Temporal expression of progesterone and proteins associated with prostaglandin F2alpha-induced luteolysis in the sheep

Darren E. Ferguson

University of New Hampshire, Durham

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Abstract
To better understand the events associated with the functional and structural regression of the corpus luteum (CL), the temporal expression of steroidogenic acute regulatory (StAR) protein, progesterone (P4), matrix metalloproteinase (MMP)-2 and -9, tissue inhibitor of metalloproteinase (TIMP)-1 and -2, cyclooxygenase (COX)-1 and -2 was determined following three systemic infusions of PGF2alpha, in sheep. Luteectomies were performed at 0 hour (controls) or at -1, 1, 8, 16, and 24 hours relative to the third infusion of PGF2alpha. Plasma samples were also collected. StAR decreased 60 and 45% at the 16 and 24 hour time points, respectively, while P4 reached a nadir (25%) after the 8 hour time point before rebounding at the 24 hour time point. MMPs and TIMP-2 were unchanged. TIMP-1 decreased 30% at the 1 hour time point. COX-1 was undetectable while COX-2 results were inconclusive. Overall, 25% of sheep underwent luteolysis after three infusions of PGF2alpha.

Keywords
Biology, Animal Physiology

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TEMPORAL EXPRESSION OF PROGESTERONE AND PROTEINS ASSOCIATED WITH PROSTAGLANDIN $\text{F}_{2\alpha}$-INDUCED LUTEOLYSIS IN THE SHEEP

BY

Darren E. Ferguson
B.A. University of New Hampshire, 2006

THESIS

Submitted to the University of New Hampshire
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Master of Science
in
Animal Science

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Thesis Director, Paul C.W. Tsang, Professor of Molecular and Cellular Biomedical Sciences

David H. Towson, Associate Professor of Molecular and Cellular Biomedical Sciences

John A. McCracken, Professor-in-Residence, University of Connecticut

2/24/2009
Date
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ABSTRACT

TEMPORAL EXPRESSION OF PROGESTERONE AND PROTEINS ASSOCIATED WITH PROSTAGLANDIN F$_{2\alpha}$-INDUCED LUTEOLYSIS IN SHEEP

by

Darren E. Ferguson

University of New Hampshire May 2009

To better understand the events associated with the functional and structural regression of the corpus luteum (CL), the temporal expression of steroidogenic acute regulatory (StAR) protein, progesterone (P$_4$), matrix metalloproteinase (MMP)-2 and -9, tissue inhibitor of metalloproteinase (TIMP)-1 and -2, cyclooxygenase (COX)-1 and -2 was determined following three systemic infusions of PGF$_{2\alpha}$ in sheep. Luteectomies were performed at 0 hour (controls) or at -1, 1, 8, 16, and 24 hours relative to the third infusion of PGF$_{2\alpha}$. Plasma samples were also collected. StAR decreased 60 and 45% at the 16 and 24 hour time points, respectively, while P$_4$ reached a nadir (25%) after the 8 hour time point before rebounding at the 24 hour time point. MMPs and TIMP-2 were unchanged. TIMP-1 decreased 30% at the 1 hour time point. COX-1 was undetectable while COX-2 results were inconclusive. Overall, 25% of sheep underwent luteolysis after three infusions of PGF$_{2\alpha}$.
CHAPTER I

Literature Review

Introduction

One of the major benefits of improving fertility among mammals is increasing livestock production. Large domesticated animals such as sheep, cows, pigs, goats, and bison are raised for meat, dairy products, leather, wool, and fertilizer. Other uses include their use as grazing animals and mechanical labor on farms. Thus, factors that decrease their fertility can lead to severe economic losses for producers. Therefore, it is essential to understand the physiological mechanisms that dictate reproductive cycles so that offspring production is maximized in these animals.

Mammals require sexual reproduction to produce offspring. They typically exhibit either a menstrual (humans and other primates) or estrous (non-primates) cycle. Mammals that follow an estrous cycle can be further divided into animals that are polycyclic, monocyclic, spontaneous ovulators, induced ovulators, spontaneous luteal phase, or induced luteal phase. Despite such a variety of reproductive modes, the major organs/glands and physiological events that occur over the reproductive cycle are common to all mammals. These major events include follicular development, ovulation, luteinization, and luteolysis. Therefore, studying the reproductive cycle of a specific species allows us to translate that knowledge across a variety of species.
The present study focuses on the sheep estrous cycle with emphasis on luteolysis. Before we can study the mechanisms involved in sheep reproduction, this chapter will review some of the basic components of its estrous cycle. As an overview, the sheep estrous cycle lasts about 17 days and consists of a follicular phase and a luteal phase (McCracken et al., 1999). The follicular phase is dominated by structures called follicles and the steroid hormone estradiol (E₂). The end of this phase is characterized by estrus and ultimately marked by ovulation. Following ovulation is the beginning of the luteal phase (14 days) which is dominated by the corpus luteum (CL), a structure that secretes the steroid hormone progesterone (P₄). Progesterone production is essential for maintaining pregnancy. However, if pregnancy does not occur, it is crucial that the CL regresses so that another cycle can begin.

The process of CL regression is termed luteolysis and is initiated by a hormone called prostaglandin F₂α (PGF₂α). However, the mechanisms by which PGF₂α act on the CL during luteolysis are not fully elucidated. Therefore, the present study focuses on the expression of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), steroidogenic acute regulatory protein (StAR), progesterone (P₄), and cyclooxygenase (COX) enzymes by the sheep corpus luteum following physiological administration of PGF₂α.
The Follicular Phase

The ovarian follicle housing the egg is the dominant structure of the follicular phase. In primates and ruminants, a female is born with a set number of primordial follicles. At birth, Blackface and Welsh sheep have approximately 100,000 primordial follicles per ovary (Land, 1970). At the beginning of the follicular phase, a group of these follicles is recruited for maturation in response to follicle stimulating hormone (FSH; Hansel and McEntee, 1977). As they develop, several of them become primary and secondary follicles; however, only one will mature into a tertiary follicle and finally into a Graafian follicle while the rest die through a process called atresia. Mature follicles consist of three main cell types (granulosa, theca interna, and theca externa), a basement membrane, and an antrum (Hirshfield, 1991). The granulosa cells line the inner periphery of the follicular basement membrane and they also surround the egg, i.e. cumulus oophorus. This layer of cells is avascular and is bathed in the follicular fluid of the antrum (Fraser, 2006). The vascularized theca interna line the outside of the basement membrane with the theca externa surrounding it.

The hallmark of the mature ovarian follicle is production of the steroid hormone estradiol (E$_2$). For a long time, it was thought that theca cells alone from the follicle wall were the site of E$_2$ synthesis (Mossman, 1937; Corner, 1938; Stafford, 1942; Deane and Barker, 1952). Later, Falck (1959) showed through a series of elegant experiments in which various combinations of theca, granulosa and vaginal tissues were transplanted into the anterior chamber of the eye, that the theca and granulosa cells cooperate to synthesize this hormone. Subsequently, Short (1961) coined the phrase “two-cell theory”
to describe this interaction. According to this theory, theca interna cells produce androstenedione which then diffuses through the basement membrane of the follicle wall where granulosa cells convert it to E₂ by the enzyme aromatase (Ryan et al., 1968; Dorrington et al., 1975; Makris and Ryan, 1975; Fortune and Armstrong, 1977; Moon et al., 1978). In addition, granulosa cells are capable of producing the steroid hormone progesterone (P₄) which diffuses through the basement membrane of the follicle wall where it can be converted into androstenedione by theca cells. Androstenedione is then used as a precursor by granulosa cells to make E₂ (Hsueh et al., 1984). The production of androstenedione by theca cells is driven by luteinizing hormone (LH) whereas the conversion of androgens to E₂ in granulosa cells is driven by FSH (Dorrington et al., 1975; Makris and Ryan, 1975; Moon et al., 1978). Follicle stimulating hormone and LH are gonadotropins produced in the anterior pituitary gland and they are responsible for follicular maturation and ovulation (Schally et al., 1971; Clarke et al., 1987).

Production of FSH and LH depends on the hypothalamic hormone called gonadotropin-releasing hormone (GnRH), which is a part of the hypothalamo-pituitary-gonadal feedback loop (Schally, 1971). Gonadotropin-releasing hormone is produced within the arcuate nucleus of the hypothalamus. In sheep, GnRH is released in low frequency, high amplitude pulses early in the follicular phase (Clarke et al., 1987; Moenter et al., 1991). Therefore, FSH and LH production is relatively low. Gonadotropin-releasing hormone increases to high frequency, low amplitude pulses in response to increasing E₂ concentrations throughout the follicular phase. As a result, gonadotropin secretion by the anterior pituitary increases, driving the Graafian follicle toward ovulation.
Production of GnRH is regulated mainly by steroid hormones (E_2 and P_4) and a light-dark cycle in domestic animals (Downey, 1980). Generally, E_2 induces GnRH production while P_4 inhibits it (Moenter et al., 1990; Kasa-Vubu et al., 1992). Since sheep are short day breeders, reducing amounts of light, either naturally or artificially, will initiate their estrous cycle by stimulating GnRH synthesis, while increasing the amount of light will inhibit it (Hansel and McEntee, 1977).

The regulation of FSH throughout the estrous cycle of the sheep is similar to that of GnRH in that it is also controlled by E_2 and P_4. Depending on its concentration and duration during the estrous cycle, E_2 either stimulates or inhibits FSH production (Downey, 1980). During the luteal phase (Days 0-14), when P_4 is the dominant hormone and E_2 is at basal concentrations, low E_2 decreases hypothalamic GnRH and reduces anterior pituitary receptivity to GnRH, resulting in suppression of FSH production (Baird et al., 1975; Goodman, 1994). As E_2 increases during the follicular phase, it stimulates the hypothalamus to increase GnRH production, and consequently, a rise in FSH is observed (Jonas et al., 1973; Reeves et al., 1974; Pant, 1977; Dobson and Ward, 1977; Goodman et al., 1981). Estradiol treatment of anestrous and ovariectomized ewes yields a similar increase in FSH production (Jonas et al., 1973; Reeves et al., 1974; Pant, 1977; Dobson and Ward, 1977; Goodman et al., 1981). As follicles mature, there is a positive feedback loop between E_2 and FSH, which continues until they both peak in the middle of the follicular phase. Increasing E_2 concentrations beyond this point by addition of exogenous E_2 will be inhibitory.

In addition to being controlled by E_2, FSH production is influenced by a 31 kDa protein hormone called inhibin, which is present in the follicular fluid of the follicle.
(Martin et al., 1986). Immunization against inhibin resulted in a rise in FSH concentrations (Al-Obaidi, 1986; Martin et al., 1986; Clarke et al., 1986; Findlay et al., 1987; Mann et al., 1990; Larson et al., 1991). Treatment with inhibin decreases pituitary and peripheral concentrations of FSH, but has no effect on LH (Martin et al., 1986). In addition to decreasing FSH concentration, treatment with follicular fluid containing inhibin to cows delays estrus (Quirk and Fortune, 1986). Therefore, FSH is essential for follicular development.

Follicle development is a complex process. Robertson (1969) suggested that during the late stages of the follicular phase in sheep, a Graafian follicle is present along with another follicle that is less mature (subordinate follicle). At ovulation, the Graafian follicle ruptures and involutes to become the corpus luteum (CL). Meanwhile, the subordinate follicle persists through the luteal phase and ovulates in the next cycle. Similarly, Quinlan and Mare (1931) proposed that the subordinate follicle grows rapidly just after ovulation from the previous cycle and then grows slowly throughout the “inter-estrus” period. In contrast, Grant (1934) suggests the subordinate follicle steadily grows throughout the estrous cycle. These results were obtained by measuring the diameter of follicles from sheep killed at different stages of the estrous cycle and imply that there is one wave of follicles per cycle.

A subsequent study involved marking follicles with carbon at different stages of the sheep estrous cycle to determine their growth pattern was conducted (Smeaton and Robertson, 1971). Laparotomies were performed early in the cycle (days 6-9) and large follicles were marked with carbon. Subsequently, laparotomies on the same animals were performed later in the cycle (days 13-15) and new large follicles were marked.
Ovariectomies were then performed 3-9 days after estrus and ovaries were sectioned and fixed onto slides for observation by microscopy. All carbon-marked follicles regressed while the corpus luteum contained no trace of carbon, suggesting that it came from a follicle that rapidly matured into a Graafian follicle after the second laparotomy and ovulated during estrus. These results suggest that there are three follicular waves during the estrous cycle in sheep. This hypothesis was subsequently supported by transrectal ultrasonography (Souza et al., 1998; Bartlewski et al., 1999; Vinoles et al., 1999; Evans et al., 2000; Duggavathi et al., 2003; Evans, 2003). The transrectal ultrasonography procedure includes inserting a transducer into the rectum and held face down to maintain contact with the rectal mucosa. The ovary is then located by hand and scanned at different cross sections. The images captured by the transducer are used to examine follicles. The first cohort of follicles results in one follicle (5 mm diameter) enlarging from days 6-9, while the second cohort results in one follicle (5 mm diameter) enlarging from days 13-15 (Smeaton and Robertson, 1971). However, the follicles in both waves become atretic before the next estrous cycle. The third cohort results in one follicle (5 mm diameter) rapidly growing to 12 mm in diameter between the time of CL regression and ovulation. This mature follicle is the one that ovulates while the others undergo atresia. In sheep, there are typically 3-4 waves of follicular growth during each estrous cycle, with waves emerging every 3-5 days. In addition, transient peaks of FSH precede each wave (Bartlewski et al., 1998; Evans et al., 2001).

It is thought that dominant follicles acquire certain attributes that enable them to grow while subordinate ones are destined for atresia. Dizerega et al. (1982) suggested that the mature or dominant follicle secretes follicle regulatory protein (FRP), which...
causes the regression and the eventual death of less mature (subordinate) follicles. Although the granulosa cells of the follicle secrete FRP, this hypothesis was not supported by subsequent research. An alternative and more likely hypothesis proposed by Erickson and Hsueh (1978) is that the mature follicle secretes inhibin, which decreases FSH through a negative feedback loop. The resulting low concentrations of FSH can not induce maturation of the subordinate follicles, but are enough to maintain growth of the dominant follicle. Zeleznik and Kubic (1986) provided support for this hypothesis by showing that low concentrations of FSH are incapable of recruiting follicles, but are capable of maintaining growth of the dominant follicle in cynomolgus monkeys. More recently, Knight and Glister (2006) propose that an autocrine/paracrine role of granulosa cell-derived activin and bone morphogenetic protein-6 (BMP-6), and a paracrine role of oocyte-derived growth and differentiation factor-9 (GDF-9), BMP-15, and BMP-6, induce granulosa cell proliferation and modulate FSH-dependent follicle function. Differential exposure to these proteins may be one way that follicles become sensitized to FSH, thus giving rise to a dominant follicle.

Ovulation

In order for the Graafian follicle to rupture, LH initiates a cascade of events which include degradation of the follicle wall, an inflammatory response, and an immune response (Walton and Hammond, 1928; Espey, 1980). Preceding ovulation, the apex of the follicle is present on the ovary surface and a stigma forms. The LH surge causes
rupture of the follicle and release of the egg into the oviduct for fertilization (Gaytan et al., 2005).

In sheep, while several hormonal factors contribute to ovulation, the central component is the LH surge at the end of the follicular phase (Hansel and McEntee, 1977). During the estrous cycle, there is interplay between steroid hormones and gonadotropins. Exogenous P₄ suppresses the FSH and LH response to GnRH during the post estrus period in sheep (Hooley et al., 1974). Similarly, Karsch et al. (1977) found that P₄ inhibits the LH surge during the estrous cycle in sheep. Despite a study by Martin (1988) that suggests P₄ has no effect on gonadotropin concentrations, it is largely accepted that P₄ inhibits LH and FSH production (Hooley et al., 1974; Baird et al., 1975; Karsch et al., 1977; Sarkar and Fink, 1980; Martin et al., 1983). Luteinizing hormone concentrations are basal throughout most of the follicular phase, but begin to increase 5 hours or 22-26 hours before ovulation and reach a peak (45 ng/ml or 100-200 ng/ml) at the time of follicular rupture (Karsch et al., 1977; Goodman, 1994, respectively). To achieve the LH surge, an increase in E₂ from maturing follicles acts to alter the pattern of hypothalamic GnRH from low frequency and high amplitude pulses to high frequency and low amplitude pulses (Moenter et al., 1991). As a result, LH production is increased by the anterior pituitary. In addition, E₂ also acts directly on the anterior pituitary gland to increase GnRH receptors which increases its responsiveness to GnRH (Reeves et al., 1971). Blocking GnRH action inhibits ovulation (Evans et al., 1996). Therefore, E₂ acting on both the hypothalamus and the anterior pituitary is necessary for the LH surge and ovulation.
One pathway set into motion by the LH surge is structural degradation of the follicle wall and the surrounding extracellular matrix (ECM). The Graafian follicle consists of an inner layer of granulosa cells bathed in follicular fluid, the oocyte, a basement membrane, and several layers of theca cells (Hirshfield, 1991). The only way the oocyte can exit the follicle is for the basement membrane to be breached and for the surrounding ECM to be disrupted. The ECM of the ovary is composed of fibronectin, entactin, nidogen, vitronectin, and collagens IV and XVIII (Ruoslahti and Pierschbacher, 1987; Luck et al., 1995; Irving-Rodgers and Rodgers, 2006), while the basement membrane is composed of type IV collagen, laminin, heparin sulfate proteoglycans, and fibronectin (Birkedal-Hansen et al., 1993). Dispersed throughout the thecal layers are fibroblasts that become active and start to proliferate in response to the LH surge. These cells secrete the latent form of the collagenase enzyme matrix metalloproteinase-1 (MMP-1; Bauer et al., 1975). Collectively, matrix metalloproteinases are a family of enzymes that degrade all components of the ECM (Roy, 2006) and they are activated by serine proteases, such as plasmin, trypsin, and chymotrypsin as well as active MMPs (Woessner, 1991). Tadakuma et al. (1993) treated rabbits with human chorionic gonadotropin (hCG) to induce ovulation and examined the presence of MMPs around and within the follicle. They found that MMP-1 is present in theca interna, theca externa, and around the apex of the preovulatory follicle. Since it is known that MMP-1 degrades laminin, fibronectin, vitronectin and collagens I, II, III, VII, VIII, X, XI, the increase in MMP-1 and corresponding decrease in collagen concentrations at the apex of the preovulatory follicle (Murdoch and McCormick, 1992; Tadakuma et al., 1993) lead to weakening of the follicle wall. Indeed, the addition of the synthetic collagenase inhibitor
SC 44463 prevents ovulation in the rat ovary (Butler et al., 1991). Similar to MMP-1, other MMPs such as MMP-2 and -9 also have the ability to degrade several components of the ovarian ECM and the follicle wall. Curry et al. (2001) reported that MMP-2 and -9 activities are localized at the apex of the rat follicle around the time of ovulation. Similarly, MMP-2 in the sheep ovary is a crucial enzyme for ovulation. Russell et al. (1995) showed that MMP-2 expression increases in ovine follicular fluid as the follicle approached ovulation. A later study demonstrated that ovaries of sheep that were immunized against MMP-2 never formed a stigma and failed to ovulate whereas the control animals formed a stigma and ovulation occurred (Gottsch et al., 2002). Subsequently, Smith et al. (2005) found that bovine theca cells secrete MMP-2 and -9, and that both theca and granulosa cells secrete MMP-13 in response to LH. Overall, these findings and those from others (Espey and Lipner, 1994; Hullboy et al., 1997; Smith et al., 1999; Fata et al., 2000), show that MMP activity is a component of follicular rupture.

An inflammatory response (Parr, 1974; Espey, 1980; Espey, 1994; Espey and Litner, 1994, Espey, 1994b, Richards et al., 2002), as manifested by redness and swelling, accompanies the proteolytic activity during the ovulatory cascade. Inflammation, whether brought on by infection or disease, involves vasodilation and increased capillary permeability resulting in an increase in blood flow and an abundance of serum proteins (Willcox, 1963; Ebert and Grant, 1974). Lee and Novy (1978) showed that rabbits treated with LH experienced increased ovarian blood flow when compared to rabbits treated with the anti-inflammatory indomethacin. Additionally, Zachariae (1958) and Espey (1978) demonstrated that there is increased capillary permeability in preovulatory follicles.
To induce this inflammatory response in the ovary, the LH surge stimulates prostaglandin production by granulosa and thecal cells as well as fibroblasts (Plunkett et al., 1975; Erickson et al., 1977; Espey, 1980). Prostaglandins (PGs) are a group of eicosanoid hormones that cause pain and inflammation (Vane and Botting, 1998; McCracken, 2005). In 1973, LeMaire et al. found that PGF and PGE synthesis increases after gonadotropin stimulation in rabbits. Prostaglandin F reaches a maximum concentration in the rabbit follicle by the time of ovulation, while PGE concentrations continue to increase several hours after ovulation (Yang et al., 1974). Murdoch et al. (1981) measured the amount of PGF$_{2\alpha}$ and PGE$_2$ in the largest follicles from sheep ovaries 12 hours before estrus, at the onset of estrus, and 12 hours after estrus. Prostaglandin E$_2$ concentrations increased from 12 hours before estrus to the onset of estrus, and then decrease 12 hours after estrus. In contrast PGF$_{2\alpha}$ concentrations increased from pre-estrus to the onset of estrus, and continued to increase 12 hours after estrus.

The prostaglandins, particularly PGF$_{2\alpha}$, play a central role in ovulation. Inhibiting prostaglandin production by administration of aspirin or indomethacin prevents rupture of the ovarian follicle (Orczyk and Behrman, 1972); however, treatment with exogenous PGF$_{2\alpha}$ reestablishes ovulation (Armstrong et al., 1973). Interestingly, PGE$_2$ inhibits ovulation in the rabbit (Richman et al., 1974). The difference between PGF$_{2\alpha}$ and PGE$_2$ was further shown by Murdoch et al. (1986), who found that sheep ovaries treated with the anti-inflammatory drugs isoxazol or indomethacin failed to ovulate. However, the addition of exogenous PGF$_{2\alpha}$ to ovaries exposed to either treatment restores the ovulatory process, while exogenous PGE$_2$ has the same effect only on indomethacin-treated ovaries suggesting that PGF$_{2\alpha}$ is the primary prostaglandin that regulates ovulation. It is
suggested that PGF$_{2\alpha}$ stimulates quiescent fibroblasts to the proliferative and active states (Espey, 1978). As a result, MMP production by these fibroblasts increases (Dayer et al., 1976; Dowsett et al., 1976; Lupulescu, 1977; Pettigrew et al., 1978) and, in turn, components of the follicle wall and surrounding ECM are degraded, leading to rupture of the follicle.

