α treatment. Due to the fact that StAR protein only has a half-life of 3-5 minutes (Epstein and Orme-Johnson 1991), a reduction in StAR will lead to a sudden and drastic reduction of P synthesis.

Another fundamental step of luteal regression involves apoptosis, or programmed cell death. PGF2α is, in part, believed to initiate this process throughout luteolysis (Sawyer et al. 1990). Apoptotic cells have distinguishable characteristics including: nuclear and DNA fragmentation (oligonucleosome formation), formation of membrane bound vesicles, chromatin condensation, and cellular shrinkage (Earnshaw et al. 2000); all of which have been noted in luteal cells throughout luteolysis (Juengel et al. 1993; Zheng et al. 1994).

Another common feature of apoptosis is loss of cell adhesion to the ECM due to the decay of microtubule structures within the cell (Murdoch 1996). ECM stability

is correlated to the balance of tissue inhibitors of metalloproteinases (TIMPs) and their enzymatic counter-parts, matrix metalloproteinases (MMPs) (see next section). Researchers suggest that TIMPs promote ECM preservation (Smith et al. 1999) and subsequent down-regulation of TIMPs throughout luteolysis could promote structural demise of the gland in response to PGF2α (Duncan et al. 1996; McIntush et al. 1997; Towle et al. 2002). Although there are many immune and cytokine factors that have important roles in luteolysis, they are beyond the scope of this discussion.

Tissue Inhibitors of Metalloproteinases (TIMPs)

Tissue inhibitors of metalloproteinases (TIMPs) comprise a family of proteins that form tight specific and reversible non-covalent complexes with latent and active forms of MMPs in a 1:1 ratio (Bode and Maskos 2001). There are four known TIMPs that have been cloned and sequenced to date. **Table 2** summarizes some basic features of these inhibitors.

Table 2. Biochemistry and molecular features of TIMPs

TIMPs	Molecular Weight (kDa)	Glycosylation	Cellular localization of protein	Chromosome localization	mRNA size (kb)
TIMP-1	~29	Glycosylated	Diffusible	Xp11.3-11.23	0.9
TIMP-2	~19-21	Unglycosylated	Diffusible	17q25	3.5 and 1
TIMP-3	~24-27	Glycosylated	ECM bound	22q12.1-13.2	4.5, 2.8, 2.4
TIMP-4	~23-24	Unresolved	Diffusible	3p25	1.2

Adapted from (Greene et al. 1996; Salamonsen and Woolley 1996)

These TIMPs share only 40% homology in their cDNA sequence (Douglas et al. 1997), suggesting that each TIMP has a distinct identity, even though all TIMPs can bind active MMPs. All four TIMPs have 12 cysteine residues in common, forming six disulfide bonds that segregate both the C- and N-terminal domains with three disulfide bonds each (Williamson et al. 1990). The N-terminal domain is the portion of all TIMP proteins that interacts with the catalytic domain of MMPs which leads to inhibition of MMP enzymatic activity (Woessner and Nagase 2000). The variability of the C-terminus may contribute to preferred inhibition of MMP targets (Bode and Maskos 2001).

TIMP-1 is an ~28.5 kDa N-linked glycoprotein (Gasson et al. 1985) that inhibits all known MMPs (Edwards 2001). The C-terminal domain of TIMP-1 preferentially binds MMP-9 at the hemopexin domain (Goldberg et al. 1989). TIMP-2 is an ~19 kDa protein that, unlike other TIMPs, has an extended C-terminal domain that is negatively charged (Murphy and Willenbrock 1995) and preferentially binds MMP-2 (Goldberg et al. 1989). TIMP-3 is an N-glycosylated protein that varies in molecular mass (24-27 kDa) depending on the degree of glycosylation (Apte et al. 1995). TIMP-3 is the only TIMP that is strongly associated with the ECM (Leco et al. 1994) and preferentially binds MMP-1, -2, -3, -9, and -13 (Apte et al. 1995). TIMP-4 is an ~24 kDa protein and its glycosylation state is unknown. TIMP-4 is suggested to be an important regulator of ECM turnover (Leco et al. 1997) due to the fact that TIMP-4 binds MMP-1, -2, -3, -7, -8, -9, -12, -13, and -14 (Stratmann et al. 2001a; Stratmann et al. 2001b).

Other TIMP Functions

TIMPs have a large role in regulating ECM remodeling and homeostasis, however, they possess a variety of other functions. Evidence suggests that TIMPs regulate MMP activity and activation by forming TIMP/proMMP complexes (Wang et al. 2000). TIMP-1 has also been associated with the proliferation of endothelial cells and fibroblasts, thus acting as a pseudo growth factor (Hayakawa et al. 1992). Further, TIMP-1 stimulates steroidogenesis in both leydig and granulosa cells (Boujrad et al. 1995). Although there has been no direct link between TIMP-1 and StAR expression, they share a partial (124 base pairs) homologous DNA sequence (Hartung et al. 1995). This relationship has yet to be fully elucidated.

Matrix Metalloproteinases (MMPs)

The matrix metalloproteinases (MMPs) are a family of zinc and calcium dependent proteolytic enzymes that collectively digest all ECM components (Birkedal-Hansen 1995b). MMPs are classified into groups based on their preferred matrix protein substrate (Birkedal-Hansen 1995a; Matrisian 1990). Currently there are 26 known MMP family members composing 6 classification groups **(Table 2)**: collagenases, gelatinases, stromelysins, MT-MMPs (membrane type), matrilysins, and other type (Johnson et al. 1998; McCawley and Matrisian 2001). These MMPs regulate ECM remodeling, and thus mediate cellular events such as cell proliferation, migration, differentiation and apoptosis (Smith et al. 1999; Sternlicht and Werb 2001). These enzymes typically demonstrate low activity in normal tissues, but are activated/upregulated during inflammation and physiological remodeling processes

(Johnson et al. 1998). These processes include follicular growth, ovulation, luteinization, luteolysis, menstruation, and placental development (Smith et al. 2002).

MMP Characteristics

There are several domains preserved in all MMPs. They are: (1) the signal peptide domain, which directs the enzyme to the rough ER during synthesis; (2) the propeptide domain, which maintains latency until activation; (3) the catalytic domain, which contains a highly preserved zinc (Zn^{2+}) binding region; (4) the hemopexin domain, which determines substrate specificity; and (5) the small hinge region, which enables the hemopexin region to present substrate to the active core of the catalytic domain. The MT-MMPs possess an additional transmembrane domain containing ~20 hydrophobic amino acids and a 24 amino acid intracellular domain (Bode and Maskos 2001; Sternlicht and Werb 2001). These MMP domains are configured in a way that makes the zymogen form of the enzyme more stable.

MMP Families

As mentioned earlier, there are six classifications of MMPs, categorized by substrate preferences. Currently the subsets of MMP families are: (1) collagenases, including MMP-1, MMP-8, MMP-13; (2) gelatinases; including MMP-2, and MMP-9; (3) stromelysins, including stromelysin-1 and -2; (4) membrane type metalloproteinases (MT-MMPs), including MT1-6 MMP; (5) matrilysins, containing MMP-7 and MMP-26; and (6) other MMPs, including MMP-19, MMP-18, and MMP-7, with most still being characterized. Currently the MMP family consists of at

least 26 MMPs. **Table 3** shows the families and characteristics of MMPs (not all discussed in this review).

Table 3. Matrix Metalloproteinase Family Characteristics

Subfamilies	Trivial Name	MMP #	Molecular mass (kDa)		Chromosome localization	
			Latent	Active	Human	Mouse
	Collagenase-1	MMP-1	52	42	11q22-q23	
Collagenases	Collagenase-2	MMP-8	85	64	11q21-q22	
	Collagenase-3	MMP-13	52	42	11q22.3	9(A1-A2)
	Collagenase-4	MMP-18	53	42		
Gelatinases	Gelatinase A	MMP-2	72	66	16q13	8(42.9)
	Gelatinase B	MMP-9	92	84	20q11.2-q13.1	2(H1-H2)
Stomelysins	Stromelysin-1	MMP-3	57	45	11q23	9(1.0)
	Stromelysin-2	MMP-10	54	44	11q22.3-q23	
MT-MMP	MT-MMP1	MMP-14	66	54	14q11-q12	14(12.5)
	MT-MMP2	MMP-15	72	60	16q13-q21	8(45.5)
	MT-MMP3	MMP-16	64	53	8q21	4(3.6)
	MT-MMP4	MMP-17	57	53	12q24.3	5(1.0)
	MT-MMP5	MMP-24	73	62	20q11.2-q12	2(1.0)
	MT-MMP6	MMP-25	63	62	16p13.3	
Matrilysins	Matrilysin-1	MMP-7	28	19	11q21-q22	9(1.0)
	Matrilysin-2	MMP-26	29	19	11p15	
Other MMPs	Stromelysin-3	MMP-11	64	46	22q11.2	10(40.9)
	Metalloelastase	MMP-12	54	22	11q22.2-q22.3	9(1.0)
	RASI-1	MMP-19	54	45	12q14	10(71.0)
	Enamelysin	MMP-20	54	22	11q22.3	9(1.0)
	XMMP	MMP-21	70	53		
	CMMP	MMP-22	51	43	11q24	
	CA-MMP	MMP-23	44	31	11p36.3	
	Epilysin	MMP-28	59	45	17q11.2	

Adapted from (McCawley and Matrisian 2001; Smith et al. 1999).

Gelatinases

Since the MMP family is so large, a complete description of each individual is beyond the purview of the present review. Thus, this discussion is limited to the gelatinases A & B, also known as MMP-2 & MMP-9, respectively. Gelatinases degrade collagens type IV and V, in addition to gelatin, a byproduct of denatured collagen (Matrisian 1990; McCawley and Matrisian 2001). The gelatinases are among the most highly researched MMPs due to their roles in degradation of basement membrane components. Gelatinase activity was first detected in rheumatoid synovial fluid in 1972 (Harris and Krane 1972). Initial experiments suggested that gelatinases digest soluble type IV collagen (Liotta et al. 1979; Murphy et al. 1981). This activity remains questionable because neither MMP-2 nor MMP-9 degrade full-length type IV collagen (Mackay et al. 1990). However, gelatinases are characterized by three repeats of type II fibronectin-like gelatin binding regions, which enable them to bind to the denatured form of collagen (O'Farrell and Pourmotabbed 1998). Gelatinases are of great interest due to their roles in cancer (Liotta et al. 1979), luteal regulation (Goldberg et al. 1996), and many other reproductive and pathological processes (Birkedal-Hansen 1995b; Birkedal-Hansen et al. 1993; Hulboy et al. 1997).

Gelatinase A: MMP-2

Gelatinase A, also known as MMP-2 is an ~72 kDa protein in its latent form that is cleaved to its ~62 kDa active form via a membrane-dependent mechanism (Brown et al. 1990). MMP-2 degrades gelatin, type IV, type I (Aimes and Quigley

1995), type V (Okada et al. 1990), type VII (Seltzer et al. 1989), and type X (Welgus et al. 1990) collagens as well as numerous other components of the ECM such as elastin and laminin (Giannelli et al. 1997; Senior et al. 1991). This wide range of substrates enables MMP-2 to participate in a variety of cellular processes, including cell proliferation, differentiation, adhesion of cells to the ECM, and cellular migration. Due to these actions, MMP-2 is thought to have a significant role in tumor invasion (Brown et al. 1990), angiogenesis (Brooks 1996), and Alzheimer's disease (LePage et al. 1995).

