DISSERTATION

PROSTAGLANDIN METABOLISM IN THE OVINE CORPUS LUTEUM

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In partial fulfillment of the requirements
for the Degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Spring 2000

COLORADO STATE UNIVERSITY

November 10, 1999

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY PATRICK JAMES SILVA ENTITLED: "PROSTAGLANDIN METABOLISM IN THE OVINE CORPUS LUTEUM" BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

PROSTAGLANDIN METABOLISM IN THE OVINE CORPUS LUTEUM

The goal of the research comprising this dissertation was to gain insight into the relative importance of luteal biosynthesis of prostaglandin and possible catabolism of $PGF_{2\alpha}$ by the corpus luteum. Expression patterns of two enzymes (cyclooxygenase-2 and 15-hydroxyprostaglandin dehydrogenase) important to prostaglandin synthesis and catabolism are described herein. On day 4 post estrus and day 13 postcoitus, the ovine corpus luteum is resistant to the luteolytic effects of $PGF_{2\alpha}$. Thus, the primary biological question that inspired this research is: "What mechanisms allow the corpus luteum early in the estrous cycle and early in pregnancy to be resistant to the luteolytic activity of $PGF_{2\alpha}$?". Two potential mechanisms were addressed, decreased synthesis of $PGF_{2\alpha}$ and catabolism of $PGF_{2\alpha}$ in the corpus luteum.

The first hypothesis tested was: "PGDH enzymatic activity is greater on day 4 of the estrous cycle and day 13 of pregnancy than on day 13 of the estrous cycle". Enzymatic activity of PGDH, measured by rate of conversion of $PGF_{2\alpha}$ to PGFM, was greater in corpora lutea on both day 4 of the estrous cycle ($p=0.03$) and day 13 of pregnancy ($p=0.05$) than on day 13 of the estrous cycle.

The inability of the corpus luteum to produce $PGF_{2\alpha}$ may also play a role in resistance of the corpus luteum to the luteolytic actions of uterine $PGF_{2\alpha}$. Thus, the

second hypothesis tested was that expression of mRNA encoding COX-2 will be attenuated on day 4 of the estrous cycle and day 13 of pregnancy relative to day 13 of the estrous cycle". Messenger RNA encoding COX-2 was undetectable (<3 amol/ μg poly A^+ RNA) in corpora lutea from ewes on day 13 of the cycle. In corpora lutea from ewes on day 4 of the estrous cycle, levels of mRNA encoding COX-2 were 43.77± 27.75 amol/μg poly A^+ RNA, while on day 13 of pregnancy levels of mRNA encoding COX-2 were 10.57± 4.41 amol/μg poly A^+ RNA.

The results of this research suggest that a novel mechanism, enhanced catabolism of $PGF_{2\alpha}$, plays a role in resistance of the corpus luteum to the luteolytic activity of $PGF_{2\alpha}$. The fact that increased PGDH activity occurs when resistance of the corpus luteum to the luteolytic activity of $PGF_{2\alpha}$ is known to occur is an exciting advance in understanding luteal physiology and adaptations in luteal function during early pregnancy.

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ACKNOWLEDGEMENTS

There are many people that deserve credit and recognition; this work was as much a result of their efforts and sacrifice, as it was mine. First and foremost, I wish to acknowledge Danette, the love of my life for her endless support and patience, she has made the rigors of the past year more pleasant. I thank my parents for being supportive and encouraging, even when they didn't understand the nature of my discouragements and triumphs. My two younger brothers, Greg and Dusty, deserve credit; they helped inspire me to be the best. I would also like to thank the best 'roomdog' I ever had, just because he wants to be mentioned! Without the friendship of BJ Shipp, I would have driven myself insane long ago; he taught me the value of relaxing, laughing, and seeing the lighter side of things. These are the people that tolerated me during difficult times, and that was perhaps the hardest work of all!

Many of my colleagues also deserve credit because their efforts contributed to my success. Dr. Jenny Juengel and Bernadette Meberg were a tremendous asset to all of the graduate students at ARBL, they were technically talented and were always willing to share their expertise, no matter how busy they might be. Dr. Eric McIntush and Dr. Jenny Juengel played the most sustained and influential role in my development as a scientist, they are the ones that ensured that I was always thinking, that I 'dotted every *i*', and 'crossed every *t*'. I thank them for their intellectual contribution to each of my

endeavors, but most of all, I thank them for their friendship. Dr. Hans Mayan was never in a bad mood and that was a rare quality I admired. Hans, thanks for always providing luteal cells on demand and helping with surgery when we were shorthanded. The staff of the endocrine lab analyzed countless blood and culture media samples; I thank Xaoming Sha, Kit Sutherland, Tina Gardner, and Bonnie Martin. I thank Kathy Thomas for protecting me from the mountains of paperwork required for my job, and especially ensuring I got my paycheck when the bureaucrats forgot about me. Ruth Howard was the most helpful and pleasant person I had the privilege of meeting while here. Ruth made my life so much easier; I admire and respect the pride and professionalism that characterized her. Dr. Dave Haworth and Keith Rollyson looked out for me and showed me the ropes until I became accustomed to the workings of the lab. I'll always remember the chats Keith and I would have while bleeding sheep at 4 AM when it was 10 below outside. Ironically, these will be the some of the warmest memories of my graduate school experience. Mike Gallegos was always willing and helpful to provide sheep for experiments, perform surgery, prepare sheep for surgery, was never afraid to get down and dirty wrestling sheep for tissue collection, and finally graciously allowing me to heat check when it was 30 below outside! Mike is a hell of a good hand. I also appreciate the friendship of Jennifer Malvey-McCallum and Willie Mellion. Jeremy Adler deserves thanks for letting me crash at his place for almost a year. One of the most therapeutic activities I engaged in during my tenure here at CSU was sneaking off work early, every couple months, with Sean Limesand and Ryan Orbus to get in 9 holes before the sun set.

I also thank the whole crew in the animal science department for adopting me and helping me get through statistics. Henry Zerby, Julian Garcia, Jason Ahola, John Evans,

and Eric Downing have all been great friends and colleagues. Dr. Ronnie Green was always a source of encouragement and is a tremendous asset to the university, not just his department; he even talked my boss into letting me work cattle when I needed a break from the lab! Dr. Jason Bruemmer has been a friend and colleague since my sophomore year of college at New Mexico State University. After all of the big experiments, cruel practical jokes, water fights, ski trips, and constant harassment, I still respect him and consider him one of my closest friends.

I would also like to thank Dr. Milo Wiltbank and the students and staff in his laboratory at the University of Wisconsin at Madison. Dr. Wiltbank was instrumental in measurements of mRNA encoding COX-2 and assisted in developing the QC-RT-PCR assay for mRNA encoding PGDH.

Finally I would like to recognize a distinguished group of scholars that provided me with a superior education, the faculty of ARBL and members of my graduate advisory committee. My advisor, Dr. Gordon Niswender, ensured that I thought critically and demanded a standard of work that would never be labeled inferior or substandard, if it passed his scrutiny. He taught me to be the very best and nothing less is acceptable. These lessons will ensure my success and prosperity in the future. Dr. Russ Anthony, Dr. Heywood Sawyer, Dr. Jack Whittier, and Dr. Alan Tucker have all been invaluable as committee members and I thank them for their time and effort, which was plentiful despite their busy schedules. I would also like to thank some other faculty at ARBL that contributed significantly to my intellectual development. Dr. George Siedel and Dr. Terry Nett were not on my committee; however, their contributions to my research and my understanding of the most fundamental principles of reproductive biology were as

valuable as the contributions of my committee. Dr. Siedel is one of the great, legendary intellects in reproductive biology and it was a great privilege to attend his lectures and study in his classroom. Dr. Nett was patient and deliberate in teaching me proper surgical methodology and the sheep thank him!

My experiences at ARBL will prove to be valuable and useful throughout the remainder of my professional life. I appreciate the hard work, tolerance, patience, and generosity of those who made my success possible.

DEDICATION

This dissertation is dedicated to my late grandfather, James Ernest Seymour. He taught me the value of hard work, discipline, and perseverance. He taught me to never give up. He provided me with a foundation of values and traditions that inspired me to excel, not just succeed. Without his encouragement and the confidence he instilled in me, I would have never have been capable of realizing this triumph.

TABLE OF CONTENTS

TABLE OF FIGURES

Chapter 1

Review of the Literature

Introduction to the Corpus Luteum

The corpus luteum is a transient endocrine organ that secretes progesterone, a steroid hormone essential for growth (Owens, 1991; Geisert et al., 1992) and survival (Fraenkel, 1903) of all mammalian embryos. Coiter (1573) first described the corpus luteum in the 16th century. Coiter described yellow structures enmeshed in the human ovary but never assigned functionality to these structures. However, Regnier de Graff (1641-1673; 1672) later described these structures in rabbits and noted that the number of structures was usually related to the number of fetuses *in utero*. Soon after de Graff's studies, Marcello Malpighi (1628-1694; 1689) noted the structures as well, and named the structure the corpus luteum (Latin meaning: yellow body; yellow = luteum, body = corpus).

Biological Importance of the Corpus Luteum

At the dawn of the twentieth century, interest in the corpus luteum intensified. Two centuries after Malpighi's work, Beard (1897) recognized that the corpus luteum was present throughout pregnancy and was coincident with an absence of ovulatory and estrous activity. These observations led Beard to postulate that the corpus luteum somehow suppressed ovulation and estrus. Working independently, Prenant (1898) hypothesized that the corpus luteum had a glandular function based on its histological

characteristics. Three years later, Fraenkel (1901, 1903) and Magnus (1901) demonstrated that removal of ovaries or corpora lutea in pregnant rabbits resulted in abortion of the embryos. If Fraenkel and Magnus had been using sheep after 50 days of pregnancy (Casida and Warwick, 1945) as their experimental subjects, they would have likely generated dramatically different and erroneous conclusions about the biological role of the corpus luteum. By the turn of the century it was clear that the corpus luteum was a gland that secreted a substance that facilitated pregnancy. Magnus (1901) suggested supplementation of pregnant, ovariectomized rabbits with extracts from corpora lutea to confirm these suppositions. These experiments were conducted, thirty years later, by Allen and Corner (1929; 1930) who used an alcohol soluble extract from the corpora lutea of sows which was capable of maintaining pregnancy in ovariectomized rabbits. Four years later the elusive biological agent was crystallized and characterized by four independent research laboratories (Allen and Wintersteiner, 1934; Hartmann and Wettstein, 1934; Slotta et al., 1934; Butenandt et al., 1934a; Butenandt et al., 1934b). The structure of the factor was determined and it was named progesterone by Slotta et al. (1934). Subsequently, Butenandt and Westphal (1934) synthesized a biologically active preparation of progesterone.

Biological Actions of Progesterone

The biological functions of progesterone during pregnancy are myriad. Progesterone acts primarily in the uterus and the hypothalamic-pituitary axis. In general, progesterone serves to prepare and maintain the reproductive tract for pregnancy, facilitates mammary development, suppresses milk synthesis, and hypothalamic-pituitary stimulation of ovulatory processes (Reviewed in Graham and Clarke, 1997). There is

also a significant body of evidence that progesterone has a role in bone remodeling (Reviewed by Prior, 1990), but this function will not be explored further in this dissertation.

Progesterone acts directly at the level of the nucleus in target cells by strategically regulating the transcription of genes. The mechanism of progesterone action is similar to that utilized by other steroids: activation of nuclear receptors that are ligand-inducible transcription factors (Reviewed in Tsai and O'Malley, 1994; see review for primary references). Upon binding of progesterone to its receptor, the receptor initiates a complex sequence of events termed *transformation*, which culminates in activation of the receptor-ligand complex. Activation is defined as the ability of the receptor ligand complex to DNA and modulate transcription of target genes. In the absence of progesterone, progesterone receptors are associated with heat shock proteins (HSP) HSP56, HSP 70, and HSP 90 (Reviewed in Tsai and O'Malley, 1994) and are therefore unable to bind DNA; i.e. unable to stimulate or suppress transcription. Upon binding of progesterone, the receptor dissociates from the HSPs, dimerizes, and becomes active. Progesterone receptor dimers, bound to progesterone, then associate with progesterone response elements on DNA and regulate the transcription of target genes.

In most tissues, estrogen priming is required for progesterone responsiveness (Rosser et al., 1993). Estrogens, especially estradiol-17β, upregulate progesterone receptors via estrogen receptor-stimulated transcription of progesterone receptor messenger ribonucleic acid (mRNA; Ing and Tornesi, 1997; Kaneko et al., 1993; Kraus and Katzenellenbogen, 1993). However, progesterone indirectly down-regulates its own receptors, and consequently progesterone responsiveness, in target tissues. Progesterone

also down-regulates estrogen receptors (Brenner et al., 1974; Evans and Leavitt, 1980; West et al., 1987) and estrogen receptor mRNA (Evans and Leavitt, 1980, Iwai et al., 1995), thus removing the stimulus for progesterone receptor synthesis. Many tissues outside the central nervous system gradually experience a decline in sensitivity to progesterone after prolonged exposure. Progesterone also serves to desensitize tissues to the biological actions of estrogens.

Progesterone acts in a variety of reproductive tissues in mammals, including endometrium (Perrot-Applanat et al., 1985; Clarke et al., 1987; Garcia et al., 1988; Lessey et al., 1988; Press and Greene, 1988; Press et al., 1988), myometrium (Press et al., 1988; Janne et al., 1976), granulosa cells (Duffy and Stouffer, 1995; Parke-Sarge et al., 1995; Reviewed in Pinter et al., 1996), corpus luteum (Parke-Sarge et al., 1995; Reviewed in Pinter et al., 1996), and oviduct (Brenner et al., 1974; Brenner et al., 1983; Sawyer et al., 1984). In the endometrium, progesterone elicits differentiation of stromal and epithelial cells and a variety of morphological changes to facilitate pregnancy (Reviewed in Cummings and Yochim, 1984). Estrogens secreted by the developing follicle stimulate mitotic activity in the uterine epithelial cells (Clarke, 1971; Tachi et al., 1972); however, the onset of elevated progesterone suppresses uterine proliferation (Bigsby and Cunha 1988; Padykula et al., 1989). Progesterone also stimulates differentiation of stromal cells (Moulton and Koenig, 1984). Thus, progesterone inhibits proliferation of epithelial cells but stimulates proliferation of stromal cells (Clarke 1971). Some characteristics of stromal differentiation include accumulation of vacuoles in the glandular epithelium (Maslar et al., 1986), changes in DNA synthesis (Moulton and Koenig, 1984), and changes in endometrial secretory activity (Strinden and Shapiro,

1983; Maslar et al., 1986). In the human, progesterone promotes decidualization of the uterus and differentiation of stromal cells into polynuclear tetraploid decidual cells (Moulton and Koenig, 1984). In general, progesterone acts to alter the glandular structure and secretory activity of the endometrium in preparation for pregnancy.

Progesterone has an important biological effect on the myometrium during pregnancy; it causes suppression of muscular activity. Progesterone promotes myometrial quiescence by preventing electrical coupling of myometrial cells (Parkington 1983). In addition, progesterone inhibits calcium uptake by myometrial cells (Batra, 1986) by suppression of genes encoding subunits of voltage gated calcium channels (Tezuka et al., 1995). Moreover, progesterone induces expression of calcitonin in the myometrium (Ding et al., 1994); calcitonin is believed to lower intracellular calcium levels in the myometrium. Progesterone also blocks estradiol-induced expression of alpha-adrenergic receptor subunits (Bottari et al., 1983) and stimulates expression of beta-adrenergic receptors (Vivat et al, 1992), both effects that reduce the excitability of the myometrium. Finally, progesterone acts to prevent prostaglandin $F_{2\alpha}$-induced myometrial contraction (Fuchs and Fuchs, 1984; Graham and Clarke, 1997). Progesterone interferes with estradiol-induced biosynthesis of $PGF_{2\alpha}$ in the endometrium (Abel and Baird, 1980; reviewed in Silvia et al., 1991) and stimulates 15-hydroxyprostaglandin dehydrogenase (PGDH) activity in the myometrium (Thornburn and Challis, 1979; reviewed in Okita and Okita, 1996). Prostaglandins of the E and F series are rendered biologically inactive by PGDH (reviewed in Okita and Okita, 1996). More discussion of steroidal regulation of prostaglandin metabolism in reproductive tissues will appear later in this dissertation. In general, progesterone acts to reduce

myometrial excitability by inhibiting the stimulatory inputs and the cellular and molecular mechanisms of myometrial contraction.

In equids, 5-alpha-reduced progestogens of placental origin, not progesterone, are postulated to be responsible for promoting myometrial quiescence (Thornburn, 1993). Ovariectomy in the mare after day 70 does not result in abortion (Holtan et al., 1979). Thus, it appears that progesterone secretion of luteal origin is important for a limited time, until placental progestogen secretion can provide adequate progestational support. In the ewe, progesterone of placental origin appears to take the place of luteal progesterone after 50 days of pregnancy (Ricketts and Flint, 1980).

A role for progesterone has been proposed in granulosa (Greenberg et al., 1990) and luteal (Park-Sarge et al., 1995) cells. Progesterone is hypothesized to play a role in the differentiation of granulosal to luteal phenotype (Greenberg et al., 1990). Consistent with this notion, it appears that progesterone receptor expression is induced by the preovulatory LH surge in the granulosa cells of preovulatory follicles (Park and Mayo, 1991). Furthermore, expression of progesterone receptors is cAMP mediated (Clemens et al., 1998). It is unclear what genes are regulated by progesterone in granulosa cells. Levels of the A-isoform of progesterone receptor gradually decline throughout the menstrual cycle in macaques (Duffy et al., 1997). Progesterone receptors appear to be induced by progesterone in the corpora lutea of macaques (Duffy and Stouffer, 1995), in contrast to the effects of progesterone in other tissues. Interestingly, binding sites for progesterone have been localized to the plasma membranes of luteal and granulosa cells, suggesting a nongenomic role for progesterone in ovarian steroidogenic cells (Rae et al., 1998).

In the oviduct, progesterone has an effect on ovum transport mechanisms and secretory activity of the oviductal epithelium; i.e. quiescence of estrogen-induced oviductal activity. Progesterone inhibits oviductal capacity to transport the ovum by signaling deciliation (Brenner et al., 1983; Sawyer et al., 1984) of the oviductal epithelium. Progesterone also prevents estrogen-stimulated protein secretion in the baboon oviduct (Verhage and Fazleabes, 1988). Biologically, this is logical because during the mid-luteal phase, the oocyte is no longer in the oviductal environment and secretory activity and movement of cilia are no longer necessary. Progesterone possibly has secondary roles, as a chemotactic agent for sperm (Villanueva-Diaz et al., 1995) and induces sperm hyper-activation (Jaiswal et al., 1999).

In summary, progesterone acts in a variety of tissues to prepare the female reproductive tract for pregnancy (reviewed in Graham and Clarke, 1997). Throughout pregnancy, the female physiological milieu is regulated by progesterone to support pregnancy. In the absence of progesterone, pregnancy is terminated.

Progesterone also acts at the hypothalamic-pituitary axis to indirectly and directly regulate the estrous cycle and estrous behavior. To describe the role of progesterone in regulating the hypothalamic-pituitary neuroendocrine pathway, a brief description of the roles of the hypothalamus and pituitary gland in reproductive processes is merited.