The inflammatory response, as part of the ovulatory cascade, is linked to an immune response involving a variety of leukocytes (Espey, 1980). Granulocytes, a group of leukocytes that include basophils, eosinophils and neutrophils, secrete matrix degrading enzymes such as collagenase, elastase and plasminogen activator. Zachariae et al. (1958) histologically showed that blood vessels of rabbit follicles contain basophils two hours before ovulation (during the LH surge), whereas those of precoital rabbits (no LH surge) lacked basophils. Macrophages, another leukocyte, have also been implicated in the ovulatory process. After hCG treatment, macrophages increased 5-fold when compared to controls and were found mostly around blood vessels in the theca region (Brannstrom et al., 1993; Brannstrom et al., 1994). Similar to other leukocytes, macrophages aid in matrix degradation, phagocytosis and destruction of invading antigens. They also secrete cytokines and chemokines which regulate local immune and inflammatory responses (Gordon, 1999). The idea that leukocytes play a critical role in ovulation is further strengthened by Hellberg et al. (1991). Perfused rat ovaries were treated with LH, leukocytes, or both. Ovulation was not seen in any ovaries treated with either LH or leukocytes alone; however, ovulation occurred in all ovaries treated with LH and leukocytes.
Luteinization

Following ovulation, the ruptured follicle collapses and undergoes a structural and functional transformation to become the corpus luteum (CL; Channing, 1975), a process known as luteinization. Before ovulation, the FSH peak induces LH receptors on granulosa cells (Zeleznik, 1974; Channing, 1975; Marsh, 1976). Since the follicular fluid contains luteinizing inhibitor (LI), there is no effect when LH diffuses into the antrum of the follicle (Ledwitz-Rigby, 1977; Channing et al., 1980). With the LH surge, the egg ovulates and the follicular fluid, along with LI, is released. As a result, LH binds to its receptors on granulosa cells to stimulate the production of cAMP which, in turn stimulates P₄ production. At this point, the granulosa and theca cells have undergone a change to become cells of the corpus luteum.

During luteinization, granulosa and thecal cells transform into large and small luteal cells of the CL, respectively. In sheep, small luteal cells contain LH receptors and produce relatively low concentrations of P₄. However, this production is increased by 5-15 fold in response to LH (Niswender, 2002). Large luteal cells also contain LH receptors and produce high basal concentrations of P₄. They produce about 80% of the P₄ within the CL (Niswender et al., 1985); however, they do not respond to an increase in LH.

To support the proliferation and function of the steroidogenic luteal cells, fibroblasts, endothelial cells, pericytes, and immune cells (Smith et al., 1994; Stocco et al., 2007) within the CL, a considerable blood supply is needed (Reynolds et al., 2000). In the follicle, the thecal layer of the preovulatory follicle is vascularized while the granulosa layer is not. As involution of the follicle and transformation of follicle cells to
luteal cells occur, angiogenesis, the formation of new blood vessels from pre-existing ones, is initiated. The main angiogenic factors within the corpus luteum are vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), endocrine gland-derived growth factor (EG-VEGF), and angiopoietins (Ferrara et al., 1998; LeCouter, 2002). These growth factors are made by luteal cells, and they increase MMP production to break down the remainder of the follicle basement membrane and act as chemotactic agents to promote endothelial cell proliferation and migration resulting in vascularization of the new gland (Gospodarowicz et al., 1985; Mignatti et al., 1989; Berish et al., 2000). Angiogenesis of the ruptured follicle results in a “bloody body”, also known as corpus hemorrhagicum (Shapiro et al., 1980). When angiogenesis is complete, every luteal cell is in contact with multiple capillaries allowing for maximum delivery of nutrients to keep up with the demand for P4 production during the luteal phase and the gland is now a “yellow body” or corpus luteum (Reynolds et al., 2000). Progesterone production throughout the luteal phase and into pregnancy is essential for inhibiting uterine contractions (Allen, 1935), thickening cervical mucus, and building the endometrium for the implantation of the fetus (Zarrow, 1965).

**Luteolysis**

If pregnancy does not occur, the CL must regress so that another ovarian cycle can begin. Although the mechanisms of CL regression remain to be fully elucidated, the hormone that initiates luteolysis in many species is PGF2α (McCracken et al., 1972; Nancarrow et al., 1973; Gleeson et al., 1974; Kindahl et al., 1976; Moeljono, 1976;
Following PGF$_{2\alpha}$, the CL undergoes functional and structural luteolysis, which are defined as a decrease in progesterone (P$_4$) production and apoptosis, respectively (Hoyer, 1998; Stocco et al., 2007). It is reported by some that functional luteolysis precedes structural luteolysis. In sheep, a decrease in P$_4$ concentration is observed 5 hours after PGF$_{2\alpha}$ treatment whereas evidence of apoptosis does not occur until 12 hours after treatment (McGuire et al., 1994). Similarly, functional regression in cows occurs between 1-8 hours after PGF$_{2\alpha}$ treatment whereas structural regression is not observed until 12-24 hours (Juengel et al., 1993; Tian et al., 1994). In addition, a decrease in P$_4$ concentrations can be reversed 	extit{in vivo} and 	extit{in vitro} as long as the structural integrity of the CL is not compromised (Bender et al., 1978; Fraser et al., 1985; Hutchinson and Zeleznik, 1985). Although these findings suggest that functional luteolysis precedes structural luteolysis, the temporal sequence of these two events or the mechanisms of PGF$_{2\alpha}$ action require further study. Therefore, in the remaining sections of this Literature Review, the current knowledge of prostaglandins, their synthesis and the mechanisms of PGF$_{2\alpha}$ action within the reproductive tract, with emphasis on the ovine CL, will be discussed.

**Prostaglandins:**

Prostaglandins (PGs) are members of the eicosanoid family and have a wide variety of actions within the body (McCracken, 2005). Some are involved in gastric cytoprotection, luteolysis, vasodilation, and inhibition of platelet aggregation, while others are involved in glaucoma, vasoconstriction, platelet aggregation, and inducing sleep and fever. The first two prostaglandins, PGE$_1$ and PGF$_{1\alpha}$, were isolated from sheep.
prostate glands (Bergstrom and Sjovall, 1957; Bergstrom and Sjovall, 1960; Bergstrom and Sjovall, 1960). As more prostaglandins were isolated from sheep prostate glands, bovine lung, and human seminal plasma, it was later determined that all prostaglandins are composed of a 20 carbon lipid skeleton called prostanoic acid (Figure 1; Bergstrom et al., 1962; Samuelsson, 1963; Samuelsson, 1964; Hamberg and Samuelsson, 1965; Bergstrom, 1968). Modifications to this structure, such as addition of hydroxyl and oxygen groups, as well as double bonds, determine the structure of specific prostaglandins. Examples of specific prostaglandins include PGD$_2$, PGE$_2$, and PGF$_{2\alpha}$ (Figure 1; McCracken, 2005). The letter after the abbreviation “PG” is based on the fraction of the extraction in which the prostaglandin resides. For example, PGF$_{2\alpha}$ resides in the “Fosfat” fraction while PGE$_2$ resides in the “Ether” fraction. The number associated with it indicates the number of double bonds within the structure, while the Greek letter denotes the orientation of the hydroxyl groups.

Figure 1. Prostaglandins
Prostaglandin Biosynthesis:

Prostaglandins can be synthesized by any cell that has a phospholipid bilayer and the appropriate enzymes (McCracken, 2005). Therefore, it is not surprising that prostaglandins are secreted by a wide variety of tissues, organs, and glands, such as fat (Shaw, 1966; Ramwell and Shaw, 1967), adrenals (Ramwell et al., 1966; Ramwell and Shaw, 1967), ovaries (Euler, 1937), stomach (Bennett et al., 1967; Cochani et al., 1967), intestines (Sukuki and Vogt, 1965; Ambache et al., 1966), spinal and phrenic nerves (Ramwell et al., 1965; Ramwell et al., 1966). They are also present in the uterus (Karim and Devlin, 1967), lung (Bergstrom et al., 1962; Samuelsson, 1964), thymus (Bergstrom and Samuelsson, 1963), brain and spinal cord (Samuelsson, 1964; Cochani and Wolfe, 1965; Ambache et al., 1966; Holmes and Horton, 1967; Horton and Main, 1967), kidney (Lee et al., 1965; Lee et al., 1966; Strong et al., 1966; Daniels et al., 1967), iris (Anngard...
Prostaglandin synthesis begins with the enzyme phospholipase A\(_2\) (PLA\(_2\)) cleaving a 20 carbon structure called arachidonic acid (AA) from the cell membrane. The free AA serves as the precursor for all eicosanoids (McCracken, 2005). The PLA\(_2\) family includes three forms: cytosolic PLA\(_2\) (cPLA\(_2\)), secretory PLA\(_2\) (sPLA\(_2\)), and calcium-independent PLA\(_2\) (iPLA\(_2\); Diaz and Arm, 2003). Cytosolic PLA\(_2\) and sPLA\(_2\) are considered to be the primary enzymes that regulate eicosanoid production. Cytosolic PLA\(_2\) preferably cleaves AA from phospholipids. Then, the cyclooxygenase (COX) enzymes convert AA into the unstable endoperoxide intermediate PGG\(_2\) by a cyclooxygenase reaction (oxygenation) and subsequently to PGH\(_2\) by a peroxidase reaction (removal of 2e\(^-\)). Prostaglandin H\(_2\) is next converted into prostanoids (PGD\(_2\), PGE\(_2\), PGF\(_{2\alpha}\), thromboxane A\(_2\), and PGI\(_2\)) by specific isomerase enzymes (e.g. PGD synthase, PGE synthase, PGF synthase, Tx synthase, PGI synthase; Flower and Vane, 1972; Smith and Lends, 1972; Needleman et al., 1986; Smith et al., 1991). The rate-limiting step in prostaglandin biosynthesis is believed to be either the cleaving of AA from the cell membrane or the conversion of AA to the PG intermediates (van der Bosch, 1980; Levy et al., 2000, respectively). This pathway is depicted in Figure 2.
Alternatively, free AA can be converted into other eicosanoids such as leukotrienes (LTs), lipoxins (LPXs) and hydroxyeicosatetraenoic acids (HETEs) by an enzyme called 5-lipoxygenase (5-LO; McCracken, 2005). Unlike the COX enzymes, 5-LO requires activation by Ca$^{2+}$ and translocation to the cell membrane. Leukotrienes and HETEs are involved in inflammation, vasoconstriction and blood flow associated with
asthma and allergic rhinitis (Currie et al., 2005; Graeme et al., 2005; Miyata and Roman, 2005) whereas LPXs are anti-inflammatory compounds (Claria and Planaquama, 2005).

Cyclooxygenase-1:

Cyclooxygenase-1 (COX-1), also known as PGH2 synthase-1, is a 600-602 amino acid enzyme (MW~70 kDa) that is glycosylated at Asn68, Asn144, and Asn410, and is involved in prostaglandin synthesis (Garavito and Mulichak, 2003). It was purified from seminal vesicles of sheep (Hemler and Lands, 1976; Van der Ouderaa et al., 1977) and cow (Miyamoto et al., 1976) and was later cloned from the same tissue (DeWitt and Smith, 1988; Merlie et al., 1988). The COX-1 gene is 22 kb and is located on chromosome 9 (Robertson, 1998). The mRNA transcript is 2.8-3 kb and is relatively stable. The promoter region, which contains SP1-like elements, has low inducibility so the enzyme is constitutively expressed in most cell types and tissues, e.g. smooth muscle, fibroblasts, stomach, and kidney, but is expressed in relatively large amounts in fibroblasts and endothelial cells (DeWitt et al., 1983; Funk et al., 1991; Kraemer et al., 1992). It was shown through immunocytochemistry that COX-1 is associated with the membranes of the endoplasmic reticulum and nucleus, but not the plasma or mitochondria of cultured fibroblasts (Rollins and Smith, 1980). Cyclooxygenase-1 plays more of a “housekeeping” role because of its association with PGI2 and PGE2 production, which are involved in vasodilation and maintaining gastric mucosa and kidney function (Moncada et al., 1976; Whittle et al., 1978; Smith, 1992). The cyclooxygenase-1 protein consists of 5 regions (Figure 3); (1) signal peptide, (2) dimerization domains, (3) membrane binding domain, (4) catalytic domain and (5) membrane targeting sequence.
(Picot et al., 1994; Simmons et al., 2004). The signal peptide (22-26 amino acids) resides at the N-terminus and serves to direct the enzyme into the lumen of the endoplasmic reticulum or nuclear membrane. Following this domain are 8 amino acids that are not found in COX-2. The function of these residues is unknown. The dimerization domain, also called the epidermal growth factor-like domain, is approximately 50 amino acids in length and allows for dimerization with COX-1 but not COX-2. The homodimer is held together by four disulfide bonds; three by the dimerization domains and one by the catalytic domain. The membrane binding domain is composed of 50 amino acids that form four helices whose surfaces are hydrophobic. These helices penetrate the hydrophobic core of the lumen side of the endoplasmic reticulum/nuclear phospholipid bilayer. As a result, the dimers float on the surface of the lumen. The catalytic domain composes most of the enzyme (480 residues) and contains two active sites; cyclooxygenase and peroxidase active sites. The cyclooxygenase active site is a long, narrow, hydrophobic channel that contains tyrosine 385 which forms a tyrosyl radical. This radical removes a hydrogen from carbon 13 of AA, creating an arachinonyl radical that can now be converted in PGG₂ by an oxygenation reaction. This reaction is governed by two other residues that reside in the channel: serine 530 and valine 349. The peroxidase active site is represented by a shallow cleft in the active site. Here, heme is bound to iron which is bound to His388 in sheep COX-1. A large portion of the heme is exposed allowing for interaction with PGG₂. Subsequently, PGG₂ is converted into PGH₂ by the peroxidase active site.
Cyclooxygenase-2:

The COX-2 (PGH$_2$ synthase-2) gene was first isolated and cloned from chicken embryo cells (Simmons et al., 1989; Robertson, 1998). It is 8 kb and is located on chromosome 10 (Kulkarni et al., 2000). The mRNA transcript is 4-4.5 kb and is degraded relatively quickly. The enzyme was purified by Sirois and Richards in 1992 from pre-ovulatory rat follicles and is composed of 604 amino acids which yield a molecular weight of approximately 72 kDa. Cyclooxygenase-2 shares 63% amino acid homology with COX-1 (Yokoyama and Tanabe, 1989; Kraemer et al., 1992; Garavito and Mulichak, 2003) and has the same glycosylated residues as COX-1 with an additional one at Asn588. Like COX-1, COX-2 is bound to the membranes of the nucleus and the endoplasmic reticulum and is also involved in prostaglandin synthesis. Cyclooxygenase-2 comprises the same five regions as COX-1 (Figure 4) and functions in similar ways. The active site of COX-2 converts AA to PGG$_2$ by oxygenation and subsequently to PGH$_2$ by
peroxidation, which is a heme-dependent reaction. However, a substitution of Val523 in COX-2 for Ile523 in COX-1 opens up a hydrophobic pocket in COX-2 that allows COX-2 selective drugs to bind (Kurumbail et al., 1996). While COX-1 and -2 have the same affinity for AA, the hydrophobic pocket in COX-2 allows for bulkier substrates such as endocannabinoids to be converted to endocannabinoid-derived prostanoids (Kozak et al., 2002, 2003). Another distinct difference is that COX-2 is inducible whereas COX-1 is not. The promoter region of the COX-2 gene contains a cAMP response element, CREB/NF-IL-6 element and NFkB sites which are critical for maximum transcription of COX-2 (Sirois and Richards, 1993; Crofford et al., 1997; Herschman et al., 1997; Kulkarni et al., 2000). Additionally, PPARγ response elements, Ets sites and PEA sites have been identified in the promoter region of the COX-2 gene; however, their function remains unknown (Smith et al., 2000). Cyclooxygenase-2 is induced in fibroblasts by growth factors (epidermal growth factor, platelet derived growth factor and transforming growth factor β), phorbol esters, interleukin-1 (IL-1); lipopolysaccharide (LPS) in monocytes and macrophages, and tumor necrosis factor (TNF) in endothelial cells (Lafyatis et al., 1989; Lin et al., 1989; Smith et al., 1994). Unlike COX-1, COX-2 is inhibited by glucocorticoids such as dexamethasone. Because COX-2 is an inducible enzyme, large concentrations of prostaglandins are produced leading to diseases involving pain and inflammation (Dubois et al., 1998; Mollace et al., 2005). For example, COX-2 is associated with pathological conditions such as arthritis and cancer. Individuals with arthritis have increased COX-2 expression and prostaglandin production (Anderson et al., 1996). Human and animal colorectal tumors express high concentrations of COX-2 whereas normal intestinal mucosa does not (Kutchera et al., 1996). However,
physiological events such as ovulation and luteolysis are also accompanied by an increase in prostaglandins which is the result of COX-2 induction (Richards et al., 1995).

**Figure 4.**

**COX-2**

![Image of COX-2](image)

**Cyclooxygenase-3:**

The COX-3 isoform (~65 kDa) is a COX-1 gene derivative isolated and characterized by Chandrasekharan et al. (2002). It contains the same regions as COX-1 and -2 isozymes, but it retains intron 1 (**Figure 5**). Glycosylation by the endoplasmic reticulum is essential for activation and is expressed predominantly in the brain. The inhibition of COX-3, but not COX-1 or -2, by a therapeutic dose of acetaminophen, phenacetin, antipyrine, and dipyrone (antipyretic and analgesic drugs) suggests that the primary function of COX-3 is to induce pain and fever (Warner et al., 2004).
COX Expression during the Estrous Cycle and Luteolysis:

Studying the expression of COX enzymes, especially COX-2, during luteolysis is important because they are necessary for PG production. If COX expression within the CL increases during luteolysis, not only will uterine PGF$_{2\alpha}$ be exerting its effects on the CL, but intraluteal PGF$_{2\alpha}$ will be acting on the CL to cause its eventual demise. Several \textit{in vivo} studies have investigated the expression of luteal COX-2 expression during the estrous cycle or in response to PGF$_{2\alpha}$. Expression of luteal COX-2 increases from day 7-17 during the bovine estrous cycle (Arosh et al., 2004). Similar results in the cow were observed by Hayashi et al. (2003). In another study, sheep were infused with 1 $\mu$mol of PGF$_{2\alpha}$ through the ovarian artery on day 11 or 12 of the estrous cycle and corpora lutea were removed at 1, 4, 12 and 24 hours afterwards (Tsai and Wiltbank, 1997). Expression of COX-2 mRNA increased at the 1 and 4 hour time points when compared to controls before returning to basal concentrations. Levy et al. (2000) supported these findings. In the pseudopregnant rabbit, a 200 $\mu$g i.m. infusion of the PGF$_{2\alpha}$ analogue alfaprostol on days 4 or 9 resulted in an increased expression of COX-2 mRNA 1.5-6 hours after
treatment (Zerani et al., 2007). It is not surprising that COX-1 expression does not change in response to PGF$_{2\alpha}$ treatment because it is non-inducible (Arosh, 2004; Zerani et al., 2007).

Administering a luteolytic dose of PGF$_{2\alpha}$ may not mimic events associated with the physiological onset of luteolysis in sheep. Therefore, Allen (UNH Master’s Thesis) and Ricketts (UNH Master’s Thesis) used a model in which sheep received infusions of PGF$_{2\alpha}$ that mimic physiological conditions during the onset of luteolysis to determine the expression of COX-1 and COX-2 in the CL. Following a single 1 hour infusion of PGF$_{2\alpha}$ (0.22 ug/kg/min), COX-2 protein increased 16 and 24 hours after treatment when compared to untreated sheep (Allen, UNH Master’s Thesis). This increase was maintained 1, 8, 16, and 24 hours following a second infusion of PGF$_{2\alpha}$ (0.22 ug/kg/min) when compared to untreated sheep (Ricketts, UNH Master’s Thesis). Cyclooxygenase-1 expression remained unchanged after the first and second infusions of PGF$_{2\alpha}$.

**Prostaglandin F$_{2\alpha}$ as the Luteolytic Agent:**

If pregnancy does not occur, the CL undergoes luteolysis, allowing for a new cycle to begin. For a long time, it was unknown what factor(s) signal luteolysis; however, several studies involving uterine effects on the ovary identified the hormone prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) as the signal. Early studies in the hysterectomized guinea pig (Loeb, 1923, Denamur et al., 1966), sheep (Demers et al., 1972), cow (Demers et al., 1972), and mare (Ginther, 1971) during the luteal phase extended the lifespan of the CL that was equal to or longer than pregnancy. This strongly suggested that the luteolytic agent was of uterine origin. To confirm this finding, autotransplant experiments in sheep
were performed. One ovary of a sheep was removed from the abdominal cavity and attached to the jugulo-carotid loop in the neck (Goding et al., 1967; McCracken and Baird, 1969). As a result, the CL in the transplanted ovary remained for greater than 100 days. Conversely, if both the ovary and ipsilateral uterine horn are transplanted to the neck, normal luteal regression occurs (McCracken et al., 1973). Collectively, these experiments support the notion that the luteolytic agent is of uterine origin and that it acts locally, not systemically.