MMP-2 is constitutively expressed in most cell types (Yeow et al. 2001) and its transcription appears to be only mildly induced or repressed (2-4 fold), (Huhtala et al. 1991) compared to other MMPs that can be more significantly induced (up to 100 fold) (Brown et al. 1990). This suggests that MMP-2 regulation is primarily controlled by extracellular activation or inhibition by TIMPs. Pro-MMP-2 is often associated with TIMP-2 at their respective C-termini (Goldberg et al. 1989). A proposed mechanism of activation couples proMMP-2 and TIMP-2, with the N-terminus of TIMP-2 bound to MT1-MMP (Butler et al. 1998). Upon binding, MT1-MMP becomes inactivated, however, other local MT1-MMPs activate the associated MMP-2 molecule through cleavage of the N-terminus of proMMP-2, thus revealing the zinc-dependent active site (Butler et al. 1998; Hernandez-Barrantes et al. 2000). MMP-1 (Crabbe et al. 1994a) and MMP-7 (Crabbe et al. 1994b) have also been linked to MMP-2 activation through cleavage of the proMMP-2 N-terminus. MMP-2 then in turn is capable of binding and activating other MMPs such as proMMP-1, proMMP-2 (Crabbe et al. 1994a), and proMMP-13 (Knauper et al. 1996).

Gelatinase B: MMP-9

Gelatinase B, also known as MMP-9 is an ~92 kDa protein in its latent form that is cleaved to its ~84 kDa active form via a membrane-dependent mechanism (Brown et al. 1990). It is processed with N- and O-linked glycosylation (Murphy et al. 1994). Like MMP-2, MMP-9 degrades gelatin, type IV, and type V collagens. MMP-9 is more effective than MMP-2 at degrading type V collagen, however, MMP-9 is incapable of degrading type I collagen or fibronectin (Yu et al. 1998). MMP-9 was first characterized in rabbit bone culture medium (Murphy et al. 1981) and in human and porcine neutrophils (Murphy et al. 1981).

Much like MMP-2, MMP-9 is secreted as a proMMP-9/TIMP-1 complex associated at the C-terminus (O'Connell et al. 1994; Ward et al. 1991). The activation of MMP-9 depends on its association with TIMP-1, meaning that MMP-9 only undergoes one cleavage when bound to TIMP-1. When TIMP-1 is not bound, MMP-9 undergoes a series of cleavages from the C-terminus to result in ~67 kDa and ~83 kDa forms. If there is no association with TIMP-1, then MMP-3 processes the active ~67 kDa form to an inactive ~50 kDa form (Shapiro et al. 1995). ProMMP-9 is activated by MMP-1, MMP-7, MMP-2 (Fridman et al. 1995), mast cell chymase (Fang et al. 1996), trypsin, MMP-3, and cathepsin G (Okada et al. 1992). In addition to cytokine stimulation, MMP-9 can be upregulated by component fragments of the ECM, such a laminin and fibronectin (Corcoran et al. 1995; Huhtala et al. 1991)

Unlike MMP-2, MMP-9 is only constitutively expressed by several tissues including neutrophils, macrophages, osteoclasts, and trophoblasts. MMP-9 is found in high levels in physiological processes and pathologic conditions such as bone

development (Engsig et al. 2000; Vu and Werb 2000), implantation (Reponen et al. 1995), and inflammation (Leppert et al. 1995). It is strongly believed that MMP-9 helps to regulate physiological responses such as inflammation, tissue injury and tissue remodeling and may play a role in subendothelial basement membrane reorganization, allowing immune cell infiltration into an area (Vu and Werb 2000; Werb et al. 1996).

MMPs and TIMPs in the CL

Since the CL is a unique tissue that undergoes routine formation and regression, the turnover and remodeling of the ECM must be tightly controlled. It is believed that ECM remodeling and reorganization is controlled, in part, by the balance between TIMP and MMP activity. *In vitro* studies using follicle and luteal cells demonstrated that ECM components direct differentiation, particularly follicular to luteal cells, and that loss of ECM may cause apoptosis and dedifferentiation (Smith et al. 1999; Sternlicht and Werb 2001).

MMPs and TIMPs in the CL

During the estrous cycle, TIMPs and MMPs play important roles in luteal angiogenesis, CL growth and development, and luteolysis. Several TIMPs and MMPs have been characterized in the ovaries of several species. TIMP-1 is a major secretory product of the sheep CL shortly following ovulation, particularly days 3-10 of the estrous cycle (Smith et al. 1994a; Smith et al. 1994b). TIMP-2 also is expressed at its highest levels early in CL development, days 3-7 (Smith et al. 1994b;

Zhang et al. 2003). Evidence shows that TIMPs, particularly TIMP-1, may be a stimulator of cell proliferation of various cell types within the CL, and steroidogenesis, particularly in LLCs, where TIMP-1 mRNA is found in concentrations up to 15 times higher than in SLCs (Smith et al. 1994b). Likewise, TIMP-1 has been found to stimulate steroidogenesis in rat leydig cells and granulosa cells (Boujrad et al. 1995). TIMPs have also been linked to luteinization (Edwards et al. 1996; Nothnick et al. 1997). In turn, evidence suggests that these proteins, particularly TIMP-1, may be under hormonal control (O'Sullivan et al. 1997).

In rats, MMPs are found at their highest levels in the early stages of CL development compared to any other stage (Nothnick et al. 1996). A possible link is suggested between MMP activity allows for blood vessel formation and successful CL development (Goldberg et al. 1996; Zhang et al. 2002). It has also been determined that deficient levels of MMP-2 lead to reduced fertility. This was demonstrated in ewes when immunization with an MMP-2 antibody led to incomplete formation of the CL (Gottsch et al. 2001). During CL formation, intense tissue remodeling occurs, which explains the increased expression of MMP-2, MMP-9, and MMP-1 in the mid stage porcine CL (Pitzel et al. 2000). As the CL wanes, P concentrations correlate to MMP expression. For example, MMP-2, MMP-9, and MMP-13 levels increase dramatically following a decrease in peripheral P levels (Duncan et al. 1998; Liu et al. 1999; Towle et al. 2002), suggesting a role for these enzymes in luteolysis.

Summary

In the developing CL there is a tight balance between TIMPs and MMPs that promote the rapid growth of the tissue. Following this rapid period of growth, it appears that the expression of some luteal TIMPs decrease while MMP expression increases, coinciding with peripheral P decline associated with the action of PGF2α. In the following chapter, select functional (COX-1, COX-2, StAR, P) and structural (TIMP-1, TIMP-2, MMP-2, MMP-9) mediators of luteolysis are investigated. Specifically, the goal of the present study is to use an established *in vivo* model to determine the temporal relationship among these mediators following two sequential pulses of PGF2α during luteolysis in sheep.

CHAPTER II

TEMPORAL EXPRESSION OF PROTEIN MEDIATORS DURING PGF2α-INDUCED LUTEOLYSIS IN SHEEP

Introduction

The corpus luteum (CL) is an ephemeral endocrine gland that is crucial for reproduction. The functionality of the CL is marked by the production of the steroid hormone progesterone (P), which is necessary for successful implantation of the embryo and maintenance of pregnancy in most mammals (Ellicott and Dziuk 1973). If pregnancy does not occur, the CL undergoes luteolysis, a process that is initiated by prostaglandin F2α (PGF2α). Defects in luteal function have been associated with infertility, abortion, and ovarian cycle disorders (Niswender et al. 2000).

It was determined in the 1970's that PGF2α is the primary agent responsible for initiating luteolysis in estrous cycling animals (McCracken et al. 1972; McCracken et al. 1970). This notion was first indicated by the prolonged lifespan of the CL after removal of the uterus (hysterectomy) in guinea pigs (Loeb 1923a), and further supported by work showing that the uterus is a primary source of endogenous PGF2α responsible for initiating luteolysis in estrous cycling animals (McCracken et al. 1972). Luteolysis has been described as a two step process: a decline in P production that leads to functional demise, and luteal involution that leads to structural regression (Diaz et al. 2002; McCracken et al. 1999)

In the sheep, luteolysis is caused by a sequential series of four to five one-hour-long pulses of uterine PGF2α, which activates a cascade of events that ultimately result in the functional and structural demise of the CL (McCracken et al. 1999). This pulsatile infusion of PGF2α induces luteolysis with only $1/40^{th}$ of the amount required when given by continuous infusion (Schramm et al. 1983). Thus, in order to study progressive biochemical changes within the CL in vivo, a model was established in which sheep, during the mid-luteal phase, received systemic infusions of PGF2α (20ug/min/1hr) at intervals mimicking the frequency of PGF2α pulses at the onset of natural luteolysis. This dosage provides enough PGF2α systemically to escape metabolism by the lung to a point equivalent to physiological conditions.

We have successfully used this physiological model to determine dynamic changes in the protein expression of several molecular mediators of luteolysis after a 1 hr infusion of PGF2α (Allen et al. *in review;* Towle et al. 2002). Specifically, we reported that TIMP-1 and TIMP-2 proteins decreased drastically at 1 hr, which was accompanied by an increase in MMP-2 activity at 4 and 8 hrs following PGF2α infusion. The increase in MMP-2 at 8 hrs was coupled with a nadir in StAR and peripheral plasma progesterone (P) concentrations. In addition, there was a delayed induction of COX-2 protein at 16 and 24 hrs following PGF2α infusion.

Collectively, these data provided the impetus for the present study, which was to determine the response of these proteins following two 1 hr infusions of PGF2α. We chose to continue monitoring the zinc- and calcium-dependent enzymes, matrix metalloproteinases (MMPs), and their inhibitors (TIMPs) because of their influence on the structural remodeling of the extracellular matrix (ECM). The ECM influences

cellular processes such as migration, differentiation, gene expression, and apoptosis (Ricke et al. 2002a). Furthermore, in domestic ruminants, luteolysis is marked by loss of cell adhesion to the ECM and loss of progesterone synthetic capacity (Ricke et al. 2002b). Besides the MMPs and TIMPs, we also monitored other proteins that mediate luteal function. These mediators include the cyclooxygenase (COX) enzymes -1 and -2, and steroidogenic acute regulatory protein (StAR). Prostaglandin endoperoxidase H synthases, or COX, are membrane bound enzymes (Smith and Dewitt 1996) capable of both peroxidase and oxygenase activity (Smith et al. 1996). These combined activities convert arachidonic acid to produce PGG2 (Smith and Dewitt 1996; Smith et al. 1996), a precursor of PGF2α. In response to PGF2α, the CL upregulates COX synthesis (and thus prostaglandin production) in a amplification and positive feedback manner (Tsai and Wiltbank 1997).

Another protein of interest is StAR, which is central to steroidogenesis. StAR transports cholesterol from the cytoplasm across the mitochondrial membranes (Stocco 2001), thus providing substrate for the steroidogenic pathway . In the porcine CL, StAR protein expression is decreased after PGF2α treatment (Diaz and Wiltbank 2005).

Therefore, to gain further insight into the temporal response of the sheep CL to PGF2α, the specific objective of our study was to determine the protein expression patterns of TIMP-1 and TIMP-2, COX-1 and COX-2, and StAR after two pulses of PGF2α.

Materials and Methods

Animal Model of PGF2α-Induced Luteolysis

To mimic the physiological onset of luteolysis in sheep, a single 1 hr systemic infusion of PGF2α (~.22 μg/kg/min) can be administered at mid-cycle (Custer et al. 1995). This rate of infusion provides a sufficient level of PGF2α that reaches the ovary even after metabolism by the lungs, resulting in a 40% decline in P (Towle et al. 2002). This decline in P levels is similar to that seen following an endogenous pulse of PGF2α (Zarco et al. 1988). This established model was used to study the in vivo changes in molecular mediators of luteolysis following two systemic infusions of PGF2α, with the second infusion given 16 hrs after the first.