During the follicular phase of the estrous cycle, concentrations of estradiol are increasing and enhancing pituitary responsiveness to gonadotropin releasing hormone (GnRH; reviewed in Leung and Peng, 1996; Anderson, 1996). Increased pituitary responsiveness to GnRH is due to increased expression (Turzillo et al., 1998a; Kirkpatrick et al., 1998a) and surface density (Nett et al., 1981; Khalid et al., 1987) of

GnRH receptors in gonadotroph cells. The sum of these effects on the GnRH system is that estradiol-17β stimulates the release of LH, and to a lesser extent, FSH (Reeves et al., 1981). The pattern of release of LH during the follicular phase is low amplitude, high frequency pulses (reviewed in Tonetta and diZerega, 1989). This pattern of LH secretion drives follicular development (in essence, rescues the dominant follicle from atresia; reviewed in Fortune, 1994; Lucy et al., 1992). As follicular development progresses, increasing quantities of estradiol-17β stimulate pituitary release of high amplitude pulses of LH culminating in the LH surge (reviewed in Fortune et al., 1991).

Progesterone acts at the level of the pituitary gland and the hypothalamus to suppress GnRH stimulated LH secretion and attenuate expression of receptors for GnRH (Laws et al., 1990; Bauer-Dantoin et al., 1995; Kirkpatrick et al., 1998b; Turzillo et al, 1998b). Progesterone also inhibits secretion of GnRH from neurons in the hypothalamus (Attardi and Happe, 1986; Kasa-Vubu et al., 1992). A nongenomic mechanism of progesterone action is inhibition of Ca^{++} mobilization in cultured pituitary gonadotrophs (reviewed in Wiebe, 1997). Influx of calcium into gonadotrophs is a known mechanism of GnRH's signal transduction pathway (reviewed in Stojilkovic et al., 1994). The sum of these actions of progesterone results in attenuated pituitary responsiveness to GnRH manifest as decreased LH secretion (Janovick and Conn, 1996).

Progesterone modulates GnRH-stimulated gonadotropin secretion and production of gonadotropins. Expression of the β subunits of both FSH (Brann et al., 1993;) and LH (Brann et al., 1993; Digregorio and Nett, 1995), and the common α subunit (Brann et al., 1993; Digregorio and Nett, 1995) is suppressed in the presence of progesterone. However, progesterone, in some situations, facilitates gonadotropin secretion (Attardi and

Happe, 1986; Brann and Mahesh, 1991; Bauer-Dantoin et al., 1995). In additional to actions in reproductive tissues, it appears that progesterone plays a key role in modulated reproductive functions in the central nervous system and pituitary gland.

Importance of Luteal Physiology- Animal Agriculture

The National Animal Health Monitoring System (NAHMS) reported that infertility in cattle costs producers $381.9 million in 1996 (USDA, 1996). Costs to beef producers totaled $249 million while costs to dairy producers totaled $132.9 million. This report identifies infertility in cattle as a major economic problem and a significant factor in the efficient production of food. The NAHMS report defines infertility as an inability to become and remain pregnant upon first service. There are two primary causes of infertility, failure to achieve fertilization and early embryonic loss. Casida (1953) estimated that 50 percent of matings result in failed fertilization or embryonic loss. Roche (1981) reported that up to 40 percent of bovine embryos are lost by day 25 of pregnancy. Roche did not identify the physiological mechanism of embryonic death; however, he proposed that spontaneous regression of the corpus luteum is involved.

Supplementation of cows with exogenous progesterone during early pregnancy decreases the incidence of early embryonic mortality (Reviewed in Diskin and Sreenan, 1986; Zavy, 1994). Changes in embryonic mortality range from a 13 percent reduction (Macmillan et al., 1991) to a 30 percent reduction (Wiltbank, 1956). It is evident that inadequate secretion of progesterone by the corpus luteum or untimely luteal regression during early pregnancy accounts for a significant percentage of embryonic wastage in cattle.

The economic losses derived from embryonic loss are the direct or indirect result of: delayed return to estrus, nonpregnant females, late calves, and decreased weaning weights. There is a loss of 4 million pounds of beef production (in the US alone) that would occur *at no added cost*! In addition to loss of beef production, similar losses occur in the dairy industry.

Importance of Luteal Physiology- Human Medicine

The corpus luteum merits study because of its relevance to two important issues in human medicine: infertility and cancer. The importance of the corpus luteum in infertility is evident based upon the preceding discussion. Embryonic loss in humans is perhaps more prevalent than it is in domestic ruminants. In the United States, 61.9 percent of human conceptuses are lost before 12 weeks of pregnancy (Edmonds et al., 1982), 91.7 percent of these pregnancies are undetected by the mother.

The corpus luteum is an excellent model to study basic biological processes involved in tumor proliferation: apoptosis (reviewed by Amsterdam et al., 1998), rapid mitosis, and angiogenesis (reviewed in Augistin et al., 1995; Neeman et al., 1997). For example, the rate of cell proliferation in the corpus luteum exceeds that of the most rapidly proliferating tumors (Jablonka-Shariff et al., 1993). Angiogenesis is a biological process that is similar between luteal development and tumor development; this will be discussed in detail in the next section.

Luteal Development

The Follicle

Since the corpus luteum is formed from cells of the follicle, it is prudent to briefly discuss the structure and function of the precursor tissue to the corpus luteum. The

Graafian, or preovulatory follicle consists of three distinct compartments: the thecal layer, the granulosal layer, and the antrum.

The thecal layer is subdivided into the theca interna and the theca externa. The thecal layer is a vascularized tissue fixed to a collagenous basement membrane (reviewed in Monniaux et al., 1997). The primary function of cells of the theca interna is to produce androgens that granulosa cells ultimately convert to estrogens (reviewed in Richards et al., 1987; Bao and Garverick, 1998). A basement membrane provides a barrier between the blood supply and the granulosa compartment.

The granulosa cell layer is an avascular layer of follicular cells enclosing the antrum of the follicle. Granulosa cells can be of several subtypes in the antral follicle, depending on their localization relative to the oocyte. Granulosa cells are actively involved in estrogen biosynthesis (reviewed in Bao and Garverick, 1998), progesterone biosynthesis (reviewed in Bao and Garverick, 1998), and oocyte maturation (reviewed in Buccione et al., 1990).

Theca and granulosa cells coordinate to produce estrogens (Henderson and Swanston, 1978; reviewed in Fortune and Quirk, 1988). Theca cells possess the steroidogenic machinery (cytochrome P450 sidechain cleavage/adrenodoxin enzyme complex [P450 SCC], 3β-hydroxysteroid dehydrogenase/Δ^5,Δ^4-reductase [3β-HSD], and cytochrome P450 17α-hydroxylase [P450c17]) to produce androgens. However, theca cells are devoid of P450 cytochrome aromatase (P450arom) activity (reviewed in Fortune and Quirk, 1988; Bao and Garverick, 1998), the enzyme complex that catalyzes conversion of androgens to estrogens. Conversely, granulosa cells are devoid of

P450c17, therefore cannot produce androgens from progestogens (Conley et al., 1995). Androgens are transported from the theca to the granulosa cells, which do express P450arom, and are converted to estrogens (Henderson and Swanston, 1978). Granulosa cells have the capacity to synthesize progesterone; however, granulosa cells do not express all of the steroidogenic components until they begin to assume a luteal phenotype. In support of this assertion, mRNA encoding the steroidogenic acute regulatory protein (StAR) is not expressed in granulosa cells until luteinization occurs after the preovulatory LH surge (Bao et al., 1998). Furthermore, mRNA encoding $P450_{SCC}$ is unregulated in response to hCG in the rat corpus luteum (Goldring et al., 1987). Finally, expression of messenger RNA encoding 3β-HSD is confined to the theca interna of preovulatory follicles of sheep (O'Shea et al., 1980) and rats (Dupont et al, 1990) until the periovulatory period (Hay and Moore, 1975; Dupont et al., 1990).

Ovulation and Luteinization

As a result of the preovulatory LH surge, the follicle ruptures, releasing the ovum into the infundibulum leading to the oviduct. The cells remaining in the ruptured follicle differentiate, and change in phenotype (reviewed in Smith et al., 1994; Richards et al., 1998), eventually gaining the capacity to secrete large quantities of progesterone. In fact, the granulosa cells of the follicle gain the capacity to secrete progesterone prior to ovulation (McNatty and Sawers, 1975; McNatty, 1979). Estradiol-17β and FSH are partially responsible for initiating the differentiation of granulosa cells via induction of expression of LH receptors late in follicular development (Richards et al., 1980). The differentiation of the follicular cells and obtaining the capacity to secrete high amounts of progesterone is termed *luteinization.*

In response to the preovulatory surge of LH, cells of the follicle have decreased capacity to produce androgen and estrogens due to decreased expression of P450c17 and $P450_{arom}$ (Voss and Fortune, 1993a,b). In a biological context, this is reasonable; the LH surge facilitates luteinization, and therefore, facilitates a shift in steroid production from androgens and estrogens to progesterone.

Another significant event associated with luteinization is degradation of the basement membrane (reviewed in Smith et al., 1994). The basement membrane consists of several structural proteins including laminin, fibronectin, and especially type IV collagen and is hypothesized to be degraded by proteolytic enzymes (i.e. metalloproteinases; Smith et al., 1994). Degradation of the basement membrane allows migration of endothelial cells and fibroblasts into the granulosa layer of the ruptured follicle (Cavander and Murdoch, 1988; Forsman and McCormack, 1992).

Luteinization is a very complex biological process that bears many similarities to tumor growth. The avascular follicular antrum becomes quite vascularized during luteinization (reviewed in Grazul-Bilska et al., 1997; Neeman et al., 1997). The vascular remnant of the follicle is called the corpus hemmoragicum. First, the capillaries of the forming corpus luteum invade the intercellular spaces of the luteinized follicular cells (reviewed in Redmer and Reynolds, 1996). Then the microcapillary network of the luteal parenchyma develops (Zheng et al., 1993). In fact, 75 percent of cells in the corpus luteum are vascular cells (Azmi et al., 1984) and 20 percent of the volume of the corpus luteum is capillary lumen (Doraiswamy et al., 1995; Dharmarajan et al., 1985). Consistent with this, nearly all parenchymal luteal cells are in contact with capillaries in the midcycle corpus luteum (Zheng et al, 1993; Dharmarajan et al., 1985). In fact, an

average of 60 percent of the surface of steroidogenic luteal cells is adjacent to capillaries (Dharmarajan et al., 1985). The midcycle corpus luteum receives a greater blood flow than any other organ in the body (Bruce and Moor, 1976; Niswender et al., 1976) on a mass basis (6-10 ml blood/g tissue/min). This rate of blood flow allows the corpus luteum to consume far more oxygen than any other tissue in the body (197 ml/min/kg vs. 94 for heart and 33 for brain; Swann, 1987).

Capillary infiltration involves mitosis and migration of endothelial cells. There are many mitogenic and angiogenic factors involved in angiogenesis of the corpus luteum: basic fibroblastic growth factor (bFGF; Gospodarowicz et al., 1985; Neufeld et al., 1987; Stirling et al., 1991; Grazul-Bilska et al., 1992), heparin-binding growth factor-2 (HBGF-2 is also known as bFGF, while HBGF-1 is also known as acidic FGF [aFGF]), platelet-derived growth factor (PDGF; Khachigain et al., 1996), and vascular endothelial growth factor (VEGF; reviewed by Redmer and Reynolds, 1996).

Basic FGF (or HBGF-2) is produced by the corpus luteum (reviewed in Reynolds et al, 1992; Grazul-Bilska et al., 1992b), among other tissues (reviewed in Klagsbrun and D'Amore, 1991), and probably acts locally in the corpus luteum (Grazul-Bilska et al., 1992b; Zheng et al., 1993). Heparin binding growth factors are hypothesized to be the primary angiogenic agents in the corpus luteum (Reynolds et al., 1994). Heparin binding growth factor-1 is also produced by the corpus luteum but likely plays a minor role in luteal angiogenesis relative to HBGF-2 (Zheng et al., 1993). Furthermore, greater levels of HBGF-2 expression occur at the stage of luteal development in which angiogenesis is progressing at the greatest rate (Zheng et al., 1993).

Platelet-derived growth factor (PDGF) is a cytokine produced by platelets and fibroblasts that promotes endothelial cell proliferation and chemotaxis (reviewed in Miyazono and Takaku, 1991). It is reasonable that PDGF exerts this effect during luteal angiogenesis; however, this has not been investigated. Platelet derived growth factor has also been shown to stimulate progesterone secretion from cultured mixed luteal cells, *in vitro* (Batista et al., 1989), supporting a nonangiogenic role of PDGF.

Vascular endothelial growth factor (VEGF) enhances mitotic rates in endothelial cells (reviewed in Ferenra, 1993; Ferenra et al., 1999), and specifically, endothelial cells of the corpus luteum (Ferenra et al., 1998). Expression of VGEF is induced by LH in granulosa cells (Garrido et al., 1993; Koos, 1995). Ablation of bioactive VEGF results in complete inhibition of luteal angiogenesis and ischemic necrosis in the developing corpus luteum (Doraiswamy et al., 1995; Ferrara et al., 1998). These observations contradict the hypothesis forwarded by Reynolds et al. (1994), who proposed that HBGF's are the primary angiogenic agents during luteal development.

In summary, the importance of angiogenesis in luteal development becomes apparent when reflecting on the luteal growth and cellular proliferation that occurs over such a short period. A better understanding of these angiogenic mechanisms might foster the development of technologies that allow manipulation of luteal development or tumor growth, the implications of which are immense.

Cellular Composition of the Corpus Luteum

The corpus luteum is comprised of five primary types of cells: two types of steroidogenic parenchymal cells (reviewed in Niswender and Nett, 1994;Wiltbank, 1994; Smith et al., 1994): 1) large luteal cells and 2) small luteal cells, and 3) endothelial cells,

4) fibroblasts, and 5) pericytes. Large and small luteal cells are hypothesized to be derived from follicular granulosa and theca cells, respectively (Donaldson and Hansel, 1965; McClellan et al., 1975; O'Shea et al., 1980). Consistent with this, the number of granulosa cells within preovulatory follicles (O'Shea et al., 1985) is approximately the same as the number of large luteal cells in the corpus luteum (Rodgers et al., 1984; O'Shea et al., 1986). Furthermore, monoclonal antibodies raised against surface antigens of granulosa cells bind primarily to large luteal cells (Alila and Hansel, 1984), suggesting that granulosa and luteal cells at least express similar cell surface antigens. It has also been proposed that small luteal cells can differentiate into large luteal cells (Warbritton, 1934; Enders and Lyons, 1964; Donaldson and Hansel, 1965). There is experimental evidence supporting this view: administration of superphysiological doses of LH increases the number of large luteal cells in the corpus luteum and stimulates the appearance of cells intermediate in size between large luteal cells and small luteal cells (Farin et al., 1986). However, this apparent differentiation of small luteal cells into large luteal cells is not observed upon administration of LH at physiological concentrations (Farin et al., 1990). More recently, it has been proposed that large luteal cells can differentiate into small luteal cells (Fisch et al., 1989). Finally, it has been proposed (Niswender et al., 1985a) that the corpus luteum contains undifferentiated stem cells, intermediate in phenotype between fibroblasts and small luteal cells (O'Shea et al., 1980) that can differentiate into small luteal cells that can further differentiate into large luteal cells. In conclusion, the follicular lineage of steroidogenic luteal cells is still quite controversial and inconclusive.

The development of the corpus luteum involves rapid cell proliferation and a dramatic increase in tissue mass. In fact the corpus luteum increases 25-30-fold in mass in just 10 days (Farin et al., 1986; Jablonka-Shariff et al., 1993). Small luteal cells, fibroblasts, and endothelial cells have the greatest mitotic rate during luteal development (McClellan et al., 1975) while large luteal cells increase in size with little division (Farin et al., 1986); large luteal cells are limited to hypertrophy (McClellan et al., 1975). From day 4 to day 16 of the ovine estrous cycle, the numbers of small cells, endothelial cells and fibroblasts increase 5-fold, 6.5-fold and 2-fold, respectively (Farin et al., 1986).

There are a variety of ultrastructural changes in luteal cells during luteinization which allow for the secretory and steroidogenic functions of the corpus luteum throughout the estrous cycle. During luteinization, there is an increase in the number and density of several organelles within large luteal cells, including: Golgi apparatus, smooth endoplasmic reticulum, and especially mitochondria (Priedkalns et al., 1968; Cavazos et al., 1969; Fawcett et al., 1969; Enders et al., 1973; McClellan et al., 1975; Sawyer, 1995). The increases in mitochondrial numbers and density probably facilitate the high level of progesterone biosynthesis (discussed in detail in the next section) that occurs in the large luteal cells of the midcycle corpus luteum (Kenny et al., 1989). Analysis of luteal tissue sections by electron microscopy has revealed the presence of secretory granules in large luteal cells but not in small luteal cells (Sawyer, 1995). These secretory granules contain oxytocin (Theodosis et al., 1986; Fields et al., 1992), metalloproteinases (Goldberg et al., 1996), inhibitors of metalloproteinases (Goldberg et al., 1996; McIntush et al., 1996), and inhibin and relaxin in some nonruminant species (reviewed in McCracken et al., 1999). Specifically, relaxin-containing secretory granules have been localized in large luteal

cells of the rat (Goldsmith et al., 1981; Fields et al., 1992), human (Weiss, 1981), and pig (Sherwood and O'Bryne, 1974). Interestingly, secretory granules are distributed differently in the large luteal cells of sheep and cows (reviewed in Sawyer, 1995). Small luteal cells of the ovine corpus luteum contain lipid droplets while large luteal cells are devoid of lipid droplets (Sawyer, 1995), possibly because the high basal level of progesterone biosynthesis in large luteal cells prevents lipid accumulation. In contrast, both steroidogenic cell types in the bovine corpus luteum contain lipid droplets (Sawyer, 1995). In general, the microanatomical features of the corpus luteum are dynamic and evolve in a manner consistent with the function of the gland throughout the luteal lifespan.

The number of endothelial cells increases throughout the estrous cycle in the ovine corpus luteum until luteolysis commences (Farin et al., 1986). In fact, the number of endothelial cells in the ovine corpus luteum increases four-fold between days 4 and 8 of the estrous cycle and increases an additional two-fold between days 8 and 16 but decreases in regressing day 16 corpora lutea (Farin et al., 1986). These observations are consistent with the importance of a well-developed vascular system in corpus luteum function (Doraiswamy et al., 1995; Ferrara et al., 1998). One interesting aspect of the luteal vasculature is the high degree of fenestration of the capillaries making them permeable to many large proteins (Ellinwood et al., 1978) and some immune cells (Tizard et al., 1992).

Fibroblasts are elongated cells that remain static in morphology and size throughout the estrous cycle. Their role in luteal development and function has not been extensively investigated, but it has been hypothesized that they serve as a pool of stem

cells that differentiate into small luteal cells during luteal development (Niswender et al., 1985a).

Several types of immune cells have been identified in the corpus luteum but are normally present in small numbers in all phases of the luteal lifespan except luteolysis (reviewed in Pate et al., 1994). Eosinophils and macrophages are the primary types of immune cells found in the corpus luteum. Immune cells are probably involved in mediating luteolysis and thus will be discussed in a later section.