Pharriss and Wyngarden (1969) suggested that PGF$_{2\alpha}$ was the luteolytic agent in mammals because it was abundant in the rat uterus. Prostaglandin F$_{2\alpha}$ was later determined to be the luteolytic agent in sheep (McCracken et al., 1972). Similarly, PGF$_{2\alpha}$ is the luteolytic agent in the cow (Nancarrow et al., 1973; Kindahl et al., 1976; Milvae and Hansel, 1983a; Jarowszewski et al., 2003), the pig (Gleeson et al., 1974; Moeljono, 1976), and the guinea pig (Poyser, 1976). Furthermore, PGF$_{2\alpha}$ is secreted from the uterus in a pulsatile manner in ruminants (McCracken et al., 1973; Kindahl et al., 1976a; Kindahl et al., 1976b; Kindahl et al., 1980; Kindahl et al., 1984), the sow (Kindahl et al., 1981), and the guinea pig (Elger et al., 1994). In ewes, Zarco et al. (1988) determined that multiple, sequential pulses of PGF$_{2\alpha}$ are required for luteolysis. Specifically, eight one-hour pulses of PGF$_{2\alpha}$ on average, are noted during the luteal phase in which the first one starts on day 13 of the estrous cycle. The interval between the first two pulses is 16 hours while all intervals after that are 8 hours. Although eight pulses of PGF$_{2\alpha}$ occur, 4-5 pulses are usually adequate to induce full regression of the CL (McCracken et al., 1999; Schramm et al., 1983).
These pulses of uterine PGF$_{2\alpha}$ in sheep act locally on the CL because a systemic infusion of PGF$_{2\alpha}$ (25 ug/hr) did not result in regression of the CL (McCracken, 1971). The failure of systemic PGF$_{2\alpha}$ can be accounted for by a dilution effect and the fact that >99% of PGF$_{2\alpha}$ is readily metabolized by one passage through the lungs (Davis et al., 1980). If PGF$_{2\alpha}$ is metabolized by the lungs, then there must be another route that uterine PGF$_{2\alpha}$ travels to exert its effects on the ovary. Such a hypothesis was tested over a series of experiments in sheep (McCracken et al., 1971; McCracken et al., 1972; McCracken et al., 1973). Given that the utero-ovarian vein and the ovarian artery are twisted around each other, it was thought that substances secreted by the uterus could diffuse into the ovarian artery and act on the ovary. To test this hypothesis, [$^3$H]PGF$_{2\alpha}$ (0.1 μCi/min) was infused into the uterine vein for one hour. Afterwards, 1% of it was recovered in the adjacent ovarian artery. Since each uterine horn releases 25 μg/hour PGF$_{2\alpha}$ into their respective uterine veins (McCracken, 1972; Green et al., 1972), the same dose of PGF$_{2\alpha}$ was infused into the uterine vein in situ or was given systemically (control animals). As a result, the in situ CLs underwent premature luteolysis whereas the systemic infusions did not induce luteolysis. Collectively, these studies support the theory behind the counter-current transfer in which uterine PGF$_{2\alpha}$ traveling through the utero-ovarian vein diffuses into the ovarian artery where it can subsequently act on the CL to initiate luteolysis.

Although PGF$_{2\alpha}$ is the central hormone that initiates luteolysis, it is important to review some of the factors that trigger its release from the uterus as well as within the CL. To explain the release of PGF$_{2\alpha}$ from the uterus, experiments based on the Ferguson reflex (Ferguson, 1941), the mechanical stimulation of the female reproductive tract to stimulate neurohypophysial release of oxytocin, were pursued. Roberts and Share (1968,
1970) demonstrated that vaginal distension in sheep and goats causes an increase in blood oxytocin concentrations while it was shown that mechanical stimulation of the uterus resulted in secretion of PGF$_{2\alpha}$ early and late in the sheep estrous cycle (Wilson et al., 1974). This hypothesis was strengthened by the work of Armstrong and Hansel (1959) in which they found that exogenous oxytocin resulted in premature luteolysis in cows. However, the direct link between oxytocin and uterine PGF$_{2\alpha}$ production was not shown until McCracken (1980) demonstrated that a physiological dose of oxytocin (200 pg/min) infused into the uterine artery of sheep for 10 minutes on different days of the ovine estrous cycle (days 3, 8, 13 and 14) resulted in an increase in PGF$_{2\alpha}$ secretion on days 3 and 14. The lack of PGF$_{2\alpha}$ increase on days 8 and 13 can be explained by the decrease in oxytocin receptors on the endometrium and myometrium of the uterus during those days of the estrous cycle. Regulation of oxytocin receptors on the uterus is controlled primarily by E$_2$ and P$_4$. Estradiol enhances expression of oxytocin receptors in the sheep (Sharma and Fitzpatrick, 1974) and rat (Soloff, 1975) uterus; however, this effect was blocked in sheep by the addition of P$_4$.

In addition to being secreted by the uterus, PGF$_{2\alpha}$ is produced by the CL in several species. Prostaglandin F$_{2\alpha}$ is produced by corpora lutea of women (Shutt et al., 1976; Swanston et al., 1977; Patwardhan and Lanthier, 1980), sows (Guthrie et al., 1978), ewes (Rexroad and Guthrie, 1979; Tsai and Wiltbank, 1997), cows (Milvae and Hansel, 1983b; Pate, 1988), and rodents (Olofsson et al., 1992). Guthrie and Rexroad (1980a) showed that intraluteal PGF$_{2\alpha}$ increases from mid (days 8, 12, and 14) to late (days 16 and 18) luteal phase in pigs. Similarly, in the cow, DelVecchio et al. (1995) showed that cells from late luteal stage CLs (days 17-18) produced more PGF$_{2\alpha}$ than cells from mid...
luteal stage CLs (days 10-12). In the pseudopregnant rabbit, a 200 μg i.m. infusion of a PGF$_{2\alpha}$ analogue induced an increase in PGF$_{2\alpha}$ production by luteal cells (Zerani et al., 2007). Tsai and Wiltbank (1997) found that ovine luteal cells in culture increased COX-2 production after addition of PGF$_{2\alpha}$, which increases PGF$_{2\alpha}$ production by the CL. In addition, Milvae (2000) suggests that in ruminants, endothelin-1 (ET-1) increases luteal PGF$_{2\alpha}$. However, Milvae and Hansel (1983b) showed that CLs from day 5 of the bovine cycle produced more PGF$_{2\alpha}$ than day 10, 15, and 18 CLs. To explain these high concentrations of PGF$_{2\alpha}$ early in the luteal phase without regression of the CL, it has been suggested that the young CL is not responsive to PGF$_{2\alpha}$. Gadsby et al. (1990) showed that day 6-8 CL had less high affinity binding sites for PGF$_{2\alpha}$ than the day 12, 13, 14, 16, and 17 porcine CL, suggesting that as the CL ages, it becomes more sensitive to PGF$_{2\alpha}$.

Collectively, these studies indicate that uterine as well as intraluteal PGF$_{2\alpha}$ production contribute to the eventual demise of the CL.

**Matrix Metalloproteinases:**

Matrix metalloproteinases (MMPs) are a family of divalent cation-dependent proteases that break down the components of extracellular matrix (ECM) to aid in tissue remodeling (Roy, 2006). The components of the ECM include proteins such as collagen, gelatin, fibronectin, nidogen, laminin, aggrecan, and elastin (Table 1). Physiological events within the body that require tissue remodeling include embryonic development, morphogenesis, angiogenesis, reproduction, and tissue reabsorption. Pathological events that require this process include tumorigenesis/metastasis and arthritis (Vincenti et al., 1994; Nagase and Woessner, 1999; Roy et al., 2006). Currently, 24 MMPs have been
identified, 23 of which are present in humans, and are categorized into the following families: collagenases, gelatinases, stromelysins, membrane type-MMPs (MT-MMPs), matrilysins, and other MMPs (Visse and Hideaki, 2003; Clark et al., 2008). These families are defined by the substrates (ECM components) they bind and degrade.

The basic structure of MMPs consists of five domains (Figure 6; Woessner, 1991; Visse Nagase, 2003; Roy et al., 2006). The first is a signal peptide (19-29 amino acids) that targets the enzyme for secretion by the cell by sending it to the rough endoplasmic reticulum during synthesis. The second is the propeptide domain (80 amino acids) which maintains the latent form of the enzyme. The third is the catalytic domain (170 amino acids) which contains a highly conserved Zn$^{2+}$ binding region (HExGHxxGxxHS/T) and is the site of enzyme activity. The zinc ion is bound to three residues from the catalytic domain and one (cysteine) from the propeptide (Figure 7; Vallee and Auld, 1990b). The fourth is the hemopexin domain (210 amino acids) which determines substrate specificity. This domain is required by MMPs for them to cleave triple helical collagens (Bode, 1995) and is an essential structure involved in the activation of proMMP-2 by MT-MMP (Murphy et al., 1992). The fifth is the hinge region which presents the hemopexin domain, with bound substrate, to the catalytic domain for degradation.
In order to understand how these enzymes function, it is necessary to discuss their activation process. One notable characteristic of MMPs is that they are synthesized and secreted as latent enzymes and are activated extracellularly. In the latent form, the propeptide domain forms a cysteine-zinc bridge with the catalytic domain, blocking enzyme activity (Woessner, 1991). Upon secretion by the cell, the enzyme can be activated by trypsin, plasmin, kallikrein, and MMPs (Figure 7; Murphy et al., 1991). Plasmin is a serine protease secreted as plasminogen from the brain and liver and is activated by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA; Raum et al., 1980; Mignatti et al., 1986; Alexander and Werb, 1992; Sappino et al., 1993). These activators break the cysteine-zinc bridge and cleave the propeptide domain exposing the zinc core within the catalytic domain (Van Wart and Birkadel-Hansen, 1990). This allows water to bind to zinc, a crucial ligand in activation of this enzyme, resulting in degradation of the ECM by protein hydrolysis (Vallee and Auld, 1990a; Vallee and Auld, 1990b). Furthermore, MMPs can degrade their own propeptide region.
by autocatalytic cleavage. In other words, once an MMP has been activated, it can continue to cleave the remainder of the propeptide. Depending on how much of the propeptide is cleaved off, an individual MMP (e.g. active MMP-2) can have various molecular weight forms.

Matrix metalloproteinase-1 was the first member of this enzyme family to be isolated from tadpole tail, gill, and gut during natural and hormone-induced metamorphosis (Gross and Lapiere, 1962). It was subsequently isolated from rat bone (Walker et al., 1964). Since then, other members have been characterized giving rise to the six families previously mentioned. The gelatinase family comprises MMP-2 and MMP-9 and will be the focus of the next sections.
Matrix metalloproteinase-2:

Matrix metalloproteinase-2 (Gelatinase A) was first found to be present in
medium of cultured explants of a mouse tumor (Liotta et al., 1979). It was partially
characterized by Liotta et al. (1981) and then purified and characterized by Collier et al.
(1988). It has subsequently been found in various tissues and cell types such as bovine
and sheep corpora lutea (Goldberg et al., 1996; Towle et al., 2002, respectively), follicle (Curry et al., 2001), uterus (Bany et al., 2000), endothelial cells (Hanemaaijer et al., 1993), rat granulation tissue (Nakagawa et al., 1987), human skin (Seltzer et al., 1981), synovial fluid (Koolwijk et al., 1995), tumors (Iurlaro et al., 1999), bone (Kusano et al., 1999), fibroblasts (Saed et al., 2000) and other tissues that undergo structural remodeling. The human MMP-2 gene (27 kb), found on chromosome 16 (Huhtala et al., 1990b, Huhtala et al., 1991), consists of 2733 nucleotides which yield a protein of 621 amino acids. The promoter region does not contain a TATA or CAAT box; however, it does contain a GGGCGG consensus sequence that binds the transcription factor SP-1 (Huhtala et al., 1990a). It also contains a CCCCAGGC (CG box) consensus sequence and is a potential binding site for activator protein-2 (AP-2). The MMP-1 and -3 promoters contain the 12-O-tetradecanoylphorbol 13-acetate (TPA) response element sequence (TGAGTCAG) while it is not present in MMP-2. This is an interesting finding because Salo et al. (1985) showed that TPA increases MMP-2 activity and mRNA concentrations. However, the effects of TPA on MMP-2 remain uncertain because Collier et al. (1988) showed that it has no influence on MMP-2 production. Matrix metalloproteinase-2 is constitutively expressed in most cell types (Salo et al., 1985, Templetone et al., 1990; Huhtala et al., 1991; Yeow et al., 2001) and is only moderately induced (transforming growth factor-β; TGF-β) or repressed 2-4 fold, suggesting that it is regulated primarily by their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs; Huhtala et al., 1991; Overall et al., 1991; Salo et al., 1991). Matrix metalloproteinase-2 contains the previously mentioned five domains in addition to another one called fibronectin type II repeat (Figure 6; Collier et al., 1988), a region specific to gelatinases that binds collagens.
and gelatins (Allan et al., 1995; Steffensen et al., 1995). This region is encoded by three additional exons of the MMP-2 gene that are lacking in MMP-1 and -3 (Huhtala et al., 1991). The bovine MMP-2 gene consists of 2350 base pairs and shares 94.7%, 94.1%, 93.5%, 93%, and 95.9% homology to that of human, rat, mouse, rabbit and pig, respectively (Zhang et al., 2005). The latent and active forms of this enzyme have molecular weights of approximately 72 kDa and 66 kDa, respectively.

**Matrix metalloproteinase-9:**

Matrix metalloproteinase-9 (Gelatinase B) was first found to be present in human leukocytes (Sopata and Dancewicz, 1974). It was later purified and characterized by Wilhelm et al. (1989) and Morodomi et al. (1992). The human MMP-9 gene (7.7 kb), found on chromosome 20, consists of 2334 nucleotides yielding a protein of 707 amino acids (Wilhelm et al., 1989; Collier et al., 1991; Huhtala et al., 1991). It has subsequently been found in a variety of tissues such as bovine and sheep corpora lutea (Goldberg et al., 1996; Towle et al., 2002, respectively), follicle (Curry et al., 2001), uterus (Bany et al., 2000), endothelial cells (Hanemaaijer et al., 1993), synovial fluid (Koolwijk et al., 1995), tumors (Iurlaro et al., 1999), bone (Kusano et al., 1999), fibroblasts (Saed et al., 2000) and other tissues that undergo structural remodeling. The preferred substrates for MMP-2 and -9 are collagen IV and gelatin, but they also degrade elastin and fibrillin. A list of MMPs and their substrates is outlined in Table 1. Similar to MMP-2, the MMP-9 promoter lacks TATA and CAAT boxes (Huhtala et al., 1991). However, the MMP-9 promoter contains a TATA-like sequence (TTAAA) whereas MMP-2 does not. It also
contains the CG box found in MMP-2 that allows SP-1 to bind. Unlike MMP-2, the MMP-9 promoter contains 2 sequences that serve as activator protein-1 (AP-1) binding sites, which is stimulated by TPA. In addition, it contains a TPA response element, which is lacking in MMP-2. Transcription of MMP-9 is increased up to 8-fold by TGF-β (Salo et al., 1991; Overall et al., 1991). The latent and active forms of this enzyme have molecular weights of approximately 92 kDa and 84 kDa, respectively. Matrix metalloproteinase-9 is composed of the same domains as MMP-2, but it also has a 54 residue sequence located adjacent to the carboxyl-terminal side of the zinc-binding domain (Wilhelm et al., 1989; Huhtala et al., 1991). This sequence shares 30-50% homology to part of the α2(V) chain in collagen. Wilhelm et al. (1989) suggested that it is the result of genetic recombination of the functional domains between MMP-9 and collagen.

Table 1.

Matrix Metalloproteinase Families

<table>
<thead>
<tr>
<th>Family</th>
<th>MMP number</th>
<th>Molecular mass (kDa)</th>
<th>Chromosomal location</th>
<th>Prototypical ECM substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td>MMP-1</td>
<td>52</td>
<td>42</td>
<td>11q22–q23, 9 (A1–A2), Collagen I, II, III, VII, VIII, X, XI; gelatin, entactin/nidogen, fibronectin, laminin,</td>
</tr>
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</table>

L = Latent, A = Active
<table>
<thead>
<tr>
<th>Family</th>
<th>MMP number</th>
<th>Molecular mass (kDa)</th>
<th>Chromosomal location</th>
<th>Prototypical ECM substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>A</td>
<td>Human</td>
<td>Mouse</td>
</tr>
<tr>
<td>Gelatinases</td>
<td>MMP-8</td>
<td>85</td>
<td>64</td>
<td>11q21–q22</td>
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<td>MMP-13</td>
<td>52</td>
<td>42</td>
<td>11q22.3</td>
</tr>
<tr>
<td>Stromelysins</td>
<td>MMP-2</td>
<td>72</td>
<td>66</td>
<td>16q13</td>
</tr>
<tr>
<td></td>
<td>MMP-9</td>
<td>92</td>
<td>84</td>
<td>20q11.2–q13.1</td>
</tr>
<tr>
<td></td>
<td>MMP-3</td>
<td>57</td>
<td>45</td>
<td>11q23</td>
</tr>
<tr>
<td>Family</td>
<td>MMP number</td>
<td>Molecular mass (kDa)</td>
<td>Chromosomal location</td>
<td>Prototypical ECM substrates</td>
</tr>
<tr>
<td>--------------</td>
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<td>-----------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>A</td>
<td>Human</td>
<td>Mouse</td>
</tr>
<tr>
<td>MT-MMPs</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-10</td>
<td>54</td>
<td>44</td>
<td>11q22.3–q23</td>
<td>9p24.1</td>
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<td>54</td>
<td>14q11–q12</td>
<td>14 (12.5)</td>
</tr>
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<td>60</td>
<td>16q13–q21</td>
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<tr>
<td>MMP-16</td>
<td>64</td>
<td>53</td>
<td>8q21</td>
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<td>53</td>
<td>12q24.3</td>
<td>5 (1.0)</td>
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<td>62</td>
<td>16p13.3</td>
<td></td>
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<td>28</td>
<td>19</td>
<td>11q21–q22</td>
</tr>
<tr>
<td>Family</td>
<td>MMP number</td>
<td>Molecular mass (kDa)</td>
<td>Chromosomal location</td>
<td>Prototypical ECM substrates</td>
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<tr>
<td>------------</td>
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<td>---------------------</td>
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<td>-----------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>L A</td>
<td>Human</td>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Other MMPs</td>
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<td></td>
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<td></td>
</tr>
<tr>
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<td>29 19</td>
<td>11p15</td>
<td></td>
<td>Laminin, vitronectin, aggrecan, osteonectin</td>
</tr>
<tr>
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<td>64 46</td>
<td>22q11.2</td>
<td>10 (40.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fibronectin, laminin, aggrecan</td>
</tr>
<tr>
<td>MMP-12</td>
<td>54 22</td>
<td>11q22.2-2q22.3</td>
<td>9 (1.0)</td>
<td>Fibronectin, elastin, laminin, proteoglycan, fibrin/fibrinogen</td>
</tr>
<tr>
<td>MMP-19</td>
<td>54 45</td>
<td>12q14</td>
<td>10 (71.0)</td>
<td>Collagen IV, gelatin, laminin, fibrin/fibrinogen</td>
</tr>
<tr>
<td>MMP-20</td>
<td>54 22</td>
<td>11q22.3</td>
<td>9 (1.0)</td>
<td>Amelogenin, aggrecan</td>
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<tr>
<td>MMP-23</td>
<td>44 31</td>
<td>1p36.3</td>
<td></td>
<td>Gelatin</td>
</tr>
</tbody>
</table>

Adapted from Roy et al. (2006).

**Tissue Inhibitors of Metalloproteinases:**

Matrix metalloproteinases are regulated at several levels including gene activation, transcription, mRNA stability, translation and secretion of latent proenzymes,
binding of proenzymes to cell membranes, proenzyme activation, inactivation by endogenous inhibitors, and degradation of the active site. Two endogenous inhibitors are $\alpha_2$-macroglobulin which is found in plasma, and tissue inhibitors of metalloproteinases (TIMPs) which are typically associated with the ECM (Birkedal-Hansen et al., 1993; Stetler-Stephenson et al., 1993; Baker et al., 2002). Currently, four members of the TIMP family have been identified in vertebrates; TIMP-1 (Sellers et al., 1977; Murphy et al., 1981; Carmichael et al., 1986), TIMP-2 (Stetler-Stevenson et al., 1989), TIMP-3 (Yang and Hawkes, 1992), and TIMP-4 (Greene et al., 1996). All TIMPs inhibit all MMPs with the exception that TIMP-1 can not inhibit MMP-14, -16, or -24 (Visse and Nagase, 2003). They form a 1:1 complex through reversible non-covalent binding with active and less commonly, latent forms of MMPs resulting in inhibition. A balance of MMPs and TIMPs maintains the integrity of the ECM. However, if MMP concentrations exceed TIMP concentrations for example, the result is an increase in net proteolytic activity, allowing for cell migration, angiogenesis, and tissue remodeling. Understanding the structure and function of TIMPs and how they interact with MMPs will give us a better idea of how the ECM remodeling is regulated. Due to the relationship between TIMP-1 and MMP-9 and TIMP-2 and MMP-2, TIMP-1 and -2 will be emphasized.

**Tissue Inhibitor of Metalloproteinase-1:**

Tissue inhibitor of metalloproteinase-1 is an approximately 29 kDa glycoprotein first described in rabbit bone (Sellers et al., 1977), and was subsequently isolated and characterized (Murphy et al., 1981). It was then cloned from normal human fibroblasts by Docherty et al. (1985) and Carmichael et al. (1986). The human TIMP-1 gene consists of
811 nucleotides giving rise to a protein of 206 amino acids. The ovine gene consists of 887 nucleotides yielding a 207 amino acid protein (Smith et al., 1994). The primary structure of ovine TIMP-1 is 95%, 86%, and 77% homologous to bovine, human, and mouse, respectively. The first 23 amino acids from the N-terminus serve as a signal peptide and are cleaved off upon secretion. The 3-dimensional structure contains six disulfide bonds which form six loops making it highly stable (Figure 8; Williamson et al., 1990). It preferentially binds to MMP-3, active MMP-1, and both active and latent forms of MMP-9 (Welgus et al., 1983; Welgus et al., 1985; Wilhelm et al., 1989). The inhibitory action of TIMP-1 resides in the three N-terminal loops which bind to the active site of an MMP (Woessner and Nagase, 2000). Inhibitors of TIMP-1 include serine proteinases such as trypsin, chymotrypsin, and elastase (Okada et al., 1988). Tissue inhibitor of metalloproteinase-1 is found in aorta, gingival, dental pulp, cartilage, embryonic bone, tendon, synovium, uterus, corpus luteum, follicles, fibroblasts, epithelial cells, endothelial cells, osteoblasts, chondrocytes, smooth muscle, platelets, monocytes/macrophages, and tumor cells (Takahashi et al., 1993; Hayakawa, 1994; Smith et al., 1994; Pitze1 et al., 2000; Goldman and Shalev, 2004; Lind et al., 2006; Bogusiewicz et al., 2007; Skrzypczak et al., 2007).
Tissue Inhibitor of Metalloproteinase-2:

Tissue inhibitor of metalloproteinase-2 is an approximately 21 kDa non-glycosylated protein (Stetler-Stevenson et al., 1989). The human gene consists of 1062 nucleotides which are translated into a 201 amino acid protein (Stetler-Stevenson et al., 1990). Sheep mRNA for TIMP-2 was identified in ovine follicles and corpora lutea by Smith et al. (1995). The primary structure of TIMP-2 has also been sequenced in calf (Boone et al., 1990) and mouse (Shimizu et al., 1992). Human and mouse TIMP-2 sequences share 97% homology. Tissue inhibitor of metalloproteinase-1 and -2 share 43% homology so it is not surprising that TIMP-2 is composed of 201 amino acids, of which the first 24 from the N-terminus serve as a signal sequence (Stetler-Stevenson et
al., 1989). Similar to TIMP-1, the 3-dimensional structure of TIMP-2 contains six disulfide bonds which form six loops (Figure 9); the three N-terminal loops binding to the active site of MMPs. Although TIMP-2 binds all MMPs, it preferentially binds to the active and latent forms of MMP-2 (Howard et al., 1991). It has been identified in rabbit brain capillary endothelial cells (Herron et al., 1986), bovine aortic endothelial cells (DeClerck et al., 1989), bone marrow cells (Zdzisińska et al., 2008), tumors (Xu et al., 2002), sheep CL (Towle et al., 2002), cow CL (Goldberg et al., 1996; Zhang et al., 2003), uterus (Zhang and Salamonsen, 1997), ovarian follicle (Bakke et al., 2002), skeletal muscle (Singh et al., 2000), Leydig cells (Blavier and DeClerck., 1997), placenta (Marzusch et al., 1996), and brain (Giraudon et al., 1995).