Tissue Collection and Preparation

Luteal tissue was collected and prepared as previously described (Towle et al. 2002). A group of mixed Suffolk and Dorset ewes (*Ovis aries;* approximately 90 kg) were housed at the University of Connecticut (Storrs, CT) and their estrous cycles synchronized using two intramuscular injections of Lutalyse (5mg; UpJohn Co.; Kalamazoo, MI) given at 4 hour intervals. Ewes were observed twice daily for estrus using a vasectomized ram following treatment (Day 0 = estrus). On the eleventh day post estrus ewes were placed in metabolism cages and 16 gauge cannulae were inserted into both jugular veins, while under local anesthesia (2% [w/v] lidocaine). Two 1 hr systemic infusions of PGF2α (UpJohn Co.) were administered, 16 hrs apart to mimic endogenous frequency, to the right jugular vein via a Harvard Infusion Pump (model no. 600-910/920; Harvard Apparatus Co.; Holliston, MA) at a rate of

20 μg/min (Custer et al. 1995). Control animals received no treatment. CL were removed surgically via flank laparotomy under local anesthesia (2% lidocaine) before the second PGF2α infusion, designated as time 0 (controls), and 1, 8, 16, and 24 hrs post PGF2α infusion (n=4 sheep for each time point; see **Figure 5**). All experimental animal procedures received prior approval by the University of Connecticut Animal Care and Use Committee. After collection, luteal tissue was immediately placed on dry ice, and stored at -80°C. For analysis, individual samples representing each of the five time points were analyzed in series (n=4).

Protein Extraction

Luteal tissue proteins (TIMP-2, StAR, COX-1, and COX-2) were extracted with a buffer (50mM Tris-HCl, 150mM NaCl, 0.02% [w/v] sodium azide, 10mM EDTA, 1% [v/v] Triton X-100, pH 7.4) containing protease inhibitors (1μg/ml AEBSF, 1μg/ml pepstatin A, 10μg/ml aprotinin) in a ratio of 1g tissue : 8ml extraction buffer, as previously described (Zhang et al. 2002). Tissue was homogenized with a Kinematica Polytron, and the homogenate was then sequentially centrifuged at 800 x g for 10 minutes and at 10,000 x g for 10 minutes at 4°C. The supernatant between the pellet and fat layer was then removed and stored at -20°C until used for immunoblot analysis of TIMP-2 and StAR. For COX-1 and COX-2 analysis, a portion was removed after homogenization, and sonicated twice for 5 seconds (Sonifier Cell Disruptor 350, Branson Sonic Power Co.) prior to centrifugation, as described above. Complete extraction protocols are found in **Appendix A.**

Luteal TIMP-1, MMP-2, and MMP-9 proteins were extracted in 2 M NaCl, 0.01M HEPES, 0.02% NaN3, pH 7.6, as previously described (Moses et al. 1990; Murray et al. 1986; Towle et al. 2002). Briefly, conditions were normalized using an 8:1 (v/w) ratio of extraction buffer to tissue (8ml:1g). Samples were minced with scalpel blades and homogenized before being placed on a Clay Adams Nutator Mixer (Beckton Dickinson, Sparks, MD) to extract for 24 hours at 4°C. Extracts were then centrifuged initially at 2,500 x *g* followed by a final centrifugation at 15,000 x *g* to further remove debris. Afterwards, the samples were ultrafiltered through Amicon Ultra 4 concentrators (10,000 M.W.C, Amicon, Beverly, MA) at 3,500 x *g* before dialysis with CAB+ buffer (0.2M NaCl, 1mM $CaCl_2$, 50mM Tris, 0.02% NaN_3, pH 7.6). The samples were ultimately concentrated 4-fold before storage at 4°C until analysis. Details are in **Appendix B.**

Protein Determination (Bradford Assay)

Protein concentrations were determined by the Bradford Method (Bradford 1976) using Coomassie Blue. Bovine serum albumin (Sigma; St. Louis, MO) dissolved in CAB+ buffer (0.2M NaCl, 1mM $CaCl_2$, 50mM Tris, 0.02% NaN_3, pH 7.6) was the protein standard. Optical densities were read at 595nm using a spectrophotometer (model DU640; Beckman Instruments; Fullerton, CA). All luteal tissue protein samples were diluted 1:10 or 1:20 with CAB+ buffer and assayed in triplicate. Details are in **Appendix C.**

Immunoblot Analysis

Equivalent amounts of luteal tissue protein extracts were loaded on SDS-PAGE gels (10% [w/v] for COX-1, COX-2, TIMP-1 and TIMP-2, and 12.5% [w/v] for StAR). Except for TIMP-1, all were run under reducing conditions, (5% [v/v] 2-mercaptoethanol, 100°C for 5 minutes), at 150 V for 45-60 minutes, along with the dual color, prestained SDS-PAGE marker standards (Bio-Rad Laboratories, Hercules, CA, USA). Separated proteins were transferred to a Protran nitrocellulose membrane (Schleicher & Schuell; Whatman Group; Keene, NH, USA) for 2 hrs at 200mA. Non-specific binding was blocked with 5% [w/v] non-fat dried milk in TBST buffer (0.01M Tris-HCl, 0.15M NaCl, 0.05% [v/v] Triton X-100, pH 8.0) for either 2 hrs (COX-1 and COX-2) or 1 hr (StAR, TIMP-1, and TIMP-2) at room temperature. See **Appendix D** for immunoblot and zymography recipes. Respective primary antibodies were then added, and allowed to incubate either overnight at 4°C (COX-1, COX-2, TIMP-1, and TIMP-2) or for 1 hr at room temperature (StAR). The membrane was then washed with TBST buffer before incubation with a secondary antibody for 1 hr at room temperature. Following TBST washes, blots were visualized using an enhanced chemiluminescent (ECL) detection system (SuperSignal West Pico Chemiluminescent Substrate, Pierce). The blots were exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY, USA), which was developed with a Konica (Wayne, NJ, USA) automatic developer. Each sample was run in duplicate. Two negative controls were performed for each protein; one with either normal mouse or rabbit non-specific IgG in lieu of primary antibody, and the second was exclusion of the primary antibody. Details are in **Appendix E.**

Immunoblot Antibodies and Positive Controls

All COX antibodies and proteins were purchased from Cayman Chemical (Ann Arbor, MI, USA). The COX-1 monoclonal antibody was used at a concentration of 4μg/ml, and was raised in mice against purified ovine COX-1. An ovine recombinant COX-1 protein (0.5μg) served as the positive control. The COX-2 polyclonal antibody, used at 1:1000, was raised in rabbits against a synthetic peptide from the C-terminus region of mouse COX-2, and cross-reacts with ovine, murine, and rat COX-2, but does not cross-react with COX-1 (Tsai and Wiltbank 1997). An ovine COX-2 recombinant protein (0.5μg) served as the positive control. The StAR protein polyclonal antibody (1:1000) was a generous gift from Dr. Douglas Stocco (Texas Tech University, TX, USA). The antibody was raised in rabbits against a synthetic peptide created from amino acids 88-98 of the mouse StAR protein (Clark et al. 1994). The bovine CL was used as a positive control, co-migrating with the 30 kDa protein in each sample (Pescador et al. 1996). The TIMP-1 and TIMP-2 primary antibodies were purchased from Oncogene Research Products (Cambridge, MA). The TIMP-1 monoclonal antibody was raised in mice against human TIMP-1 and was used at a concentration of 1μg/ml (Cat# IM32L). The TIMP-2 monoclonal antibody was also raised in mice against human TIMP-2 and was used at a concentration of 5μg/ml (Cat# IM56). Furthermore, a recombinant bTIMP-1, or a human TIMP-2 protein standard (Oncogene Research Products; Cambridge, MA) was included where appropriate. The secondary antibodies were immunoglobulin G conjugated to horseradish peroxidase (Pierce, Rockford, IL, USA). Anti-rabbit secondary antibody (1:10,000) was used for

StAR and COX-2 immunoblots, while anti-mouse (1:10,000) was used for COX-1, TIMP-1, and TIMP-2 immunoblots.

Gelatin Zymography

Zymography was used to detect gelatinase (MMP-2 and MMP-9) activity using previously described methods (Towle et al. 2002) with minor modifications. Luteal extracts were mixed 1:1 with sample buffer (10% SDS, 4% sucrose [w/v], 0.1% bromophenol blue [w/v], 0.25M Tris, pH 6.8) and electrophoresed under non-reducing conditions at ~200 volts, using the Mini-Protean II system (Bio-Rad, Melville, NY) in 10% polyacrylamide gels containing 0.05% gelatin. Gels were then washed twice (15 minutes each) in 2.5% Triton X-100 (v/v) to remove SDS, rinsed with distilled water and incubated for 17-18 hours at 37°C in substrate buffer (5mM $CaCl_2$, 50mM Tris, pH 8.0). After incubation, gels were stained with Coomassie Blue R250 solution (0.5% in a 1:3:6 ratio of acetic acid, isopropanol, distilled water) for 30 minutes and destained with distilled water for 2 days. MMP activity was observed as zones (bands) of clearance against the blue background of the gel. Adjacent lanes contained Perfect Protein markers (Novagen, Madison, WI) and the positive control, HT-1080 (Cat# CCL-121; American Type Culture Collection; Manassas, VA), which is conditioned medium of a human fibrosarcoma cell line known to produce several MMPs (e.g., MMP-2 and MMP-9, as well as TIMPs (e.g., TIMP-1 and TIMP-2). Details are in **Appendix F.**

To verify that the bands of clearing were the result of metal-dependent proteinases (MMPs), gels were incubated in substrate buffer containing 1,10-phenanthroline (10mM; Sigma; St. Louis, MO). Latent and active forms of the

MMPs were distinguished by *incubating* the samples with 2mM p-aminophenylmercuric acetate (APMA; Sigma; St. Louis, MO) for two hours prior to electrophoresis.

Quantification and Statistical Analysis

Photographic films of immunoblots and zymograms were densitized using UN-SCAN-IT version 6.1 (Silk Scientific Industries, Orem, UT, USA). Total pixel counts for every sample duplicate at each time point for individual sheep were determined, and averaged before analysis of variance was performed using the General Linear Model (GLM) subroutine of the Statistical Analysis System [SAS Institute Inc., 1989]. Differences in the treatment means were evaluated with least square differences (LSD) with appropriate corrections for multiple comparisons. A value of $p < 0.05$ was considered to be significant.

Results

Analysis of TIMPs and MMPs

As for the ECM mediators, immunoblotting revealed the presence of an ~30 kDa protein, consistent with the reported M_r of TIMP-1 and which also co-migrated with the TIMP-1 recombinant protein **(Fig. 6)**. TIMP-1 expression decreased 60% ($p<0.05$) at 1 hr before recovering by 8 hrs and reaching a 30% increase ($p<0.05$) at 24 hrs. A TIMP-2 protein was also identified in all samples, co-migrating with a 19 kDa human TIMP-2 protein standard **(Fig. 7)**. Like TIMP-1, TIMP-2 protein levels decreased by nearly 90% ($p<0.05$) by the 1 hr time point and remained low throughout the 24 hr sampling period.

Gelatinase (MMP-2 and MMP-9) activity was identified by zymography in all samples. Besides co-migration with the HT-1080 positive control, true MMP activity was verified with 1,10-phenanthroline, while latent and active species were distinguished after incubation with APMA (data not shown). In sheep CL, active MMP-2 (~64 kDa), latent MMP-2 (~70 kDa), and active MMP-9 (~85 kDa) were identified **(Fig. 8)**. Active MMP-2 increased ($p<0.05$) at the 1 hr time point before returning to basal levels by 8 hrs, while latent MMP-2 did not change at any time point ($p>0.05$). MMP-9 displayed a rising, but non-significant ($p>0.05$) trend towards the later time points.