Progesterone Biosynthesis

The synthesis of progesterone requires the least number of enzymatic steps (two) of any steroid hormone. First, the side chain of cholesterol is cleaved by the cytochrome P450 sidechain cleavage (P450 SCC)/ adrenodoxin/adrenodoxin reductase enzyme complex (Stone et al., 1954) to form pregnenolone. This reaction, catalyzed by P450 SCC, occurs in three monoxygenations and finally cleavage of the C-20, 22 bond (reviewed in Hanukoglu, 1992). This enzyme complex is localized to the matrix face of the inner mitochondrial membrane (Yago and Ichii, 1969). Then pregnenolone is converted to progesterone by the 3β-hydroxysteroid dehydrogenase/Δ^4, Δ^5-isomerase enzyme complex (reviewed in Hanukoglu, 1992).

Cholesterol is the substrate for biosynthesis of all steroid hormones including progesterone. The liver is the primary site of cholesterol biosynthesis (reviewed in Gwynne and Strauss, 1982; Krisans, 1996); however, cholesterol can be synthesized *de novo* in steroidogenic tissues (Hechter et al., 1953; reviewed in Johnson et al., 1997). In the liver and steroidogenic tissues, cholesterol is synthesized in a series of 11 enzymatic reactions (reviewed in Krisans et al., 1996). The rate-limiting step in *de novo* cholesterol

synthesis is catalyzed by HMG CoA reductase (reviewed in Beg et al., 1987). Cholesterol is synthesized from acetate in the corpus luteum (Cook et al., 1967; Kaltenbach et al., 1968; Cook and Kaltenbach, 1968) in the absence of blood borne lipoprotein.

Circulating lipoproteins are the primary source of cholesterol for progesterone biosynthesis (reviewed in Gwynne and Strauss, 1982; Pate and Condon, 1982; Ohashi et al., 1982; Hwang and Menon, 1983). High density (HDL) and low-density (LDL) lipoproteins are the most common cholesterol source in domestic animals (Pate and Condon, 1982; Ohashi et al., 1982; Hwang and Menon, 1983; reviewed in Grummer and Carroll, 1988). Circulating levels of HDL are greater than levels of LDL in cattle (Grummer and Davis, 1984) and sheep (Diskin et al., as cited in Wiltbank, 1994). The primary function of HDL is to traffik cholesterol to the liver, while LDL traffiks cholesterol from the liver to the periphery (reviewed in Hill and McQueen, 1997).

Biochemical and structural differences between HDL and LDL include differences in apolipoprotein composition and cholesterol content of the particle (reviewed in Gwynne and Strauss, 1982). Apolipoprotein A-1 is the primary apolipoprotein in HDL (Gwynne and Strauss, 1982) but apolipoprotein E is absent (reviewed in Grummer and Carroll, 1988). Apolipoprotein B is the primary apolipoprotein in LDL (reviewed in Gwynne and Strauss, 1982). Lipoproteins transport cholesterol in the form of both cholesterol esters and free cholesterol (reviewed in Johnson et al., 1997). The cholesterol load of LDL is ~1600 cholesterol ester molecules and ~1400 free cholesterol molecules, totaling ~3000 cholesterol molecules (reviewed in Ginsberg, 1998). The cholesterol load of HDL is less than that of LDL (~1200

cholesterol molecules); however, the lipid core of HDL is far more dense than that of LDL particles (reviewed in Ginsberg, 1998).

The mechanism of delivery of cholesterol to the intracellular compartment is different for LDL and HDL. The delivery of LDL is via receptor-mediated endocytosis (Rajan and Menon, 1985; Brown and Goldstein, 1986). Upon binding to its receptor, the LDL particle bearing cholesterol is internalized and transported to lysosomes (Brown and Goldstein, 1986). In the lysosome the LDL receptor is catabolized or recycled and cholesterol is released and readily available as steroidogenic substrate (reviewed in Grummer and Carroll, 1988). Delivery of cholesterol by HDL is facilitated by binding of HDL to plasma membrane receptors (Ferreri and Menon, 1992); however, receptor mediated endocytosis does not appear to be the mechanism of cholesterol delivery (reviewed in Lestavel and Fruchart, 1994). To date the mechanism of HDL delivery of cholesterol to cells has not been described.

Upon entry into steroidogenic cells, cholesterol is subject to many possible fates, storage in the form of cholesterol-fatty acid esters in lipid droplets or immediate utilization as steroidogenic substrate (reviewed in Johnson et al., 1997). It has been postulated that cells devoid of lipid droplets do not store cholesterol esters (Sawyer, 1995). These stored cholesterol esters are mobilized as steroidogenic substrate via conversion to free cholesterol by cholesterol esterase (hormone sensitive lipase; reviewed in Ramsay, 1996). In fact, lipid droplets are transported through the cell by cytoskeletal components (Ray and Strott, 1978; reviewed in Niswender and Nett, 1994) and can associate with mitochondria (Merry, 1975). It has also been proposed that mitochondria are transported and lipid droplets are immobile (Thomson, 1998). These cytoskeletal

components are believed to be primarily intermediate filaments (Almahbobi and Hall, 1990; Almahbobi et al., 1992a,b; Almahbobi et al., 1993). Intermediate filaments associate with both the lipid droplets (Almahbobi and Hall, 1990; Almahbobi et al., 1992a) and mitochondria (Almahbobi et al., 1992b; Almahbobi et al., 1993) of adrenal cells (Almahbobi and Hall, 1990; Almahbobi et al., 1992a,b) and Leydig cells (Almahbobi et al., 1993).

Until recently, it was believed that conversion of cholesterol to pregnenolone by the cytochrome P450 sidechain cleavage enzyme complex was the rate limiting and regulated step in steroid biosynthesis. Experimental evidence supporting this notion was derived from experiments in which steroidogenic cells were able to synthesize large quantities of progesterone when supplemented with pregnenolone but not when supplemented with cholesterol (reviewed in Stocco and Clark, 1996). However, supplementation with hydrophilic cholesterol analogs (25-hydroxycholesterol, 22R-hydroxycholesterol, and 20α- hydroxycholesterol) results in high rates of steroidogenesis without tropic hormonal input (Jefcoate et al., 1973; Jefcoate et al., 1974; Lin et al., 1995). These cholesterol analogs are able to freely diffuse to the inner mitochondrial membrane and readily access the sidechain cleavage active site (Jefcoate et al., 1974; Toaff et al., 1982). Based on these observations it was concluded that cholesterol was actively transported to the inner mitochondrial membrane and that this was the rate-limiting step in steroidogenesis (reviewed in Thomson, 1998).

Tropic stimulation of adrenal cortical cells with ACTH in the presence of cycloheximide (protein synthesis inhibitor), does not increase steroid output (Fergeson, 1963; Garren et al., 1965; Cooke et al., 1975). In the presence of protein synthesis

inhibitors, cholesterol accumulates in the outer mitochondrial membrane and is not available for pregnenolone biosynthesis (Simpson et al., 1978). The factor facilitating cholesterol delivery to the inner mitochondrial membrane was found to be a protein rapidly synthesized upon trophic stimulation in adrenal cells.

The cycloheximide-sensitive proteins were found to be a family of 30 kD phosphoproteins rapidly induced after trophic stimulation of many steroidogenic cell types including adrenal cells (Krueger and Orme-Johnson, 1983;Pon et al., 1986a), Leydig cells (Pon et al., 1986b), corpus luteum (Pon and Orme-Johnson, 1986), MA-10 mouse Leydig tumor cells (Stocco and Chaudhary, 1990; Stocco and Chen, 1991), and R2C rat Leydig tumor cells (Stocco and Chen, 1991). These proteins are posttranslationally modified variants of a 37-kD precursor molecule (Stocco and Sodeman, 1991). The protein was named **St**eroidogenic **A**cute **R**egulatory protein (StAR; Clark et al., 1994).

Analysis of StAR by two-dimensional polyacrylamide gel electrophoresis (PAGE) reveals that there are at least four processed (30 kDa) isoforms of the protein in the mouse (Stocco and Sodeman, 1991; Epstein and Orme-Johnson, 1991) and human (Arkane et al., 1997). There are two precursor molecules of 32 and 37 kDa that have been described in the mouse (Stocco and Sodeman, 1991). There are acidic and basic isoforms of murine StAR that can be detected in phosphorylated and unphosphorylated states (Alberta et al., 1989). The biochemical characteristics resulting in acid and basic isoforms have not been described. The role of phosphorylation will be discussed in another section.

If StAR were the elusive protein that facilitated delivery of cholesterol to the inner mitochondrial membrane, it would be expected that mutation of StAR would result in a dramatically reduced capacity to synthesize steroids. Indeed, it has been known for quite some time that humans born with congenital adrenal lipoid hyperplasia (lipoid CAH) do not have the capacity for gonadal and adrenal steroidogenesis (Degenhart et al., 1972; Hauffa et al., 1985). It has since been shown that lipoid CAH is due to a mutation in StAR (Lin et al., 1995), specifically a single amino acid mutation (A218V; Nakae et al., 1997) that prevents StAR from folding properly (Bose et al., 1998). One of the most striking morphological characteristics of individuals afflicted with lipoid CAH is the accumulation of lipid droplets and hyperplasia in steroidogenic cells (Camacho et al., 1968; Hauffa et al., 1985; reviewed in Miller, 1997) possibly due to an imbalance of cholesterol intake and cholesterol conversion to steroids in these cells. Interestingly, the human placenta does not depend on StAR expression for progesterone biosynthesis (Sugwara et al., 1995), explaining why individuals afflicted with lipoid CAH are able to survive to term (Saenger et al., 1995). It has recently been proposed that a StAR homologue (MLN64) functions in a role similar to StAR in human placenta (Watari et al., 1997).

The mechanism of StAR action has not been accurately described to date but several hypotheses have been proposed (King et al., 1995; reviewed in Waterman, 1995; Stocco and Clark, 1996; Miller, 1997; Thomson, 1998; Niswender et al., 1999). The original hypothesis was that cholesterol delivery to the inner mitochondrial membrane involved import of StAR into mitochondria and concurrent cleavage of the mitochondrial targeting sequence (Waterman, 1995). To date, there is no conclusive evidence in

support of this hypothesis. In fact, there is some evidence that contradicts this notion. Truncation of the N-terminal mitochondrial targeting sequence of human StAR yields a protein that can associate with but cannot be imported into the mitochondria; however, this construct is able to stimulate cholesterol delivery to P450 SCC (Arkane et al., 1996; Arkane et al., 1998; Wang et al., 1998). It is reasonable that association of StAR with the outer mitochondrial membrane mechanically drives the formation of contact points in which the outer and inner mitochondrial membrane become continuous. Thomson (1998) proposes that the cholesterol contained within the inner mitochondrial membrane is available as a steroidogenic pool while cholesterol in the outer mitochondrial membrane needs only to be transferred to the inner membrane to become available for steroidogenesis. Consistent with this, the concentration of cholesterol in the outer mitochondrial membrane is 10-fold greater than that of the inner mitochondrial membrane (della-Cioppa et al., 1986). Thus, the formation of contact points between the inner and outer mitochondrial membranes would allow cholesterol to diffuse down a concentration gradient to the inner mitochondrial membrane and P450 SCC, *without ever leaving the lipophilic environment of the mitochondrial membranes.* It has also been proposed (Eastbrook and Rainey, 1996) that import of StAR into the mitochondria is a mechanism of attenuating cholesterol delivery to the inner mitochondrial membrane by sequestering StAR, thus rendering it inactive. If this were true it would be expected that StAR without a mitochondrial targeting sequence would have much greater activity than wild-type StAR. This remains to be determined.

Several other proteins are believed to be associated with the formation of contact points in mitochondria of steroidogenic cells (reviewed in Papadopolous et al., 1993).

Some of these proteins include the peripheral-type benzodiazapine receptor (PBR; Muhkin et al., 1989), the peptide diazapam binding inhibitor (DBI; the endogenous ligand for PBR, also known and *endozepine*; Papadopolous and Brown, 1995), and voltage-dependant anion channel (VDAC; McEnery et al., 1992). Abrogation of expression of endozepine in R2C cells (rat Leydig tumor cell line which constitutively produces high levels of progesterone) by treatment with cholesterol-linked phosphorothioate antisense oligonucleotides results in cessation of progesterone secretion (Garnier et al., 1994). Furthermore, utilization of the same antisense strategy has resulted in evidence that endozepine is requisite for hormone-stimulated progesterone secretion in MA-10 mouse Leydig tumor cells (Boujrad et al., 1993). The antisteroidogenic effects of endozepine antisense oligonucleotides were reversed by addition of 22-hydroxycholesterol (Boujrad et al., 1993; Garnier et al., 1994), suggesting that endozepine plays a role in cholesterol delivery to the inner mitochondrial membrane. Finally, deletion of the gene encoding PBR in R2C rat Leydig tumor cells results in a pronounced reduction of basal progesterone secretion which can be reversed by reintroduction of a functional PBR gene (Papadopolous et al., 1997). From these observations one can conclude that the endozepine/PBR system has *at least* a permissive role in steroidogenesis; whether this ligand/receptor system represents a regulated component of the steroidogenic pathway remains to be revealed.

Regulation of Progesterone Biosynthesis

The corpus luteum is subject to both tropic and inhibitory regulation of progesterone biosynthesis, primarily by LH (Schomberg et al., 1967; Niswender et al., 1976; reviewed in Niswender and Nett, 1994; Wiltbank, 1994) and $PGF_{2\alpha}$, (Pate and

Nephew, 1988; Wiltbank et al., 1990), respectively (reviewed in Niswender and Nett, 1994; Niswender et al., 1999). Small luteal cells produce relatively low basal levels of progesterone but dramatically increase progesterone output in response to luteinizing hormone (Williams and Marsh, 1978; Fitz et al., 1982). Conversely, small luteal cells do not respond to $PGF_{2\alpha}$ by decreasing their progesterone output, probably due to the lack of $PGF_{2\alpha}$ receptors on the surface of small luteal cells (Fitz et al., 1982). Pharmacological activation of the protein kinase C pathway (the second messenger system utilized by $PGF_{2\alpha}$) with phorbol myristate acetate (PMA) does elicit a decrease in progesterone biosynthesis in cultured small luteal cells (Wiltbank et al., 1993; Belfiore et al., 1994). Furthermore, $PGF_{2\alpha}$ does not elicit a calcium influx into small luteal cells as it does in large luteal cells (Wiltbank et al., 1989a; Wegner et al., 1990). Thus, PKC could mediate negative regulation of progesterone biosynthesis in small luteal cells, but the effector of this response remains to be identified.

Large luteal cells produce basal levels of progesterone up to 40 fold greater than small luteal cells (reviewed in Niswender et al., 1999) and large luteal cells are estimated to be responsible for 80 percent of the progesterone output of the corpus luteum (reviewed in Niswender et al., 1985a). Consistent with these observations, morphometric analysis of the corpus luteum has revealed that large luteal cells have a greater concentration of mitochondria that do small luteal cells (Kenny et al., 1989). Additionally, in large luteal cells, a greater percentage of total RNA is 3β-HSD mRNA (Hawkins et al., 1993). Large luteal cells are not significantly responsive to LH (Hoyer and Niswender, 1985; Hansel et al., 1991) although they have a similar number of LH receptors as small luteal cells on a per cell basis (Harrison et al., 1987). The relative

surface area of small and large luteal cells allows for a greater density of LH receptors on small luteal cells and this *potentially* is partially involved in the differential responsiveness between the two steroidogenic cell types.

Tropic stimulation of the corpus luteum appears to be primarily due to LH (reviewed in Niswender and Nett, 1994; Niswender et al., 1999) and to a lesser extent, GH (Juengel et al., 1995). The importance of pituitary hormones in luteal development and function has been well established (Astwood et al., 1941; Denamur et al., 1973; Farin et al., 1990). Hypophysectomy, which ablates circulating concentrations of both GH and LH, on day 5 of the estrous cycle in the ewe, arrests luteal growth and halts the increase in progesterone biosynthesis (Farin et al., 1990; Juengel et al., 1995a), and ultimately results in luteolysis (Denamur, 1966; Denamur et al., 1973). In addition, passive immunoneutralization of LH in ewes results in inhibition of progesterone biosynthesis (Niswender et al., 1976) and loss of luteal mass (Fuller and Hansel, 1970). Furthermore, administration of GnRH antagonists (suppress pulsatile, but not tonic LH secretion) results in suppressed progesterone secretion (Baird, 1992; Peters et al., 1994), although to a lesser extent than in hypophysectomized animals. The observed decrease in progesterone secretion in hypophysectomized ewes appears to be the result of lack of tropic support that maintains expression of several steroidogenic components including StAR, P450 SCC and 3βHSD (Juengel et al., 1995a,b). The removal of tropic input also results in decreases in the size of small and large luteal cells, as well as a decrease in the number of small luteal cells and fibroblasts (Farin et al., 1990). However, replacement of LH in hypophysectomized ewes restores luteal function (Kaltenbach et al., 1968; Niswender et al., 1976; Farin et al., 1990) including expression of mRNA encoding P450

SCC, StAR, and 3β-HSD (Farin et al., 1990; Juengel et al., 1995a,b. In general, it appears tonic LH acts to maintain the integrity and steroidogenic capacity of luteal cells.

Interestingly, during the midluteal phase, when progesterone secretion is at its greatest, LH pulse frequency is at its lowest (Baird et al., 1978). This is likely due to the negative feedback effects of progesterone on GnRH (Attardi and Happe, 1986; Kasa-Vubu et al., 1992) and LH (Laws et al., 1990; Bauer-Dantoin et al., 1995; Kirkpatrick et al., 1998b; Turzillo et al, 1998b). However, between days 4 and 12 of the estrous cycle, the 2-4 fold increase in progesterone biosynthesis concurs with an increase in LH receptor number in the corpus luteum (Diekman et al., 1978a). In the rat, hCG-induced luteinization is followed by a constitutive pattern of expression of P450 SCC (Goldring et al., 1987; Hickey et al., 1989). It appears that LH provides tonic support for luteal function; however it has a less critical role in acute regulation of luteal biosynthesis of progesterone (Suter et al., 1980; Baird et al., 1976).

More recently it has been shown that replacement of LH in hypophysectomized ewes only partially restores the function of the corpus luteum (Juengel et al., 1995a). Supplementation of hypophysectomized ewes with GH in addition to LH results in full restoration of luteal function (Juengel et al., 1995a). Supplementation with GH alone allows mRNA encoding StAR and P450 SCC, but not 3βHSD, to remain at normal levels (Juengel et al., 1995b). There is some evidence that suggests prolactin is also requisite for normal luteal function (Denamur, 1973); however, ablation of endogenous prolactin secretion with bromoergocriptine has no detrimental effect on luteal function (Bevers et al., 1988; Niswender, 1974). Therefore, it remains questionable whether prolactin is essential for luteal function, but LH and GH definitely play a key role.

The actions of LH in regulating progesterone biosynthesis are primarily by modulating expression and activity of key components of the steroidogenic pathway (reviewed in Niswender et al., 1999). Expression of mRNA encoding steroidogenic proteins, specifically 3β-HSD, P450 SCC, and StAR, are attenuated in the absence of trophic input by LH (Juengel et al., 1995a, b).