Figure 9.

TIMP-2

Hayakawa (1994)
**Other actions of TIMPs:**

Although the primary function of TIMPs is to inhibit MMPs, a function unique to TIMP-2 actually aids in the activation of proMMP-2. This event includes the formation of a complex between proMMP-2, MT-1MMP, and TIMP-2 on the cell surface. Membrane-type-2, and -3 also aid in the activation of proMMP-2, but the complex involving MT-MMP1 has been studied most extensively (Overall and Sodek, 1990; Sato et al., 1994; Butler et al., 1997; Takino et al., 1995). Butler et al. (1998) demonstrated that addition of MT-1MMP and TIMP-2 increased proMMP-2 activation; however, an excess of TIMP-2 inhibited activation of proMMP-2. Furthermore, TIMP-2 lacking the C-terminal domain did not enhance activation of proMMP-2. Therefore, Butler et al. (1998) proposed that TIMP-2 and MT1-MMP form a complex that acts as a receptor for proMMP-2. When proMMP-2 binds to this “receptor”, free/active MT1-MMP activates proMMP-2. In this three-way interaction, the N-terminus of TIMP-2 bind to the active site of MT1-MMP while its C-terminus binds to the hemopexin domain of proMMP-2 resulting in proMMP-2 activation by free/active MT1-MMP (Strongin et al., 1995).

Tissue inhibitor of metalloproteinase-1 may have a role in regulating the activation of proMMP-9. As proMMP-9 is secreted by cells, it can form a complex with MMP-1 which results in its activation by MMP-3 yielding an 82 kDa protein (Goldberg et al., 1992; Ogata et al., 1992; O’Connell et al., 1994). However, if TIMP-1 binds to proMMP-9, the complex between proMMP-9 and MMP-1 can not occur and activation is prevented.

An additional role of TIMP-1 within the CL may be to regulate steroidogenesis. Boujrad et al. (1995) found that a 70 kDa complex consisting of TIMP-1 and cathepsin L
activates steroidogenesis. Furthermore, Nothnick (2003) showed that serum P₄ concentration was lower in TIMP-1 null mice than wild type.

**MMP and TIMP Expression during the Estrous Cycle and Luteolysis:**

In order for luteolysis to occur, tissue remodeling is essential. The mRNA and protein expression of MMPs and TIMPs throughout the estrous cycle, along with their regulation by PGF₂α in various species, have been reported. Xinlei et al. (2006) showed an increase in staining for MMP-2 and -9 in the late luteal stage (day 15) in rhesus monkey corpora lutea when compared to the early (day 5) and mid (day 10) luteal stages. Also, mRNA for MMP-2 and -9 were assessed using *in situ* hybridization and MMP-2 mRNA expression matched the protein profile from the tissue staining; however, MMP-9 mRNA was too low to be detected. Similar results were reported in other species (Liu K et al., 1999; Pitzel et al., 2000; Young et al., 2002; Kliem et al., 2007). However, Li et al. (2002) showed that MMP-2 protein expression is undetectable on day 1 postpartum in the rat. In the cow, there was no difference in MMP-2 mRNA or latent MMP-2 between early (day 4), mid (day 10), and late (day 16) cycle CLs (Zhang et al., 2005). Goldberg et al. (1996) found that MMP-2 protein does not change throughout the bovine estrous cycle. However, they found that proMMP-9 is highly expressed on day 4 of the cycle, but decreases on days 8, 10, 12, 14 and 16. In sheep CL, MMP-2 mRNA and activity was highest on day 10 of the estrous cycle, whereas MMP-9 mRNA was undetectable (Ricke et al., 2002).

Xinlei et al. (2006) assessed TIMP-1, -2 mRNA using *in situ* hybridization in the rhesus monkey. The TIMP-1 mRNA expression decreased from day 5 to 15 while TIMP-
2 mRNA expression increased during the same time period. Similar results were found by Inderdeo et al. (1996) and Young et al. (2002). However, Smith et al. (1995) demonstrated that TIMP-2 mRNA concentrations decrease from early luteal phase (days 3-7) to late luteal phase (day 16) in corpora lutea of sheep. Expression of TIMP-1 and TIMP-2 mRNA during early, mid and late pseudopregnancy were determined in the rat (Nothnick et al., 1995). While TIMP-2 remained unchanged, TIMP-1 expression decreased to almost 0% from early to mid stage pseudopregnancy. In the late stage of pseudopregnancy (which is associated with CL regression), it increased to about 50% of the expression seen in the early stage. In contrast, gene expression of TIMP-1 and -2 in pig CL is high in the early luteal phase (days 6-8) and increases by the late luteal phase (days 13-15; Pitzel et al., 2000). In the cow, TIMP-1 mRNA and protein decreased from early (day 4) and mid (days 10-12) cycle CL to late (day 16) cycle CLs (Zhang et al., 2003). In contrast, TIMP-2 mRNA and protein increased from early cycle CLs to mid and late cycle CLs. Kliem et al. (2007) showed that TIMP-1 mRNA from bovine follicles increased in CL during the luteal phase (days 0-12).

Multiple factors such as PGF$_{2\alpha}$, endothelin-1 (He et al., 2007), nitric oxide (Nee et al., 2008), and cytokines (Nee et al., 2007) are known to regulate MMPs and TIMPs. Since the focus of the present study is to determine the effects of physiological pulses of PGF$_{2\alpha}$ on MMPs and TIMPs, this aspect of PGF$_{2\alpha}$ action will be reviewed. Kliem et al. (2007) reported that MMP-2 mRNA expression in the cow was up-regulated 24 hours after a luteolytic dose (500 ug i.m.) of PGF$_{2\alpha}$ and continued to increase through 64 hours. On the other hand, MMP-9 mRNA expression increased 12-64 hours after PGF$_{2\alpha}$ treatment. Liu et al. (1999) profiled MMP-2 mRNA expression during the formation and
regression of the CL in the pseudopregnant rat. They found that MMP-2 was expressed in the CL on day 1 of pseudopregnancy and drastically decreased on day 7. A single 5 ug s.c. injection of cloprostenol, a stable PGF$_{2\alpha}$ analogue, was given on day 8 to initiate regression of the CL and the low MMP-2 expression found in day 7 CL was maintained in day 10, 13, 16, and 19 CLs. However, Endo et al. (1993) concluded that both the active and latent forms of MMP-2 increased during structural luteolysis in the rat. Similarly, in rats injected with GnRH, which is known to induce luteolysis, MMP-2 expression increases during structural regression of the CL (Goto et al., 1999). In sheep, MMP-2 mRNA does not change in response to PGF$_{2\alpha}$ treatment (15 mg i.m.), whereas MMP-9 mRNA was undetectable (Ricke et al., 2002).

In the cow, TIMP-1 and -2 mRNA expression decreased 12 hours and 12, 48, and 64 hours, respectively, after PGF$_{2\alpha}$ treatment (500 ug i.m.; Kliem et al., 2007). Similar results were observed by McIntush and Smith (1997) and Ricke et al. (2002) in sheep. In contrast, Juengel et al. (1994) reported luteal TIMP-1 and -2 mRNA expression in the cow CL increased eight hours after PGF$_{2\alpha}$ treatment. It then increased 4 and 6 hours after treatment when compared to controls. Although TIMP-2 mRNA was detected, it did not change. Similarly, in the rat, TIMP-1 mRNA is present in the forming and regressing CL, but not the functional CL, after a single 5ug (s.c.) injection of cloprostenol on day 8 of pseudopregnancy (Liu et al., 1999).

The single systemic infusions of PGF$_{2\alpha}$ administered to animals in some of the aforementioned studies are given in relatively high concentrations. However, natural luteolysis is driven by multiple pulses of PGF$_{2\alpha}$ that are relatively lower in concentration. Thus, we wanted to determine whether the expression of MMPs and TIMPs in response
to a single luteolytic dose of PGF$_{2\alpha}$ were different from the response to physiological infusions of PGF$_{2\alpha}$. Therefore, Towle et al. (2002) and Ricketts (UNH Master's Thesis) used a model in which sheep received one or two infusions of PGF$_{2\alpha}$ respectively, that mimic physiological conditions during the onset of luteolysis. Tissue inhibitor of metalloproteinase-1 protein levels decreased by 55% one and eight hours following a single one-hr infusion of PGF$_{2\alpha}$ (~0.22 ug/kg/min) when compared to controls. Tissue inhibitor of metalloproteinase-2 decreased 1, 8, and 24 hours by 80%, 80%, and 70%, respectively, after the infusion when compared to controls. Meanwhile, active MMP-2 increased by 165% at the 8 hour time point while active MMP-9 remained unchanged. Similarly, TIMP-1 protein decreased by 60% one hour following two one-hour infusions of PGF$_{2\alpha}$ (~.22 ug/kg/min) and increased to 120% 24 hours after the infusion. In addition, TIMP-2 decreased by 80% one hour after the infusions and remained low through 24 hours. Active MMP-2 increased to 120% one hour following the second infusion while latent MMP-2 and active MMP-9 remained unchanged throughout 24 hours after the second infusion.

It is clear that MMPs and TIMPs are involved in the structural regression of the CL in several species. However, luteolysis also involves the functional demise of the CL, which encompasses a decline in P$_4$ production. Progesterone production is dependent, in part, on the steroidogenic acute regulatory (StAR) protein. Therefore, the ensuing discussion is a review of StAR and P$_4$ in the CL.
General Characteristics of the Steroidogenic Acute Regulatory (StAR) Protein:

The steroidogenic acute regulatory (StAR) protein is an approximately 30 kDa phosphoprotein (259-287 amino acids) associated with mitochondria (Clark et al., 1994). It is processed from two precursor proteins (32 kDa and 37 kDa) and is a key component in steroid biosynthesis (Krueger and Orme-Johnson, 1983; Pon et al., 1986; Pon and Orme-Johnson 1988; Stocco and Kilgore, 1988; Epstein and Orme-Johnson, 1991; Stocco and Sodeman, 1991). In 1994, it was sequenced and cloned in MA-10 mouse Leydig tumor cells by Clark et al. (1994). The human StAR gene (855 nucleotides) resides on chromosome 13 and is predominantly found as a 1.6 kb transcript, but has also been described as a 4.4 kb and 7.5 kb transcript (Sugawara et al., 1995). The cDNA used to probe for StAR mRNA in this experiment was about 1.6 kb which explains why the 1.6 kb transcript was most abundant. The 4.4 kb and 7.5 kb transcripts most likely represent full length transcripts of StAR. The StAR gene was later cloned from rat (Sandhoff et al., 1998), cow (Hartung et al., 1995), pig (Pilon et al., 1997), mare (Kerban et al., 1999), and hamster (Fleury et al., 1996). Interestingly, the StAR and TIMP-1 genes share a homology sequence of 124 bases in a noncoding region, suggesting that these two proteins may be regulated similarly (Hartung et al., 1995). Naturally occurring mutations of the StAR gene in patients with congenital lipoid adrenal hyperplasia result in severely reduced adrenal and gonadal steroid synthesis (Lin et al., 1995). Once translated, the mRNA yields a 37 kDa protein that is phosphorylated at residues 194 and 195 in mouse and human, respectively; an important characteristic for the function of StAR (Arkane et al., 1997). The StAR protein is selectively expressed in all steroidogenic tissues, such as adrenal fasciculata, corpus luteum, gonads, adrenal glomerulosa cells, and the placenta of
cows, pigs, and rodents (Strauss et al., 1996). It is also expressed in the brain, nerves, and kidney (Carroll et al., 1986; Clark et al., 1995).

**StAR Protein Structure and Function:**

Once the 37 kDa form of StAR protein is synthesized and phosphorylated, it transports cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, the rate-limiting step in steroid biosynthesis. During this event, the 37 kDa form of StAR is cleaved to yield the 32 kDa and ultimately the 30 kDa form and becomes inactive while cholesterol is converted into pregnenolone by the side chain cleavage enzyme (P450scc; Farkash et al., 1986; Clark et al., 1994; Pescador 1996; Paz et al., 2002). Understanding how StAR functions has come from studying its protein structure. The N-terminus of the 37 kDa form contains a mitochondrial targeting sequence, which directs it to the mitochondria (Figure 10). Amino acids 63-193 serve as a protease resistant region (Song et al., 2001). The C-terminus contains a START (StAR-related lipid transfer) domain consisting of 210 amino acids and appears to be the key functional part of this protein because steroidogenesis is inhibited in cells in which the C-terminal region is truncated by 28 amino acids (Arkane et al., 1996; Wang et al., 1998). The START domain has also been found in several other proteins involved in steroidogenesis such as one found in the lymph node called metastatic lymph node 64 (MLN64; Watari et al., 1997; Kallen et al., 1998; Potter and Aravind, 1999; Soccio and Breslow, 2003). The function of MLN64 is to transport cholesterol out of vesicles formed by endocytosis of LDL (Strauss et al., 2003). The START domain of MLN 64 shares 35% homology with the one in StAR and allows both proteins to bind cholesterol in a 1:1 ratio (Tsujishita and
The StAR protein is typically found in gonads while MLN 64 is found in the brain and placenta; however, the START domain of both proteins contains a hydrophobic channel in which one molecule of cholesterol binds. Furthermore, mutant forms of MLN64 which contain only the START domain binds cholesterol and increases steroidogenesis by P450scc (Watari et al., 1997; Tsujishita and Hurley, 2000).

**Signal Transduction Pathways of StAR:**

Steroidogenesis is regulated primarily by cyclic adenosine monophosphate-dependent (cAMP-dependent), and to a lesser degree, by cAMP-independent pathways (Stocco et al., 2005). In the cAMP-dependent pathway, LH or human chorionic gonadotropin (hCG) binds to a 7-transmembrane, G protein coupled receptor of a steroidogenic cell and activates adenylate cyclase, the enzyme that converts adenosine triphosphate (ATP) to cAMP (Oon and Johnson, 2000). Cyclic AMP then phosphorylates protein kinase A (PKA), which subsequently initiates transcription and translation of StAR. In addition, PKA phosphorylates StAR protein that is already translated (Stocco and Kilgore, 1988; Clark et al., 1994; Arkane et al., 1997). The increased cAMP that
results from activation of this pathway also induces arachidonic acid (AA) release which, in turn, increases intracellular Ca$^{2+}$ (Alila et al., 1990; Jamaluddin et al., 1992; Kumar et al., 1994). Inhibition of AA synthesis reduces P$_4$ production, StAR promoter activity, StAR mRNA, and StAR protein (Wang et al., 2002). These results were reversed when AA was added to cells. Although cAMP induces release of AA, addition of AA metabolites to MA-10 cells with low concentrations of cAMP increased StAR expression and steroidogenesis (Wang et al., 2003). Furthermore, steroidogenic cells incubated in Ca$^{2+}$ free medium and treated with maximal stimulating concentrations of LH/hCG resulted in a 50% decrease in steroid production and decreased StAR expression (Sullivan and Cooke, 1986; Ramnath et al., 1997; Manna et al., 1986). Therefore, it seems that these pathways regulate steroidogenesis in different ways. Sullivan and Cooke (1986) suggest that sub-maximal concentrations of steroid hormones are regulated primarily by the Ca$^{2+}$ messenger system while maximal testosterone concentrations are regulated by both the Ca$^{2+}$ messenger system and the cAMP-dependent pathway.

It is important to note that the cAMP-mediated pathway alone does not yield maximum steroid production; rather it is the co-regulation of cAMP-dependent and cAMP-independent pathways (Wang X et al., 2002). The cAMP-independent pathways have been studied to a lesser degree. Manna et al. (2006) showed that insulin-like growth factor (IGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor $\alpha$ (TGF$\alpha$), interleukin-1 (IL-1), and colony-stimulating factor-1 (CSF-1) increase StAR mRNA expression and P$_4$ secretion without stimulating cAMP. Transforming growth factor $\alpha$ increases StAR mRNA and P$_4$ by binding to the epidermal growth factor receptor (EGFR), a single transmembrane receptor. Therefore, it was
hypothesized that TGFα increases steroidogenesis through the mitogen-activated protein kinase (MAPK) pathway. This sequence of events includes TGFα binding to EGFR causing homodimerization of the receptors and autophosphorylation of their tyrosine tails. As a result, growth factor receptor-bound protein 2 (Grb2) and Son of Sevenless (SOS) form a complex and dock on the phosphorylated tyrosine tail. The SOS protein becomes activated and removes guanosine diphosphate (GDP) from Ras so that it becomes activated by binding GTP. Activated Ras phosphorylates the protein kinase RAF, which, in turn, phosphorylates MAPK/ERK kinase (MEK). The MEK protein activates mitogen-activated protein kinase (MAPK), inducing transcription of StAR. However, inhibition of this pathway resulted in different effects on steroidogenesis; stimulation (Seger et al., 2001; Tajima et al., 2003), inhibition (Gyles et al., 2001; Manna et al., 2002; Martinelli et al., 2004), or no effect (Tai et al., 2001; Seto-Young et al., 2003; Tajima et al., 2005).

While the stimulation of steroidogenesis is driven primarily by LH and hCG through the cAMP-dependent pathway, inhibition of steroidogenesis is driven by the protein kinase C (PKC) pathway (Conley and Ford, 1989; Wiltbank et al., 1989; McGuire et al., 1994). Ovine luteal cells deficient in PKC do not respond to PGF2α with a decrease in P₄ (Wiltbank et al., 1990). Therefore, it appears that PGF2α exerts its anti-steroidogenic effect through this pathway. In order to elicit a response, PGF2α binds to its receptor (FPr), a 368 amino acid 7-transmembrane receptor (Fitz et al., 1982; Kyte and Doolittle, 1982; Gadsby et al., 1990; Graves et al., 1995). The FPr is present on large, but not small luteal cells (Fitz et al., 1982; Balapure et al., 1989; Coleman et al., 1994; Juengel et al., 1996). This binding leads to activation of the G-coupled protein which, in
turn, activates phospholipase C (PLC). Phospholipase C is an enzyme that cleaves phosphotidylinositol 4,5-biphosphate (PIP2), which is found in the cell membrane, into two products: diacylglycerol (DAG), which remains in the cell membrane, and inositol triphosphate (IP3), which acts on the endoplasmic reticulum causing the release of Ca\(^{2+}\) into the cytoplasm. Diacylglycerol and Ca\(^{2+}\) activate PKC which then inhibits steroidogenesis possibly by decreasing P450scc and 3β-HSD mRNA expression (Cooke and Robaire, 1988; Hawkins et al., 1993) or by decreasing cholesterol transport into the mitochondria (Wiltbank et al., 1993). Interestingly, PKC increases intracellular Ca\(^{2+}\) and the transcription and translation of StAR, similar to the response of the cAMP/PKA pathway; however, the protein produced in response to PKC is not phosphorylated (Jo et al., 2005).

**StAR Gene Regulation:**

The StAR gene is transcriptionally regulated by several factors. Steroidogenic factor-1 (SF-1) is a member of the steroid hormone receptor family of nuclear transcription factors and is essential for protein-DNA interactions and maximal transcription of the StAR gene (Wooton-Kee and Clark, 2000). It binds to five sites in the mouse promoter and six in humans (Caron et al., 1997; Sugawara et al., 1997; Sandhoff et al., 1998). Another group of proteins that helps regulate StAR expression is the CCAAT/enhancer binding proteins (C/EBP), a family of leucine zipper transcription factors (Johnson and Williams, 1994). Two specific members include C/EBPα and C/EBPβ, which are phosphoproteins targeted by PKA (Roelser et al., 1998). The StAR promoter contains two binding sites for C/EBPs. It was found that C/EBPβ binds to at
least one of these sites and induces transcription of the StAR gene. These functional
binding sites are also essential for transcription of the StAR gene by SF-1, suggesting that
SF-1 and C/EBPβ form a complex on the promoter (Stocco DM et al., 2001). A third
factor involved with the regulation of StAR is SP-1, a member of the zinc finger family
of transcription factors (Berg, 1992). This factor is involved in up-regulating several
P450scC genes as well as StAR (Stocco et al., 2001). It interacts with the estrogen
receptor (Sun et al., 1998) and C/EBPβ (Lee et al., 1997) to stimulate the expression of
StAR. Another important activator of the StAR gene is thought to be GATA-4, which is
present in porcine corpora lutea and is expressed constitutively in granulosa cells (Gillio-
Menia et al., 2003). Silverman et al. (1999) located the binding sequences of GATA-4
and C/EBPβ on the StAR promoter. Mutagenesis of both sites resulted in a lack of
promotor activation. Since GATA-4 is constitutively expressed while C/EBPβ expression
increases in response to LH and FSH, it is likely that GATA-4 plays a more permissive
role in StAR regulation while C/EBPβ plays more of a central role (Sugawara et al.,
1997). The steroid regulatory element binding protein (SREBP) was also shown to
increase transcription of the StAR gene; however, no binding sites on the StAR promoter
for this protein have been found (Christenson et al., 1998). Similarly, cAMP response
element binding protein (CREB) increases steroidogenesis, StAR promoter activity, and
StAR mRNA expression, but no consensus cAMP response element has been found on
the StAR promoter, suggesting that CREB helps regulate StAR expression possibly
through a non-consensus sequence or by indirectly binding to the promoter (Stocco et al.,
2001). Unlike the stimulators, the inhibitors of the StAR promoter are less studied. One
of them is DAX-1, a protein that is part of the nuclear hormone receptor family.
Overexpression of DAX-1 in mouse adrenal tumor cells blocks steroid biosynthesis by inhibiting P450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD; Zazopoulos et al., 1997; Lalli et al., 1998). It has been shown that DAX-1 interacts with SF-1 to impair transcription (Ito et al., 1997). In addition, DAX-1 binds directly to the StAR promoter, blocking expression of the gene (Manna et al., 2008).