Analysis of COX enzymes

In all luteal tissue samples, visual observations of immunoblots revealed an ~70-kDa protein, which co-migrated with a COX-1 recombinant protein **(Fig. 9)** and a single ~72-kDa protein, which co-migrated with a COX-2 recombinant protein **(Fig. 10)**. Densitometric analysis revealed no change ($p>0.05$) in the protein expression of COX-1 or COX-2 protein over time.

Analysis of StAR and Progesterone

In all ovine luteal tissue samples, immunoblotting revealed an ~30-kDa protein, which co-migrated with the previously characterized StAR protein from bovine luteal tissue (Pescador et al., 1996) **(Fig. 11)**. Peripheral progesterone concentrations **(Fig. 12)** displayed a 40% decline by 8 hrs before rising above pre-infusion control levels at 24 hrs following two systemic infusions of PGF2α.

Discussion

The ovine physiological model used in this study allowed us to determine the temporal expression of protein mediators during the onset of PGF2α induced luteolysis. The molecular mediators chosen in this study are considered to participate or serve as indicators of functional and structural demise of the CL. Previously, we have used this model to study dynamic in vivo changes in TIMP and MMP (Towle et al. 2002), and COX and StAR (Allen et al. *in review*) protein expression after a single infusion of PGF2α. In the present study, we examined the response of these same molecular mediators following a second 1 hr systemic infusion of PGF2α. Thus, this provides a unique opportunity to assess successive changes that occur in the CL during the early stages of luteolysis. At this point, it is important to mention that two physiological pulses/infusions of PGF2α are not sufficient to induce luteolysis (McCracken et al. 1999).

PGF2α of uterine origin is the luteolysin in sheep, and is known to stimulate a number of biochemical pathways that mediate both structural and functional regression (McCracken et al. 1999). Evidence suggests that very low concentrations of uterine PGF2α may be adequate to stimulate luteolysis (McCracken et al. 1984) through an auto-amplification feedback loop that upregulates luteal PGF2α, which is mediated by cyclooxygenase (COX) enzymes via PKC activation and Ca^{++} mobilization (Wu and Wiltbank 2001a). This positive feedback system may act in an autocrine manner to inhibit P production by the large luteal cells (LLCs) and the subsequent loss of function of the CL (Wu and Wiltbank 2001b). Indeed, we previously reported that COX-1 protein expression was unchanged, while COX-2 was

upregulated approximately 300% by 24 hrs after a single systemic infusion of PGF2α (Allen et al. *in review*). Interestingly, in the current study, COX-2 protein expression did not appear to change within the 24 hr sampling period following a second infusion of PGF2α. This could be explained by the fact that COX-2 protein levels are already elevated 300% following a single 1 hr infusion of PGF2α, suggesting that COX-2 protein may have already reached maximal expression following a single infusion of PGF2α **(Fig 10, Fig 13)**.

In addition to a delayed induction of COX-2 expression, PGF2α depresses P synthesis. Peripheral P concentrations declined 40% at 8 hrs following a single infusion of PGF2α (Towle et al. 2002), which parallels the drop in StAR protein (Allen et al. *in review*). In the current study, peripheral P concentrations again dropped 40%, reaching a nadir at 8 hrs following a second infusion of PGF2α. Interestingly, StAR exhibited a transient, but non-significant drop in expression that did not occur until 16 hrs following PGF2α infusion. The biological significance of the asynchrony between peripheral P concentrations and StAR protein expression is open to speculation. MMP-2 may be involved. The more rapid increase in MMP-2 activity (by 1 hr following a second infusion of PGF2α) perturbs the communication between the ECM and integrins, and in turn affects the trafficking of cholesterol by the cellular cytoskeleton. This is coupled with reports that membrane fluidity may be altered by PGF2α (Buhr et al. 1983; Goodsaid-Zalduondo et al. 1982). Therefore, it is possible that these changes in membrane fluidity may act in concert with the alterations of the ECM, leading to a decreased mobility of both LH and cholesterol receptors could account for the drop in P concentrations independent of changes in

StAR protein. MMP-2 may also act directly on StAR, as MMP-2 is thought to cleave StAR from an ~37 kDa active form to an ~30 kDa inactive form upon entering the mitochondria (Epstein and Orme-Johnson 1991), which may account for the decrease in P synthesis.

The TIMP and MMP family members are mediators of structural remodeling events. The balance of MMPs and TIMPs, therefore, determines the rate of turnover of the ECM, and it is believed that these mediators may alter the rate of cell adhesion and apoptosis in the CL (Sternlicht and Werb 2001). Following a single infusion of PGF2α, TIMP-1 and TIMP-2 protein levels plummeted at 1 hr (Towle et al. 2002) while MMP-2 activity increased at 4 hrs (Allen et al. *in review*). In the current study, TIMP-1 and TIMP-2 proteins continued to respond acutely following two 1 hr infusions of PGF2α, with a drastic decrease in expression at 1 hr, coinciding with an elevation of MMP-2 activity. These results are consistent with previous reports in which TIMP mRNA levels declined significantly following a luteolytic dose of PGF2α in the ovine models (McIntush et al. 1997). However, other findings indicate that both TIMP-1 and TIMP-2 mRNA in the bovine CL increase significantly above control levels 8 hrs following a luteolytic dose of PGF2α (Juengel et al. 1994). The dosage of PGF2α, the animal model, and the endpoint measured (mRNA versus protein) may account for differences between the current study and that of Juengel et al. (1994). In the current study, of note was the significant increase in TIMP-1 protein 24 hrs following two 1 hr infusions of PGF2α. Because the rise in TIMP-1 parallels peripheral P concentrations, and TIMP-1 has been shown to be a promoter of steroidogenesis (Boujrad et al. 1995), this could suggest that the CL may upregulate

TIMP-1 expression as a mechanism to recover from the insult of PGF2α. Further study is necessary.

Regarding the gelatinases, while MMP-9 was unchanged, MMP-2 activity increased sooner after two infusions of PGF2α than after one (Allen et al. *in review;* Towle et al. 2002). Previously reported data suggests that MMPs are upregulated and TIMP mRNA is downregulated, increasing the ratio of MMP:TIMP-1 mRNA and protein levels in response to PGF2α, thus creating a microenvironment that favors proteolysis (Ricke et al. 2002b). Our finding of an early increase in MMP-2 activity following PGF2α infusion may shift the balance between TIMPs and MMPs towards proteolysis of the ECM as luteal regression ensues. In fact, low levels of TIMP-2 stimulate MMP-2 activation by membrane type 1-MMP (Hernandez-Barrantes et al. 2000). This is due to preferential binding of TIMP-2 preferentially binds MMP-2 at the C-terminus (Goldberg et al. 1989), forming a membrane type 1-MMP/MMP-2/TIMP-2 triplex, which activates MMP-2 at the cell surface (Strongin et al. 1995). The imbalance between TIMPs and MMP-2 throughout two infusions of PGF2α is shown in **Fig 14**.

During PGF2α induced luteolysis in sheep, the interplay among these mediators is complex. Our current working model is depicted in **Fig 15**. Upon binding of PGF2α to the large luteal cell, nitric oxide synthase (NOS) is upregulated, which generates nitric oxide (NO). In turn, NO stimulates PGF2α production as shown in cultured bovine luteal cells (Skarzynski et al. 2003). Thus, there is a positive feedback mechanism that links PGF2α and NO in luteal regression (Estevez et al. 1999). This is supported by studies that show treatment with a NOS inhibitor

(N-ω-nitro-L-arginine methyl ester) prolongs the lifespan of the bovine CL (Jaroszewski and Hansel 2000; Jaroszewski et al. 2003). In addition, NO is produced by macrophages (Knight 2000), an important component of the immune system that plays a central role in luteolysis (Pate 1994). Furthermore, the NO that is generated can react with a superoxide anion (O_2^-) to yield peroxynitrite ($ONOO^-$), which inactivates TIMP-1 through catabolism (Frears et al. 1996). As a result of peroxynitrite action, TIMP-1 and probably TIMP-2 sharply decline.

PGF2α also increases COX-2 protein expression in the ovine CL (Allen et al. *in review*). Since the peroxynitrite generated from NO also activates COX through stimulation of its active site (Mollace et al. 2005), this culminates in the increased synthesis of PGF2α, downregulating StAR (Allen et al. *in review*) and subsequent P concentrations. Besides the effects of PGF2α on StAR, P biosynthesis may also be affected by perturbations of the cytoskeleton. It is known that the cellular cytoskeleton is connected to the ECM via transmembrane integrins (Calderwood et al. 2000; Choquet et al. 1997). When the ECM is disrupted due to decreased TIMP and increased MMP-2 activity, this leads to instability of the cytoskeleton, affecting the intracellular trafficking of cholesterol (Niswender et al. 2000).

Conclusions

In conclun, following the second of two 1 hr systemic infusions of PGF2α, luteal COX-2 protein remained elevated while COX-1 and StAR protein were unchanged. TIMP expression continued to be negatively influenced by PGF2α with an apparent inability of TIMP-2 to recover during the early stages of luteolysis.

Notably, MMP-2 activity was increased sooner from a second dose of PGF2α than from a single 1 hr infusion of PGF2α. In addition, studies using this model are currently underway to determine the relationship between NO and the above mentioned mediators. Thus, future investigations on the fate of these mediators following additional infusions of PGF2α are necessary.

Figures

Figure. 5: Experimental design: Bracketed arrows depict the one hour long infusions of PGF2α. The triangles indicate the time of luteectomy before (0), and 1, 8, 16, or 24 hrs following the second infusion of PGF2α.

Figure. 6: TIMP-1: Representative immunoblot and densitometric analysis of TIMP-1 protein in sheep luteal extracts. The time points are before (0 hr), and at 1, 8, 16, and 24 hrs from the onset of a second one-hr PGF2α infusion. On the left, molecular weight markers are indicated in kilodaltons (kDa). The arrow indicates the relative molecular mass of the TIMP-1 protein (~30 kDa), which is consistent with a TIMP-1 recombinant protein (RP) used as a positive control. For each sheep (n=4), total pixel counts for each sample (in duplicate) and for all time points were averaged and expressed as a percentage of the total pixel count for each time point over the pixel count for the control (time 0) (± SEM). Asterisks denote significance $p<0.05$.

Figure. 7: TIMP-2: Representative immunoblot and densitometric analysis of TIMP-2 protein in sheep luteal extracts. The time points are before (0 hr), and at 1, 8, 16, and

24 hrs from the onset of a second one-hr PGF2α infusion. On the left, molecular weight markers are indicated in kilodaltons (kDa). The arrow indicates the relative molecular mass of the TIMP-2 protein (~19 kDa), which is consistent with a TIMP-2 recombinant protein (RP) used as a positive control. For each sheep (n=4) total pixel counts for each sample (in duplicate) and for all time points were averaged and expressed as a percentage of the total pixel count for each time point over the pixel count for the control (time 0) (± SEM). Asterisks denote significance $p<0.05$.

Figure. 8: Representative zymogram and densitometric analysis of MMP-2 and MMP-9 in sheep luteal extracts. The time points are before (0 hr), and at 1, 8, 16, and 24 hrs from the onset of a second one-hr PGF2α infusion. On the left, molecular weight markers are indicated in kilodaltons (kDa). The arrow indicates the relative molecular mass of latent MMP-2 (~70 kDa), active MMP-2 (~64 kDa), and active MMP-9 (~85 kDa). For each sheep (n=4) total pixel counts for each sample (in duplicate) and for all time points were averaged and expressed as a percentage of the total pixel count for each time point over the pixel count for the control (time 0) (± SEM). Asterisks denote significance $p<0.05$.