The intracellular signaling pathway utilized by LH is the protein kinase (PK) A second messenger system (Mendelson et al., 1975; Marsh, 1975; Jordan et al., 1978). Binding of LH to its extracellular receptor, which is coupled to adenylate cyclase by a G-protein, causes adenylate cyclase to increase intracellular concentrations of cAMP (reviewed in Marsh, 1975). Binding of cAMP to the regulatory domains of PKA result in dissociation of the regulatory subunits from the catalytic subunits and subsequent activation of PKA (reviewed in Nimmo and Cohen, 1977; Flockhart and Corbin, 1982). The activated subunit of PKA catalyses the phosphorylation of target proteins resulting in altered biological activity (reviewed in Nimmo and Cohen, 1977).

A significant function of LH is to promote luteal biosynthesis of progesterone by induction of PKA and phosphorylation and activation of proteins involved in steroidogenesis (reviewed in Niswender and Nett, 1994). Small and large luteal cells have similar levels of PKA activity (Wiltbank et al., 1989b); however, cAMP or LH do not stimulate progesterone secretion in purified cultures of large luteal cells (Hoyer et al., 1984). Pharmacological activation of PKA in small luteal cells results in enhanced progesterone biosynthesis (Wiltbank et al., 1993). The stimulatory effects of LH on steroidogenesis are first at the level of intracellular cholesterol flux. Activation of PKA results in phosphorylation and activation of cholesterol esterase (hormone sensitive

lipase), which removes cholesterol esters from the storage pool and liberates free cholesterol that can be used as steroidogenic substrate (Trzeciak and Boyd, 1974; Pittman et al., 1975; Caffrey et al., 1979). Furthermore it appears that phosphorylation of intermediate filaments by PKA results in the association of lipid droplets and mitochondria (reviewed in Boyd et al., 1983). It has been hypothesized that PKA might modulate the activity of StAR, thus LH might regulate the delivery of cholesterol to the inner mitochondrial membrane through PKA (Niswender et al., personal communication). Thus LH acts via PKA to increase cholesterol flow to the mitochondria.

Based on the deduced amino acid sequence of ovine StAR, there are three putative PKA phosphorylation sites in the StAR protein (Juengel et al., unpublished observation). It is likely that the LH-induced expression of StAR mRNA (Juengel et al., 1995b) is mediated by PKA. Additionally, since large luteal cells are not very responsive to LH, it is unclear whether StAR plays a significant role in progesterone biosynthesis in large luteal cells. It is reasonable to speculate that the LH-induced expression of StAR mRNA (Juengel et al., 1995b) observed in the ewe was primarily in small luteal cells, but this remains to be investigated. Increases in expression of mRNA encoding StAR occurs more than 24 hours after LH administration (Juengel et al., 1995b), therefore acute regulation of StAR by LH is likely at the translational or posttranslational levels. Indeed, activation of PKA does result in phosphorylation of StAR (Pon and Orme-Johnson, 1986; Epstein and Orme-Johnson, 1991; Arkane et al., 1997) and this appears to increase steroidogenic activity, presumably by enhancing cholesterol delivery to the inner mitochondrial membrane.

It does not appear that the activity of P450 SCC is affected by PKA. Neither pharmacological (Wiltbank et al., 1993) nor hormonal (Belfiore et al., 1994) activation of PKA results in increased P450 SCC activity in cultured luteal cells, although progesterone biosynthesis is stimulated. These observations weaken the argument that P450 SCC catalyzed conversion of cholesterol to pregnenolone is the rate-limiting step in steroidogenesis. Furthermore, it is evident that LH does not acutely regulate enzymatic conversion of cholesterol to pregnenolone.

There is indirect evidence that 3β-HSD expression is upregulated by LH (reviewed in Niswender et al., 1994). Levels of mRNA encoding 3β-HSD are greatest in ovine corpora lutea during the mid luteal phase of the estrous cycle (d 6-12; Hawkins et al., 1993; Juengel et al., 1994); however, 3β-HSD mRNA levels in small luteal cells remain constant throughout this period while levels in large cells increase (Juengel et al., 1994). In addition, removal of LH (hypophysectomy; Juengel et al., 1995a, b) results in decreased levels of mRNA encoding 3β-HSD. Thus, it appears that the major point of regulation of steroidogenesis by LH is delivery of cholesterol to the mitochondria, and possibly to the inner mitochondrial membrane.

A variety of other hormones regulate progesterone biosynthesis in the corpus luteum, albeit to a lesser extent than LH (reviewed in Niswender et al., 1999). Growth hormone also appears to be a major tropic regulator of luteal function. Messenger RNA encoding GH receptor is expressed in the ovine (Juengel et al., 1997) and bovine (Lucy et al., 1993) corpus luteum. Whether the effects of GH are direct or mediated by IGF-1 has not been well established. Growth hormone stimulates IGF-1 expression in many tissues and IGF-1, known to be produced by the corpus luteum (Juengel et al., 1997), stimulates

luteal progesterone biosynthesis (McArdle and Haltorf, 1989; Constantino et al., 1991; Parmer et al., 1991). Progesterone biosynthesis is also enhanced by insulin (Sauerwein et al., 1992; Wiltbank et al., 1992). Indeed, expression of IGF-I in the corpus luteum appears to be modulated by growth hormone (Juengel et al., 1997). Because of the effects of IGF-I on the corpus luteum, it has been difficult to determine the direct effects of GH on luteal function.

Prostaglandins also appear to be key modulators of luteal function. Prostaglandins of the E and I series are probably the primary luteotropic prostaglandins (reviewed in Hansel et al., 1991), while $PGF_{2\alpha}$ has the opposite role (reviewed in Niswender et al., 1999). Prostaglandin E_2 stimulates progesterone secretion from bovine (Alila et al., 1988; Shelton et al., 1990) and ovine (Fitz et al., 1984a,b) luteal cells. The primary action of PGE_2 appears to be directed to large luteal cells, as these cells possess a majority of the receptors for PGE_2 (Fitz et al., 1982). However the mechanism of PGE_2 stimulation of progesterone biosynthesis remains controversial (reviewed in Niswender et al. 1999).

An important role for PGI_2 in luteal development has been demonstrated (Homeida and El-Eknah, 1992), while others have shown PGI_2 induced stimulation of progesterone biosynthesis in the corpus luteum of cows (Milvae and Hansel, 1980; Alila et al., 1988), women (Bennegard-Eden et al., 1990), and ewes (Fitz et al., 1984a). The luteotropic effects of PGI_2 are mediated by adenylate cyclase that results in PKA activation (Bennegard-Eden et al., 1990). Both small and large cells of bovine corpora lutea possess binding sites for PGI_2 (Chegini et al., 1990; Chegini et al., 1991); however, it is not known if these are specific PGI_2 receptors. Progesterone biosynthesis is

stimulated by PGI_2 in both small and large ovine luteal cells (Fitz et al., 1984a), but this is not likely mediated by PKA in large luteal cells because PKA activation in large luteal cells does not result in increased progesterone biosynthesis (Fitz et al., 1984; Hoyer et al., 1984). It has been shown that LH activates phospholipase C (reviewed in Davis et al., 1996) which results in liberation of arachadonic acid, the primary substrate for PGI_2 and PGE_2 biosynthesis (reviewed in Vane et al., 1998). This is a potential mechanism by which LH might indirectly stimulate progesterone biosynthesis in an autocrine/paracrine manner.

Luteolysis

Luteolysis is defined as the loss of function and structural demise of the corpus luteum. The process of luteolysis can be divided into two distinct but related components: loss of the capacity to synthesize and secrete progesterone (McGuire et al., 1994) followed by structural luteolysis (reviewed in Pate 1994; Knickerbocker et al., 1988; McCracken et al., 1999).

In domestic ruminants, the luteolytic agent has been identified to be of uterine origin; hysterectomy of heifers (Wiltbank and Casida, 1956; Anderson et al., 1961; Malven and Hansel, 1964) and ewes (Wiltbank and Casida, 1956) resulted in delayed luteolysis. However, in many primate species, hysterectomy does not delay luteolysis (Neill et al., 1969; Beling et al., 1970; Castracane et al., 1979). Prostaglandin $F_{2\alpha}$ is the compound from the uterus that initiates luteolysis (McCracken et al., 1970; Hansel et al., 1973). Initiation of luteolysis by $PGF_{2\alpha}$ appears to be a local effect of $PGF_{2\alpha}$ of uterine origin (reviewed in Ginther et al., 1974; Del Campo and Ginther, 1972; Del Campo and

Ginther, 1973a,b; Del Campo and Ginther, 1974). Prostaglandin $F_{2\alpha}$ enters the ovarian artery from the utero-ovarian vein, possibly by diffusion, involving a countercurrent exchange mechanism (reviewed in Ginther, 1974). This countercurrent exchange mechanism allows $PGF_{2\alpha}$ to be shunted directly to the ovarian artery without entering the pulmonary circulation where it would be inactivated by 15-hydroxyprostaglandin dehydrogenase in the lungs (Piper et al., 1970).

Since the identification of the gross physiological effects of $PGF_{2\alpha}$ (decreased progesterone secretion followed by disappearance of the tissue), the mechanisms of luteolysis have been studied extensively. More recently, the biochemical and cellular facets of $PGF_{2\alpha}$ action on the corpus luteum have been characterized (Micheal et al., 1994). The biochemical and cellular actions of $PGF_{2\alpha}$ in the corpus luteum will be discussed later in this section.

The factors initiating the luteolytic cascade are complex and poorly understood. It has been hypothesized that estradiol-17β produced from the preovulatory follicle initiates luteolysis by stimulating the release of oxytocin from the neurohypophysis (reviewed in McCracken et al., 1996; McCracken et al., 1999). Prostaglandin $F_{2\alpha}$ of uterine origin initiates a positive feedback loop involving oxytocin of luteal (Walters et al., 1984; Hooper et al., 1986; Moore et al., 1986) and hypophyseal (reviewed in McCracken et al., 1996; McCracken et al., 1999) origin and $PGF_{2\alpha}$ of both luteal (Tsai et al., 1997) and uterine origin (reviewed in Silvia et al., 1991). Oxytocin stimulates synthesis and secretion of $PGF_{2\alpha}$ from the uterus of guinea pigs (Leaver and Seawright, 1982), heifers (Roberts and McCracken, 1976; Lafrance and Goff, 1985), mares (Goff et al., 1987), sows (Kieborz et al., 1991) and ewes (Walker et al., 1997). Consequently,

$PGF_{2\alpha}$ of uterine origin can stimulate release of luteal oxytocin that elicits the synthesis and release of yet more uterine $PGF_{2\alpha}$, resulting in a rapidly amplified luteolytic signal. The ability of oxytocin to stimulate release of $PGF_{2\alpha}$ is regulated by estradiol-17β (reviewed in Silvia et al., 1991). Administration of estradiol-17β to anestrous ewes results in enhanced uterine secretion of $PGF_{2\alpha}$ in response to oxytocin (Sharma et al., 1974). It is likely that the effect of estradiol-17β is due to estrogen receptor-mediated stimulation of transcription of mRNA encoding oxytocin receptors and subsequent oxytocin binding (Soloff et al., 1975). During luteolysis, $PGF_{2\alpha}$ is secreted in pulses with increasing amplitude (Thornburn et al., 1973; Barcikowski et al., 1974; Peterson et al., 1974; Zarco et al., 1984; reviewed in McCracken et al., 1999).

It has recently been proposed that the luteolytic signal in domestic ruminants is also locally amplified by luteal $PGF_{2\alpha}$ via an ultrashort positive feedback loop involving a paracrine/autocrine mechanism (Tsai et al., 1997). The sum of the effects of estradiol-17β, $PGF_{2\alpha}$ and oxytocin results in rapid amplification of the luteolytic signal and sustained high local concentrations of $PGF_{2\alpha}$ in the luteal vasculature and parenchyma.

Blood flow and vascular changes

Originally, $PGF_{2\alpha}$ was believed to cause luteolysis by reducing blood flow to the corpus luteum (Phariss et al., 1970), depriving the structure of nutrients, substrates for steroidogenesis, and luteotropic support by gonadotropins. Administration of $PGF_{2\alpha}$ to ewes dramatically causes a reduction of blood flow to the corpus luteum (Niswender et al., 1973; Nett, et al., 1976). A marked reduction in capillary density in corpora lutea exposed to $PGF_{2\alpha}$ is also a potential mechanism by which $PGF_{2\alpha}$ can reduce blood flow to the luteal parenchyma (Nett et al., 1976; Azmi and O'Shea, 1984; Braden et al., 1988).

The arteriole and capillary lumens of corpora lutea exposed to $PGF_{2\alpha}$ are littered with fragments of degenerative endothelial cells (O'Shea et al., 1977; Sawyer et al., 1990) resulting in the demise of the capillary system that supports the corpus luteum. This is likely a direct effect of $PGF_{2\alpha}$ on endothelial cells because endothelial cells express mRNA encoding receptors for $PGF_{2\alpha}$ (Mamluk et al., 1998). In support of this, $PGF_{2\alpha}$ induces apoptosis in capillary endothelial cells in the ovine corpus luteum (Juengel et al., 1999). Collectively, vasoconstriction and capillary deletion by apoptosis of endothelial cells, in response to $PGF_{2\alpha}$, results in a hypoxic state in the luteal parenchyma, a condition known to result in endothelin-1 release (Rakugi et al., 1990).

More recently, endothelin-1 has been implicated as a possible mediator of the effects of $PGF_{2\alpha}$ on luteal blood flow (Usuki et al., 1991; Girsh et al., 1996a; Girsh et al., 1996b; reviewed in Meidan and Girsh et al., 1997; Ohtani et al., 1998). Prostaglandin $F_{2\alpha}$ stimulates vascular endothelial cells of corpora lutea to produce endothelin-1 *in vitro* (Girsh et al., 1996b) and *in vivo* (Ohtani et al., 1998). In addition to potent vasoconstriction activity (reviewed in Huggins et al., 1993), endothelin-1 inhibits the *in vitro* steroidogenic activity of enriched steroidogenic luteal cells (Girsch et al., 1996b). Levels of mRNA encoding receptors for endothelin-1 are down regulated by LH and IGF-1, but endothelin-1 is expressed at the greatest levels around the time of luteolysis (Mamluk et al., 1999). Furthermore, angiotensin II is produced by the corpus luteum in response to $PGF_{2\alpha}$ and angiotensin II stimulates luteal production of endothelin-1 (Hayashi and Miyamoto, 1999). The antiluteolytic properties of PGE_2 (Pratt et al., 1977) may, in part, be manifest in its ability to attenuate the vasoconstrictive actions of endothelins (Silldorf et al., 1995).

Intracellular signaling

Prostaglandin $F_{2\alpha}$ acts by binding to specific receptors localized primarily to large steroidogenic luteal cells (Fitz et al., 1982; Juengel et al., 1996). These receptors belong to the seven-transmembrane family of G-protein coupled receptors (Abramovitz et al., 1994; Sugimoto et al., 1994; Sakamoto, et al., 1994; Graves et al., 1995). Upon binding to high affinity receptors, $PGF_{2\alpha}$ induces activation of the membrane bound enzyme phospholipase C (Berridge and Irvine, 1984) via a stimulatory G-protein (Miwa et al., 1990). Phospholipase C catalyzes the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP_2) to inositol 1, 4, 5-trisphosphate (IP_3) (Davis et al., 1988) and 1, 2-diacylglycerol (DAG; Berridge and Irvine, 1984). Increased cytosolic concentrations of IP_3 result in the flux of free Ca^{++} sequestered in the smooth endoplasmic reticulum to the cytoplasmic compartment (Berridge and Irvine, 1984). Increased cytosolic free Ca^{++} and DAG (localized to the plasma membrane) stimulate the catalytic activity of Ca^{++}-dependent protein kinase (PKC; also localized to the plasma membrane) (Nishizuka, 1986).

Protein kinase C refers to a family serine/threonine protein kinases (Nishizuka, 1988, 1992) that exist in 11 isoforms identified to date (reviewed in Quest, 1996). Differences among isoforms include subcellular localization and Ca^{++}-dependence (Quest, 1996). The alpha (cytosolic) and epsilon (plasma membrane) isoforms of PKC are immunochemically detectable in the bovine corpus luteum (Orwig et al., 1994) while the delta isoform is the predominant form in the corpus luteum of pseudopregnant rats (Cutler et al., 1993). Protein kinase C-α is calcium-dependent but PKC-δ and PKC-ε are not calcium dependent (Nishizuka, 1992). Both calcium-dependent and calcium-

independent isoforms of PKC have highly conserved, cysteine-rich domains involved in binding of DAG and pharmacological activators of PKC such as PMA (Bell and Burns, 1991). Translocation of PKC-α from the cytosol to the nucleus is stimulated by phorbol esters in some cell types (Thomas et al., 1988; Leach et al., 1989; Divecha et al., 1991). The variety of PKC isoforms, their subcellular distribution, and their relative importance in luteal function have received little attention to date.

Protein kinase C is believed to mediate many of the actions of $PGF_{2\alpha}$ in the large luteal cell (Wiltbank et al., 1990; Wiltbank et al., 1991; McGuire et al., 1994). In support of this, purified large luteal cells cultured with $PGF_{2\alpha}$ exhibit an influx of extracellular calcium into the cytosolic compartment similar to the influx observed in response to the Ca^{++} ionophore, A23187 (Wiltbank et al., 1989a). Prostaglandin $F_{2\alpha}$–induced accumulation of microsomally derived and extracellular-derived Ca^{++}, in the cytosolic compartment, enhances the catalytic activity of PKC. Activation of PKC in large luteal cells is believed to result in post-translational modification of cellular proteins involved in steroidogenesis (Wiltbank et al., 1990; McGuire et al., 1994), cholesterol availability (Behrman et al., 1971), apoptosis (reviewed by Schwartzman and Cidlowski, 1993), and maintenance of the extracellular matrix (reviewed in Lum and Malik, 1996; Wojtowicz-Praga et al., 1997). Although pharmacological activation of PKC does not induce apoptosis in the corpus luteum (Wiltbank et al., 1989a; McGuire et al., 1994), PKC induces expression and activation of proteins in other cell types that are involved in apoptosis (reviewed in Schwartzman and Cidlowski, 1993). It is possible that PKC, along with other effectors, facilitates apoptosis in large luteal cells. Alternatively, isoforms of PKC activated by $PGF_{2\alpha}$ in luteal endothelial cells might be different than isoforms

activated in large luteal cells, causing apoptosis only in endothelial cells at first. It is possible that apoptosis in large luteal cells is not a direct effect of $PGF_{2\alpha}$, but may be due to hypoxia or some other general effect of $PGF_{2\alpha}$ that can only occur *in vivo*.