**Possible Mechanisms of Cholesterol Transport by StAR:**

The mechanism of how StAR transports cholesterol into the mitochondria is not fully elucidated, but several hypotheses have been suggested. Kallen et al. (1998a) proposed that the StAR C-terminus, which contains the START domain, induces changes in the outer mitochondrial membrane resulting in the transport of cholesterol to the inner mitochondrial membrane. Furthermore, Kallen et al. (1998b) suggest that StAR interacts directly with the outer mitochondrial membrane and not through an intermediate protein. Similarly, Stocco and colleagues suggest that once StAR anchors itself to the mitochondrial membrane via the targeting sequence, a lipid bridge forms between the inner and outer mitochondrial membranes (Clark et al., 1994; Stocco and Clark, 1996). Subsequently, the 37 kDa form of StAR transports cholesterol into the mitochondria and is cleaved by matrix processing proteases in the mitochondria resulting in the 32 kDa intermediate form. The inner and outer mitochondrial membranes separate and the 32 kDa form of StAR is cleaved to yield the 30 kDa form, which is no longer able to aid in cholesterol transport. However, Arkane et al. (1996) showed that steroidogenic capacity was not affected in cells containing StAR proteins with deletion of 62 residues on the N-
terminus (the mitochondrial targeting sequence), which suggests that steroid synthesis is possible when StAR remains on the outside of the mitochondria.

An alternative hypothesis, called the molten globule hypothesis, has been proposed by Bose et al. (1999). A protein that is in the molten globule state is slightly denatured to yield a different conformation than its native state. They suggest that StAR undergoes a conformational change near the mitochondria because of the acidic microenvironment produced by the proton pump. As a result, the positively charged C-terminal domain is exposed and may interact with the negatively charged mitochondrial membrane or a receptor. This suggestion was further strengthened when Papadopoulos (1993) proposed that a peripheral benzodiazepine receptor (PBR), which is found in high abundance on the outer mitochondrial membranes of steroidogenic cells, is involved with cholesterol transport into mitochondria. Disruption of PBR in rat tumor Leydig cells resulted in a 90% decrease in steroid secretion, while reintroduction of this receptor resulted in normal steroid secretion (Papadopoulos et al., 1997). To show that PBR is linked to cholesterol uptake by mitochondria and not linked to the secretion of steroids by cells, Li and Papadopoulos (1998) transfected *E. coli* DE3 cells with PBR to induce mitochondrial uptake of cholesterol. Since StAR and PBR increase steroidogenesis, West et al. (2001) inquired if these two proteins interact to transport cholesterol into mitochondria. Their results led them to hypothesize that cholesterol binds to StAR either in the cytoplasm or to StAR already imbedded in the mitochondrial membrane, which then presents cholesterol to PBR so that it transports cholesterol into the inner mitochondrial membrane. However, Kallen et al. (1998a) found that StAR transported cholesterol into the mitochondria whose outer membrane proteins were removed or
partially lysed with trypsin. Subsequently, Christensen et al. (2001) supported this work when they found that recombinant StAR bound to mitochondrial membranes without any associated proteins.

Although there are many hypotheses of how cholesterol is transported into mitochondria, the one proposed by Stocco’s group is the most plausible. Their three pieces of evidence are the most compelling: (1) The START domain of StAR binds cholesterol in a 1:1 ratio. (2) Mitochondria lacking membrane associated proteins, such as PBR, still exhibit transport of cholesterol by StAR. (3) The active form (37 kDa) of StAR is found in the cytoplasm while the intermediate (32 kDa) and inactive (30 kDa) forms are found within the mitochondria. Not only is StAR primarily responsible for cholesterol transport into the mitochondria, but it is also the acute regulatory protein involved in steroidogenesis and it is important to understand why. The following criteria define an acute regulatory protein: 1) a protein newly synthesized within minutes to hours (de novo synthesis) in response to a hormonal stimulus, 2) the protein’s synthesis has to be sensitive to cycloheximide (an inhibitor of de novo protein synthesis), and 3) the protein is localized in the mitochondria.

**Other Cholesterol Carrying Proteins involved in Steroidogenesis:**

Although StAR is known as the acute regulator in steroid biosynthesis, it is important to keep in mind that there are other cholesterol transporting proteins that affect steroidogenesis. One of these proteins is sterol carrier protein 2 (SCP2), which is a 13 kDa protein found in the liver, adrenal, and corpus luteum (Chanderbhan et al., 1982; Vahouny et al., 1987). The role of this cycloheximide resistant protein is to transport
lipids, one of which is cholesterol. It binds cholesterol in a 1:1 molar ratio within the cell; it is concentrated in peroxisomes, and it is minimally detected on mitochondria (Mendis-Handagama et al., 1995). However, when treated with LH, SCP2 is concentrated in peroxisomes, mitochondria, and lipid droplets in luteal cells suggesting that SCP2 transports cholesterol from lipid droplets to mitochondria for steroidogenesis. Another protein is the steroidogenesis activator protein (SAP), a 3.2 kDa protein found only in steroidogenic cells (Pedersen and Brownie, 1987; Mertz and Pederson, 1989). It is a cAMP-dependent protein. Pedersen (1987) found that SAP and P450scc activity is upregulated by hCG treatment suggesting that SAP is a key regulator of P450 activity.

**Progesterone Biosynthesis:**

Progesterone (P₄) is a 21 carbon steroid hormone discovered by Corner and Allen (1929) and subsequently isolated from rabbit ovary (Allen, 1935). It maintains pregnancy by inhibiting uterine contractions (Allen, 1935), thickening cervical mucus, and building the endometrium for implantation of the fetus (Zarrow, 1965). Steroid biosynthesis encompasses a cascade of enzymatic reactions which begins with cholesterol, the precursor for all steroid hormones (Christenson and Devoto, 2003). A steroidogenic cell has three sources of cholesterol; 1) LDL/HDL from the bloodstream, 2) lipid droplets within the cell, and 3) intracellular synthesis of cholesterol from acetate (Grummer and Carroll, 1988). The steroidogenic cells of most species utilize exogenous sources of cholesterol found in LDL and HDL rather than synthesizing cholesterol from acetate. Mice, rats, and ruminants primarily utilize HDL as a source of cholesterol while humans,
When a steroidogenic cell engulfs LDL or HDL by receptor-mediated endocytosis, cholesterol is cleaved by acid lipases and is transported into the mitochondria by StAR. Luteinizing hormone drives cleavage of cholesterol from lipid droplets in steroidogenic cells by binding to its 7-transmembrane receptors on small and large luteal cells. As a result, the G-protein activates adenylate cyclase and produces cAMP. This activates PKA which activates cholesterol ester hydrolase, also known as hormone sensitive lipase. This enzyme cleaves cholesterol from cholesterol esters that are stored in lipid droplets within the cell allowing free cholesterol to be transported into the mitochondria by StAR. Although both small and large luteal cells produce P₄ in response to LH, it is important to keep in mind that P₄ production is increased 5-15 fold in response to elevated concentrations of LH by small, but not large luteal cells (Niswender, 2002). During the luteal phase of the estrous cycle, high concentrations of circulating P₄ result in low frequency secretion of LH (Lucy et al., 1992). If there is not enough cholesterol available either in the bloodstream or in lipid droplets, the steroidogenic cell synthesizes cholesterol from acetate. Obtaining cholesterol by this method requires a lot of energy so the cells use it as a last resort for steroid production.

Once a steroidogenic cell obtains cholesterol from one of these three sources, StAR transports it into the mitochondria where 20,22 lyase (P450scc) converts it to pregnenolone (Figure 11; Geslin and Auperin, 2004). Pregnenolone is then converted to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) in the smooth endoplasmic reticulum (Christenson and Devoto, 2003).
Figure 11.

**Progesterone Biosynthesis**

Cholesterol

\[ \text{Cholesterol} \rightarrow \text{P450scc} \rightarrow \text{Pregnenolone} \rightarrow \text{3β HSD} \rightarrow \text{Progesterone} \]

**StAR and Progesterone Expression during the Estrous Cycle and Luteolysis:**

During functional luteolysis in a variety of species, PGF\(_{2\alpha}\) initiates a cascade of events that results in decreases in StAR expression and P\(_4\) concentrations. Edqvist et al.
(1975) injected a single 25 mg dose of PGF$_{2\alpha}$ into cows on either day 8 or 14 of the estrous cycle and found that plasma P$_4$ concentrations decreased in all cows 24 hours after treatment. Pescador et al. (1996) measured StAR protein levels in bovine corpora lutea and found that they were low during CL development, high during the midluteal phase, and disappeared 24 hours after a single 500 ug injection of a PGF$_{2\alpha}$ analogue.

Similarly, Fielder et al. (1999) found that StAR protein and P$_4$ concentrations are lower in day 7 post ovulatory rat corpora lutea after a single 250 ug (i.p.) injection of PGF$_{2\alpha}$ when compared to controls. However, the level of StAR mRNA in PGF$_{2\alpha}$-treated rats was not different from controls suggesting that the decrease in StAR protein is not due to a decrease in StAR gene expression, but rather post transcriptional inhibition. In addition, rat luteal cells treated with increasing amounts of PGF$_{2\alpha}$ resulted in a dose-dependent decrease of StAR protein (Liu et al., 2007) In the mare, plasma P$_4$ decreases 24 hours after a single (i.m.) injection of either 0.8 mg/100 kg, 0.4 mg/100 kg, or 0.2 mg/100 kg of PGF$_{2\alpha}$ on day 7 of the estrous cycle when compared to controls (Handler et al., 2004). A similar experiment involving mares treated with 10 mg of PGF$_{2\alpha}$ on day 9 after ovulation showed a decrease in progesterone (<1 ng/ml) 24 hours after treatment when compared to controls (Vanderwall et al., 2000). Liu et al. (2003) found that the expression of StAR in the rhesus monkey corresponded to the decline in P$_4$ in the late luteal stages. Plasma P$_4$ and total protein decrease during natural luteolysis in women when compared to early and mid luteal phases (Del Canto et al., 2007). Similarly, StAR mRNA and P$_4$ decreased in human CLs treated with PGF$_{2\alpha}$ (Chung et al., 1998). Juengel et al. (1995) treated sheep with two (i.m.) injections (10 mg or 25 mg) of PGF$_{2\alpha}$ on days 11-12 of the estrous cycle. Blood samples and CLs were taken 4, 12, or 24 hrs after treatment and from control
animals. Serum P₄ concentrations and StAR mRNA were decreased at all time points in treated animals.

Because of the pulsatile release of PGF₂α in ruminants, would the temporal pattern of StAR expression and progesterone concentration differ in animals receiving a bolus dose? To answer this question, Allen (UNH Master's thesis), Towle et al. (2002), and Ricketts (UNH Master's Thesis) used a model in which sheep receive one or two infusions of PGF₂α to mimic physiological conditions during the onset of luteolysis. Progesterone transiently decreased by 40% (p<0.05) 8 hours after a single one hour systemic infusion of PGF₂α (0.22 ug/kg/min), while StAR expression remained unchanged. Eight hours following a second one hour systemic infusion of PGF₂α (0.22 ug/kg/min), P₄ concentrations decreased by 40% (p<0.05). It then rose above pre-infusion levels 24 hours after PGF₂α treatment (p<0.05) while StAR expression remained unchanged throughout the experiment.

**Other Factors Contributing to Luteolysis:**

While the present study focused on the effects of PGF₂α on MMPs, TIMPs, StAR, P₄ and COX expression, it is important to note that other factors are also involved in luteolysis. They are briefly reviewed below.

Structural regression of the CL is accompanied by DNA fragmentation and apoptosis. Juengel et al. (1993), McGuire et al. (1994), Rueda et al. (1995), and Juengel et al. (2000) found that increased intracellular Ca²⁺ causes DNA fragmentation in the cow and ewe during luteal regression. Niswender et al. (2007) proposed that PGF₂α binds to
large luteal cells to increase PKC and oxytocin in large luteal cells (Flint and Sheldrick, 1982). As a result, oxytocin binds to small luteal cells to increase PKC which decreases P₄ production. Along with the increase in PKC in both cell types, there is an increase in intracellular Ca²⁺ which causes DNA fragmentation and apoptosis, marking structural luteolysis. Although oxytocin is secreted by large luteal cells (Cosola-Smith et al., 1990; Wathes and Denning-Kendall, 1992), Hoyer (1998) found that oxytocin causes DNA fragmentation in large, but not small ovine luteal cells suggesting that large luteal cells use an autocrine signal for apoptosis. Okuda et al. (1992) found that bovine luteal cells contain oxytocin receptors, but were unable to distinguish small from large luteal cells. However, Niswender at al. (2007) reported that small luteal cells isolated from ewes showed a dose-dependent response to oxytocin suggesting that they have oxytocin receptors. Another effect of oxytocin is the stimulation of uterine PGF₂α (Sharma and Fitzpatrick, 1974; Roberts and McCracken, 1976). As a result, uterine and luteal PGF₂α are produced to enhance regression of the CL.

Nitric oxide (NO) has been implicated in CL function. Keator et al. (2008) have shown that NO has a luteolytic effect in sheep. In their study, ovaries were transplanted to the neck so uterine PGF₂α could not induce luteolysis. Sheep with a day 11 CL received a single 1, 10, 100, or 1000 µg infusion of dipropylentriamine NONOate (DPTA), a fast-acting nitric oxide donor. Progesterone concentrations decreased 24 hours after infusion of 1000 µg/min of DPTA. Microdialysis was used to perform an in vivo perfusion nitric oxide donor spermine NONOate (100 mg/4 hrs) into the CL of cows on day 12 of the cycle resulted in no change in P₄ concentration (Jaroszewski et al., 2003). However, Jaroszewski and Hansel (2000) infused cows with the nitric oxide synthase
(NOS) inhibitor L-NAME on days 11 or 12 and days 17 and 18, and found that P₄ concentrations remained increased in all three groups through day 25 of the cycle, suggesting that NO inhibits P₄ secretion by the CL. When a mixture of small and large bovine luteal cells in culture were treated with 100 μg/4 hrs spermine NONOate, P₄ concentration decreased. Similar results were also reported by Skarztnski and Okuda (2000) and Korzekwa et al. (2004).

Reactive oxygen species (ROS), which are free radicals, have also been suggested to play a role in regression of the CL. Xanthine oxidase, an enzyme that generates the ROS superoxide, inhibits P₄ production by rat luteal cells (Gatzuli et al., 1991). Furthermore, hydrogen peroxide, another ROS, blocks hormone-sensitive cholesterol transport into the mitochondria of rat luteal cells (Behrman and Aten, 1991). In addition to being involved in functional regression, ROS may contribute to structural regression. Tilly and Tilly (1995) showed that ROS are associated with apoptosis of granulosa cells undergoing atresia. Sawada and Carlson (1989) showed that ROS increase in response to PGF₂α in the rat. Furthermore, superoxide dismutase, an enzyme that protects against ROS, is reduced during luteolysis in cows and sheep (Rueda et al., 1995b; Hoyer, 1998).

Immune cell products such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and IL-2 may also contribute to CL regression. Interleukin-1β-converting enzyme mRNA and TNFα are upregulated during luteolysis in cows (Shaw and Britt, 1995; Rueda et al., 1997). These cytokines, especially TNFα, inhibit basal and hCG-induced P₄ production in pigs (Pitzel et al., 1993). In addition, TNFα, IL-1β, and interferon γ (IFγ) inhibit P₄ production in cultured bovine luteal cells (Townson and Pate, 1994; Pate, 1995). Immune cells also contribute to the structural demise of the CL. Hehnke et al.
(1994) found that macrophages increased 24 hours after PGF$_{2\alpha}$-induced luteolysis in the pig. Paavola (1979) reported that macrophages ingest luteal cells by phagocytosis in the guinea pig CL. Similarly, macrophages, leukocytes, and T-lymphocytes increase during regression of the CL in the cow (Penny et al., 1999). The products of these immune cells include TNF$\alpha$, IL-1 and IFN$\gamma$, which increase PGF$_{2\alpha}$ production (Townson and Pate, 1994; Pate, 1994) and promote DNA fragmentation and apoptosis of luteal cells.

The 21 amino acid protein endothelin-1 (ET-1) has also been associated with the functional demise of the CL (Hinckley and Milvae, 2001). It is part of a family of proteins that includes ET-2, ET-3, and sarafotoxin (Inoue et al., 1989). Endothelin-1 is a potent vasoconstrictor; it is present in a variety of tissues and is produced by endothelial cells (Yanagisawa et al., 1988). Endothelin-1 also stimulates phospholipase C, phospholipase A$_2$, phospholipase D, cAMP accumulation, arachidonic acid release, and prostanoid production (Muldoon et al., 1989; Resnick et al., 1989; VanRenterghem et al., 1989). The two receptors for endothelins have been identified as ET$_A$ and ET$_B$ (Maggi et al., 1989), with ET$_A$ having a higher affinity for ET-1 and -2 than it does for ET-3. All three endothelins have the same affinity for the ET$_B$ receptor (Naruse et al., 1994). Mamluk et al. (1999) showed that both small and large luteal cells contain mRNA for the ET$_A$ receptor, making ET-1 a likely mediator of luteolysis. To investigate the function of ET-1 in the corpus luteum, Girsch et al. (1996a) conducted an in vitro study involving ovine and bovine luteal cells treated with ET-1. The result was a decrease in basal and LH-stimulated P$_4$ production. Hinckley et al. (1996) showed that in vitro treatment of bovine luteal cells with the ET$_A$ antagonist BQ123 before addition of ET-1 resulted in no change in basal or LH-stimulated P$_4$ production. Thus, the inhibitory action of ET-1 on P$_4$
production is mediated through the ET$_A$ receptor. Given that ET-1 decreases P$_4$
production, it is not surprising that ET-1 content is low in the young CL (days 5-6),
higher in the mature CL (days 9-16), and highest in the late CL (days 17-21) during the
bovine estrous cycle (Milvae, 2000). Since ET-1 production increases around the same
time as PGF$_{2\alpha}$ (late luteal phase), it is also not surprising that Miyamoto et al. (1997)
showed that intraluteal administration of PGF$_{2\alpha}$ to bovine luteal tissue in vitro resulted in
an increased output of ET-1 as well as a decrease in P$_4$ concentration. Further, in vivo
treatment with either 100 ug of ET-1 or a subluteolytic dose of PGF$_{2\alpha}$ resulted in
decreased P$_4$ production, but cycle length was not affected in midcycle sheep (Hinckley
et al., 1998). However, when sheep were treated with both ET-1 and a subluteolytic dose
of PGF$_{2\alpha}$, a greater decrease in P$_4$ was observed which was accompanied by a shortened
cycle length (10.3 days). Furthermore, pretreatment with BQ123 mitigated the luteolytic
effects of PGF$_{2\alpha}$ in cows (Girsh et al., 1996b). These results suggest that the effects
PGF$_{2\alpha}$ are, in part mediated through ET-1 binding to ET$_A$ receptors.
CHAPTER II

TEMPORAL EXPRESSION OF PROGESTERONE AND PROTEINS DURING PROSTAGLANDIN F$\textsubscript{2\alpha}$-INDUCED LUTEOLYSIS IN THE SHEEP

INTRODUCTION

The luteal phase of the sheep estrous cycle is 14 days long and is dominated by the corpus luteum (CL), a transient endocrine gland that secretes the steroid hormone progesterone (P$_4$; Casida and Warwick, 1945; Edgar and Ronaldson, 1958). Progesterone thickens cervical mucus, and builds the endometrium for implantation of the fetus (Zarrow, 1965). During pregnancy, the CL continues to produce P$_4$ to inhibit uterine contractions (Allen, 1935; Dutta and Sanyal, 1969; Morishita, 1986; Kostrzewska et al., 1993; Ruddock et al., 2008). However, if pregnancy does not occur, the uterus secretes the eicosanoid hormone prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) in a pulsatile manner to cause regression of the CL, a process known as luteolysis (McCracken et al., 1972; Zarco et al., 1988). On average, four to five sequential pulses of PGF$_{2\alpha}$ are required for complete luteal regression (Schramm et al., 1983; McCracken et al., 1999). Following PGF$_{2\alpha}$, the CL undergoes structural regression, which is characterized by degradation of the extracellular matrix (ECM) and apoptosis of luteal cells (Umo, 1975; Hoyer, 1998; Stocco et al., 2007), and functional demise, which is defined as a decrease in P$_4$ production.
The ECM is composed of many proteins, which serve as anchorage points for cells and aid in cell-to-cell interactions, cell migration, cell replication, and morphogenesis (Gumbiner, 1996; Yamada and Geiger, 1997; Geiger et al., 2001). The ECM of the CL is composed primarily of collagen types I and IV, fibronectin and laminin (Silvester and Luck, 1999; Irving-Rogers et al., 2006). The structural integrity of the ECM is regulated by the net balance between proteolytic enzymes called matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Matrix metalloproteinases are a diverse family of divalent cation-dependent enzymes that degrade all the components of the ECM (Roy, 2006) while TIMPs inhibit MMPs by forming reversible, non-covalent complexes with them in a 1:1 ratio. When the balance between MMPs and TIMPs tips in favor of net proteolysis, the integrity of the ECM is compromised, i.e. structural regression of the CL occurs (Zhao and Luck, 1995; Kliem et al., 2007). Among the members of these enzyme and inhibitor families, the gelatinases, MMP-2 and -9, and TIMP-1 and -2 are among the most well studied. Several studies in a number of animals have shown that MMP-2 and -9 expression increases during natural luteolysis (Pitzel et al., 2000; Xinlei et al., 2006; Kliem et al., 2007) and in TNFα treated corpora lutea (Zhang et al., 2005). The same has been done following prolactin-induced (Endo et al., 1993) and PGF2α-induced (Kliem et al., 2007) luteolysis, while TIMP-1 (Zhang et al., 2003) and TIMP-2 decrease (Smith et al., 1995; Inderdeo et al., 1996; McIntush and Smith, 1999; Kliem et al., 2007). Fewer studies have determined the protein expression of these endpoints during CL regression (Li et al., 2002; Young et al., 2002; Chen et al., 2006; Ribeiro et al., 2006). Collectively, the increase in enzymes along with the decrease in inhibitors results in net
proteolysis, which is an integral part of the complex processes that contribute to the structural demise of the CL.