Figure. 9: COX-1: Representative immunoblot and densitometric analysis of COX-1 protein in sheep luteal extracts. The time points are before (0 hr), and at 1, 8, 16, and 24 hrs from the onset of a second one-hr PGF2α infusion. The positive control is COX-1 recombinant protein (RP). On the left, molecular weight markers are indicated in kilodaltons (kDa). The arrow indicates the relative molecular mass of the

COX-1 protein (~70 kDa). For each sheep (n=4) total pixel counts for each sample (in duplicate) and for all time points were averaged and expressed as a percentage of the total pixel count for each time point over the pixel count for the control (time 0) (± SEM).

Figure. 10: COX-2: Representative immunoblot and densitometric analysis of COX-2 protein in sheep luteal extracts. The time points are the untreated time zero prior to any infusion (UT), before (0 hr), and at 1, 8, 16, and 24 hrs from the onset of a second one-hr PGF2α infusion. COX-2 recombinant protein served as a positive control (RP). On the left, molecular weight markers are indicated in kilodaltons (kDa). The arrow indicates the relative molecular mass of COX-2 (~72 kDa). For each sheep (n=4) total pixel counts for each sample (in duplicate) and for all time points were averaged and expressed as a percentage of the total pixel count for each time point over the pixel count for the control (time 0) (± SEM).

Figure. 11: StAR: Representative immunoblot and densitometric analysis of StAR protein in sheep luteal extracts. The time points are before (0 hr), and at 1, 8, 16, and 24 hrs from the onset of a second one-hr PGF2α infusion. The positive control is untreated, mid-cycle bovine luteal tissue (BLT). On the left, molecular weight markers are indicated. The arrow indicates the relative molecular mass of the (~30 kDa) StAR protein. For each sheep (n=4) total pixel counts for each sample (in duplicate) and for all time points were averaged and expressed as a percentage of the

total pixel count for each time point over the pixel count for the control (time 0) (± SEM).

Figure. 12: Mean concentration of progesterone in mid-cycle ewes (n=20) administered two one-hour infusions of PGF2α (20 μg/min/hr). Open triangles indicate times of luteectomies (n=4), performed at specific intervals (0, +1, +8, +16, +24 hours) relative to the second infusion of PGF2α.

Figure. 13: COX-2 protein expression after two infusions of PGF2α. Data are expressed as a percent of controls (0 hr time point, untreated) versus time in hours after the onset of PGF2α infusion. Arrow brackets indicate infusions of PGF2α.

Figure. 14: Ratio of TIMP and MMP expression after two infusions of PGF2α. Data are expressed as a ratio of MMP-2:TIMP-1 and MMP-2:TIMP-2, based on percent of controls (0 hr time point, untreated) versus time in hours after the onset of PGF2α infusion. Arrow brackets indicate infusions of PGF2α

Figure. 15: Our working cell model: 1) PGF2α binds a FP receptor and stimulates NOS and NO production. 2) Peroxynitrite catabolizes TIMP-1 and TIMP-2. 3) Imbalance of MMP to TIMP leads to ECM disruption and perturbations in steroidogenesis. 4) Delayed induction of COX-2 facilitates an auto-amplification feedback loop of PGF2α, possibly initiating cell death machinery.

Fig. 1

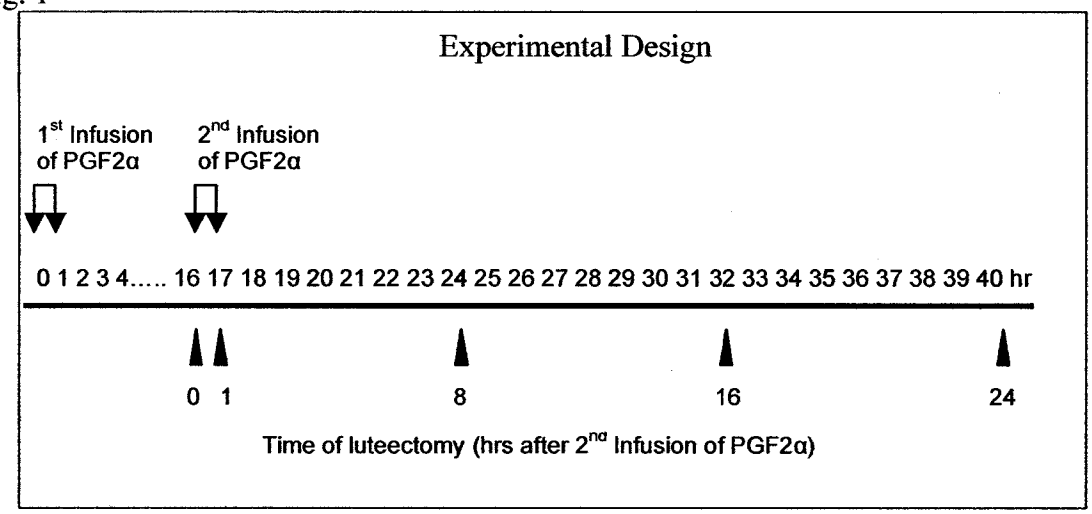


Fig. 2

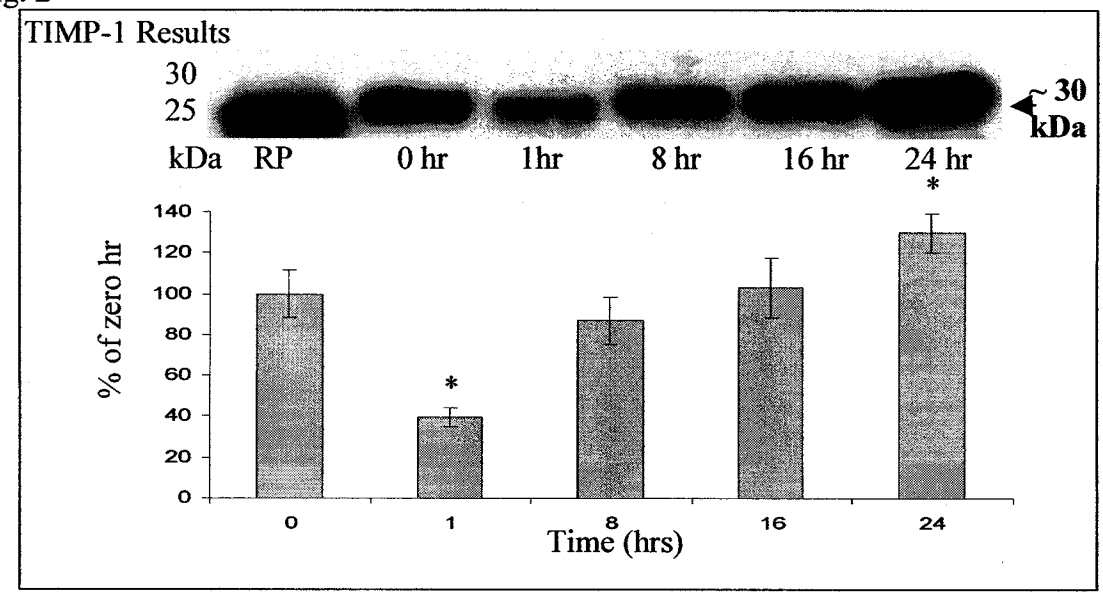


Fig. 3

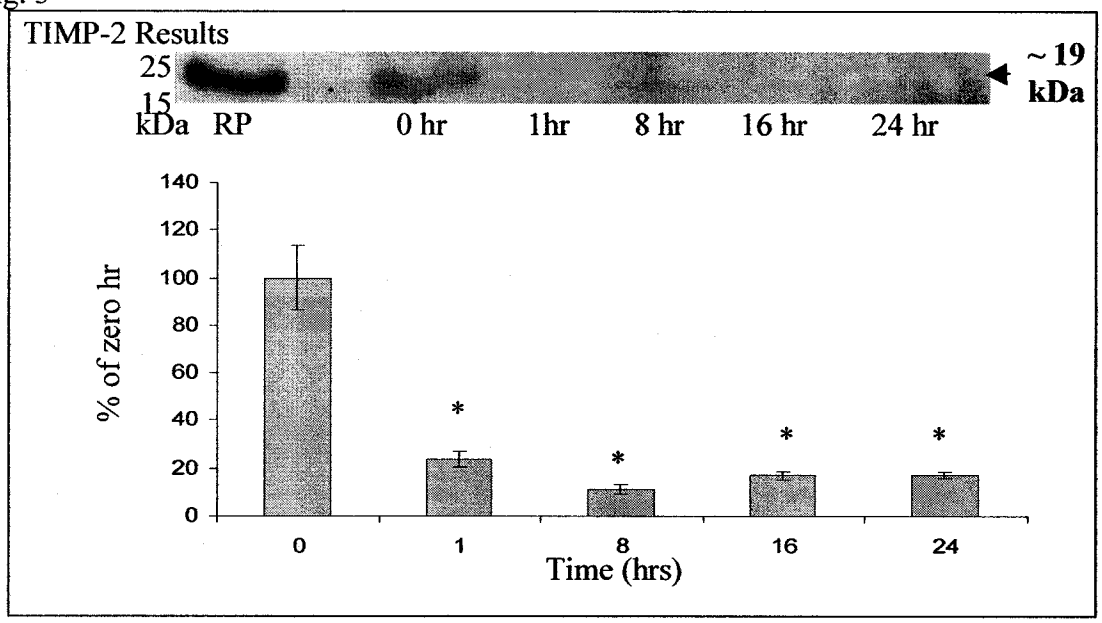
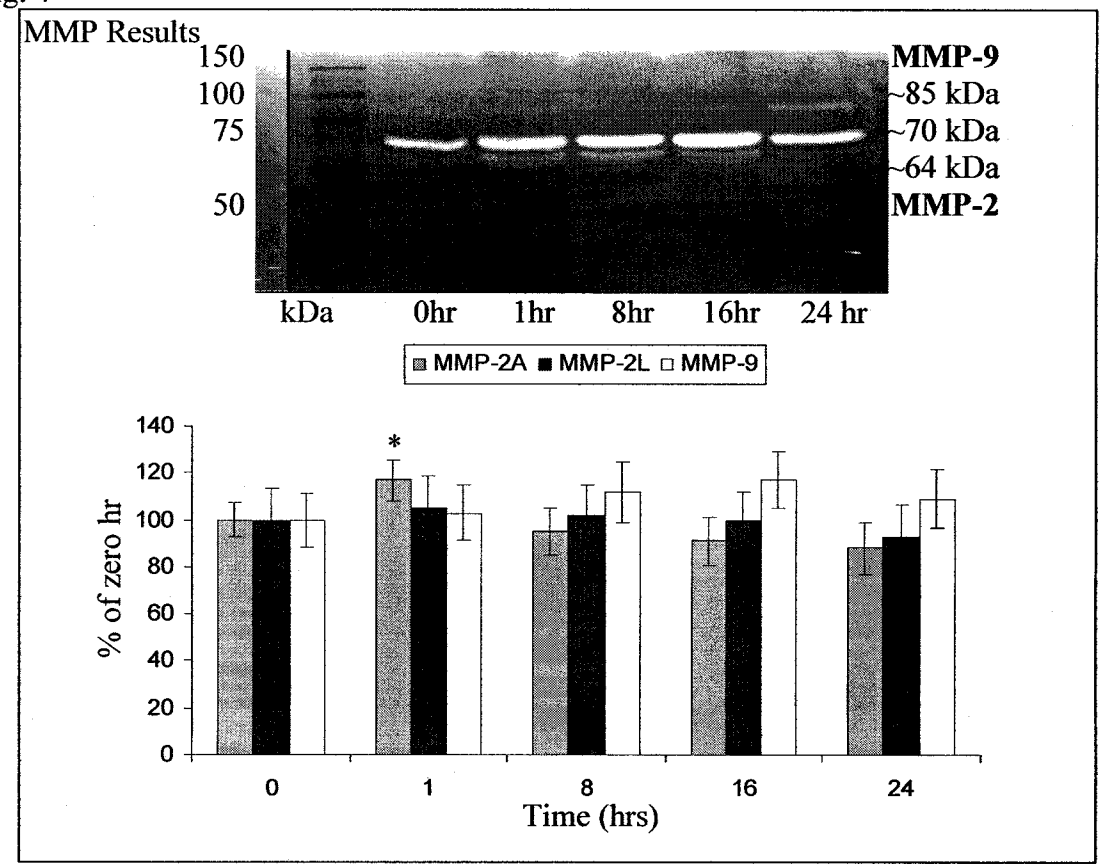