Luteal Prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ can be synthesized by corpora lutea of women (Schutt et al., 1976; Swanston et al., 1977; Sugimoto et al., 1977; Patwarden et al, 1980), sows (Guthrie et al., 1978), ewes (Rexroad and Guthrie, 1979; Tsai et al., 1997), cows (Pate, 1988), and rodents (Olofsson et al., 1992). During the late luteal phase of the estrous cycle, $PGF_{2\alpha}$ from peripheral sources can stimulate an increase in levels of $PGF_{2\alpha}$ in corpora lutea of ewes (Tsai and Wiltbank, 1997). In contrast to ruminants, luteolysis in primates is not mediated by uterine $PGF_{2\alpha}$ (Beling et al., 1970), but, likely by $PGF_{2\alpha}$ produced locally in the corpus luteum acting via a paracrine and/or autocrine mechanism (Auletta and Flint, 1988). Prostaglandins E_2 and $F_{2\alpha}$ are synthesized using membrane phospholipids as substrates in a three-step series of reactions termed the cyclooxygenase pathway. Phospholipases A_2 and C, localized to the plasma membrane, hydrolyze membrane phospholipids liberating arachadonic acid that can be utilized as substrate for $PGF_{2\alpha}$ synthesis (Kawai and Clark, 1986). Cyclooxygenase-2 (COX-2; also called prostaglandin G/H synthase-2, PGHS-2) catalyzes the rate-limiting step in prostaglandin biosynthesis, conversion of arachadonic acid to PGH_2 (reviewed in Dewitt and Smith, 1995). Finally, PGH_2 is rapidly converted to $PGF_{2\alpha}$ by prostaglandin F synthase (Watanabe et al., 1985; Barski and Watanabe, 1993).

Phospholipases A_2 and C exhibit increased enzymatic activity in the presence of elevated intracellular free Ca^{++} (Flower and Blackwell, 1976; Abdel-Latif, 1986). The

ovine corpus luteum expresses COX-2 in response to $PGF_{2\alpha}$ (Tsai and Wiltbank, 1997; 1998). To further illustrate the importance of luteal $PGF_{2\alpha}$ in luteolysis, one should consider the luteolytic competence of corpora lutea with one of these mechanisms absent. First, removal of immune cell-derived cytokines by splenectomy (Matsuyama et al., 1987) results in delayed luteolysis. This effect is reversed by injection of isolated splenocytes. Furthermore, immunosuppressive doses of dexamethasone also prevent luteolysis (Wang et al., 1993). It appears that the presence of immune cell-derived cytokines, that stimulate luteal $PGF_{2\alpha}$ biosynthesis, are a necessary component of the luteolytic machinery. In addition, the presence of COX-2 appears to be necessary for luteolytic competence. Corpora lutea between days 1 and 5 in cows and ewes cannot undergo luteolysis in response to $PGF_{2\alpha}$. Furthermore, corpora lutea on day 10 of the estrous express mRNA encoding COX-2 in response to $PGF_{2\alpha}$; however, on day 4 of the estrous cycle, $PGF_{2\alpha}$ actually attenuates COX-2 expression in ewes (Tsai and Wiltbank, 1997) and cows (Tsai and Wiltbank, 1998). Levels of mRNA encoding COX-2 are identical on days 1, 2, and 10 of the estrous cycle in heifers (Tsai et al., 1996).

These observations support the notion that $PGF_{2\alpha}$ can autoregulate its synthesis by stimulating the liberation of arachadonic acid by hydrolysis of membrane phospholipids as a result of 1) $PGF_{2\alpha}$ receptor coupled G-protein activation of phospholipase C and conversion of arachadonic acid to PGH_2, and 2) increased cytosolic Ca^{++}. Availability of arachadonic acid and cyclooxygenase activity are both increased in corpora lutea in response to $PGF_{2\alpha}$. Both of these events result in an increased capacity for the corpus luteum to synthesize $PGF_{2\alpha}$.

Prostaglandin $F_{2\alpha}$ Regulation of Progesterone Biosynthesis

Prostaglandin $F_{2\alpha}$ acts to attenuate synthesis and secretion of progesterone, by a mechanism independent of the mechanism $PGF_{2\alpha}$ utilizes to facilitate cytotoxicity, reduced blood flow and decreased luteal mass (McGuire et al., 1994; Juengel et al., 1999). Indeed, decreased synthesis and secretion of progesterone precedes the loss of luteal tissue (Knickerbocker et al., 1988; Juengel et al., 1999). The differential effects of $PGF_{2\alpha}$ on progesterone synthesis and secretion, and loss of luteal tissue can likely be explained by the second messenger systems activated by $PGF_{2\alpha}$. Pharmacological activation of protein kinase C with PMA, which does not result in elevated intracellular calcium, suppresses the steroidogenic capacity of luteal cells without promoting cell death (Wiltbank et al., 1989b; Hoyer and Marion, 1989; McGuire et al., 1994). From these observations, it can be concluded that PKC likely acts to phosphorylate and reduce the steroidogenic capacity of proteins that promote progesterone biosynthesis.

The first steps in progesterone biosynthesis, involving stimulation of luteal cells by luteotropic hormones and delivery of cholesterol to the cell, are affected by $PGF_{2\alpha}$ (reviewed in Niswender et al., 1999). Luteal responsiveness to LH is attenuated by $PGF_{2\alpha}$; mRNA encoding LH receptor is down regulated by $PGF_{2\alpha}$ (Guy et al., 1995; Smith et al., 1996). The downregulation of mRNA encoding LH receptor likely results in decreased synthesis of LH receptors, in fact, $PGF_{2\alpha}$ causes decreased binding of LH to luteal cells (Diekman et al., 1978b). However, the decrease in synthesis and secretion of progesterone in response to $PGF_{2\alpha}$ is not primarily due to decreased sensitivity to tropic input because the decrease in LH binding does not occur until after the precipitous decline in progesterone output (Diekman et al., 1978b; Spicer et al., 1981). Activation of

PKC also may interfere with trophic input by increasing the activity of phosphodiesterase (Agudo et al., 1984; Garverick et al., 1985), resulting in depressed intracellular concentrations of cAMP. Because large luteal cells produce 80 percent of luteal progesterone, it would be expected that degradation of cAMP would have a dramatic effect on luteal progesterone output.

Delivery of cholesterol to the intracellular compartment of luteal cells is possibly reduced by $PGF_{2\alpha}$ because mRNA encoding receptors for LDL is downregulated by $PGF_{2\alpha}$ (Rodgers et al., 1987; Tandeski et al., 1996). However, mRNA encoding HDL receptor is not downregulated by $PGF_{2\alpha}$ (Tandeski et al., 1996), and HDL is the primary transporter of cholesterol to ovine luteal cells (Wiltbank et al., 1990). Thus, it is unclear whether decreased cholesterol uptake by luteal cells is a mechanism by which $PGF_{2\alpha}$ attenuates progesterone biosynthesis and secretion.

Mobilization of intracellular cholesterol stores does not appear to be stringently regulated by $PGF_{2\alpha}$ (reviewed in Niswender et al., 1999). Cholesterol esterase activity is not affected by $PGF_{2\alpha}$ (Wiltbank et al., 1993). It appears $PGF_{2\alpha}$ might cause disruption of the cytoskeleton (Murdoch et al., 1996), but the effect of cytoskeletal disruption on progesterone biosynthesis remains unclear (reviewed in Niswender et al., 1999). However, delivery of cholesterol to the inner mitochondrial membrane is likely attenuated by $PGF_{2\alpha}$ because of decreased mRNA encoding StAR (Juengel et al., 1995b; Pescador et al., 1996; Chung et al., 1998). It is possible that $PGF_{2\alpha}$ acts by directly phosphorylating StAR and modulating its activity. In fact, ovine StAR has five putative PKC phosphorylation sites (Juengel et al., unpublished observations). In support of this, supplementation of $PGF_{2\alpha}$ -treated luteal cells with cholesterol analogues that freely

diffuse to the inner mitochondrial membrane reverses a significant portion of the antisteroidogenic activity of $PGF_{2\alpha}$ (Grusenmeyer and Pate, 1992; Fitz et al., 1993; Wiltbank et al., 1993).

Expression of an inducible isoform of heat shock protein-70 (HSP-70) is required for $PGF_{2\alpha}$ to suppress steroidogenesis (Khanna et al., 1995a). Accumulation of HSP-70 in response to $PGF_{2\alpha}$ (Khanna et al., 1995b) occurs primarily in large luteal cells (McPherson et al., 1993; Murdoch et al., 1995). It has been postulated that HSP-70 somehow interacts with StAR to attenuate steroidogenesis (reviewed in McCracken et al., 1999). In support of this, supplementation of $PGF_{2\alpha}$ -treated rat luteal cells with 22R-hydroxycholesterol restores normal steroidogenic function (Khanna et al., 1994). It is possible that HSP-70 sequesters StAR in the cytoplasmic compartment, preventing it from interacting with mitochondria. However, this is inconsistent with the known function of HSP-70 in facilitating protein import into the mitochondria (reviewed in Stuart et al., 1994). Alternatively, HSP-70 could facilitate StAR import into mitochondria, where mitochondrial sequestered StAR might be removed from the cytoplasmic pool of StAR that actively facilitates steroidogenesis. It is also possible that the effects of HSP-70 on StAR are indirect. Induction of HSP-70 results in decreased synthesis of StAR (Liu and Stocco, 1997). It is possible that HSP-70 prevents nuclear translocation of transcriptional activators that accentuate StAR transcription. It seems more likely that HSP-70 might facilitate nuclear translocation of nascent transcriptional repressors that inhibit transcription of the StAR gene. Inhibitory regulation of StAR at the transcriptional level is a reasonable means of acute regulation of steroidogenesis. The half-life of StAR protein is 3-5 minutes (Epstein and Orme-Johnson, 1991; Stocco and

Sodeman, 1991), therefore transcription of StAR and StAR biological activity should be highly correlated.

Luteolytic doses of $PGF_{2\alpha}$ also downregulate expression of mRNA encoding the steroidogenic enzyme 3β-HSD (Hawkins et al., 1993; Tian et al., 1994; McGuire et al., 1994). Furthermore, $PGF_{2\alpha}$ decreases, but does not obliterate, catalytic activity of P450 SCC (Wiltbank et al., 1989). In contrast, subluteolytic doses of $PGF_{2\alpha}$ that inhibit progesterone biosynthesis, do not suppress expression of mRNA encoding P450 SCC (Belfiore et al., 1994; Grusenmeyer and Pate, 1992; McGuire et al., 1994; Tian et al.1994) or P450 SCC enzymatic activity (Grusenmeyer and Pate, 1992; Rodgers et al., 1995). Levels of P450 SCC and 3β-HSD proteins do not decline until after progesterone secretion declines (Rodgers et al., 1995); however, it is not known whether P450 remains active. Activity of 3β-HSD does not decrease when expression of mRNA is ablated (Juengel et al., 1998a). In conclusion, $PGF_{2\alpha}$ appears to regulate steroidogenic enzymes to some degree, but acute regulation of progesterone biosynthesis appears to be at the level of cholesterol delivery to the inner mitochondrial membrane.

Prostaglandin $F_{2\alpha}$ regulates the biological activity of some steroidogenic proteins by phosphorylation. However, $PGF_{2\alpha}$ also regulates expression of genes encoding steroidogenic proteins. It is possible PKC phosphorylates nuclear transcription factors known to regulate steroidogenic cell-specific gene expression, such as steroidogenic factor-1 (SF-1) and DAX-1.

Morphological Changes

Prostaglandin $F_{2\alpha}$ elicits a remarkable series of morphological changes. The proportion of steroidogenic luteal cells that occupy the corpus luteum decreases within 24

h in ewes treated with $PGF_{2\alpha}$ during the midluteal phase (Braden et al., 1988). The number of large luteal cells that can be recovered from enzymatically dispersed corpora lutea decreases, and this decrease precedes a decrease in the number of small steroidogenic luteal cells (Braden et al., 1988). However, a reduction in the size of large luteal cells occurs at this time (Gemmell et al., 1976; Braden et al., 1988) and cells that once were identified as large steroidogenic luteal cells appear in the small luteal cell fraction.

In steroidogenic luteal cells (small and large, collectively), morphological changes do not become evident until 24-36 h after exposure to $PGF_{2\alpha}$ (Sawyer et al., 1990) although the steroidogenic capacity of the cells is markedly reduced at this time. Interestingly, endothelial cells of corpora lutea from ewes treated with $PGF_{2\alpha}$ are the first population of cells to exhibit dramatic morphological changes (Sawyer et al., 1990) that are indicative of apoptosis (Kerr et al., 1972). It is suspected that degeneration of endothelial cells is a direct effect of $PGF_{2\alpha}$, but this has not been investigated.

Immune mediated events

Compelling evidence exists that the immune system plays a critical role in the process of luteolysis. Splenectomy in rats results in elevated concentrations of progesterone in serum and delayed ovulation in rats during the time of normal luteal regression and this effect is reversible by injection of spleenocytes (Matsuyama et al., 1987). Immunosuppressive doses of dexamethasone delay natural luteolysis, but not $PGF_{2\alpha}$ induced luteolysis in rats (Wang et al., 1993). Glucocorticoids inhibit leukocyte accumulation in injured tissues and abrogate the synthesis and action of many cytokines (Haynes, 1990).

Leukocyte infiltration into the corpus luteum is suggested to be involved in luteolysis (reviewed in Murdoch et al., 1988; Brannstrom and Norman, 1993). Treatment of ewes in the midluteal phase with $PGF_{2\alpha}$ (Tsai, et al., 1997; Haworth et al., 1997) and PMA (Tsai, et al., 1997) induce expression of mRNA encoding monocyte chemoattractant protein-1 in corpora lutea. Macrophages infiltrate the parenchyma and blood vessels of the porcine corpus luteum during $PGF_{2\alpha}$ induced luteolysis prior to any precipitous decline in progesterone secretion (Hehnke et al., 1994). It is proposed that the primary role of macrophages during luteal regression is phagocytosis of degenerative luteal cells (Bulmer, 1964; Adams and Hertig, 1969; Paavola, 1979; Pepperell et al., 1992) and degradation of the extracellular matrix (Parker, 1991; Tscheshe, et al., 1986). In addition, tumor necrosis factor α (TNFα), produced by macrophages, inhibits basal progesterone secretion and stimulates $PGF_{2\alpha}$ secretion in cultured bovine luteal cells (Fairchild-Benyo and Pate, 1992). The function of macrophages in the luteal parenchyma during the early stages of luteolysis is likely two-fold: 1) phagocytosis of spent luteal cells, and 2) cytokine mediated inhibition of steroidogenesis.

During luteolysis, T-lymphocytes infiltrate the corpus luteum and secrete interferon-γ (IFN–γ) which stimulates presentation of major histocompatibility complex antigens on the surface of luteal cells (Fairchild and Pate, 1989). Interleukin-1 (IL-1) produced by macrophages, fibroblasts, and endothelial cells (Paavola 1979) stimulates production of $PGF_{2\alpha}$ by cultured bovine luteal cells (Nothnick and Pate, 1990). Production of bioactive TNF-α ceases before the loss of progesterone synthesis in the ovine corpus luteum (Ji et al., 1991), but TNF-α could be serving to complement the luteolytic activity of uterine $PGF_{2\alpha}$ during luteolysis by enhancing the autoregulatory

stimulation of $PGF_{2\alpha}$ (Tsai and Wiltbank, 1997). Consistent with this notion, TNF-α and IFN-γ synergistically enhance synthesis and secretion of $PGF_{2\alpha}$ by cultured bovine luteal cells without significantly inhibiting progesterone biosynthesis (Fairchild-Benyo and Pate, 1992). To summarize, attraction and infiltration of leukocytes into the luteal parenchyma results in increased local production of cytokines (particularly IL-1, TNF-α, and IFN-γ) that ultimately stimulate luteal $PGF_{2\alpha}$ synthesis and activation of the phagocytic functions of macrophages.

Eosinophils, like macrophages, accumulate in the regressing corpus luteum before progesterone decline in response to an uncharacterized chemotaxic factor (Murdoch, 1987). Eosinophils; however,, are not requisite for $PGF_{2\alpha}$–induced luteal regression to occur (Murdoch and Steadman, 1991).

In summary, immune cells and cytokines appear to play an essential role in luteolysis by regulating $PGF_{2\alpha}$ synthesis, steroidogenesis, phagocytosis, and apoptosis/cytotoxicity (discussed next).

Apoptosis

Apoptosis (reviewed in Schwartzman and Cidlowski, 1993; Martin et al., 1994) is an active, energy-dependent process by which nonessential populations of cells remove themselves from a tissue (Kerr et al., 1972). Several fundamental biochemical and morphological changes are characteristics of apoptotic cells including nuclear fragmentation, cytoplasmic fragmentation, ladder-like fragmentation of genomic DNA, and changes in gene expression. The involution of many endocrine glands following removal of tropic hormone support or activation by a negative stimuli, is achieved by apoptosis in cells comprising the gland. Granulosa cells, deprived of FSH, are subject to

apoptosis during follicular atresia (Hurwitz and Adashi, 1992; Tilly, 1997). Epithelial cells of the human endometrium experience apoptosis when progesterone and estradiol support declines (Hopwood and Llevinson, 1976). Alternatively, $PGF_{2\alpha}$ promotes apoptosis in cells comprising the corpus luteum (Sawyer et al., 1990).

The first morphological evidence that a luteal cell is apoptotic is the appearance of nuclear fragments containing degenerate chromatin (Sawyer et al., 1990), followed by cell shrinkage, and appearance of membrane bound cytoplasmic fractions (Kerr et al., 1972). These cell fragments, or apoptotic bodies, are targets for the phagocytic cells of the immune system. Macrophages infiltrate the corpus luteum during luteolysis and augment the apoptotic process in populations of steroidogenic luteal cells by phagocytosing membrane-enclosed fragments of those cells (Gemmell et al., 1976).

Another characteristic feature of apoptosis is the internucleosomal cleavage of genomic DNA into 185-bp fragments (oligonucleosomes; Arends et al., 1990). This characteristic DNA fragmentation, seen as a ladder pattern on agarose gels (Arends et al., 1990) is the result of activation of Ca^{++}-dependent endonucleases (Wyllie, 1980). The most impressive evidence for a role of $PGF_{2\alpha}$ in apoptosis of luteal cells is the appearance of oligonucleosomes in response to $PGF_{2\alpha}$ in cattle (Juengel et al., 1993; Zheng et al., 1994), sheep (Rueda et al., 1995), human (Shikone et al., 1996), rats (Matsuyama et al., 1996), and pig (Bacci et al., 1996). Obviously, if the nuclear membranes are freely permeable to Ca^{++}, the $PGF_{2\alpha}$-induced increase in cytosolic Ca^{++} in large luteal cells (or any other cell type that responds to $PGF_{2\alpha}$ by increasing cytosolic Ca^{++}) will result in Ca^{++} dependent endonuclease activation.

The appearance of oligonucleosomes in whole luteal tissue does not indicate that that tissue is committed to luteolysis. Low doses of $PGF_{2\alpha}$ can cause the incidence of apoptotic cells in the corpus luteum to increase, but there appears to be a threshold dose of $PGF_{2\alpha}$ necessary to commit the corpus luteum to luteolysis (Juengel et al., 1999). In other words, $PGF_{2\alpha}$ can cause a subpopulation of luteal cells to be committed to apoptosis but this does not necessarily result in the corpus luteum being committed to luteolysis.