Accompanying the structural regression of the CL is its functional decline, as marked by a decrease in P₄ production (Juengel et al., 1993; Tian et al., 1994; Juengel et al., 1995; Hoyer, 1998; Stocco et al., 2007). Essential for the synthesis of P₄ is the steroidogenic acute regulatory (StAR) protein, which is responsible for transporting cholesterol into mitochondria so that it can be converted into pregnenolone and subsequently into P₄ (Stocco and Sodeman, 1991). A single (Edqvist et al., 1975; Pescador et al., 1996; Vanderwall et al., 2000) or multiple (Juengel et al., 1995) supraphysiological doses of PGF₂α administered to various animals results in a decrease in serum P₄ concentrations and luteal StAR mRNA and protein expression. Indeed, these two endpoints are good indicators of the functional status of the CL.

While PGF₂α from the uterus initiates luteolysis in several species (McCracken et al., 1972; Nancarrow et al., 1973; Gleeson et al., 1974; Poyser, 1976), it is also produced by the CL of various animals (Guthrie et al., 1978; Rexroad and Guthrie, 1979; Milvae and Hansel, 1983; Johnson et al., 1988; Pate, 1988). The synthesis of prostaglandins requires the cyclooxygenase (COX) enzymes, which are responsible for converting arachidonic acid into prostaglandins (Samuelsson et al., 1978; Pagel et al., 1983; Smith, 1992; Levy et al., 2000). Because COX-1 is constitutively expressed in the CL (DeWitt et al., 1983; Funk et al., 1991; Kraemer et al., 1992; Arosh, 2004; Zerani et al., 2007), it is COX-2, the inducible isoform, which has received considerable attention regarding its role during luteolysis. Expression of luteal COX-2 mRNA and protein increases during natural luteolysis or in response to varying doses of PGF₂α (Tsai and Wiltbank, 1997;
Hayashi et al., 2003; Arosh et al., 2004). The increase in COX-2 during luteolysis therefore leads to an increase in PGF$_{2\alpha}$ secretion by the CL (Rexroad and Guthrie, 1979; Guthrie and Rexroad, 1980b). As a result, an autocrine pathway is established in which the result is amplification of luteal PGF$_{2\alpha}$. Thus, both uterine and luteal PGF$_{2\alpha}$ contribute to the eventual demise of the CL.

Uterine PGF$_{2\alpha}$ is released in a pulsatile fashion during natural luteolysis in ruminants. In order to profile the dynamic changes in progesterone and proteins associated with luteolysis following each pulse, we developed a model in sheep whereby timed infusions of PGF$_{2\alpha}$ (~0.22 ug/kg/min) are started on day 11 of the estrous cycle. Plasma P$_4$ decreased by 40% after one and two infusions of PGF$_{2\alpha}$ (Towle et al., 2002, Ricketts, UNH Master’s Thesis), which mimics the 40% decline in P$_4$ that occurs after the first pulse of PGF$_{2\alpha}$ during natural luteolysis in sheep (Custer et al., 1997). Interestingly, StAR expression transiently declined following the first PGF$_{2\alpha}$, but did not mirror the P$_4$ profile after the second infusion. On the other hand, the TIMPs fell significantly at the onset of luteolysis before recovering, while MMP-2 activity transiently increases. As expected, there were no changes in COX-1 expression, while COX-2 increased 16 hours following the first infusion of PGF$_{2\alpha}$ and remained increased after the second infusion (Allen, UNH Master’s Thesis; Ricketts, Master’s Thesis).

Thus far, it appears that the luteolytic machinery is set in motion following one or two infusions of PGF$_{2\alpha}$, but they are not adequate to induce luteolysis in sheep. However, following three sequential infusions of PGF$_{2\alpha}$ approximately 33% of treated sheep undergo luteal regression (Schramm et al., 1983; McCracken et al., 1999). Given this, how different are the pattern of P$_4$ concentration and expression of proteins associated
with luteolysis following three infusions of PGF$_{2\alpha}$ compared to one or two? To answer this question, the current study determined the expression of MMP-2 and -9, TIMP-1 and -2, COX-1 and -2, StAR, and P$_4$ in the sheep corpus luteum following three infusions of PGF$_{2\alpha}$. 
Materials and Methods

Animal model of PGF<sub>2α</sub>-induced luteolysis:

A single one hour systemic infusion of PGF<sub>2α</sub> (~0.22 ug/kg/min) can be administered at midcycle to mimic physiological conditions during the onset of luteolysis in sheep (Custer et al., 1995). This infusion rate causes a 40% decline in P<sub>4</sub> after one and two systemic infusions of PGF<sub>2α</sub> have passed through the lungs and reached the ovary (Towle et al., 2002; Ricketts, UNH Master’s Thesis). A similar decline in P<sub>4</sub> is seen under physiological conditions during the onset of luteolysis (Zarco et al. 1988). This established model was used in the present study to determine the in vivo changes in several molecular mediators of luteolysis following three systemic infusions of PGF<sub>2α</sub>, with the second and third infusions given 16 and 24 hours after the first pulse, respectively. Below is a depiction of the experimental model (Figure 12):

Figure 12:

Day 11 of Estrous Cycle

<table>
<thead>
<tr>
<th>Infusions of PGF&lt;sub&gt;2α&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
</tr>
<tr>
<td>2nd</td>
</tr>
<tr>
<td>3rd</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time of Luteectomy (n=4 for each time point)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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</table>

Figure 12: Brackets indicate 1 hour systemic infusions of PGF<sub>2α</sub> (~22 ug/kg/min) while triangles indicate time of luteectomy. Time 0 represents untreated animals and serves the control. The -1 hour time point corresponds to animals that received two infusions of PGF<sub>2α</sub> before CLs were removed one hour prior to giving the remaining animals a third
infusion of PGF$_{2\alpha}$. Luteectomies were performed 1, 8, 16, 24 hours following the third infusion. For each time point n = 4.

**Tissue Collection and Preparation:**

Luteal tissue was collected and prepared as previously described (Towle et al., 2002). Briefly, a group of mixed Dorset and Suffolk ewes (*Ovis aries*; ~90 kg) were housed at the University of Connecticut (Storrs, CT) and their estrous cycles synchronized with two intramuscular injections of Lutalyse (5mg; UpJohn Co.; Kalamazoo, MI) given at 4 hour intervals. Ewes were observed twice daily for estrus (Day 0) using a vasectomized ram. 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). Three one hour systemic infusions of PGF$_{2\alpha}$ (UpJohn Co.) were administered to the right jugular vein via a Harvard infusion pump (model no. 600-910/920; Harvard Apparatus Co.; Holliston, MA) at a rate of ~0.22 ug/kg/min (Custer et al., 1995). To mimic physiological pulses, the second and third infusions of PGF$_{2\alpha}$ were given 16 hours and 24 hours after the first, respectively. Control animals received saline. Corpora lutea were removed surgically via flank laparotomy under local anesthesia (2% [w/v] lidocaine) from untreated animals (designated 0 hr). Corpora lutea were also removed one hour before the third infusion of PGF$_{2\alpha}$ (designated -1 hr) and 1, 8, 16, 24 hrs after the third PGF$_{2\alpha}$ infusion (n = 4 sheep for each time point). After collection, luteal tissues were immediately placed on dry ice and stored at -80°C. For analysis, individual samples representing each of the six time points were analyzed in four sets of six.
To monitor peripheral plasma progesterone, jugular blood samples (5 ml) were collected hourly into heparinized tubes via the left jugular vein cannula from time zero until the time of individual luteectomy. Blood samples were taken 4, 8, 12, 16, and 20 hours after the first infusion of PGF2α and then every hour until luteectomy. All experimental procedures received prior approval by the University of Connecticut Animal Care and Use Committee.

Protein extraction:

Luteal tissue proteins (MMP-2 and -9, TIMP-1 and -2, StAR, and COX-1 and -2) were extracted with a buffer (50mM Tris HCl, 150 mM NaCl, 0.02% [w/v] sodium azide, 10 mM EDTA, 1% [v/v] Triton X-100, pH 7.4) containing protease inhibitors (1µg/ml AEBSF, 1µg/ml pepstatin A, 10 ug/ml aprotinin) in a ratio of 1 g tissue to 8 ml extraction buffer, as previously described (Zhang et al., 2002). Tissue was homogenized with a Kinematic Polytron, and the homogenate was then centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant between the pellet and fat layer was then removed and the centrifugation step was repeated until no pellet was observed. The supernatant was then stored at -80°C until used for analysis of MMP-2 and -9 by gelatin zymography and analysis of TIMP-1 and -2 and StAR by immunoblotting. For COX-1 and -2 analysis, a portion of the sample was removed after homogenization and sonicated twice for 5 seconds (Sonifer Cell Disruptor 350, Branson Sonic Power Co.) prior to centrifugation, as described above. The complete extraction protocols are found in Appendix A.
Protein Concentration Determination:

Protein concentrations were determined by the Bradford Method (Bradford, 1976) using Coomassie Blue (Bio-Rad Bradford Assay Reagent, Hercules, CA, USA). Bovine serum albumin (Sigma; St Louis, MO, USA) dissolved in CAB+ buffer (0.2M NaCl, 1mM CaCl₂, 50mM Tris, 0.02% NaN₃, pH 7.6) was the protein standard. Optical densities were read at 595 nm using a spectrophotometer (model DU640; Beckman Instruments; Fullerton, CA, USA). All luteal tissue samples were diluted 1:10 with CAB+ buffer and 2.5 µl and 5 µl aliquots in duplicate were assayed. Details are in Appendix B.

Immunoblot Analysis:

Equal volumes of luteal tissue extract were loaded on SDS-PAGE gels (12.5% [w/v] for TIMP-1, TIMP-2, StAR, COX-1, and COX-2. Except for TIMP-1, all were run under reducing conditions (5% [v/v] 2-mercaptoethanol, 100°C for 5 minutes), at 200 volts for 45-60 minutes, along with the dual color, pre-stained 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 hours at 200mA on ice. Non-specific binding was blocked with 5% [w/v] non-fat dry milk in TBST buffer (0.01M Tris-HCl, 0.15M NaCl, 0.05% [v/v] Triton X-100, pH 8) for 1 hour at room temperature. See Appendix C for immunoblot and zymography gel recipes. Respective primary antibodies were then added and allowed to incubate either over night at 4°C (TIMP-1,-2, COX-1,-2) or for 1 hour at room temperature (StAR). The membrane was then washed quickly with TBST and then 4 x 10
minutes with TBST buffer before incubation with a secondary antibody for 1 hour at room temperature. Following a quick rinse with TBST and 4 x 10 minute washes, blots were visualized using an enhanced chemiluminescent (ECL) detection system (Super Signal 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. Two negative controls were performed for each protein; one with either mouse or rabbit non-specific IgG in lieu of primary antibody and the second was exclusion of the primary antibody. Details are in Appendix D.

**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 raised in mice against purified ovine COX-1 and used at a concentration of 5 ug/ml. An ovine COX-1 recombinant protein (0.25ug) served as the positive control. The COX-2 polyclonal antibody was raised in rabbits against a synthetic peptide from the C-terminus region of mouse COX-2 and used at 1:1000. It 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 (0.25ug) served as the positive control. The StAR 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 (Clarke et al., 1994). Bovine luteal tissue extract containing the 30 kDa protein was used as a positive control (Pescador et al., 1996). The TIMP-1 and -2
primary antibodies and positive controls were purchased from EMD Biosciences (San Diego, CA). The TIMP-1 monoclonal antibody was raised in mice against bovine TIMP-1 and was used at a concentration of 1 ug/ml. The TIMP-2 monoclonal antibody was raised in mice against human TIMP-2 and was used in a concentration of 5 ug/ml. Furthermore, recombinant bovine TIMP-1 or human TIMP-2 protein standards were 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 COX-2 and StAR immunoblots while anti-mouse (1:10,000) was used for COX-1, TIMP-2, and TIMP-2 immunoblots.

**Gelatin Zymography:**

Gelatin zymography was used to detect gelatinase (MMP-2 and -9) activities 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 system III (Bio-Rad, Melville, NY, USA) 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 CaCl₂, 50 mM Tris, pH 8.0). After incubation, gels were stained with Coomassie Blue R-250 solution (0.5% in a 1:3:6 ratio of acetic acid:isopropanol:distilled water; Fischer Scientific, Fair Lawn, NJ, USA) for 30 minutes and destained with distilled water for 1 day. Matrix metalloproteinase activity was observed as zone (bands) of clearance against the blue background of the
gel. Adjacent lanes contained Perfect Protein Markers (Novagen, Madison, WI, USA), and the positive control, which is conditioned medium of the human fibrosarcoma cell line, HT-1080 (American Culture Type Collection; Manassas, VA, USA), that is known to produce several MMPs (e.g. MMP-2 and -9) as well as TIMPs (e.g. TIMP-1 and -2). Details are in Appendix E.

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 2 hours prior to electrophoresis. To ensure equal sample loading, gels containing equal volumes of sample were stained with Coomassie Blue R-250 solution (0.5% in a 1:3:6 ratio of acetic acid:isopropanol:distilled water; Fischer Scientific, Fair Lawn, NJ, USA) for 30 minutes and destained with distilled water for 1 day.

**Radioimmunoassay for Progesterone:**

Sheep jugular blood samples were spun in a centrifuge for 25 minutes at 2500 x g at 4°C, the plasma separated immediately, and stored at -20°C. Plasma P₄ concentrations were determined after extraction with petroleum ether (Appendix F) by radioimmunoassay (Appendix G; Beal et al., 1980; Goldberg et al., 1996). The P₄ antibody used was diluted to a final concentration of 1:10,000 (no. 337, anti-progesterone-11-BSA; Gordon Niswender, Colorado State, Fort Collins, CO). Radiolabeled P₄ (³H-P₄) was obtained from Perkin Elmer Life Sciences, Boston, Mass. None radiolabeled P₄ was purchased from Steraloids, Wilton, NH.
**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). For proteins, pixel densities of every sample at each time point for individual sheep were determined and averaged. Differences in means were determined using analysis of variance (ANOVA) followed by a p-diff test using the MIXED procedure of SAS (Release 9.1). A value of p<0.05 was considered to be significant. For steroid hormone data, P₄ concentrations for each time point were averaged and differences between mean concentrations was determined using the Repeated Measures analysis using the Mixed Procedure of SAS (Release 9.1). A value of p<0.05 was considered to be significant.
Results

Analysis of Progesterone and StAR:

Prior to and following the third infusion of PGF$_{2\alpha}$, progesterone concentrations decreased (p<0.05) by 40%, 30%, and 50% at the -1 hr, 1 hr, and 8 hr time points, respectively, compared to the controls (Figure 13). It subsequently reached a nadir (decreased by 75%; p<0.05) between the 8 and 16 hr time points before returning to pre-treatment concentrations at 24 hrs. The standard error of the mean was determined for each timepoint (APPENDIX I). Individual profiles of P$_4$ concentrations in sheep that underwent luteectomy at 24 hrs following three infusions of PGF$_{2\alpha}$ are shown (Figure 14). While progesterone concentrations of sheep 1, 3, and 4 rebounded, those of sheep 2 did not. The same effect is seen when the progesterone concentration of sheep 2 is compared to the mean progesterone concentration of sheep 1, 3, and 4 (APPENDIX J).

Immunoblotting revealed a band with a relative molecular mass of ~29 kDa, which co-migrated with the previously characterized StAR protein present in bovine corpus luteum (Figure 15; Pescador et al., 1996). The expression of StAR decreased (p<0.05) by 60% at the 16 hr time point and decreased (p<0.05) by 45% at the 24 hr time point when compared to untreated control (Figure 15). To confirm that there was equal loading of samples, β-actin expression (Figure 16) and total protein concentrations (Figure 40) were determined. When StAR pixel values were divided by their respective β-actin pixel values or by their respective total protein concentrations and then expressed relative to the untreated (0 hr) samples, the same decrease in StAR expression at the 16 and 24 hr time points was observed (Figures 17 & 18). Also, immunoblots containing
luteal extracts were incubated with goat anti-rabbit secondary antibody without prior incubation with primary antibody to serve as a negative control (Figure 19). Other immunoblots containing luteal extracts were incubated with IgG (same isotype as StAR antibody) in lieu of StAR primary antibody, before incubation with goat anti-rabbit secondary antibody as another negative control (Figure 20). No bands corresponding with the relative molecular mass of StAR were detected in either negative control.

Figure 13:

**Progesterone**

![Graph](image)

*Figure 13:* Mean sheep jugular vein plasma P₄ concentrations (ng/ml). The first (1st), second, (2nd), and third (3rd) systemic infusions of PGF₂α are indicated at the top by bracketed arrows. The x-axis represents hours after the onset of the first infusion of PGF₂α. Triangles (Δ) indicate time of luteectomy (n=4 sheep per luteectomy) relative to the third infusion of PGF₂α. Asterisks indicate significance at p<0.05.
Figure 14: Individual sheep jugular vein plasma P₄ concentrations (ng/ml). The first (1ˢᵗ) second, (2ⁿᵈ), and third (3ʳᵈ) systemic infusions of PGF₂α are indicated at the top by bracketed arrows. The x-axis represents hours after the onset of the first infusion of PGF₂α.
Figure 15: Representative immunoblot and densitometric analysis of StAR from sheep corpora lutea extracts. Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion of PGF\(_{2\alpha}\)) and 1, 8, 16, 24 hrs (after third infusion of PGF\(_{2\alpha}\)). Aliquot size of tissue extracts is 5 ul. The arrows on the left of the immunoblot represent the molecular weight markers (kDa). The arrow on the right indicates the relative molecular mass of StAR (~29 kDa). Steroidogenic acute regulatory protein from corpora lutea samples co-migrated with the positive control (bStAR from day 12 CL, Lane 7). All samples are expressed as a % of untreated (0 hr) control. Asterisks indicate significance at p<0.05.
Figure 16: Representative immunoblot of β-actin from sheep luteal extracts. Arrows on the left indicate molecular weight markers (kDa). Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion PGF$_{2\alpha}$). Volume loaded in each lane is 0.2ul. The arrow on the right indicates the relative molecular mass of β-actin (~42 kDa).
Figure 17: The ratio of StAR over β-Actin pixel values expressed as a % of the untreated (0 hr) samples. Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion of PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2\alpha}$); n = 4 for each time point. Asterisks indicate significance at p<0.05.
Figure 18: The ratio of StAR over total protein concentrations expressed as a % of the untreated (0 hr) samples. Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion of PGF$_2$α) and 1, 8, 16, 24 hrs (after third infusion of PGF$_2$α); n = 4 for each time point. Asterisks indicate significance at p<0.05.
Figure 19: Negative control blot for StAR. Arrows on the left indicate molecular weight markers. Five microliters of corpora lutea extracts (0, -1, 1, 8, 16, 24 hrs) were loaded. Lane 7 was loaded with 10 ul of StAR positive control (bStAR; day 12 bovine corpora lutea extract). The membrane was incubated with goat anti-rabbit secondary antibody without prior incubation with primary antibody. The arrow on the right indicates where the ~29 kDa StAR protein would have migrated.

Figure 20: Representative negative control blot for StAR. Arrows on the left indicate molecular weight markers. Ten microliters of corpora lutea extracts (0, -1, 1, 16, 24 hrs) were loaded, while lane 6 was loaded with 10 ul of StAR positive control (bStAR, day 12
bovine corpora lutea extract). The membrane was incubated overnight with normal serum containing the same isotype, IgG, as the rabbit primary antibody prior to incubation with the goat anti-rabbit secondary antibody. The arrow on the right indicates where the ~29 kDa StAR protein would have migrated.

Expression of StAR and Progesterone Concentrations during the First Three Infusions of PGF$_{2\alpha}$:

We previously determined the expression of StAR and P$_4$ concentrations following one and two infusions of PGF$_{2\alpha}$ (Towle et al., 2002; Allen, UNH Master’s Thesis; Ricketts, UNH Master’s Thesis), respectively. The data from these experiments are integrated with those from the present study in **Figure 21**. Data are expressed relative to the untreated (0 hr) samples. Progesterone concentration decreased (p<0.05) 8 hours after the first infusion of PGF$_{2\alpha}$ and did not return to control concentrations until 24 hours after the third infusion of PGF$_{2\alpha}$. The expression of StAR was unchanged (p>0.05) over the first two infusions of PGF$_{2\alpha}$ before decreasing (p<0.05) 16 and 24 hours after the third infusion.
Figure 21: Expression of StAR and progesterone concentrations following three infusions of PGF<sub>2α</sub>. All samples are expressed as a % of untreated (0 hr) control. Time is relative to the first infusion of PGF<sub>2α</sub>. Triangles (△) indicate time of luteectomy (n=4 sheep per luteectomy) relative to the third infusion of PGF<sub>2α</sub>. Asterisks indicate significance at p<0.05.

Analysis of Matrix Metalloproteinase -2 and -9 (MMP-2 and -9):

Three major enzyme bands migrating at approximately 84, 72 and 64 kDa were identified in all luteal tissue extracts by gelatin zymography (Figure 22). Representative luteal extract samples incubated with 1,10-phenanthroline, a chelator of divalent cations (Figure 23), revealed no zones of clearing. This confirmed that the observed enzyme bands were due to true MMP activity (Figure 22). Also, all three enzyme species co-migrated with those present in the positive control, which was conditioned medium from
the fibrosarcoma cell line, HT-1080. Furthermore, incubation of representative luteal extract samples with amino phenylmercuric acetate (APMA) distinguished between active and latent forms of these enzymes (Figure 24). The presence of APMA stimulates cleavage of latent enzymes, yielding active, lower weight forms. Therefore, the ~84 kDa enzyme species is an active form of MMP-9, while the ~72 kDa and ~64 kDa enzyme species are the latent and active forms of MMP-2, respectively. Expression of MMP-9 (~84 kDa) and MMP-2 (~72 and 64 kDa) remained unchanged (p>0.05) relative to the untreated control, at all time points, following the third infusion of PGF$_{2\alpha}$. (Figure 22). To further confirm that there was equal loading of samples, Coomassie Blue stained gels were performed (Figure 25). When pixel values for each MMP were divided by their respective Coomassie Blue pixel values and then expressed relative to the untreated (0 hr) samples (Figure 26), the expression of MMP-2 and -9 remained unchanged.
Figure 22: Representative zymogram and densitometric analysis of MMP-2 and -9 from sheep corpora lutea extracts. Time points are 0 hr (untreated), -1 hr (one hour before third infusion of PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2\alpha}$); n = 4 for each time point. Arrows on the left of the zymogram indicate molecular weight markers (kDa). Aliquot size of tissue extract is 1 ul. The arrows on the right indicate the relative molecular mass of active (A) MMP-9 (~84 kDa), latent (L) MMP-2 (~72 kDa), and active MMP-2 (~64 kDa). Each MMP species co-migrated with those present in the
positive control, HT1080 conditioned medium. All time points are expressed as a % of the untreated (0 hr) control.