Fig. 4



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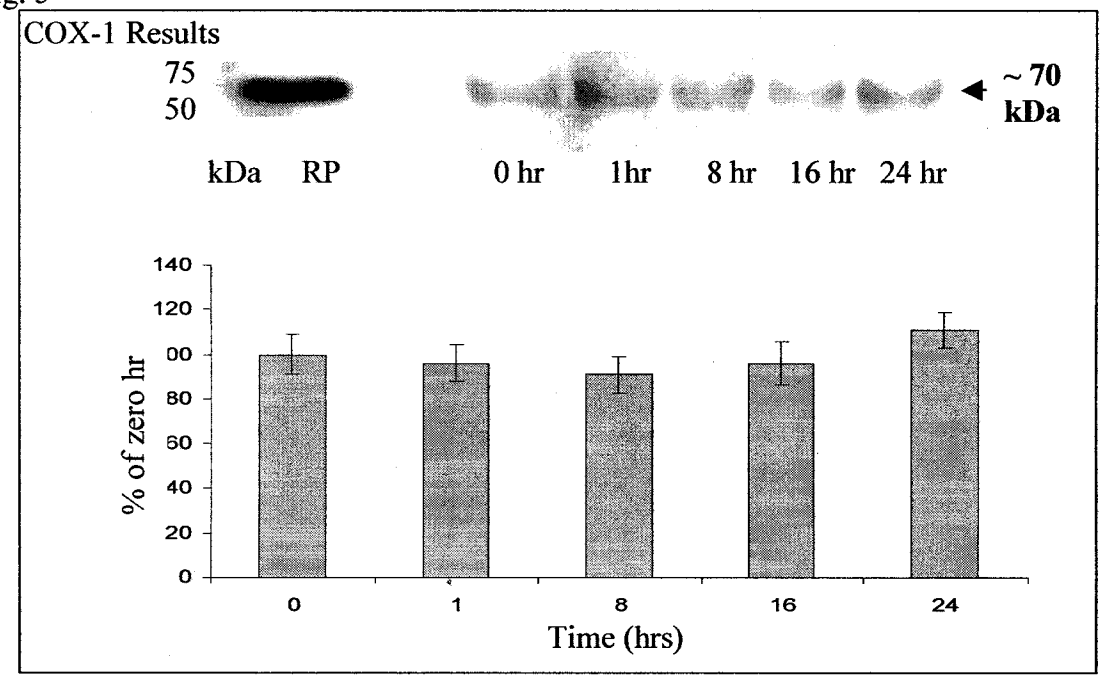

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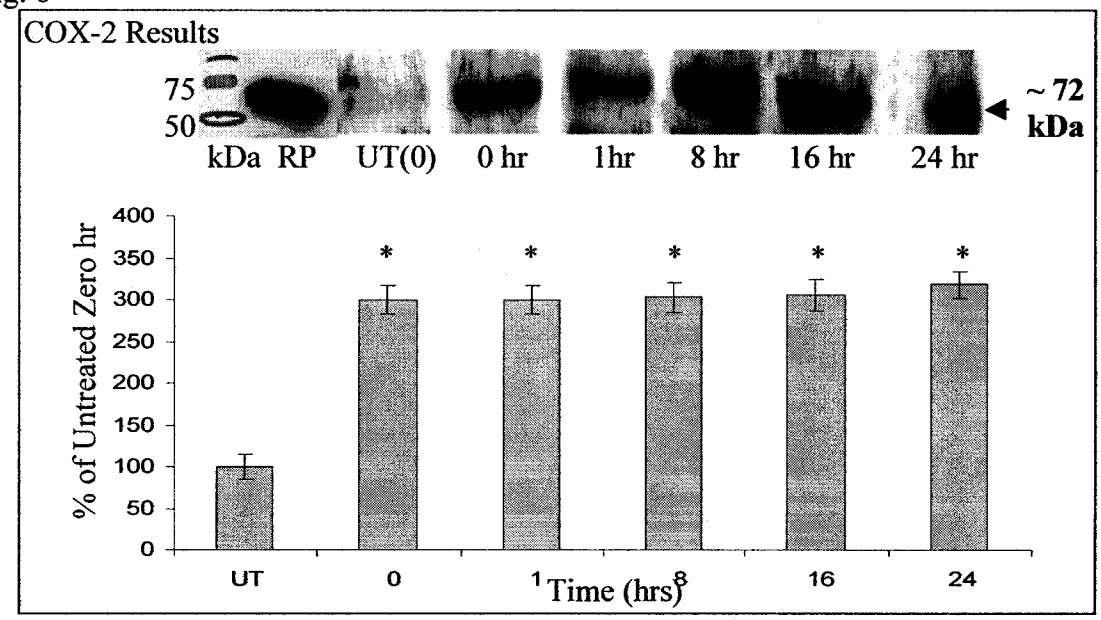