Oncogenes and Genes Involved in Cell Death

Recently, considerable interest has developed in the genes (Bcl-2 family) involved in regulating apoptosis (reviewed by Korsmeyer, 1995; Martin and Green, 1995; Reed, 1994). The first protein identified to regulate apoptosis was bcl-2 (Korsmeyer, 1992). Membrane-associated bcl-2 prevents cell death by regulating the maintenance of Ca^{++} homeostatic mechanisms (Baffy et al., 1993), attenuating oxidative stress (Hockenberry et al., 1993), and interaction with Ras (growth factor signal transduction; Fernandez and Bischoff, 1993) and bax (Korsmeyer, 1995) proteins. Bax (Bcl-2-associated-gene-x) promotes apoptosis by binding to, sequestering and antagonizing the cell survival functions of bcl-2 protein (Oltvai et al., 1993), and direct promotion of apoptosis (Korsmeyer, 1995). The ratio of bcl-2 and bax within a cell is related to that cell's potential to become apoptotic. The nuclear protein, p53, functions to stimulate apoptotic cell death by increasing transcription rates of the bax gene (Miyashita and Reed, 1995; Miyashita et al., 1994) while repressing transcription of bcl-2 (Miyashita et al., 1994a,b). Two homologs of bcl-2 have been identified, bcl-x_L and bcl-x_S, both proteins are the products of alternatively spliced variants of the bcl-x gene (Boise et al.,

1993). Bcl-x_L functions as a repressor of apoptosis (similar to bcl-2) while bcl-x_S mimics the actions of bax (Boise et al., 1993).

During luteolysis in cattle, mRNA encoding bax is elevated while mRNA encoding bcl-2 remains unchanged (Rueda et al., 1997), resulting in an increased ratio of bax to bcl-2, an event consistent with bax-mediated apoptosis. It remains unclear whether increases in bax mRNA are p53 mediated. Levels of mRNA encoding p53 do not increase during luteolysis (Trott et al., 1997; Rueda et al., 1997); however,, p53 activity may be regulated primarily at the posttranslational level (Zambetti et al., 1993).

Oxidative Stress

Reactive oxygen compounds are integrally involved in luteolysis and apoptosis (Carlson et al., 1993; Riley and Behrman, 1991). Superoxide anion radicals ($O_2^{\cdot}$-), hydroxyl radicals ($OH^{\cdot}$-), and hydrogen peroxide (H_2O_2) are the primary reactive oxygen species generated in steroidogenic cells (Hornsby and Crivello, 1983, 1983*a*). An appreciable amount of oxidative stress experienced during luteolysis is possibly caused by macrophages (Simpson et al., 1987) within the regressing corpus luteum. The potentially toxic effects of oxidative substances are attenuated by antioxidant vitamins (ascorbate, α-tocopherol; Machlin and Gabriel, 1980), enzymes (catalase, superoxide dismutase [SOD] isozymes, and glutathione peroxidase; Reuda et al., 1995), and to a lesser extent, stabilization of radicals by transfer of unpaired electrons to polyunsaturated membrane lipids (Aten et al., 1992). Superoxide dismutase enzymes catalyze the conversion of $O_2^{\cdot}$- to the more stable H_2O_2, which is then converted to H_2O by catylase or glutathione peroxidase (reviewed by Yu, 1994). Antioxidant vitamins stabilize free

radicals by virtue of their ability to form resonance structures upon acceptance of unpaired electrons (Sharma and Buettner, 1993).

Ascorbate concentrations in the ovine corpus luteum are 5-38-fold greater than in other tissues (Scheldrick and Flint, 1989) but depleted concurrent with luteal regression (Endo et al., 1993) and lipid peroxidation (Sawada and Carlson, 1985; Aten et al., 1992). Consistent with the concept of decreased cellular protection against oxidative stress, levels of mRNA encoding secreted and mitochondrial (Mn-dependant)-SOD and catalase are decreased in regressing bovine corpora lutea (Rueda et al., 1995). It remains unclear whether oxidative stress and associated changes in antioxidative mechanisms are a cause or an effect of luteolysis.

Cytotoxicity and apoptosis can be induced by interruption of the mechanisms a cell utilizes to maintain Ca^{++} homeostasis (reviewed in Orrenius et al., 1992). In conclusion, $PGF_{2\alpha}$ action in the induction of apoptosis and luteal regression involves interruption of these homeostatic mechanisms resulting in endonuclease activation, protease activation, and uncoupling of mitochondrial respiration.

Maternal Recognition of Pregnancy

If fertilization occurs upon mating, then the corpus luteum must be rescued so that progesterone secretion can continue and pregnancy can be maintained. A change in maternal physiology occurs during pregnancy so that luteolysis does not occur. These changes in maternal physiology are termed maternal recognition of pregnancy. Broad spectrums of mechanisms have been identified across species. Some of these mechanisms will be discussed briefly, but the focus of this review will be on maternal recognition of pregnancy in ruminants.

Before discussing maternal recognition of pregnancy in nonruminant species, the concept of pseudopregnancy must be illustrated. Pseudopregnancy refers to a physiological state in which the corpus luteum persists beyond the normal time of luteolysis for a given species, in the absence of an embryo.

Additionally, the distinction between luteotropic and antiluteolytic mechanisms of maternal recognition of pregnancy needs to be demonstrated. Corpora lutea of some species appear to regress by default, after luteotropic support wanes. In these species, a luteotropic mechanism of maternal recognition of pregnancy is in place. A luteotropic substance, secreted by the conceptus, acts directly at the corpus luteum to prevent luteolysis. Luteolysis in other species is actively initiated by luteolytic substances (reviewed in Silvia et al., 1991; Silvia 1992). Maternal recognition of pregnancy in these species is by an antiluteolytic mechanism. An antiluteolytic substance, secreted by the conceptus, blocks production or biological activity of the luteolysin.

Rodents

Rodents such as the rat have estrous cycles 4-5 days in length. Gestation in the rat lasts 22 days with the corpus luteum present throughout; however, the corpus luteum is only necessary through day 17 (Csapo and Weist, 1969). The corpus luteum of the rat is initially rescued from luteolysis by a neuroendocrine mechanism (reviewed in Niswender and Nett, 1994). Cervical stimulation, either mechanical or by sterile mating, extends the luteal lifespan to 12 days (Pepe and Rothchild, 1974). Cervical stimulations result in neural inputs that ultimately stimulate pituitary prolactin secretion (Spies and Niswender, 1971). Prolactin supports prolonged luteal lifespan (von Berswoldt-Wallabre et al., 1964; i.e. induces pseudopregnancy), but administration of inhibitors of prolactin

secretion terminate pseudopregnancy (Gibori et al., 1974). Interestingly, prolactin is only necessary to maintain pseudopregnancy for the first 7 days (Mosshige and Rothchild, 1974), but Niswender and Nett (1994) hypothesize that it is possible that a prolactin-like substance of placental origin (rat placental lactogen; rPL), secreted between days 8 and 15 of pregnancy (Kelly et al., 1975; Robertson et al., 1982), might replace prolactin.

The mechanism by which prolactin prevents luteolysis is not clear. There appear to be both luteotropic and antiluteolytic mechanisms. Prolactin induces expression of LH receptors (Richards and Williams, 1976). It appears that the chorionic tissues of the rat produce a luteotropic substance with LH-like activity (rCG)(Haour et al., 1976; Jayatilak et al., 1984). Progesterone secretion in the pregnant rat increases significantly between days 11 and 20 (Pepe and Rothchild, 1974), likely due to increased responsiveness to luteotropic agents. Prolactin administration induces resistance of the corpus luteum to prostaglandin $F_{2\alpha}$ (Behrman et al.,1978). In summary, it appears that the rat utilizes both luteotropic and antiluteolytic means to prevent luteolysis during early pregnancy.

Guinea Pig

Guinea pigs have a 16 day estrous cycle, are spontaneous ovulators, and luteolysis is induced by uterine $PGF_{2\alpha}$ (reviewed in Niswender and Nett, 1994). Progesterone from the corpora lutea is required through day 30 of gestation in the guinea pig (Csapo et al., 1981), then placental progesterone biosynthesis is sufficient to maintain pregnancy (Illingworth and Challis, 1973).

Luteolysis in guinea pigs normally occurs 11 days after ovulation, when uterine secretion of $PGF_{2\alpha}$ increases (Blatchley et al., 1972; Granstrom and Kindhal, 1976). Between days 12 and 16 pregnancy, the concentrations of $PGF_{2\alpha}$ in uterine venous blood

are lower than in nonpregnant females (Blatchley et al., 1975; Antonini et al., 1976). This is possibly due to decreased synthesis of $PGF_{2\alpha}$ (Maule-Walker and Poyser, 1974), in combination with catabolism of $PGF_{2\alpha}$ in the uterus (Bracken et al., 1997). The stromal cells of guinea pig endometrium express PGDH in response to progesterone (when estrogen-primed) and rapidly catabolize $PGF_{2\alpha}$ produced in the epithelial compartment of the uterus, before it reaches the uterine venous vasculature (Bracken et al., 1997). In addition, epithelial, but not stromal COX-2 expression is suppressed by progesterone (Bracken et al., 1997). Since the stromal compartment of the endometrium produces primarily PGE_2 and the epithelial compartment produces primarily $PGF_{2\alpha}$, attenuated COX-2 expression in the epithelial compartment would result in an decreased $PGF_{2\alpha}$:PGE_2 ratio. Consistent with this, between day 10 and day 20 of pregnancy, concentrations of progesterone in serum increase 100-fold (Challis et al., 1971). This increase in concentrations of progesterone in serum is achieved by increased synthesis and decreased metabolic clearance of progesterone (Heap and Deanesly, 1967; Willingworth et al., 1970). This marked increase in progesterone secretion could dramatically attenuate endometrial synthesis of $PGF_{2\alpha}$.

The changes in maternal physiology associated with pregnancy are fairly well understood; however, the mechanism by which the conceptus signals its presence has not been described. A molecule with LH-like biological (guinea-pig chorionic gonadotropin; gpCG) activity has been isolated from the guinea pig conceptus (Humphreys et al., 1981). Secretion of this molecule coincides (day 18 of pregnancy) with increased secretion of progesterone and increases in luteal mass (Babra et al., 1984). Perhaps gpCG stimulates

progesterone secretion beyond a threshold that prevents uterine secretion of biologically active $PGF_{2\alpha}$ and subsequent luteolysis.

In summary, luteolysis appears to be prevented in the pregnant guinea pig by both luteotropic and anti-luteolytic mechanisms. Mechanisms of maternal recognition of pregnancy in the guinea pig include: attenuated uterine secretion of $PGF_{2\alpha}$, gpCG is secreted, and circulating levels of progesterone increase. It is even possible that the luteotropic signals (gpCG) promote the anti-luteolytic mechanisms that occur during pregnancy. Furthermore, there is evidence that the guinea pig corpus luteum expresses PGDH (Bracken et al., 1997) and thus is potentially resistant to $PGF_{2\alpha}$.

Equids

The signal that initiates luteolysis in the mare is likely $PGF_{2\alpha}$ (Douglas and Ginther, 1975). As in other mammalian species, concentrations of $PGF_{2\alpha}$ in serum are suppressed in pregnant mares (day 10 and 14) when compared to nonpregnant mares (Douglas and Ginther, 1976). Maternal recognition of pregnancy is achieved by day 16 of pregnancy in the mare; removal of the equine embryo after day 16 does not result in luteal regression (Hershman and Douglas, 1979). The nature of the signal that alerts the maternal endocrine system of the presence of the conceptus is not definitively known.

Some speculation exists that estrogen might be involved, but there is very little convincing evidence in support of this notion. The equine conceptus can secrete estradiol *in vitro* (Mayer et al., 1977); however, the biological effects of estradiol in the pregnant mare have not been shown to prevent luteolysis. In fact, in nonpregnant mares, estradiol enhances endometrial responsiveness to oxytocin (Goff et al., 1993), perhaps by

stimulating the expression of oxytocin receptors, similarly to what occurs in ruminants (Bartol et al., 1985).

In nonpregnant mares oxytocin stimulates $PGF_{2\alpha}$ secretion to initiate luteolysis (Goff et al., 1993), but in pregnant mares, the uterus does not secrete $PGF_{2\alpha}$ in response to oxytocin (Sharp et al., 1997; Starbuck et al., 1998). This lack of responsiveness to oxytocin is due to decreased density of receptors for oxytocin (Sharp et al., 1997; Starbuck et al., 1998) and decreased affinity of oxytocin receptors for ligand (Sharp et al., 1997). In the absence of luteolytic $PGF_{2\alpha}$, the equine corpus luteum continues to secrete progesterone, which is required through 70 days of gestation (Holtan et al., 1979).

There are some interesting observations that may be relevant to maternal recognition of pregnancy in equids (reviewed in Niswender and Nett, 1994). First, the equine oocyte never leaves the oviduct unless it has been fertilized (van Niekerk and Gemeke, 1966). This indicates that the equine conceptus signals maternal tissues early in pregnancy to allow passage into the uterine compartment. Second, the equine conceptus is mobile and constantly transverses the uterus every two hours throughout the first month of pregnancy (Leith and Ginther, 1984). It is speculated that this migration occurs because the equine conceptus is spherical throughout the period of maternal recognition of pregnancy and migration allows the conceptus to communicate and signal its presence to the entire endometrial surface (Leith and Ginther, 1984).

Later in pregnancy, FSH secretion (Evans and Irvine, 1975) drives a high degree of follicular development (Allen, 1974). Around day 35, fetal derived cells of the endometrial cups secrete eCG, a FSH-like molecule with LH-like biological activity, resulting in ovulation and luteinization of the follicles (Cole et al., 1931). These

luteinized follicles are referred to as accessory corpora lutea. Growth and progesterone biosynthesis of accessory and original corpora lutea are stimulated by eCG (Squires et al., 1979).

It appears the primary mechanism of maternal recognition of pregnancy in the mare is anti-luteolytic. However the nature of the signal has not been described to date. It is unknown whether the equine corpus luteum is resistant to the luteolytic properties of $PGF_{2\alpha}$ during early pregnancy nor is it evident that there are luteotropic signals during this period.

Primates

The human corpus luteum is required to secrete progesterone through approximately six weeks of gestation (Csapo and Pulkkinen, 1978), when the placenta can replace the corpus luteum as a progesterone source. The primary luteolytic signal in primates, as in most other mammalian species, appears to be $PGF_{2\alpha}$ (Auletta et al., 1973; reviewed in Poyser et al., 1995). However, hysterectomy does not delay luteolysis in many higher primates (Neill et al., 1969; Beling et al., 1970), therefore $PGF_{2\alpha}$ must not be of uterine origin. However, human corpora lutea are capable of secreting $PGF_{2\alpha}$ (Schutt et al., 1976; Challis et al., 1976; Swanston et al., 1977; Patwardhan et al., 1980). It is postulated that $PGF_{2\alpha}$ of luteal origin causes luteolysis in higher primates (reviewed in Bazer et al., 1991; Zelenik and Fairchild-Benyo, 1994).

The lifespan of the human corpus luteum is prolonged by a glycoprotein secreted by the conceptus (hCG; Hisaw, 1944). The evidence in support of this is quite convincing: immunization against hCG results in the inability to maintain pregnancy (Prahalada et al., 1975; Thau and Sundram, 1980). The biological activity of hCG

resembles that of LH (reviewed in Segaloff and Ascoli, 1993), in fact, hCG appears to act through the LH receptor (Cameron and Stouffer, 1982). Detectable levels of hCG first appear in human urine 7 to 8 days after ovulation (Baird et al., 1991).

The molecular mechanism by which hCG rescues the corpus luteum from luteolysis remains unclear. It is interesting that hCG and LH act through the same receptor, yet hCG is the only gonadotropin that can prevent luteolysis. There are some unique properties of hCG that might be important in describing the differential effects of hCG and LH in luteal lifespan. There are some distinct biochemical differences between hCG and LH. The α-subunits of LH and hCG are identical, but the β-subunits, although somewhat similar, are different (Zelenik and Fairchild-Benyo, 1994). The β-subunit of hCG has an additional 30-amino acids, probably the evolutionary result of point mutation of a stop codon in the LHβ gene (Zelenik and Fairchild-Benyo, 1994). The glycosylation pattern of hCG is more extensive than that of LH (Morel et al., 1971). The half-life of hCG in blood is much greater (~100-fold) than that of LH (Parlow, 1968). After binding to receptor, internalization rates of hCG are also much slower than those for LH (Mock and Niswender, 1983), possibly due to the relative degree of glycosylation of each molecule (Niswender et al., 1985b). Internalization of LH receptor is related to the rate of lateral migration of ligand receptor complexes into clathrin-coated pits, where they are moved into the cell by endocytosis. In essence, the biochemical properties of LH and hCG likely explain the differential biological properties of the two hormones.

It would be useful to know the effects of hCG on luteal prostaglandin metabolism in the primate corpus luteum. Because hCG induces PGDH expression in corpora lutea of pregnant rabbits (Bedwani and Marley, 1975), it is reasonable that a similar event

might occur in the corpora lutea of pregnant women. This would result in a barrier against luteal accumulation of $PGF_{2\alpha}$ in the corpus luteum, and decrease of local concentrations of $PGF_{2\alpha}$. However, in one study, hCG failed to inhibit $PGF_{2\alpha}$ -induced hydrolysis of phosphatidylinositol 4,5-bisphosphate or intracellular calcium in corpora lutea of rhesus monkeys (Houmard et al., 1994). This could simply mean that regulation of $PGF_{2\alpha}$ by hCG occurs at the level of synthesis $PGF_{2\alpha}$ or catabolism. Indeed, *in vitro* synthesis of prostaglandins, especially $PGF_{2\alpha}$, is suppressed in hCG-treated corpora lutea of rhesus monkeys (Balmaceda et al., 1981).

Ruminants

Ruminants require progesterone from the corpus luteum to maintain pregnancy, until the luteo-placental shift occurs. In the ewe, removal of the corpus luteum prior to day 50 of pregnancy, results in embryonic death (Casida and Warwick, 1945; Denamur and Martinet, 1955). The ovine placenta secretes enough progesterone after day 50 to support pregnancy (Ricketts and Flint, 1980). However, treatment of ovariectomized pregnant ewes with progesterone before day 50, maintains pregnancy (Moore and Rowson, 1959; Bindon, 1971).

Removal of the bovine corpus luteum prior to day 200 of pregnancy results in embryonic death (Estergreen et al., 1967). As in the ewe, progesterone supplementation can maintain pregnancy in ovariectomized cows (Tanabe et al., 1968). However, unlike the ewe, adrenal progesterone and not placental progesterone, appears to be able to maintain pregnancy (Wendorf et al., 1983). Transfer of embryos to synchronized recipient cows as late as day 16 can extend luteal lifespan (Betteridge et al., 1980). However, removal of embryos after day 17 does not result in luteal regression (Northey

and French, 1980; Dalla Porta and Humblot, 1983). This indicates that the critical period of maternal recognition of pregnancy in the cow occurs on day 16-17.

Maternal recognition of pregnancy in the ewe occurs around day 12-13 of pregnancy. Transfer of ovine embryos to synchronized recipients up to day 12, but not after, will extent luteal lifespan (Moor and Rowson, 1966a). Similarly, removal of embryos prior to day 12 allows luteolysis to occur at the normal time, but removal of the embryo after day 13 results in extended luteal lifespan (Moor and Rowson, 1966b). This indicates the critical period for maternal recognition of pregnancy in the ewe occurs on days 12-13. Homogenates of day 14 to 16 ovine conceptuses also extend luteal lifespan when infused into the uterine lumen (Rowson and Moor, 1967; Elinwood et al., 1979; Martal et al., 1979). Proteins secreted by the bovine conceptus between day 16 and 26 of pregnancy also extend luteal lifespan when infused into the uterine lumen of cows (Geisert et al., 1988; Helmer et al., 1989; Thatcher et al., 1989). This embryonic component was proteinaceous, heat-labile, and possessed no LH-like or prolactin-like biological activity (Ellinwood et al., 1979a).