Figure 23:

**1,10-Phenanthroline**

<table>
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<th>0 hr</th>
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<th>1 hr</th>
<th>8 hr</th>
<th>16 hr</th>
<th>24 hr</th>
<th>HT1080</th>
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<tbody>
<tr>
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<td>50 kDa</td>
<td>92 kDa</td>
<td>72 kDa</td>
<td>64 kDa</td>
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</table>

**Figure 23:** Zymogram of sheep luteal extracts incubated with 1,10-phenanthroline. Arrows on the left indicate molecular weight markers (kDa). Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion of PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2\alpha}$). The arrows on the right indicate where active MMP-9 (92 kDa), latent MMP-2 (72 kDa), and active MMP-2 (64 kDa) would have migrated.

Figure 24:

**Amino Phenylmercuric Acetate**

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<th>Time</th>
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<td>-</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 24:** Amino phenylmercuric acetate (APMA). Arrows on the left indicate molecular weight markers (kDa). Sheep CL extracts treated with (+) or without (-) APMA were loaded. Conditioned medium from HT1080 cells was loaded in lane 7. All
lanes show active MMP-9 (~84 kDa), latent MMP-2 (~72 kDa), and active MMP-2 (~64 kDa) co-migrating with the positive control (HT1080). Samples treated with (+) APMA yielded an additional band (*), representing the ~68 kDa species of active MMP-2.

**Figure 25:**

<table>
<thead>
<tr>
<th>Molecular Weight (kDa)</th>
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<th>-1 hr</th>
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</table>

**Figure 25:** Representative gel of sheep luteal extracts stained with Coomassie Blue. Arrows on the left indicate molecular weight markers (kDa). Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion PGF$_{2\alpha}$). Volume loaded in each lane is 1ul. The arrow on the right indicates the band (~55 kDa) used for densitometric analysis.
Figure 26:

**MMP-2 and -9 Expression over Coomassie**

The ratio of each MMP over Coomassie Blue pixel values expressed as a % of the untreated (0 hr) samples. Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion of PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2\alpha}$); n = 4 for each time point.
Analysis of Tissue Inhibitor of Metalloproteinase-1 and -2 (TIMP-1 and -2):

Immunoblotting of luteal extracts revealed a protein of ~29 kDa, which co-migrated with the recombinant bovine TIMP-1 positive control (Figure 27). Tissue inhibitor of metalloproteinase-1 expression decreased (p<0.05) by 20% one hour before (-1 hr) the third PGF$_{2\alpha}$ infusion, and decreased (p<0.05) by 30% one hour after (1 hr) the third PGF$_{2\alpha}$ infusion, when compared to untreated controls (0hr). When TIMP-1 pixel values were expressed over β-actin (Figure 29) or total protein (Figure 30) to confirm equal loading and then expressed as a percent of the untreated (0 hr) samples, the same changes in expression pattern were observed. In addition, a second protein of ~19 kDa, which co-migrated with the human TIMP-2 positive control (Figure 28), was also detected. Unlike TIMP-1, there was no change (p>0.05) in TIMP-2 expression at any of the time points. When TIMP-2 pixel values were expressed over β-actin (Figure 29) or total protein (Figure 30) to confirm equal loading and then expressed as a percent of the untreated (0 hr) samples, no changes (p>0.05) were observed at any time point. As negative controls, additional membranes containing luteal extracts were incubated with goat anti-mouse secondary antibody without prior incubation with the respective primary antibodies for TIMP-1 and -2 (Figure 31), or they were incubated with mouse IgG$_1$ (same isotype as TIMP-1 and -2 antibodies) in lieu of TIMP-1 or TIMP-2 primary antibody, before incubation with goat anti-mouse secondary (Figure 32). No bands corresponding to the relative molecular masses of TIMP-1 or TIMP-2 were detected on any of the negative control membranes.
Figure 27: Representative immunoblot and densitometric analysis of TIMP-1 from sheep corpora lutea extracts. Time points are 0 hr (untreated), -1 hr (one hour before third infusion of PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2\alpha}$). Aliquot size of tissue extract was 2 ul. The arrow on the left indicates the molecular weight marker (kDa). The arrow on the right indicates the relative molecular mass of TIMP-1 (~29 kDa). Tissue inhibitor of metalloproteinase-1 from corpora lutea extracts co-migrated with the positive control, bovine TIMP-1 (bTIMP-1, Lane 7). All time points are expressed as a % of untreated (0 hr) control. Asterisks indicate significance at p<0.05.
Figure 28: Representative immunoblot and densitometric analysis of TIMP-2 from sheep corpora lutea extracts. Time points are 0 hr (untreated), -1 hr (one hour before third infusion of PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2\alpha}$). Aliquot size of tissue extracts is 10 ul. The arrows on the left indicate the molecular weight markers (kDa). The arrow on the right indicates the relative molecular mass of TIMP-2 (~19 kDa). Tissue inhibitor of metalloproteinase-2 from corpora lutea extracts co-migrated with the positive control, human TIMP-2 (hTIMP-2, Lane 7). All samples are expressed as a % of untreated (0 hr) control.
Figure 29: The ratio of TIMP-1 and -2 over β-Actin pixel values expressed as a % of the untreated (0 hr) samples. Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion of PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2\alpha}$); n = 4 for each time point. Asterisks indicate significance at p<0.05.
Figure 30: The ratio of TIMP-1 and -2 over total protein concentrations expressed as a % of the untreated (0 hr) samples. Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion of PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2\alpha}$); n = 4 for each time point. Asterisks indicate significance at p<0.05.
Figure 31: Representative negative control blot for TIMP-1 and TIMP-2. Arrows on the left indicate molecular weight markers. Aliquots (2 μl and 10 μl) of corpora lutea extracts are indicated. The membrane was incubated in the absence of primary antibody, but instead was incubated only in the presence of the goat anti-mouse secondary antibody. The arrows on the right indicate where the ~29 kDa TIMP-1 and ~19 kDa TIMP-2 proteins would have migrated.

Figure 32: Representative negative control blot for TIMP-1 and TIMP-2. Arrows on the left indicate molecular weight markers. Aliquots (2 μl and 10 μl) of corpora lutea extracts are indicated. The membrane was incubated overnight with normal serum containing the same isotype, IgG1, as the mouse primary antibody, before incubation with goat anti-
Expression of MMPs and TIMPs during the First Three Infusions of PGF$_{2\alpha}$:

We previously determined the expression of MMP-2 and -9 and TIMP-1 and -2 following one and two infusions of PGF$_{2\alpha}$ (Towle et al., 2002; Ricketts, UNH Master’s Thesis), respectively. The data from these two experiments along with data from the present experiment (third infusion) are graphed in Figure 33 to profile the expression patterns of MMPs and TIMPs throughout the first three infusions of PGF$_{2\alpha}$. Data are expressed relative to untreated (0 hr) samples. Expression of MMP-2 and -9 did not change (p>0.05). However, TIMP-1 decreased (p<0.05) 1 hour after each infusion of PGF$_{2\alpha}$ while TIMP-2 decreased (p<0.05) 1 hour after the first infusion and remained decreased (p<0.05) until 1 hour after the second infusion before returning to pre-treatment levels. The ratio of MMPs:TIMPs throughout the first three infusions of PGF$_{2\alpha}$ are depicted in Figure 34. When expressed as a ratio, active MMP-2:TIMP-2 and active MMP-9:TIMP-1 increased after the first and second infusion of PGF$_{2\alpha}$ before returning to the level similar to the control (0 hr) ratio following the third infusion.
Figure 33: Expression of MMPs and TIMPs following three infusions of PGF$_{2\alpha}$. All samples are expressed as a % of untreated (0 hr) control. Time is relative to the first infusion of PGF$_{2\alpha}$. Asterisks indicate significance at $p<0.05$.
Figure 34: The ratio of active MMP-2 (MMP-2 A) over TIMP-2 and MMP-9 (MMP-9 A) over TIMP-1 following three infusions of PGF$_{2\alpha}$. The first (1$^{\text{st}}$) second, 2$^{\text{nd}}$, and third (3$^{\text{rd}}$) systemic infusions of PGF$_{2\alpha}$ are indicated at the top by bracketed arrows.

Analysis of COX-1 and COX-2:

While immunoblotting detected the ovine COX-1 positive control migrating at ~70 kDa (Figure 35), no corresponding bands were detected in any of the lanes containing luteal extracts. In contrast, immunoblotting of luteal extracts revealed a protein of ~72 kDa, which co-migrated with the ovine COX-2 positive control (Figure 36.
A). Densitometric analysis showed no changes (p>0.05) in COX-2 expression following three infusions of PGF$_{2\alpha}$ (Figure 36 B). When COX-2 pixel values were divided by their respective β-actin pixel values to confirm equal loading, the expression pattern remained the same (Figure 37). When the first immunoblot was excluded in the analysis, there was a 300% increase (p<0.05) in COX-2 expression at the -1 and 8 hr time points (Figure 38). In turn, when COX-2 pixel values were divided by their respective β-actin pixel values to confirm equal loading, the same increase was observed (Figure 39).

**Figure 35:**

![COX-1 Expression](image)

**Figure 35:** Representative immunoblot of COX-1 from sheep luteal tissue extracts. Time points are 0 hr (untreated), -1 hr (one hour before third infusion of PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2\alpha}$). Aliquot size of tissue extracts is 20 ul. The arrows on the left indicate the molecular weight markers (kDa). The arrow on the right indicates the relative molecular mass of COX-1 (~70 kDa). Tissue extracts are in lanes 1-6 while the positive control, ovine COX-1, is in lane 7.
Figure 36 A: All four COX-2 immunoblots of sheep corpora lutea extracts. Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion of PGF$_{2a}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2a}$). Aliquot size of tissue extract is 15 ul. The arrow on the left indicates the molecular weight markers (kDa). The arrow on the right indicates the relative molecular mass of COX-2 (~72 kDa). Cyclooxygenase-2 from samples co-migrated with the positive control, ovine COX-2 (oCOX-2, Lane 7).
Figure 36 B: Densitometric analysis of COX-2 from sheep corpora lutea extracts. Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion of PGF₉α) and 1, 8, 16, 24 hrs (after third infusion of PGF₂α). All time points are expressed as a % of untreated (0 hr) samples.
Figure 37: The ratio of COX-2 over β-actin pixel values expressed as a % of the untreated (0 hr) samples. Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion of PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2\alpha}$); n = 4 for each time point.
Figure 38: Densitometric analysis of COX-2 from sheep corpora lutea extracts (Gels 2, 3, & 4). Time points are 0 hr (untreated), -1 hr (one hour before third infusion of PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2\alpha}$). All time points are expressed as a % of untreated (0 hr) control. Asterisks indicate significance at p<0.05.
Figure 39: The ratio of COX-2 (Gels 2, 3, and 4) over β-actin pixel values expressed as a % of the untreated (0 hr) samples. Time points are 0 hr (untreated samples), -1 hr (one hour before third infusion of PGF$_{2\alpha}$) and 1, 8, 16, 24 hrs (after third infusion of PGF$_{2\alpha}$); n = 3 for each time point. Asterisks indicate significance at p<0.05.

**Bradford Assay:**

Total protein concentrations were determined for each corpora lutea extract (Figure 40), using the Bradford Assay standard curve (Figure 41), to further confirm equal loading in zymograms and immunoblots. There were no differences in total protein concentrations between any of the time points (p>0.05).
**Figure 40:**

**Bradford Assay**

<table>
<thead>
<tr>
<th>Time Points</th>
<th>Corpora Lutea Protein Concentration (ug/ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.99 16.45 13.34 12.56</td>
</tr>
<tr>
<td>-1</td>
<td>13.45 15.18 12.42 15</td>
</tr>
<tr>
<td>1</td>
<td>14.58 15.87 15.64 12.23</td>
</tr>
<tr>
<td>8</td>
<td>14.97 16.45 12.92 17.03</td>
</tr>
<tr>
<td>16</td>
<td>15.22 17.48 14.35 14.97</td>
</tr>
<tr>
<td>24</td>
<td>15.07 17.58 12.83 15.3</td>
</tr>
</tbody>
</table>

**Figure 40:** Total protein concentrations of corpora lutea extracts determined by Bradford Assay. Each number represents the mean of duplicate samples from untreated (0 hr) CL, and from those removed -1 hr (one hour before third infusion of PGF

2α) and 1, 8, 16, and 24 hrs (after the third infusion of PGF

2α; n = 4 for each time point).

**Figure 41:**

**Bradford Assay Standard Curve**

**Figure 41:** Bradford Assay standard curve. Line of best fit ($y = 0.0434x + 0.0187$) and $R^2$ (0.9977) values are shown.
DISCUSSION

Luteolysis encompasses two events, functional regression as well as structural regression of the corpus luteum, but their timing may be asynchronized (Juengel et al., 1993; McGuire et al., 1994; Tian et al., 1994). Therefore, we developed a model that allows us to determine the changes in P₄ concentration, and StAR, MMP, TIMP and COX protein expression, in response to multiple physiological infusions of PGF₂α that mimic the natural frequency in sheep. As a result, we can profile the temporal expression patterns of these endpoints to better understand the relationship between the functional and structural regression of the CL during luteolysis.

Functional regression of the CL is typified by a decline in plasma P₄ concentration (Hoyer, 1998; Stocco et al., 2007). In the current study, while plasma P₄ concentrations declined between 40 and 75% following the first three infusions of PGF₂α, they nonetheless rebounded to pre-infusion levels (Figure 13). However, looking at the data in more detail revealed that the P₄ concentration in one of the four sheep remained low and did not rebound (Figure 14), suggesting that the CL was probably undergoing irreversible functional regression. Interestingly, the pattern of StAR expression did not mirror P₄ concentrations, i.e. StAR protein expression decreased by 60 and 45% 16 and 24 hours, respectively, following the third infusion of PGF₂α (Figure 15), whereas no change in StAR was observed following the first or second infusion of PGF₂α (Figure 21). Notably, the expression of StAR in the sheep whose P₄ concentrations never rebounded was 55% less than the other three animals, further supporting that irreversible functional regression of the CL was probably occurring in this animal. In this one sheep, the P₄ concentration and StAR expression results are consistent with our previous finding
that 33% of sheep undergo complete luteolysis following three infusions of PGF$_{2\alpha}$ (McCracken et al., 1999). The fact that P$_4$ concentrations decreased well before the decline in StAR expression (Figure 21) may be explained by the decrease in membrane fluidity observed during luteolysis (Goodsaid-Zalduondo et al., 1982; Carlson et al., 1984; Luborsky et al., 1984). The reduced membrane fluidity may prevent LH receptor aggregation decreasing the amount of active hormone sensitive lipase, the enzyme responsible for cleaving cholesterol from lipid droplets. Another possibility is that PGF$_{2\alpha}$ decreases the expression of sterol carrier protein-2 (SCP-2), 3β-HSD and P450scc (McLean et al., 1995). Sterol carrier protein-2 transports cholesterol to mitochondria and decreases 8 hours following PGF$_{2\alpha}$ treatment in rats. Similarly, a decline in 3β-HSD and P450scc is seen 4 hours after PGF$_{2\alpha}$ treatment. Thus the decline in P$_4$ before the decrease in StAR expression (Figure 21) may be explained by the decrease in SCP-2, 3β-HSD and P450scc. Further, ovarian blood flow following PGF may also play a role. The relationship between blood flow and P$_4$ concentrations after PGF$_{2\alpha}$ was also considered (Einer-Jensen and McCracken, 1981; Ginther, 2007). Perhaps reduced ovarian blood flow, in response to PGF$_{2\alpha}$, decreases peripheral P$_4$ concentrations. However, P$_4$ concentrations decrease in sheep (Einer-Jensen and McCracken, 1981) and mares (Ginther, 2007) before any change in capillary blood flow in the ovary was observed. In contrast, Acosta et al. (2002) found that intraluteal blood flow within a midcycle CL increased 0.5 hours, but then decreased 4 and 8 hours after 500ug PGF$_{2\alpha}$ treatment. In a later study, Acosta et al. (2008) found that peripheral P$_4$ concentrations also decreased in the midcycle CL 4 hours following the same treatment of PGF$_{2\alpha}$. Although StAR expression was not reported, the above studies suggest that the relationship between P$_4$
concentrations and StAR is complex. Overall, additional studies are needed to elucidate the mechanisms behind the temporal asynchrony between peripheral P₄ concentrations and StAR protein expression following PGF.

Structural regression of the CL is mediated, in part, by degradation of the ECM, the result of net MMP activity (Endo et al., 1993; Liu K et al., 1999; Xinlei et al., 2006; Kliem et al., 2007). We previously reported an increase in active MMP-2 following one and two infusions of PGF₂α (Towle et al., 2002; Rickets, UNH Master’s Thesis). In order to compare MMP expression between experiments in sheep that received one, two or three infusions of PGF₂α, all time points within each experiment were expressed as a percent of the control (untreated) samples. Upon doing so, no changes in gelatinase activity were revealed (Figure 33). This is consistent with the fact that MMP-2 is expressed constitutively in most tissues (Salo et al., 1985; Templetone et al., 1990; Huhtala et al., 1991; Yeow et al., 2001), although it can be moderately induced 2-4 fold (Huhtala et al., 1991; Overall et al., 1991; Salo et al., 1991) while MMP-9 can be induced 8 fold (Salo et al., 1991; Overall et al., 1991). It is possible that the differences in gelatinase expression using our physiological model in sheep and approaches used by others may be attributed to variations in dose and frequency of PGF₂α. The active MMP-2, latent MMP-2 and active MMP-9 expression in the sheep that appeared to undergo irreversible functional regression was 10, 20, and 25 % greater, respectively, than the other three sheep in that time point. This suggests that net proteolysis may have occurred in this animal; however, TIMP expression needs to be considered.

Tissue inhibitors of metalloproteinases aid in regulating the structural degradation of the luteal ECM by inhibiting MMPs (McIntush and Smith, 1999; Ricke et al., 2002c;
Zhang et al., 2003; Kliem et al., 2007). In the present study, TIMP-1 decreased by 20 and 30% 1 hour before (-1 hr) and 1 hour after (1 hr) the third infusion of PGF$_{2\alpha}$ respectively (Figure 27), which is similar to the decrease in TIMP-1 protein seen after the first and second infusions (Figure 33) and after a single luteolytic dose of PGF$_{2\alpha}$ administered to sheep (McIntush and Smith, 1997). In addition, several studies also report a decrease in TIMP-1 expression during natural or PGF$_{2\alpha}$ induced luteolysis (Inderdeo et al., 1996; Ricke et al., 2002c; Young et al., 2002; Xinlei et al., 2006; Kliem et al., 2007) or in the late-stage CL (Zhang et al., 2003). On the other hand, TIMP-2 expression was unchanged after three infusions of PGF$_{2\alpha}$ (Figures 28), in contrast to the decrease observed following the first and second infusions (Figure 33). During natural luteolysis in several species, TIMP-2 expression either increases (Pitzel et al., 2000; Young et al., 2002; Zhang et al., 2003; Xinlei et al., 2006) or is unchanged after PGF$_{2\alpha}$ treatment (Nothnick et al., 1995; Ricke et al., 2002c). However, Smith et al. (1995) reported that TIMP-2 mRNA decreases in the sheep corpus luteum from early luteal phase (days 3-7) to late luteal phase (day 16). This decrease in TIMP-2 mRNA occurs about 48 hours (day 16) after the first physiological pulse of PGF$_{2\alpha}$ (day 14). Therefore, it may take 48 hours following the first infusion of PGF$_{2\alpha}$ in our study to see a second decrease in TIMP-2 protein, which might explain why TIMP-2 returned to control (0 hr) concentrations after three infusions of PGF$_{2\alpha}$. This suggests that TIMP-2 protein expression might not decrease again until after additional pulses of PGF$_{2\alpha}$. Further, the individual expression of MMPs or TIMPs may reveal the full picture. Instead, the balance between MMP activity and TIMP expression need to be considered. The unchanged expression of MMP-2 and -9 accompanied by the decrease in TIMP-1 and -2 during the early onset of luteolysis
(Figure 33) may result in net proteolytic activity; however, histological staining of ECM components is needed to strengthen this conclusion. After the third infusion of PGF$_{2\alpha}$, TIMP-1 and -2 expression returned to pretreatment levels while MMP activity was unchanged. Thus, the balance between MMPs and TIMPs was restored (Figures 33 & 34), suggesting that the structural integrity of the CL is maintained after three infusions of PGF$_{2\alpha}$. It is worth noting that the TIMP-2 pixel value for the sheep whose P$_4$ concentrations never returned to control concentrations was 70% lower than that of the other three sheep at the 24 hour time point (but TIMP-1 was not different). With the preference of TIMP-2 for MMP-2, this sizable decrease in TIMP-2 expression in this animal may result in structural regression of the CL. However, experiments with sheep receiving additional pulses of PGF$_{2\alpha}$ along with histological staining of the CL for ECM degradation and evidence of DNA fragmentation and apoptosis would need to be performed to confirm structural regression. Furthermore, it is also important to keep in mind that other MMPs, such as MMP-1, 14, and 19, and other TIMPs, such as TIMP-3 and TIMP-4, may also play a role in structural regression of the CL (Chen et al., 2006; Kliem et al., 2007).