Fig. 7

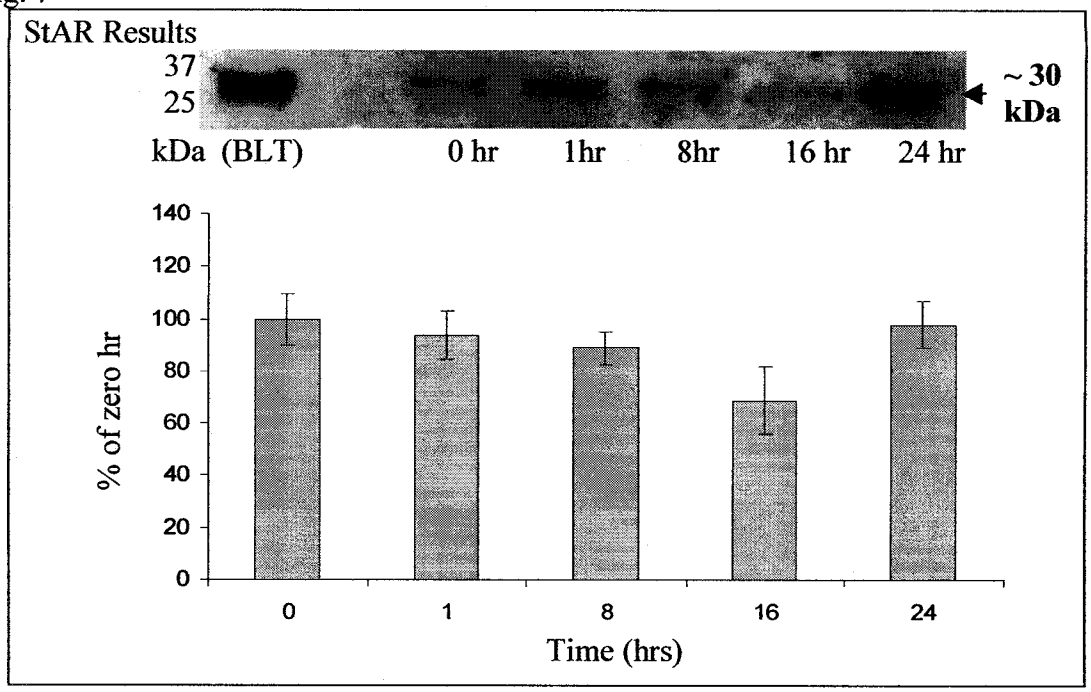


Fig. 8 Peripheral Plasma Progesterone Concentrations

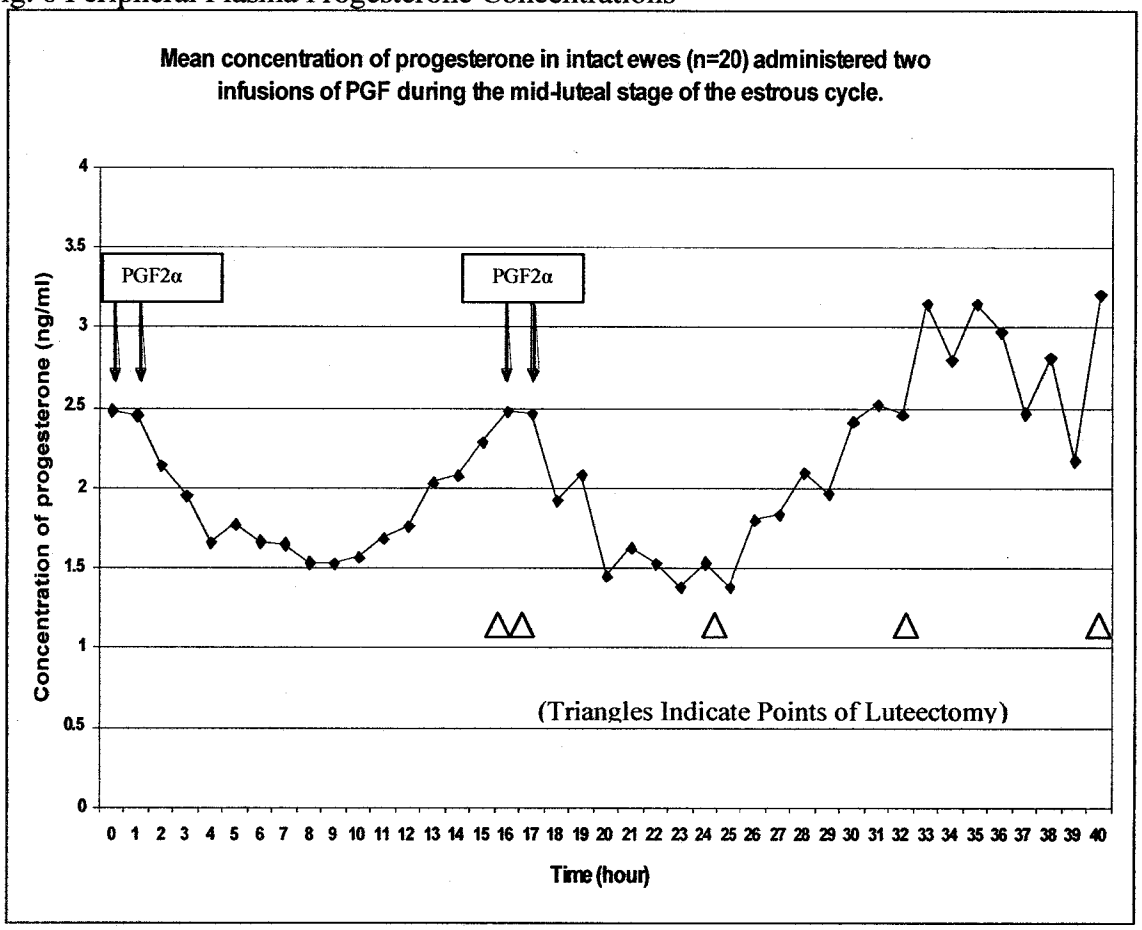


Fig. 9 Cumulative COX-2 Protein Levels Following Two Infusions of PGF2α

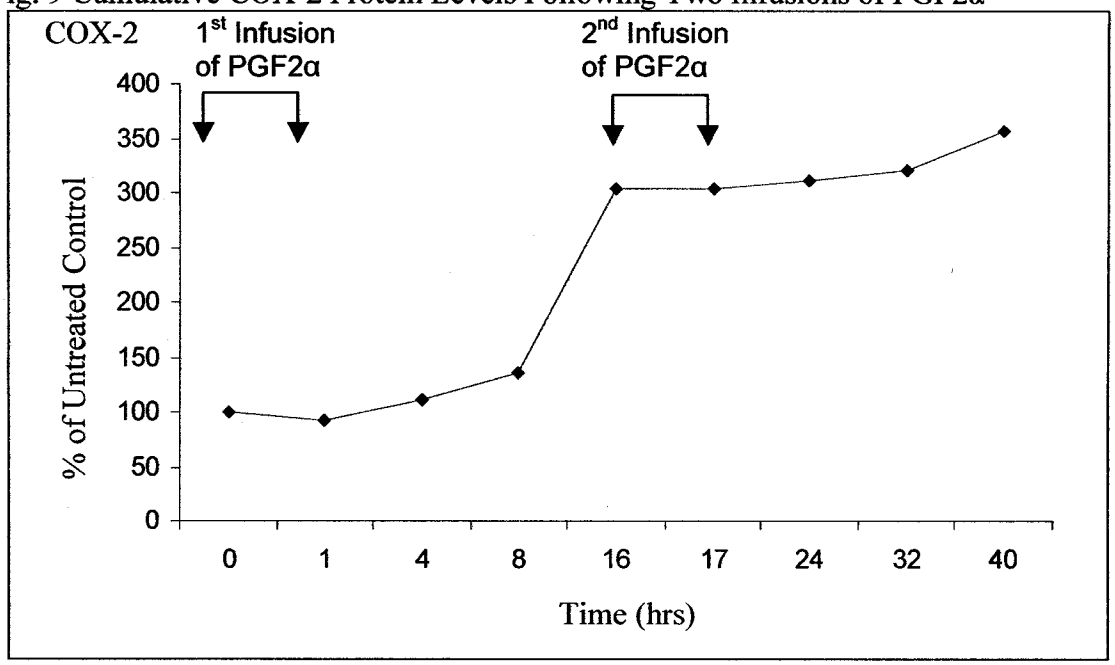


Fig. 10 Cumulative MMP:TIMP Protein Levels Following Two Infusions of PGF2α

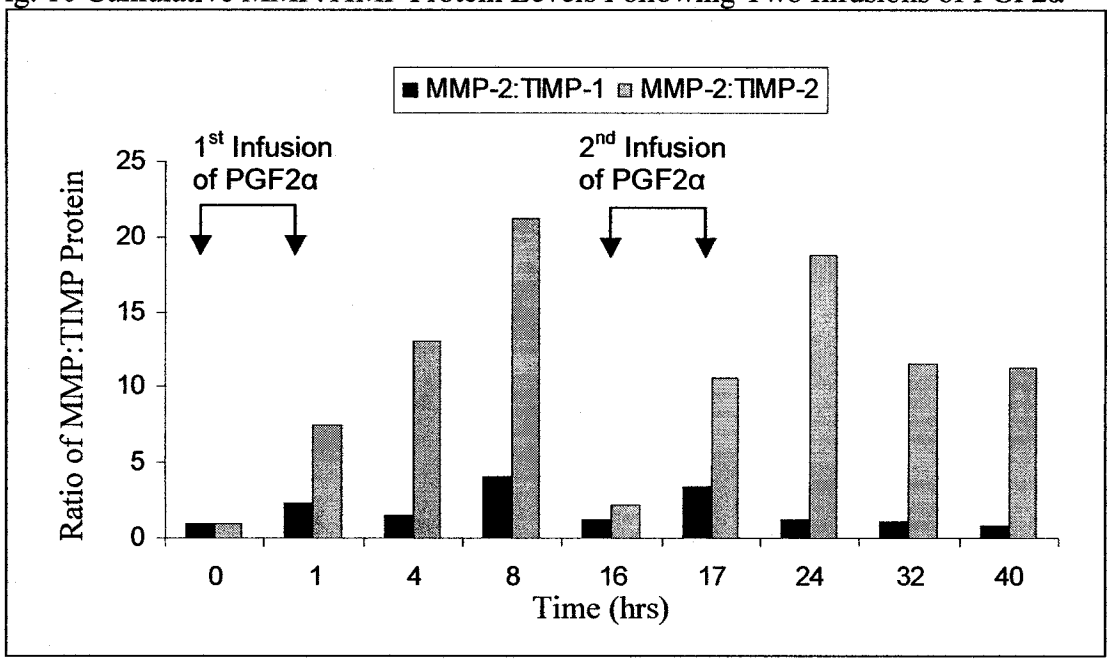
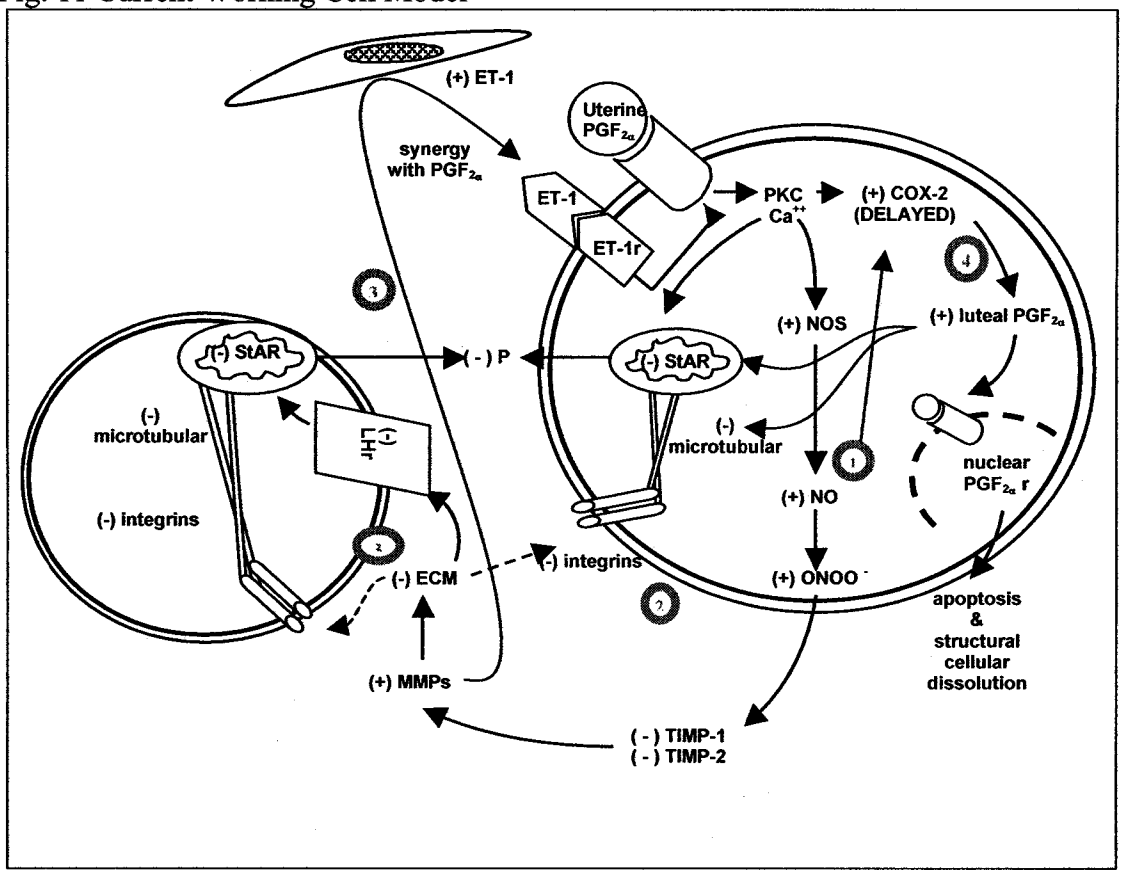


Fig. 11 Current Working Cell Model



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APPENDICES

Appendix A. Protein Extraction Method (A)

Extraction Bufer:

50mM Tris
150mM NaCl
0.02% Sodium Azide
10mM EDTA\
1% Triton X-100
pH 7.4, prior to adding Triton X-100
Bring to volume with cold ddH_2O

ALL steps are performed on ice, or @ 4°C

1. Add protease inhibitors to extraction buffer immediately before use:
   a. 1µg/ml AEBSF
   b. 1µg/ml Pepstatin A
   c. 1µg/ml Aprotinin
2. Remove tissue from freezer, weigh, and thaw on ice in Petri dish cover.
3. Add extraction buffer in a ratio of 8ml for every 1g of tissue weight. Record weight and volume of extraction buffer used.
4. Mince with scalpel. Once tissue is minced transfer to 15 ml conical tube.
5. Homogenize tissue @ speed 8 for 30 seconds on ice.
6. Rinse polytron probe with ddH_2O between samples. If necessary remove any tissue debris from polytron probe with tweezers.
7. Repeat homogenization (steps 5-6)
8. Separate into two separate tubes. One tube for COX samples and the other for StAR samples.
9. Sonicate samples that contain membrane bound molecules of interest:
   a. Transfer 0.5ml of sample into 1.0ml eppindorf tube
   b. Settings: timer = hold, continuous; output = 3
   c. On ice sonicate sample twice for 10 seconds each, rinse probe between samples with extraction buffer
10. Centrifuge @ 4°C, 800 x g for 10 minutes
11. Transfer supernatant into new tubes (discard fat layer and pellet) and centrifuge @ 4°C, 10,000 x g for 10 minutes. Repeat until samples are clear.
12. Transfer supernatant into new tubes and store @ -80°C.

Appendix B. Protein Extraction Method (B)

Solutions:

Extraction Buffer (pH 7.6)	**CAB+ Assay Buffer (10x stock; pH 7.6)**
116.9g NaCl	116.9g NaCl
2.4g HEPES	60.6g Tris
0.2g Sodium Azide	1.5g $CaCl_2$
Bring to 1L with ddH_2O	2.0g Sodium Azide
	Bring to 1L with ddH_2O; pH @ 4°C

DAY 1

Perform all procedures on ice:

1. Thaw luteal tissue on ice. Blot briefly, weigh, and record mass.
2. Mince with scalpel on Petri dish cover over ice. Add extraction buffer in a ratio of 1.0ml buffer per 0.2g of tissue weight. Record volume of buffer used
3. Put minced tissue/buffer in centrifuge tubes. Cap tightly and parafilm.
4. Tape tubes onto Clay Adams Nutator Mixer (Beckton Dickenson; Sparks, MD). Nutate in cold room (4°C) overnight (23-24 hrs).

DAY 2

Perform all procedures on ice or in 4°C centrifuge:

1. Centrifuge extract for 15 minutes @ ~3,000 x g to pellet tissue
2. Pipet supernatant into 1.5ml microcentrifuge tubes and centrifuge for 30 minutes @15,000 x g to remove particulate matter.
3. Remove supernatant to new microcentrifuge tubes and repeat centrifugation until supernatant is clear (not flocculent).
4. Pipet clear supernatant into Amicon Ultra (Amicon; Beverly, MA) ultrafiltration and concentrator device (max volume = 4ml/ concentrator)
5. Centrifuge in a fixed-angle centrifuge for ~1hr @ 3,200 x g. Continue to centrifuge until volume of retentate equals ~200 µl.
6. Wash/dialyze twice with 2 volumes of 1X CAB+ assay buffer (dilute stock 1:9) and continue to centrifuge.
7. Determine $1/4^{th}$ of original buffer used (4X concentration). Centrifuge to this volume or more. If necessary bring final volume up using CAB+.
8. Pipet retentate into sterile micofuge tubes and store @ 4°C in aliquots.
9. Replenish sodium azide (0.02%) in stored samples every 1-2 months.