The antiluteolytic protein that prevents luteolysis during early pregnancy has been characterized and identified as a novel interferon, interferon tau (IFN-τ; reviewed in Bazer et al., 1996). Interferon-τ is secreted by the trophectoderm of the ovine conceptus between days 12 and 21 of pregnancy, with maximal secretion occurring on days 14-16 (Godkin et al., 1982; Farin et al., 1989). In the cow, IFN-τ secretion must commence by days 14-16 of pregnancy (Northey and French, 1980) and is maximal around day 18 of pregnancy (Bartol et al., 1985). The endometrium expresses high affinity receptors for IFN-τ (Godkin et al., 1984; Stewart et al., 1987; Hansen et al., 1989; Knickerbocker et

al., 1989). The number and density of receptors for oIFN-τ decrease until day 14 of the estrous cycle, then begin to increase after day 16 of the estrous cycle (Knickerbocker et al., 1989). Conversely, endometrial oIFN-τ receptors are expressed through day 14 of pregnancy, expression decreases until day 16 of pregnancy, when expression ceases (Knickerbocker et al., 1989). The biological function of oIFN-τ expression after day 16 of pregnancy in the ewe is unclear since there are no endometrial receptors for this cytokine after day 16. It is possible that oIFN-τ acts on other tissues, possibly the corpus luteum.

The glandular and superficial epithelium of the endometrium are the target tissues of IFN-τ (Godkin et al., 1984; Hansen et al., 1989) Interferon-τ acts to inhibit pulsatile secretion of $PGF_{2\alpha}$ by the endometrium (reviewed in Thatcher et al., 1989; Silvia, 1992). During early pregnancy (days 14-15), high amplitude pulses of $PGF_{2\alpha}$ are attenuated (Zarco et al., 1984); however,, tonic $PGF_{2\alpha}$ secretion is actually greater than in nonpregnant ewes on days 14-15 post-estrous (Inskeep and Murdoch, 1980; Zarco et al., 1984; Fincher et al., 1984). Endometrial tissues of the pregnant ewe are capable of metabolizing $PGF_{2\alpha}$ to PGFM (Keirse et al., 1978), therefore the corpus luteum of the pregnant ewe might simply be exposed to elevated basal levels of PGFM.

Interferon-τ acts locally at the level of the uterus, not systemically, to prevent luteolysis. In support of this, intrauterine administration of conceptal homogenates (Heyman et al., 1984; Helmer et al., 1989) or roIFN-τ (Spenser et al., 1999), but not systemic administration, block pulsatile release of $PGF_{2\alpha}$ and delay luteolysis. Furthermore, luteolysis can occur in the ovary contralateral to the gravid uterine horn, but

not in the ovary ipsalateral to the gravid uterine horn (Moor and Rowson, 1966a,b; Niswender et al., 1970).

The antiluteolytic mechanism of IFN-τ action of appears to be indirect (reviewed in Silvia, 1992; Wathes and Lamming, 1995; Bazer et al., 1996; McCracken et al., 1999). Expression of mRNA encoding estrogen receptors (Spenser et al., 1995a,b,c) and estradiol-17β binding is decreased in endometrial tissues exposed to IFN-τ (Spenser et al., 1995a, b). The ultimate result of decreased estrogen receptor expression is a subsequent decrease in levels of mRNA encoding oxytocin receptor (Stevenson et al., 1994; Spenser et al., 1995a,b,c) and decreased oxytocin receptor density (McCracken et al., 1984; Spenser et al., 1995a,b,c). Consistent with this, oxytocin cannot stimulate secretion of $PGF_{2\alpha}$ in pregnant ewes to the same degree as in nonpregnant ewes (McCracken, 1980). In conclusion, INF-τ downregulates expression of endometrial estrogen receptors, thus resulting in a uterine environment devoid of oxytocin responsiveness (reviewed in Wathes and Lamming et al., 1995; McCracken et al., 1999).

It has been proposed by several investigators that maternal recognition of pregnancy involves a shift in endometrial prostaglandin synthesis from PGE_2 to $PGF_{2\alpha}$ (Silvia et al, 1984; Niswender and Nett, 1994; McCracken et al., 1999). Uterine secretion of PGE_2 increases during pregnancy (Ellinwood et al., 1979b; LaCroix and Kann, 1982), starting on day 13 (Silvia et al., 1984). Intrauterine infusion of PGE_2 results in delayed luteal regression in cyclic ewes (Pratt et al., 1977; Magness et al., 1981). Furthermore, intrauterine infusion of PGE_2 prevents $PGF_{2\alpha}$- (Mapletoft et al., 1977; Henderson et al., 1977; Reynolds et al., 1981) and estradiol-17β- (Colcord et al. 1978) induced luteolysis. Thus, it is evident that PGE_2 likely plays a key role in maternal recognition of pregnancy,

in addition to enhancement of luteal steroidogenesis, by protecting the corpus luteum from luteolytic stimuli.

The ovine corpus luteum is resistant to the luteolytic effects of $PGF_{2\alpha}$ (Inskeep et al., 1975; Mapletoft et al., 1976; Pratt et al., 1977; Silvia and Niswender, 1984; Silvia et al., 1986) during early pregnancy (day 13-16; Silvia et al., 1986), when maternal recognition of pregnancy occurs. Peak resistance to $PGF_{2\alpha}$ occurs on day 13 of pregnancy (Silvia et al., 1986). The corpus luteum of early pregnancy in the ewe is not completely resistant to the antiluteolytic actions of $PGF_{2\alpha}$, rather it requires a greater dose of $PGF_{2\alpha}$ to induce luteolysis (Silvia et al., 1986). Estradiol-17β is also ineffective as a luteolysin during maternal recognition of pregnancy in ewes (Kittok and Britt, 1977), likely due to the effects of PGE_2 (Colcord et al., 1978).

The mechanism by which the ovine corpus luteum achieves resistance to $PGF_{2\alpha}$ during early pregnancy remains unclear. Downregulation of receptors for $PGF_{2\alpha}$ does not appear to be the mechanism (Weipz et al, 1992). Expression of mRNA encoding receptors for $PGF_{2\alpha}$ is not attenuated during maternal recognition of pregnancy in ewes (Weipz et al, 1992; Juengel et al., 1998b) or cows (Sakamoto et al., 1995). In fact, levels of mRNA encoding the receptor for $PGF_{2\alpha}$ are suppressed on day 14 of pregnancy, but not day 12 of pregnancy in ewes (Hoyer et al., 1999). Furthermore, in the cow, the affinity of $PGF_{2\alpha}$ receptors for PGF_2 does not change at any time during the estrous cycle or during maternal recognition of pregnancy (Sakamoto et al., 1999). Thus the mechanism by which the ruminant corpus luteum achieves resistance to the luteolytic activity of $PGF_{2\alpha}$ does not appear to involve regulation of $PGF_{2\alpha}$ receptors.

It is possible that the intracellular signaling pathway of $PGF_{2\alpha}$ is interrupted during early pregnancy. On day 15 of pregnancy, concentrations of mRNA encoding two endogenous peptide inhibitors of protein kinase C are elevated (Juengel et al., 1998b). It is also possible that the corpus luteum achieves resistance to $PGF_{2\alpha}$ during early pregnancy by gaining the capacity to catabolize $PGF_{2\alpha}$, thus preventing local accumulation and elevated levels of $PGF_{2\alpha}$ in the corpus luteum. There is reasonable evidence that there is a threshold concentration of $PGF_{2\alpha}$ that is required for the corpus luteum to commit to luteolysis and that the corpus luteum can recover from exposure to subthreshold levels of $PGF_{2\alpha}$ (Silvia et al., 1986; Juengel et al., 1999). This, combined with the fact that luteal production of $PGF_{2\alpha}$ appears to play a key role in luteolysis, provides a compelling rationale for a role of $PGF_{2\alpha}$ catabolism in resistance of the corpus luteum to $PGF_{2\alpha}$. Catabolism of $PGF_{2\alpha}$ in the corpus luteum would keep luteal levels of $PGF_{2\alpha}$ attenuated and prevent the corpus luteum from being irreversibly committed to luteolysis.

Properties of PGDH and COX-2

Cyclooxygenase-2

Cyclooxygenase (COX; also known as prostaglandin H synthase, PGHS) catalyses the rate limiting step in prostanoid biosynthesis (reviewed in Vane et al., 1998). All prostanoids are synthesized from PGH_2, which is formed in a two-step reaction. First, oxygen molecules are complexed with the newly formed cyclopentane ring derived from arachadonic acid into a cyclic peroxide and a peroxide residue is formed at the number 15 position of the intermediate PGG_2. Second, the peroxide at the number 15 position is reduced to a hydroxyl group by a peroxidase domain that is distinct from the

cyclooxygenase domain. The cyclic peroxide at the number 15 position is reduced later in the pathway by the respective PG synthase. Because of the high degree of oxidative stress that COX is subject to, it is no surprise that the enzyme averages only about 1200 catalytic cycles per molecule (Vane et al., 1998).

There are two known isoforms of COX, an inducible isoform (COX-2) and a constitutively expressed isoform (COX-1). The active site domains of COX proteins are highly conserved within species across isoforms; however, the remaining domains of the proteins differ considerably (Otto and Smith, 1995). In fact, the human COX-1 and COX-2 genes share 63 % amino acid homology (personal observation).

There are some marked difference between COX-1 and COX-2 in substrate specificity (Otto and Smith, 1995) and pharmacological properties (Bakhle and Botting, 1996; Griswold and Adams, 1996). Aspirin, perhaps the most commonly used nonsteroidal anti-inflammatory drug (NSAID), acts by irreversibly acetylating the cyclooxygenase domain of COX, without having any effect on the peroxidase domain.

A generalized, but fairly accurate assertion is that COX-1 is involved in myriad "normal", nonpathological biological processes, but COX-2 is primarily involved in inflammatory processes. For example, COX-1 is involved in the synthesis of prostacyclin in the stomach. Prostacyclin is a potent vasodilator and increases mucosal blood flow therefore enhancing the viability of gastric mucosal stem cell populations. This is why NSAIDs specific for COX-2 have no negative side effects on the gastric mucosa. Thus, it is evident why there is profound interest in NSAIDs that specifically target COX-2.

There is also an integral role for COX-2 in reproductive processes. Female mice that have the gene encoding COX-2 inactivated are infertile (Morham et al., 1995; Dinchunk et al., 1995), probably due to the inability to ovulate (reviewed in Richards et al., 1995; Richards, 1997). The involvement of COX-2 in luteolysis has been discussed in detail and will not be visited further in this section.

15-Hydroxyprostaglandin Dehydrogenase

The rate-limiting step of inactivation of prostaglandins of the E and F series is catalyzed by 15-hydroxyprostaglandin dehydrogenase (PGDH; Anggard and Samuelsson, 1964; Anggard, 1966; reviewed in Okita and Okita, 1996); however, PGDH has a K_m for $PGF_{2\alpha}$ twofold greater than for any prostaglandin of the E series (133 μM for $PGF_{2\alpha}$ vs. 59 μM for PGE_2; Bergholte et al., 1987). Prostaglandins of the A series (Pace-Asciak and Smith, 1983) and I series (Jarabak et al., 1984) are catabolized by PGDH, but prostaglandins of the B series (Wantabe et al., 1983) and D series (Hoult et al., 1988) are not.

Prostaglandin $F_{2\alpha}$ is converted to 13, 14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) in a series of two coupled reactions (reviewed in Okita and Okita, 1996). First, Type I PGDH catalyzes reversible oxidation of the 15-hydroxyl to the metabolite 15-ketoprostaglandin $F_{2\alpha}$ concurrent with reduction of the cofactor nicotinamide adenine dinucleotide (NAD^+; Casey and Johnston, 1980). Immediately, 15-ketoprostaglandin $F_{2\alpha}$ is irreversibly converted to 13, 14-dihydro-15-keto-$PGF_{2\alpha}$ by Δ13-reductase (Tsai and Einzig, 1989). Carbonyl reductase can then reduce the 15-keto group of PGFM to a hydroxyl group, yielding to 13, 14-dihydro-$PGF_{2\alpha}$ (reviewed in Okita and Okita, 1996). Interestingly, 13, 14-dihydro-$PGF_{2\alpha}$ has been shown to be biologically active in rats (Iwata et al., 1990a,

b), rabbits (Kehl and Carlson, 1981), and heifers (Milvae and Hansel, 1983). However, PGFM nor 15-ketoprostaglandin $F_{2\alpha}$ have any luteolytic activity in ewes (Light et al., 1994).

The $PGF_{2\alpha}$ metabolite, 13, 14-dihydro-$PGF_{2\alpha}$, cannot be produced in the absence of PGDH because Δ13-reductase can only reduce 15-ketoprostaglandin $F_{2\alpha}$, not $PGF_{2\alpha}$ (reviewed in Okita and Okita, 1996). Similarly, carbonyl reductase is specific for 13, 14-dihydro-15-keto-prostaglandins and cannot convert 15-ketoprostaglandin $F_{2\alpha}$ back into biologically $PGF_{2\alpha}$. In summary, PGDH, Δ13-reductase, and carbonyl reductase are all necessary for conversion of $PGF_{2\alpha}$ to a biologically active metabolite 13, 14-dihydro-$PGF_{2\alpha}$; however, in the absence of PGDH, the pathway to produce 13, 14-dihydro-$PGF_{2\alpha}$ is blocked, and $PGF_{2\alpha}$ remains biologically active. In the absence of carbonyl reductase, $PGF_{2\alpha}$ is converted to the biologically inactive metabolite, PGFM.

Type II PGDH has been identified and characterized (reviewed in Ensor and Tai, 1995). Type II PGDH utilizes $NADP^+$ as a cofactor, has 9-ketoreductase activity, and has specificity for 13, 14-dihydro-15-keto-prostaglandins (reviewed in Ensor and Tai, 1995). Type II PGDH is really carbonyl reductase (Wermuth et al., 1988). The specificity of carbonyl reductase for 13, 14-dihydro-15-keto-prostaglandins prevents the conversion of PGE_2 to $PGF_{2\alpha}$ by carbonyl reductase catalyzed hydroxylation of the 9-keto group of PGE_2. Interestingly, in the rabbit corpus luteum, there is 9-ketoreductase activity that converts PGE_2 to $PGF_{2\alpha}$ during luteolysis (Watson et al., 1979, Schlegel and Daniels, 1989).

The M_r of immunoreactive PGDH appears to be 28 kDa and 56 kDa (Tai et al., 1990). The molecular weight as determined by matrix-assisted laser desorption/

ionization mass spectrometry revealed a molecular weight of 28,740 Da (Hohl et al., 1993), consistent with the calculated molecular weight of 28, 975 Da based on the cDNA sequence (Ensor et al., 1990). The 56 kDa species is believed to represent a dimeric PGDH particle that is associated in a covalent manner (Mak et al., 1982).

Estradiol administration appears to inhibit expression of PGDH (Chang and Tai, 1985; Chang, 1987; Erman et al., 1987). The 5'-untranslated region of the mouse PGDH gene contains a consensus estrogen receptor-binding site (Matsuo et al., 1997), thus the effects of estrogen are likely directly at the genomic level. There have been reports that estradiol can also slightly stimulate PGDH expression in human erythroleukemia cells (HEL; Xun et al., 1991a).

Other potential regulatory *cis*-elements include Sp1, ATF/CRE, multiple glucocorticoid response elements (GRE), AP1, AP2, and HF-IL6 (Matsuo et al., 1997). Expression of PGDH is induced by PMA in U937 cells at the transcriptional level (Matsuo et al., 1997). Inhibitors of protein synthesis block PMA upregulation of PGDH activity (Xun et al., 1991a,b). Conversely, concentrations of PMA greater than 10 nM actually inhibit PGDH activity by phosphorylation by protein kinase C (Xun et al., 1991b).

Progesterone appears to regulate PGDH expression in many tissues (reviewed in Okita and Okita, 1996). Indeed, PGDH expression is 20-fold greater in the lungs of pregnant rabbits than in the lung of nonpregnant rabbits (Sun and Armour, 1974; Edgerton-Vernon and Bedwani, 1975). In the corpora lutea of pregnant rabbits and pseudopregnant rabbits, PGDH activity is 15-fold greater than in nonpregnant rabbits (Okita et al., 1992). When injected into male rabbits, progesterone or progesterone and

estradiol caused an increase in PGE_2 clearance in the pulmonary vasculature (Bedwani and Marley 1975). Treatment of female rabbits with hCG also results in increased pulmonary PGDH activity (Bedwani and Marley, 1975). Furthermore, progesterone is known to induce PGDH expression in human myometrium (Thornburn and Challis, 1979). The progesterone antagonist, RU486, attenuates PGDH in endometrial stromal tissue of women (Cheng et al., 1993).

Coincidentally, the ovine corpus luteum becomes resistant to the luteolytic effects of $PGF_{2\alpha}$ after administration of hCG (Bolt, 1979). Luteolysis is delayed in dairy heifers (Howard et al., 1990) and sows (Guthrie and Rexroad, 1991; Guthrie and Bolt, 1983) treated with hCG. However, the effect of $PGF_{2\alpha}$, on *in vitro* steroidogenesis of bovine luteal cells is not prevented by hCG (Litch and Condon, 1988). Thus, the antiluteolytic and luteotropic affects of hCG, *in vivo*, appear to be in a tissue other than the corpus luteum. Interestingly, PGFM pulse frequency is reduced in heifers treated with hCG (Helmer and Britt, 1987).

The involvement of PGDH in reproductive processes is well established (reviewed in Gibb, 1998). Many $PGF_{2\alpha}$–sensitive tissues express PGDH as a mechanism of protection from $PGF_{2\alpha}$ produced by neighboring tissues. For example, decidual and amnionic tissues of the human produce large quantities of prostaglandins, primarily PGE_2 and $PGF_{2\alpha}$, during pregnancy (Okazaki et al., 1981; reviewed in Challis and Mitchell, 1994). If $PGF_{2\alpha}$ was allowed to reach the myometrium, preterm labor would occur. However, the trophoblast cells of the chorion have very high levels of PGDH activity (Keirse and Turnbull, 1975; Cheung et al., 1992) and provide a barrier preventing biologically active $PGF_{2\alpha}$ from reaching the myometrial tissues. Not surprisingly,

preterm labor is associated with suppressed PGDH activity (Van Meir et la., 1996; Van Meir et al., 1997a; Van Meir et al., 1997b). Similarly, PGDH is expressed in the endometrial epithelium of the mare, and expression is limited to the microcotyledonary regions (Han et al., 1995). Endometrial and placental tissues of the pregnant cow (Erwich et al., 1988; Janszen et al., 1994), ewe (Keirse et al., 1978), guinea pig (Keirse et al., 1978), and rat (Tsurta and Mori, 1988) also express PGDH.