Luteolysis is initially driven by pulses of uterine PGF$_{2\alpha}$ in sheep (McCracken et al., 1972; Zarco, 1988), which subsequently induces production of luteal PGF$_{2\alpha}$ (Rexroad and Guthrie, 1979; Tsai and Wiltbank, 1997). In the present study, luteal COX-1 was not detected after three infusions of PGF$_{2\alpha}$ (Figure 35), which is consistent with our previous findings following one and two infusions (Allen, UNH Master’s Thesis; Ricketts, UNH Master’s Thesis). These results are not surprising because COX-1, the non-inducible isoform, is constitutively expressed in relatively small amounts in most tissues, including
the CL (Smith et al., 2000; Arosh et al., 2004). In contrast, the COX-2 isoform is inducible (Lafyatis et al., 1989; Lin et al., 1989; Smith et al., 1994). Following three infusions of PGF$_{2\alpha}$, luteal COX-2 expression remained unchanged (Figure 36 B), which is in contrast to the 300% increase we previously observed in response to one and two infusions of PGF$_{2\alpha}$ (Allen, Ricketts, Master’s Thesis). However, if immunoblot 1 is excluded (because of unexplainable technical issues; it is inconsistent with the remaining immunoblots), COX-2 increases by 300% at the -1 hr and the 8 hr time points (Figure 38), which is similar to the increase in magnitude seen after one and two infusions of PGF$_{2\alpha}$ (Allen, Ricketts, UNH Master’s Thesis) and reported by others (Tsai and Wiltbank, 1997; Hayashi et al., 2003; Arosh et al., 2004; Zerani et al., 2007). Despite the increase in COX-2 at the 8 hr time point following the third infusion of PGF$_{2\alpha}$ (gels 2, 3, and 4), it returns to pre-infusion concentrations at the 16 and 24 hour time points (Figure 38). It is important to keep in mind that during physiological luteolysis, the fourth pulse occurs 8 hours after the third. Therefore, in order for luteal COX-2 expression to remain increased beyond the 8 hr time point, a fourth infusion may be necessary. Interestingly, the COX-2 expression of the sheep whose P$_4$ concentration never rebounded was 85% lower than that of the remaining sheep at the 24 hr time point. Perhaps complete luteal regression occurred in this animal.

In summary, throughout the first three infusions of PGF$_{2\alpha}$ in the sheep, it appears that a decrease in TIMPs accompanied by unchanged MMP expression results in local MMP activity that brings about net proteolysis. This is consistent with steps 2 and 3 of our proposed cell model of luteolysis in sheep (Appendix H). However, our present results also indicate that the balance between MMPs and TIMPs is restored following the
third infusion of PGF$_{2\alpha}$ suggesting that the overall structure of the CL is probably maintained. It therefore may take four or five infusions of PGF$_{2\alpha}$ to see another decrease in TIMP proteins and possibly an increase in MMP expression, resulting in the structural demise of the CL. With each subsequent infusion of PGF$_{2\alpha}$ the CL is also more likely to undergo functional decline. In response to the disruption of the ECM by MMP activity, the integrity of integrins within luteal cells becomes compromised (Choquet et al., 1997; Calderwood et al., 2000), cholesterol transport by StAR decreases, and as a consequence, so does P$_4$ secretion (Niswender et al., 2000). This is depicted in step 3 of our proposed cell model. Furthermore, COX-2 expression increases throughout the first three infusions of PGF$_{2\alpha}$ which increases luteal PGF$_{2\alpha}$ to aid in regression of the CL. In conclusion, three sequential pulses of PGF$_{2\alpha}$ are insufficient to cause the majority of sheep to undergo luteolysis. However, based on the results from the one sheep whose P$_4$ concentrations did not rebound after three infusions of PGF$_{2\alpha}$, the concomitant low expression of StAR and TIMP-2 proteins indicates that they may be the crucial players in the functional and structural demise of the CL. Their expression profiles, as well as other mediators of luteolysis, following four or five infusions of PGF$_{2\alpha}$ will ultimately reveal the interplay of proteins that lead to regression of the CL.
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APPENDICES
APPENDIX A. PROTEIN EXTRACTION FROM LUTEAL CELLS

SOLUTIONS:

**Extraction Buffer:**

- 50 mM Tris
- 150 mM NaCl
- 0.02% Sodium Azide
- 10 mM EDTA
- 1% Triton X-100
  
  Bring up to volume with cold ddH₂O
  
  pH to 7.4, prior to adding Triton X-100

*All steps performed on ice or at 4°C.*

1. Add protease inhibitors to extraction buffer right before use:
   a. 1 ug/ml AEBSF
   b. 1 ug/ml Pepstatin A
   c. 1 ug/ml Aprotinin

2. Remove tissue from freezer and weigh.

3. Add 8 ml of extraction buffer for every 1 g tissue, scissor mince. Clean polytron probe for homogenation:
   a. Rinse probe with EtOH, ddH₂O, and extraction buffer.

4. Transfer minced tissue (with extraction buffer) to glass tube.

5. Homogenize tissue at speed 9 for 30 seconds.

6. Rinse polytron probe with water, EtOH, and extraction buffer and remove any tissue debris if necessary.

7. Repeat homogenization for each sample.

8. Centrifuge at 4°C, 10,000 x g for 10 minutes.

9. Transfer supernatant into new 1.5 ml eppendorf tube and repeat centrifuge until no pellet forms.

10. Store at -80°C.
APPENDIX B: PROTEIN DETERMINATION (BRADFORD ASSAY)

SOLUTIONS:

Phosphate Buffered Saline:

0.25 M Na₂HPO₄
0.25 M NaH₂PO₄
8.1 g NaCl
Bring up to 1 L with ddH₂O
pH to 7.4

1. Set up standard curve (in duplicate) in 13 x 75 mm glass test tubes:

<table>
<thead>
<tr>
<th>Tube #</th>
<th>BSA</th>
<th>PBS</th>
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<tr>
<td>1,2</td>
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<td>10,11</td>
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</table>

2. Set up samples (in duplicate) in 13 x 75 mm glass tubes:

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<tr>
<th>Tube #</th>
<th>Sample</th>
<th>PBS</th>
</tr>
</thead>
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<tr>
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<td>797.5 ul</td>
</tr>
<tr>
<td>15,16</td>
<td>5 ul</td>
<td>795 ul</td>
</tr>
</tbody>
</table>

*samples diluted 1:10
*repeat for each sample

4. Read absorbance at 595 nm and record values
5. Graph standard curve (ug BSA on x-axis and absorbence on y-axis)
6. Using the regression equation, calculate the protein concentration (ug/ul) in each sample.
APPENDIX C: IMMUNOBLOTTING AND ZYMOPHOGRAPHY

SOLUTIONS

Ammonium Persulfate (100 mg/ml)
200 mg Ammonium Persulfate
2 ml ddH₂O

1.5 M Tris (pH 8.8)
18.17 g Tris
100 ml ddH₂O

4 mM APMA Solution
14.1 mg aminophenylmercuric acetate
100 ml ddH₂O

0.5 M Tris (pH 6.8)
6.06 g Tris
100 ml ddH₂O

10 mM 1,10-Phenanthroline
435 mg 1,10-phenanthroline
200 ml ddH₂O

0.25 M Tris (pH 6.8)
3.03 g Tris
100 ml ddH₂O

Stacking Gel (4% Acrylamide)
6.36 ml ddH₂O
2.52 ml 0.5 M Tris (pH 6.8)
1 ml 40% Acrylamide
100 ul 10% SDS
50 ul Ammonium Persulfate (100 mg/ml)
10 ul TEMED

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

Separation Gel (10% Acrylamide)
7.5 mg gelatin
7.275 ml ddH₂O
3.75 ml 1.5 M Tris (pH 8.8)
3.75 ml 40% Acrylamide
150 ul 10% SDS
75 ul Ammonium Persulfate
7.5 ul TEMED

Separating Gel (12% Acrylamide)
No gelatin for Westerns
6.525 ml ddH₂O
3.75 ml 1.5 M Tris (pH 8.8)
4.5 ml 40% Acrylamide
150 ul 10% SDS
75 ul Ammonium Persulfate
7.5 ul TEMED

Electrode Buffer 10x
30 g Tris
144 g Tris
5 g SDS
Bring to 1 L with ddH₂O

Substrate Buffer
6.07 g Tris
970 mg CaCl₂·2H₂O
200 mg Sodium Azide
Bring to 1 L with ddH₂O
Coomassie Blue Stain
2.5 g Brilliant Blue R-250
50 ml Acetic Acid
150 ml Isopropynol
300 ml ddH₂O

2.5% Triton X-100 Solution
5 ml Triton X-100
195 ml ddH₂O
APPENDIX D: IMMUNOBLOT (WESTERN) PROCEDURE AND SOLUTIONS

SOLUTIONS:

<table>
<thead>
<tr>
<th>TBST Buffer</th>
<th>Blotting Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 g Tris</td>
<td>800 ml ddH₂O</td>
</tr>
<tr>
<td>8.8 g NaCl</td>
<td>200 ml Methanol</td>
</tr>
<tr>
<td>0.5 ml Tween 20</td>
<td>15.03 g Glycine</td>
</tr>
<tr>
<td>Bring up to 1 L with ddH₂O</td>
<td>3.15 g Tris</td>
</tr>
</tbody>
</table>

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

PROCEDURES:

Part 1: Electrophoresis
1. Cast gels using recipes above:
   a. Separating Gel: 12% acrylamide for TIMPs, and StAR
   b. Stacking Gel: 4% acrylamide
2. Use Bio Rad Precision Plus Dual Color Protein Standards for molecular weight markers.
3. Load positive controls and samples.
4. Electrophorese for ~1 hour at 200 volts-electrophoresis is the same for zymographies.

Part 2: Prepare for Blotting
1. Cut four pieces of filter paper and prepare 4 pieces of blotting foam.
2. Cut nitrocellulose membranes to fit atop gel accordingly.
3. When gels are almost done running, prepare blotting buffer-add methanol last.
   Let filter paper, sponges and membranes soak in blotting buffer.
4. When electrophoresis is complete, cut off stacking gel and let equilibrate in blotting buffer. Repeat for 2nd gel.
5. Put notched side of membrane on the top right.

Part 3: Electroblotting
1. On black side of plastic sandwich, place in order: 1 blotting sponge, 1 piece of filter paper, gel, 1 nitrocellulose membrane, 1 piece of filter paper, 1 blotting sponge.
2. Insert sandwich into electrode cassette with black part of the sandwich facing the black part of the electrode cassette. Place Cassette into tank with blotting buffer.

3. When both sandwiches are in the tank, add a small stir bar and an ice pack into the open area. Fill the tank with blotting buffer.

4. Place tank assembly into a basin filled with ice and place onto a stir plate. Place electrode tank cover onto tank and set the power source to 200 mAm 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 in 50 ml blocking solution on shaker for 1 hour.

**Part 5: Adding Primary Antibody**

1. Before blocking is complete, thaw primary antibody and dilute to appropriate concentration in 10 ml blocking buffer.
2. When blocking is complete, discard milk.
3. Add 5 ml of antibody to each membrane.
4. Cut 2 pieces of parafilm and place over membranes.
5. Let incubate 12-16 hours at 4°C on slow nutator.

**Part 6: Washing Membranes**

1. Remove the primary antibody and freeze at 20°C.
2. Rinse quickly with TBST.
3. Rinse 4 x 10 minutes with TBST.
4. During last rinse, prepare secondary antibody in 50 ml blocking solution.

**Part 7: Adding Secondary Antibody**

1. Add 5 ul of secondary antibody to 50 ml blocking solution.
2. Remove TBST from membranes and add 25 ml of secondary antibody to each membrane.
3. Shake on low for 1 hour.

**Part 8: Second Set of Washes**

1. Remove secondary antibody and quickly rinse with TBST.
2. Rinse 4 x 10 minutes with TBST.
3. Turn on developer and press run after the second wash.

**Part 9: Chemiluminescent Detection**

1. Make Super Signal chemiluminescent solution with lights off (Pierce kit; 5 ml of each solution).
2. When washing is complete, discard buffer and add 5 ml off Super Signal to each membrane.
Part 10: Prepare membranes for Documentation

1. Place a piece of saran wrap ~12 inches x ~8 inches on a flat, clean surface.
2. Using tweezers, shake excess Super Signal off onto kimwipe and place the membrane face side down onto the saran wrap.
3. Place a piece of filter paper on top of membranes and wrap saran wrap around the filter paper.
4. Turn over and place into film cassette. Tape down to prevent movement.
5. Bring film into dark room and expose film on membranes (exposure time depends on presence of protein) and process film.
APPENDIX E: GELATIN ZYMOGRAPHY PROCEDURES

DAY 1:

1. Heat water bath to \(-90^\circ\) C.
2. Make ammonium persulfate solution in 1.5 ml tube.
3. Weigh gelatin and place into 50 ml tube.
4. Prepare gel solution:
   a. Add ddH_2O to tube followed by 1.5 M Tris (pH 8.8), 40% acrylamide (wear gloves), and SDS.
   b. Heat contents in hot water bath (30 sec. dips) until gelatin is completely dissolved.
   c. Let cool to room temperature.
5. Set up gel apparatus:
   a. Rinse and dry all components.
   b. Place rubber gasket onto tower.
   c. Place 1 small and 1 large plate into green holder and tighten.
   d. Secure assembly to the towers
6. Casting the separating Gel:
   a. Add TEMED and APS to the gel solution and swirl to mix.
   b. With Pasteur pipette, quickly load the gel solution between the two plates. Fill up to green line.
   c. With a new Pasteur pipette, add ddH_2O on top of the gel solution.
   d. Allow gel to polymerize (45 minutes).
7. Casting the Stacking Gel:
   a. When separating gel is almost polymerized, add all the ingredients for the stacking gel solution, except APS and TEMED.
   b. Dump off water on top of separating gel and dry area with filter paper.
   c. Add APS and TEMED to the stacking gel solution and swirl to mix.
   d. Load the stacking gel solution on top of the separating gel.
   e. Immediately insert a 1 mm thick 10 well comb into the stacking gel.
   f. Allow to polymerize for 35 minutes.
8. Preparing Samples and Standards:
   a. While the stacking gel is polymerizing, make up loading samples in .5 ml tubes.
   b. Use an equal volume of sample to sample buffer (1:1) unless otherwise indicated.
9. Loading Samples:
   a. When stacking gels are polymerized, carefully remove combs.
b. Remove the gel/glass plate assembly and insert into electrode cassette and place in tank.
c. Add 800 ml of 1x electrode buffer to the center area between the gels, making sure to gently rinse the wells of the gels.
d. Load samples

10. Running the Gels:
   a. Once gels are loaded, attach electrode cover in proper orientation.
   b. Plug electrode 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 150 mm Petri dishes and label.
   b. Cover gels with ~100 ml Triton X-100 solution and place on shaker vigorously for 15 minutes.
   c. Drain off Triton X-100 solution and repeat steps b-c.
   d. When second wash is complete, rinse gels with ddH2O several times.

12. Fill each dish with ~150 ml of substrate buffer.

13. Place on shaker in 37°C incubator and shake gently overnight.

Day 2:

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

2. Destaining Gels:
   a. Aspirate off Coomassie stain. Rinse gels several times with ddH2O.
   b. Shake gels in ddH2O at room temperature, changing water periodically, until clear bands are visible.
   c. When gels are of satisfactory intensity, take a picture of gels.
APPENDIX F: PROGESTERONE EXTRACTION

1. Centrifuge blood samples for 20 minutes in 4°C centrifuge and save plasma.
2. Take 100 ul of plasma from each sample and add 2 ml of petroleum ether.
   Vortex for 60 seconds.
3. Snap freeze samples in acetone/dry ice bath for 10 seconds.
4. Decant ether layer into new tube.
5. Dry with N2.
6. Repeat steps 2-5.
7. Rinse sides of new tubes with petroleum ether using a Pasteur pipette.
8. Reconstitute samples in 200 ul PBSG and vortex.
9. Use immediately in radioimmunoassay or freeze at -20°C.
APPENDIX G: RADIOIMMUNOASSAY OF PROGESTERONE

SOLUTIONS:

Phosphate Buffered Saline with Gelatin (PBSG; pH 7.0)

5.38 g NaH$_2$PO$_4$·H$_2$O
16.35 g Na$_2$HPO$_4$·H$_2$O
1.0 g Sodium Azide
9 g NaCl
1 g Gelatin

1. Add all solutes to 800 ml ddH$_2$O and stir on a warm stir plate until dissolved.
2. Adjust pH to 7.0.
3. Bring volume up to 1 L with ddH$_2$O. Store at 4°C for no longer than 2 weeks.

Charcoal Suspension

200 mg Prewashed Norit A neutral charcoal
20 mg Dextran T-70

1. Add charcoal and Dextran to 100 ml of PBSG
2. Stir on ice for 30 minutes on high speed before using. Store at 4°C for no longer than 2 weeks.

Progesterone Antibody

1. Just before using, thaw stock antibody solution (1:10).
2. Make a 1:250 dilution with PBSG for a final dilution of 1:2500. This antibody concentration typically yields 30-50% binding.

Tritiated Progesterone

1. Pipette 30 ul of $^3$H-progesterone (50 uCi/ml ethanol) into a 20 ml glass vial.
2. Dry down under 37°C water bath under air.
3. Add 15 ml PBSG, cover, vortex, and incubate in 37°C water bath for 15 minutes. Store at 4°C.

Progesterone Standards
1. Make a 1:100 dilution of stock A (800 ug/ml) to yield stock solution B (8 ug/ml). Stock solutions are dissolved in ethanol, stored at -20°C, and warmed to room temperature before using.

2. Prepare a range of standards consisting of the following concentrations: 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 ng/ml. Add 100 ul of stock B to 4.9 ml PBSG to yield a premix of 160 ng/ml. Add 2 ml of premix to 18 ml PBSG to yield a 16 ng/ml standard. Make 1:2 serial dilutions to yield the remaining standards. Store at 4°C for no longer than 2 weeks.

**Assay Procedure:**

1. Label test tubes (12x75 mm)
2. Add PBSG, standard, and samples to tubes (in duplicate) as follows:

<table>
<thead>
<tr>
<th></th>
<th>PBSG (ul)</th>
<th>Standard/Sample (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Radioactivity</td>
<td>300</td>
<td>---</td>
</tr>
<tr>
<td>Non-Specific Binding</td>
<td>300</td>
<td>---</td>
</tr>
<tr>
<td>P₄ Standards</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 ng/ml</td>
<td>200</td>
<td>---</td>
</tr>
<tr>
<td>0.125 ng/ml</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.25 ng/ml</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5 ng/ml</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2 ng/ml</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4 ng/ml</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>8 ng/ml</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>16 ng/ml</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Samples</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

3. Pipette 100 ul of antibody solution into all tubes except Totals and Non-Specific Binding.
4. Pipette 100 ul ³H progesterone into all tubes and vortex. Cover tubes with parafilm and incubate at 4°C overnight.

**Following incubation period:**
1. Pre-chill centrifuge to 4°C.
2. Place racks of test tubes into ice water baths.
3. Keep charcoal solution uniformly suspended by stirring vigorously in an ice bath. With a repeat pipeter, add 750 ul cold charcoal suspension as quickly as possible to all tubes EXCEPT Totals, which get 750 ul PBSG.
4. Start a 10 minute timer just before adding charcoal to first tube.
5. Place tubes into a 4°C centrifuge.
6. Once timer beeps, centrifuge samples at 1500 x g for 10 minutes.
7. Decant supernatant (and all Totals) into scintillation vials.
8. Add 4.5 ml Ready Safe counting cocktail (Beckman Instruments; Fullerton, CA), cap vials, number vials, and invert twice.
9. Determine counts using a liquid scintillation counter (LS 6000IC; Beckman Instruments; Port Jefferson, NY).

**APPENDIX H. PROPOSED CELL MODEL OF LUTEOLYSIS**
### APPENDIX I. PROGESTERONE STANDARD ERROR OF MEANS FOR FIGURE 13

<table>
<thead>
<tr>
<th>Hour</th>
<th>+/-SEM</th>
<th>Hour</th>
<th>+/-SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.2382</td>
<td>32</td>
<td>0.5120</td>
</tr>
<tr>
<td>4</td>
<td>0.3037</td>
<td>33</td>
<td>0.1481</td>
</tr>
<tr>
<td>8</td>
<td>0.2162</td>
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<td>0.2121</td>
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<tr>
<td>12</td>
<td>0.2982</td>
<td>35</td>
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<tr>
<td>16</td>
<td>0.3615</td>
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<tr>
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<td>0.2360</td>
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<td>24</td>
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<tr>
<td>27</td>
<td>0.3565</td>
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<td>0.8495</td>
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<tr>
<td>28</td>
<td>0.3020</td>
<td>45</td>
<td>0.8449</td>
</tr>
<tr>
<td>29</td>
<td>0.3205</td>
<td>46</td>
<td>0.6509</td>
</tr>
<tr>
<td>30</td>
<td>0.3021</td>
<td>47</td>
<td>0.8107</td>
</tr>
<tr>
<td>31</td>
<td>0.3449</td>
<td>48</td>
<td>0.9738</td>
</tr>
</tbody>
</table>
APPENDIX J. PROGESTERONE CONCENTRATIONS OF SHEEP 2 VS MEAN PROGESTERONE OF SHEEP 1,3,4

Appendix J: Mean plasma progesterone concentrations of sheep 1, 3, & 4 vs individual plasma progesterone concentration of sheep 2 following three infusions of PGF$_{2\alpha}$. Bracketed arrows indicate infusions of PGF$_{2\alpha}$.
APPENDIX K. IRB (IACUC) FORM

Univerisy of Connecticut
Office of Research Compliance

Margaret J. Sekellick, Ph.D.
IACUC Chair
860-486-3616
margaret.sekellick@uconn.edu

DATE: June 23, 2008

TO: John A. McCracken
Department of Animal Science, Unit 4040

FROM: Margaret J. Sekellick, Ph.D.

SUBJECT: Notice of IACUC Approval

This letter serves as written notice of animal use APPROVAL by the IACUC. Please note that at the April 22, 2008 IACUC meeting, the IACUC determined that the protocol required modifications to secure approval that now have been addressed. Please refer to the assigned protocol number for all animal orders and future correspondence with the IACUC; cage cards should contain this protocol number. Please advise us in the future of any necessary corrections or revisions by completing the appropriate form at http://www.iacuc.uconn.edu/forms.html.

Please Note: All investigators are required to make study records available for inspection during normal business hours. If study records are kept in locked facilities, a member of the research staff must be designated as the official contact person for record inspection.

This institution has an Assurance of Compliance on file with the Office of Laboratory Animal Welfare, National Institutes of Health (Assurance #A3124-01, 2/28/12).

Funding Source: (1) USDA, (2) UCRF

All animal use protocols must be reviewed annually from the date of IACUC approval; you will receive an e-mail notice requesting an annual update. Thank you for your efforts to help keep the University in compliance with all animal welfare regulations.

Title: 1st Year: “Molecular Mediators of Luteolysis”

Species: Sheep

Date Approved: June 23, 2008-June 22, 2009

Protocol Number: A08-015

cc: Daniel Fletcher, Department of Animal Science/Unit 4040
Richard Simonello, Office of Animal Research Services/Unit 3150
Sheryl Lohman, Office of Animal Research Services/Unit 3150
William Field, Environmental Health and Safety/Unit 4097

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Whitney Road Extension Unit 1246
Storrs, Connecticut 06269-1246
Telephone: (860) 486-8802
Facsimile: (860) 486-1044
web: http://www.compliance.uconn.edu

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