Appendix C. Bradford Protein Determination

Phosphate Buffered Saline (PBS):

27.4ml 0.25 Na_2HPO_4
13.7ml 0.25 NaH_2PO_4
8.1g NaCl
Bring to volume to 1L with ddH_2O, pH 7.4

1. Set up standard curve (in duplicate) in check tube size

Tube #	BSA	PBS
1-2	0	800µl
3-4	4µl (2µg)	796µl
5-6	8µl (4µg)	792µl
7-8	12µl (6µg)	788µl
9-10	16µl (8µg)	784µl
11-12	20µl (10µg)	780µl

2. Set up diluted (1:10) samples (induplicate) in check tubes:

Tube #	BSA	PBS
13-14	2µl	800µl
15-16	4µl (2µg)	796µl
17-18	6µl (4µg)	792µl
19-20..	8µl (6µg)	788µl

*continue dilutions as needed
* repeat for each sample

3. Add 200µl of BioRad Bradford Assay Reagent, and vortex.
4. Read absorbance at 595nm and record readings.
5. Graph standard curve with µg BSA on X-axis and absorbance on Y-axis.
6. Using the regression line equation, calculate the concentration of protein in the sample (µg/µl).
7. If absorbances fall out of range repeat using necessary dilutions or concentrations.

Appendix D. Zymography and Immunoblot Solutions

Solutions:

Ammonium Persulfate Solution (APS; 100mg/ml)
20mg ammonium persulfate
200ml ddH_2O

4mM APMA Solution
14.1mg aminophenylmercuric acetate
100ml ddH_2O

10mM 1,10-phenanthroline
435.0mg 1,10-phenanthroline
200ml ddH_2O

Stacking Gel (4% acrylamide)
6.36ml ddH_2O
2.52ml 0.5M Tris (pH 6.8)
1.0ml 40% acrylamide
100ml 10% SDS
50µl ammonium persulfate (100mg/ml)
10µl TEMED

Separation Gel (10% acrylamide)
7.5mg gelatin
7.275ml ddH_2O
3.75ml 1.5M Tris (pH 8.8)
3.75ml 40% acrylamide
150µl 10% SDS
proteins)
75µl APS (100mg/ml)
7.5µl TEMED

Electrode Buffer 10x
30g Tris
144.0g Glycine
5.0g SDS
Bring to 1L with ddH_2O

Coomassie Stain
2.5g Brilliant Blue R-250
50.0ml Acetic Acid
150.0ml Isopropyl Alcohol
300.0ml ddH_2O

1.5M Tris (pH 8.8)
18.17g Tris
100ml ddH_2O

0.5M Tris (pH 6.8)
6.06g Tris
100ml ddH_2O

0.25M Tris (pH 6.8)
3.03g Tris
100ml ddH_2O

Sample Buffer (10ml)
10ml 0.25M Tris (pH 6.8)
1.0g SDS
400mg sucrose
10mg bromophenol blue

Separation Gel (12% acrylamide)
No gelatin for Western blots
6.525ml ddH_2O
3.75ml 1.5M Tris (pH 8.8)
4.5ml 40% acrylamide
(4.688ml 40% acrylamide for COX

150µl 10% SDS
75µl APS (100mg/ml)
7.5µl TEMED

Substrate Buffer
6.07g Tris
970.0mg $CaCl_2 \cdot 2H_2O$
200.0mg Sodium Azide
Bring to 1L with ddH_2O

2.5% Triton X-100 Solution
5.0ml Triton X-100
195.0ml ddH_2O

Appendix E. Immunoblot (Western) Solutions and Protocol

Solutions:

TBST Buffer
1.2g Tris
8.8g NaCl
0.5ml Twenn 20
Bring up to 1L with ddH_2O

Blotting Buffer
800ml ddH_2O
200ml Methanol
15.03g Glycine
3.15g Tris

5% Milk
2.5g evaporated non-fat powdered milk
~50ml TBST buffer

Procedure:

Part 1. Electrophoresis.

1. Cast gels using zymography recipes:
    a. Separation gel: 12% acrylamide for TIMPs and COXs, 12.5% for StAR
    b. Stacking gel: 4% acrylamide
2. Use BioRad Precision Plus Protein Standards for molecular weight markers.
3. Load controls and samples- electrophoresis is the same as zymography

Part 2. Preparation for blotting.

1. Cut four pieces of filter paper and prepare four pieces of blotting sponge.
2. Cut nitrocellulose membranes to fit atop of gels accordingly.
3. When gels are almost done running, make blotting buffer- add methanol last. Let blotting paper/sponges and nitrocellulose membranes equilibrate in blotting buffer for several minutes.
4. When electrophoresis is complete, cut off stacking gel, and equilibrate in blotting buffer for several minutes. Repeat for 2^{nd} gel.
5. Notch the top left corner of the nitrocellulose to maintain orientation.

Part 3. Electroblotting.

1. On the black side of the plastic sandwich place in order: one white Teflon blotting sponge, and filter paper.
2. Place gel on top of filter paper and cover with nitrocellulose membrane maintaining the correct orientation.
3. Finish assembling sandwich by repeating the order filter paper > blotting sponge.
4. Insert sandwich into electrode cassette with the black part of the sandwich facing the black portion of the electrode cassette. Place cassette into tank with blotting buffer.

5. When both sandwiches are assembled in the tank, add a small stir bar, and ice pack into the open area. Fill the tank with any necessary additional blotting buffer.
6. Place tank assembly into a basin filled with ice, on top of a stir plate.
7. Place electrode cover onto tank and set power source to 200mA constant for 2 hours.

Part 4. Blocking.

1. Before blotting is complete, make blocking buffer (5% milk).
2. Once blotting is complete, remove nitrocellulose membranes and place them into ~50ml of blocking buffer on shaker for 1-2 hours.

Part 5. Adding the primary antibody.

1. Before blocking is complete, remove an aliquot of the primary antibody from the freezer, allow to thaw, and reconstitute in appropriate volume of blocking buffer.
2. When blocking is complete, discard milk.
3. Slowly add antibody onto the nitrocellulose membrane (usually ~5ml/per gel).
4. Cut two pieces of parafilm just slightly larger than the membrane and place on top, making sure to avoid air bubbles (ensures antibody contact with membrane).
5. Let incubate overnight, 12-16 hours in 4°C cold room on slow shaker.

Part 6. Washing membranes.

1. Remove the primary antibody and return the refrigerator or freezer for storage.
2. Rinse quickly with TBST to remove residual primary antibody, then replace TBST and place on shaker for 10 minutes. Repeat 3-4 times.
3. During the last rinse prepare secondary antibody in blocking buffer.

Part 7. Adding the secondary antibody.

1. Make 50ml of blocking buffer, add 5µl of secondary antibody.
2. Remove TBST from the membranes and add 25ml of secondary antibody per membrane.
3. Cover and put on a low shake for 1-2 hours.

Part 8. Second set of washes.

1. After incubation, discard secondary antibody.
2. Quickly rinse with TBST, and repeat 3-4 times.
3. Before beginning the final series of washes turn on the film processor in the dark room.

Part 9. Chemiluminescent detection.

1. During the last 5 minutes of the last wash, make up Super Signal chemilumiscent reagent with the lights off. (Pierce kit; 5ml of each solution)
2. When washing is complete, discard buffer and add 5ml of Super Signal to each membrane.
3. Let incubate for 5 minutes

Part 10. Prepare membranes for documentation.

1. Place a piece of saran wrap ~12 inches long ~8inches wide on a flat clean surface.
2. Using tweezers, shake excess Super Signal off onto kimwipe and place the membranes in reverse orientation onto the surface.
3. Place a piece of filter paper on top of the membranes then carefully wrap the saran wrap around the filter paper.
4. Turn over (should be in correct orientation now) and place into the film cassette and tape to prevent moving.
5. Bring film cassette into darkroom and expose film on membranes (time depends on protein presence) and process film.

Appendix F. Zymography Protocol:

DAY 1

1. Heat water bath (500ml beaker on hot plate) to ~90°C.
2. Make ammonium persulfate solution (APS) in 1.5ml microcentrifuge tube.
3. Weigh gelatin and place in a 50ml centrifuge tube.
4. Prepare gel solution:
    a. Add ddH_2O to tube, followed by 1.5M Tris (pH 8.8), 40% acrylamide (wear gloves), and SDS.
    b. Heat contents in hot water bath (30sec dips) until gelatin dissolves.
    c. Let cool to ~room temperature

5. Set up gel apparatus:
    a. Rinse and dry all components.
    b. Place rubber gaskets onto tower (as needed) and cover with parafilm.
    c. Assemble plates with one large plate, one small, and two 1mm spacers between them.
    d. Tighten the knobs of the assembly making sure all plates and spacers are even.
    e. Snap plates into tower with knobs facing away.
    f. To check for leaks, add ddH_2O between the plates and monitor fluid levels. If levels drop reassemble plates and repeat d-f.
    g. Make sure plates are relatively dry before continuing.

6. Casting the separation gel:
    a. Add TEMED and APS to the gel solution and swirl to mix
    b. With a Pasteur pipette, quickly load the gel solution between the two plates. Fill to the notch on the snap tower.
    c. With a clean Pasteur pipette, add ddH_2O on top of the gel solution slowly and evenly.
    d. Allow gel to polymerize (20-40 minutes).

7. Casting the stacking gel:
    a. When separation gel is almost polymerized, add all ingredients for the stacking gel solution in order, except TEMED and APS.
    b. Dump off water on top of separation gel once polymerized and dry area with Whatman filter paper.
    c. Add TEMED and APS to the solutions and swirl to mix.
    d. Load the stacking gel solution on top of the separation gel.
    e. Immediately insert a 1mm thick 10 well comb into the center of the gel.
    f. Allow to polymerize (20-40 minutes)

8. Preparing standards and samples:

a. While the stacking gel is polymerizing, make up loading samples in 0.5ml microcentrifuge tibes.
b. Use an equal volume of sample to loading buffer (1:1), unless otherwise indicated.

9. Loading samples:
    a. When stacking gels are polymerized, carefully remove combs.
    b. Remove the gels/glass assembly from tower and insert into electrode tower.
    c. Place the new assembly into gel tank and add ~800ml of 1x electrode buffer to the center area between gels, making sure to gently rinse the wells of the gel.
    d. Begin loading samples (gel loading tips):
        1. Max volume 40-50µl.
        2. Avoid using 1st and last lanes, use same volume of loading buffer as a blank to allow the gel to run consistently.
        3. Record samples contents for each lane of each gel.

10. Running the gels:
    a. Once gels are loaded, attach electrode cover in proper orientation.
    b. Plug electrode cover into power source and set volts to 200.
    c. Run until dye reaches the bottom of the gel.
    d. Prepare the Triton X-100 solution during the last 15 minutes of running.

11. Triton washes:
    a. When the gel is finished running, remove gels from the apparatus and place them into 150mm Petri dishes and label.
    b. Cover gels with ~100ml Triton solution and place on shaker vigorously for 15 minutes.
    c. Drain off Triton and repeat step b-c.
    d. When 2nd wash is complete, rinse gels with ddH_2O

12. Fill each dish with ~150ml of substrate buffer.
13. Place on shaker in 37°C incubator and shake gently overnight.

DAY 2

1. Staining the gels:
    a. Remove from incubator and pour off substrate buffer.
    b. Add 50ml of coomassie solution to each dish
    c. Shake gently at room temperature for 30 minutes.

2. Destain gels:
    a. Aspirate off coomassie stain. Rinse gels several times with ddH_2O.
    b. Shake gels in ddH_2O at room temperature, changing water periodically until zones of clearing are visible (up to 3 days).

c. When gels are of satisfactory intensity, take a picture and store gels in sealed plastic sleaves.