In light of the role of PGDH in protecting target tissues such as the myometrium from $PGF_{2\alpha}$, it is reasonable that the corpus luteum expresses PGDH and that PGDH is a mechanism by which corpora lutea achieve resistance to $PGF_{2\alpha}$.

Summary of Luteal Physiology

The corpus luteum develops from differentiation and mitosis of cells from the ruptured follicle. A variety of hormones are required for normal development and function of the corpus luteum, primarily LH and GH. The corpus luteum consists of several cell types, with small and large luteal cells of primary importance in the steroidogenic function of the corpus luteum. Progesterone output by large luteal cells accounts for a majority of luteal progesterone production and appears to happen in a constitutive manner. Conversely, progesterone production by the small luteal cells appears to be regulated. Endothelial cells appear to play a role in luteal angiogenesis and capillary destruction during luteolysis. The cell types of the corpus luteum appear to be differentially regulated by both tropic and inhibitory hormones.

In the absence of a fertile estrous cycle, the corpus luteum must regress to allow ovulation and the occurrence of another estrous cycle. Luteolysis in ruminants appears to be initiated by $PGF_{2\alpha}$ of uterine origin but occurs as the result of a complex and

interrelated sequence of events. These events include deletion of the capillary network providing oxygen and nutrients to the metabolically active corpus luteum, abrogation of the mechanisms involved in luteal steroid production, and finally death of the tissue and constituent cells. The luteolytic signal is amplified by $PGF_{2\alpha}$ of both luteal and endometrial origin and oxytocin of neurohypophyseal and luteal origin. Amplification of the luteolytic signal beyond a threshold level appears to be required for the corpus luteum to be committed to luteolysis.

The most important function of the corpus luteum is to synthesize and secrete progesterone during pregnancy. During early pregnancy, IFN-τ secreted by the trophoblast cells of the conceptus prevents the initial luteolytic signal and/or amplification of that signal beyond an apparent threshold. Pulsatile uterine secretion of $PGF_{2\alpha}$ is attenuated and the corpus luteum appears to develop mechanisms the prevent luteolysis in the event the corpus luteum is exposed to low levels of $PGF_{2\alpha}$.

Hypotheses and Objectives

Infertility is a major problem in domestic livestock production. Early embryonic loss is a major factor contributing to infertility. Inadequate secretion of progesterone by the corpus luteum is an underlying cause of early embryonic death. Luteal resistance to $PGF_{2\alpha}$ might be a mechanism to enhance luteal secretion of progesterone during early pregnancy and reduce embryonic loss. Modification of COX-2 and PGDH biological activity could be involved in luteal resistance to $PGF_{2\alpha}$.

The ultimate objectives of the research described in this dissertation were to provide insight into mechanisms of maternal recognition of pregnancy that might be

involved in embryonic death. Ideally these insights will result in refined management practices resulting in enhanced efficiency of livestock production systems.

Specifically, the following hypotheses were tested: 1) The corpus luteum has an increased capacity to catabolize $PGF_{2\alpha}$ during periods of luteal resistance to $PGF_{2\alpha}$, as the direct result of increased PGDH activity, and 2) the corpus luteum has decreased expression of mRNA encoding COX-2 during periods of luteal resistance to $PGF_{2\alpha}$.

Chapter 2

Comparison activity of 15-hydroxyprostaglandin dehydrogenase in the ovine corpus luteum on days 4 and 13 of the estrous cycle and day 13 of pregnancy

Abstract

To gain insight into the mechanisms by which the corpus luteum of early pregnancy in the ewe achieves resistance to the luteolytic actions of $PGF_{2\alpha}$, enzymatic activity of 15-hydroxy-prostaglandin dehydrogenase (PGDH) was measured in corpora lutea during periods when the corpus luteum is resistant to $PGF_{2\alpha}$ [day 4 postestrus (n=11) in nonpregnant ewes and day 13 postcoitus (n=10) in pregnant ewes] and when it is responsive to $PGF_{2\alpha}$ [day 13 postestrus (n=7) in nonpregnant ewes]. Enzymatic activity was measured by determining the rate of conversion of $PGF_{2\alpha}$ to PGFM. Enzymatic activity of PGDH was greater in corpora lutea obtained on day 4 of the estrous cycle ($p < 0.05$) and on day 13 of pregnancy ($p < 0.05$) than on day 13 of the estrous cycle in nonpregnant ewes.

The data from this experiment support the hypothesis that PGDH activity is greater in corpora lutea that are resistant to $PGF_{2\alpha}$ (day 4 of the estrous cycle and day 13 of pregnancy) than in corpora lutea that are $PGF_{2\alpha}$-sensitive. Finally, these data are the first to demonstrate a role for catabolism of $PGF_{2\alpha}$ in luteal resistance to $PGF_{2\alpha}$.

Introduction

The ovine corpus luteum is required throughout the first 50 days for maintenance of a normal pregnancy (Casida and Warwick, 1945; Denamur and Martinet, 1955). This requires that the corpus luteum of pregnancy be rescued from the luteolytic effects of $PGF_{2\alpha}$ by 1) suppression of pulsatile secretion of $PGF_{2\alpha}$ from the uterus by IFN-τ (reviewed in Thatcher et al., 1989; Silvia, 1992), and 2) establishment of mechanisms that promote resistance of the corpus luteum to the luteolytic effects of $PGF_{2\alpha}$ (Inskeep et al., 1975; Mapletoft et al., 1976; Pratt et al., 1977; Silvia and Niswender, 1984; Silvia et al., 1986). The biochemical mechanisms involved in luteal resistance to the actions of $PGF_{2\alpha}$ are poorly understood, but interference with a second messenger pathway of $PGF_{2\alpha}$ action is believed to be involved (Juengel et al., 1998b). It is also possible that catabolism of $PGF_{2\alpha}$ by the corpus luteum could account for the resistance of the corpus luteum of early pregnancy to $PGF_{2\alpha}$.

The enzyme that performs the rate-limiting enzymatic step in inactivation of prostaglandins of the E and F series is 15-hydroxyprostaglandin dehydrogenase (PGDH; Anggard and Samuelsson, 1964; Anggard, 1966; reviewed in Okita and Okita, 1996). Many tissues express PGDH to provide local protection from the actions of $PGF_{2\alpha}$. For example, the human chorion expresses high levels of PGDH to provide a barrier to $PGF_{2\alpha}$ (of amnionic origin; Keirse and Turnbull, 1975; Cheung et al., 1992) destined for the myometrium. The lungs of pregnant rabbits increase expression of PGDH in response to progesterone (Sun and Armour, 1974; Edgerton-Vernon and Bedwani, 1975), presumably to protect the corpora lutea from circulating $PGF_{2\alpha}$.

The objective of this study was to determine if luteal expression and enzymatic activity of PGDH occur during early pregnancy and early in the estrous cycle, times when luteal resistance to the luteolytic effects of $PGF_{2\alpha}$ is known to occur.

Materials and Methods

Unless otherwise indicated, all reagents and materials were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Denver, CO).

Experimental Design

To test the hypothesis that enzymatic activity of PGDH in ovine corpora lutea is greater on day 4 postestrus in nonpregnant ewes and day 13 postcoitus in pregnant ewes than on day 13 postestrus in nonpregnant ewes, 28 ewes were randomly assigned to three groups (day 4, n=11; day 13 cycle, n=7; day 13 pregnancy, n=10). Based on the mean and variability figures for PGDH activity measurements in previous experiments (Keirse et al., 1978), power calculations indicated that a sample size of 7 ewes was necessary to detect differences in PGDH activity with a power of 0.96. Because of the minute amount of tissue in the day 4 corpus luteum, tissue was collected from 11 ewes to increase the probability of collecting multiple corpora lutea from a single ewe to ensure that enough tissue would be available for all analyses without combining tissue and compromising the experimental unit (the ewe). Similarly, 10 ewes were bred for collection of corpora lutea on day 13 of pregnancy in anticipation of 70% pregnancy rates; all 10 ewes became pregnant so tissue was collected from each ewe.

Tissue collection

Western range ewes exhibiting normal estrous cycles (17 ± 1 days) were used in the experiment. Ewes were checked for estrous behavior twice daily by a vasectomized

ram and the first day of estrus, was assigned day 0 of the estrous cycle. To collect tissue from pregnant ewes on day 13 after breeding, ewes were anesthetized with sodium pentobarbitol (25 mg/ kg BW) and reproductive organs exposed through a midventral abdominal incision. Pregnancy was confirmed during surgery by flushing the uterus and observing the presence of an embryo. Corpora lutea were removed from ovaries and immediately snap frozen in liquid nitrogen and stored at –70°C for future analysis. Luteal tissue was collected from ewes on day 4 and day 13 of the estrous cycle in the manner described for tissue collection for day 13 of pregnancy without flushing of the uterus. Tissues were used for determination of steady state levels of mRNA encoding PGDH, COX-2, and PGDH enzyme assays. Blood samples were collected before surgery and assayed for progesterone by radioimmunoassay (Niswender et al., 1973) to confirm the functionality of the corpus luteum. Concentrations of progesterone were determined in a single radioimmunoassay with an intraassay coefficient of variation of 9.2%. All experimental procedures and protocols were reviewed and approved by the Colorado State University Animal Care and Use Committee.

Cloning of PGDH

A 394 bp cDNA encoding ovine 15-hydroxyprostaglandin dehydrogenase (PGDH394) was generated utilizing reverse transcription (RT), polymerase chain reaction (PCR; Perkin Elmer Cetus, Norwalk, CT). Polyadenylated messenger ribonucleic acid (Poly A^+ RNA) was isolated from ovine lung and luteal tissue collected on day 4 of the estrous cycle with oligodeoxythymidine (dT) cellulose as previously described (Badley et al., 1988; modified by Guy et al., 1995) and reverse transcribed using Ready-To-Go RT-PCR beads (Amersham-Pharmacia Biotech, Uppsala, Sweden).

The reverse transcription reaction was incubated at 37°C for 1h and 95°C for 10 min using random hexamers as primers. Upon completion of reverse transcription, PCR primers were added and PCR carried out according to the following protocol: [(95°C, 1min; 48°C, 1min; 72°C, 1min; 30 cycles), (95°C, 1 min; 48°C, 1 min; 72°C, 10 min; 1 cycle)]. Primers were designed based on the sequence of *Bovis bubalus* (water buffalo; Genebank Accession Number:AJ222837) PGDH, with 5'-PGDH (5'-ATGCACGT-GAACGGCAAAGTG-3') and 3'-PGDH (5'-TGCCACCTTCGCCTCC-ATTTT-3') corresponding to bases 1-21 and 373-394, respectively. The resulting 394-bp cDNA was gel purified and ligated into pGEM-Easy (Promega, Madison, WI) then competent *E. Coli* (DH5α) cells were transformed with the resulting construct. Transformed colonies were screened by PCR for plasmid containing PGDH insert using the original PGDH primers. Plasmid containing the 394-bp PGDH cDNA was sequenced (Geneseek, Lincoln, NE). The partial cDNA encoding ovine PGDH was amplified using PCR and the original PGDH primer pair and the product was gel purified and used as template for radiolabeling([^{32}P]-dCTP; 3000 Ci/mmol) a cDNA probe (random primer method; Feinberg and Vogelstein, 1983) for PGDH.

Northern Analysis

Poly (A^+) mRNA was isolated from brain (negative; Lee and Levine, 1975), caruncular tissue (positive; Keirse et al., 1978), endometrium (positive; Keirse et al., 1978), myometrium (positive; Keirse et al., 1978), luteal tissue from days 4, and 10 of the estrous cycle, kidney (positive; Wright and Corder, 1979), liver (positive; Iwata et al., 1990a), and lung (positive; Piper et al., 1970). The Northern blot was performed with three objectives: 1) to determine if the PGDH cDNA probe hybridized only to mRNA

isolated from tissues known to express PGDH, 2) determine the size of the PGDH transcript in ovine tissues, and 3) ensure that the probe hybridized to a single transcript. Five μg of poly (A^+) mRNA from each tissue were loaded onto a 1.5% agarose/4.5% formaldehyde/3-[N-morpholino] propanesulfonic (MOPS) acid gel, run at 70 V for 3 h in 1X MOPS, and transferred overnight by capillary action using 20X saline sodium citrate (SSC) to a Hybond nylon membrane (Amersham, Arlington Heights, IL). Messenger RNA was crosslinked to the membrane by 1200 kjoules/cm^2 of ultraviolet light. The membrane was prehybridyzed for 30 min at 42°C in Ultrahybe (Ambion, Austin, TX) hybridization buffer. The [^{32}P]-dCTP labeled PGDH cDNA probe (1-2 x 10^6 dpm/ng cDNA) was then added at a concentration of 1 x 10^6 dpm/ml of Ultrahybe hybridization buffer for Northern and slot-blot hybridizations at 42°C, overnight (16-24 h). Membranes were then washed in 2X SSC/0.1% sodiumdodecyl sulfate (SDS) two times for 5 minutes at 42°C, and twice in 0.2X SSC/0.1%SDS at 42°C for 15 min. Membranes were then exposed overnight in a phosphorimager cassette and the image analyzed (Molecular Dynamics, Sunnyvale, CA).

PGDH Enzyme Assay

Enzymatic activity of PGDH was measured in luteal homogenates utilizing a modified version of an assay previously described by Keirse et al. (1978). Luteal tissue was homogenized on ice in cold 100mM phosphate buffer/2mM nicotinamide adenine dinucleotide (NAD; PGDH assay buffer pH 7.5), for 30 sec using a polytron tissue homogenizer on setting 4. Tissue was homogenized in 1 ml of buffer per 100 mg tissue and homogenates were centrifuged for 5 min at 5000 x g at 4°C and the supernatant saved and stored at –70°C until assayed. Freezing of lung tissue at –70°C did not decrease

PGDH activity after a single thaw (Silva, personal observation; Critchley et al., 1998). To determine PGDH activity, 300 µl of luteal homogenate (equivalent to 30 mg of tissue) were added to a 5 ml borosilicate glass tube containing 50 ng of $PGF_{2\alpha}$-Tris salt in PGDH assay buffer and 700 µl of PGDH assay buffer, for a total reaction volume of 1 ml (all reagents were prewarmed to 37°C before initiation of an assay). The assay was conducted at 37°C and aliquots of 100 µl of the reaction mixture were added to 5 ml borosilicate glass tubes containing 300 µl of 200 mM formic acid to stop enzymatic activity. The pH of the stopped reaction was approximately 2.5. Concentrations of PGFM were measured at 5, 10, 15, 30, 45, 60, 90, and 180 min. The initial PGFM levels were determined by addition of 30 µl tissue homogenate, 5 µl of $PGF_{2\alpha}$, and 65 µl of PGDH assay buffer added to 300 µl of 200 mM formic acid. After terminating the enzymatic activity in the reaction aliquots, 1 ml of PBS was added to each aliquot to change the pH from 2.5 to 4.5 for PGFM extraction. Samples were extracted twice with 5 ml of ethyl acetate, dried, and reconstituted in 1 ml of phosphate buffered saline (PBS)/0.1% gelatin and allowed to sit overnight at 4°C. Concentrations of PGFM in the aliquots were determined using a previously validated radioimmunoassay (Homanics and Silvia, 1988) in three separate radioimmunoassays. The average intra-assay coefficient of variation was 5.9 % at 20 % binding and 11.8 % at 80% binding and the inter-assay coefficient of variation was 1.7 % at 50% binding.

To assure that PGFM appearance was due to enzymatic catabolism of $PGF_{2\alpha}$ and not PGFM contamination or spontaneous conversion to PGFM, every enzyme assay contained duplicate samples of buffer alone or $PGF_{2\alpha}$ and buffer. No PGFM was detected in any of these samples at any time. To ensure that conversion of $PGF_{2\alpha}$ to

PGFM proceeded to completion with no appreciable reverse reaction (PGFM to $PGF_{2\alpha}$), 100 µl of lung homogenate (10 mg tissue equivalent) was incubated with 50 ng of PGFM. These samples contained 50 ng PGFM throughout the incubation period. To establish with a greater degree of certainty that conversion of $PGF_{2\alpha}$ to PGFM was a direct result of tissue specific PGDH activity, and not some other mechanism, tissues known to possess or be devoid of PGDH activity were assayed for PGDH activity.

Statistical Analyses

Enzyme activity was expressed as average rate of conversion of $PGF_{2\alpha}$ to PGFM and normalized for the protein content of the homogenates (ng PGFM/min/mg protein) during the first 30 minutes of the enzyme assay. These data were then used for statistical analysis of PGDH activity; comparisons between days of the cycle were analyzed by Fisher's F-protected least significant difference test. Two pairwise comparisons were preplanned: 1) day 4 of the estrous cycle vs. day 13 of the estrous cycle, and 2) day 13 of pregnancy vs. day 13 of the estrous cycle. F-statistics yielding a probability of type I error less than 0.05 were considered statistically different.

Results

PGDH Cloning and Sequence Analysis

The [^{32}P]-dCTP labeled PGDH cDNA probe hybridized to mRNA from lung, corpus luteum (day 4 postestrus), endometrium, and to a lesser extent, corpus luteum (day 10 postestrus), liver, kidney, and myometrium (**Figure 2.1**). Sequence analysis (Geneworks; Intelligenetics, Upland Park, CA) led to the determination that the cDNA was 93.1%, 98.2%, 87%, and 89.3% homologous to human, water buffalo, rat, and guinea pig PGDH, respectively (**Figure 2.2**).

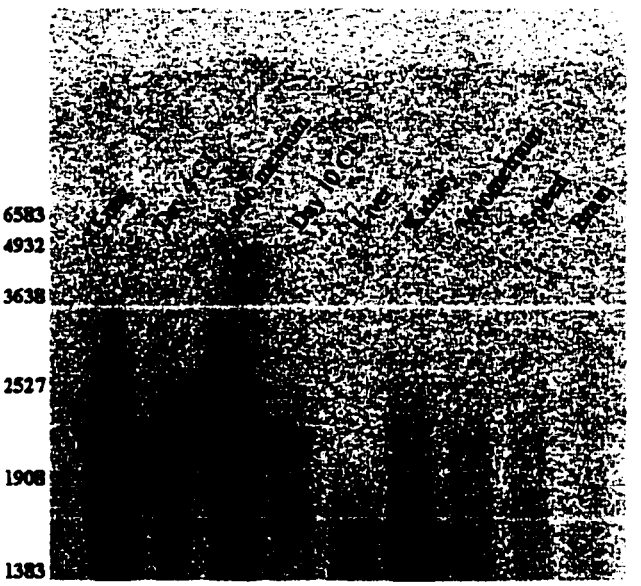

Figure 2.1. *Northern hybridization with ^{32}P-labeled PGDH cDNA probe.* Poly A RNA (10 μg) from the respective tissue was subjected to electrophoresis on a 1.5% agarose/4.5% formaldehyde/3-[N-morpholino] propanesulfonic (MOPS) acid gel, run at 70 V for 3 h in 1X MOPS, and transferred overnight by capillary action using 20X saline sodium citrate (SSC) to a Hybond nylon membrane.

The probe did not hybridize to mRNA from brain, a tissue that does not express significant levels of PGDH. The probe hybridized to a single transcript 2.2 kb in length (**Fig 2.1**), similar to the transcript observed in guinea pig ovary (2.4 kb; Bracken et al., 1997).

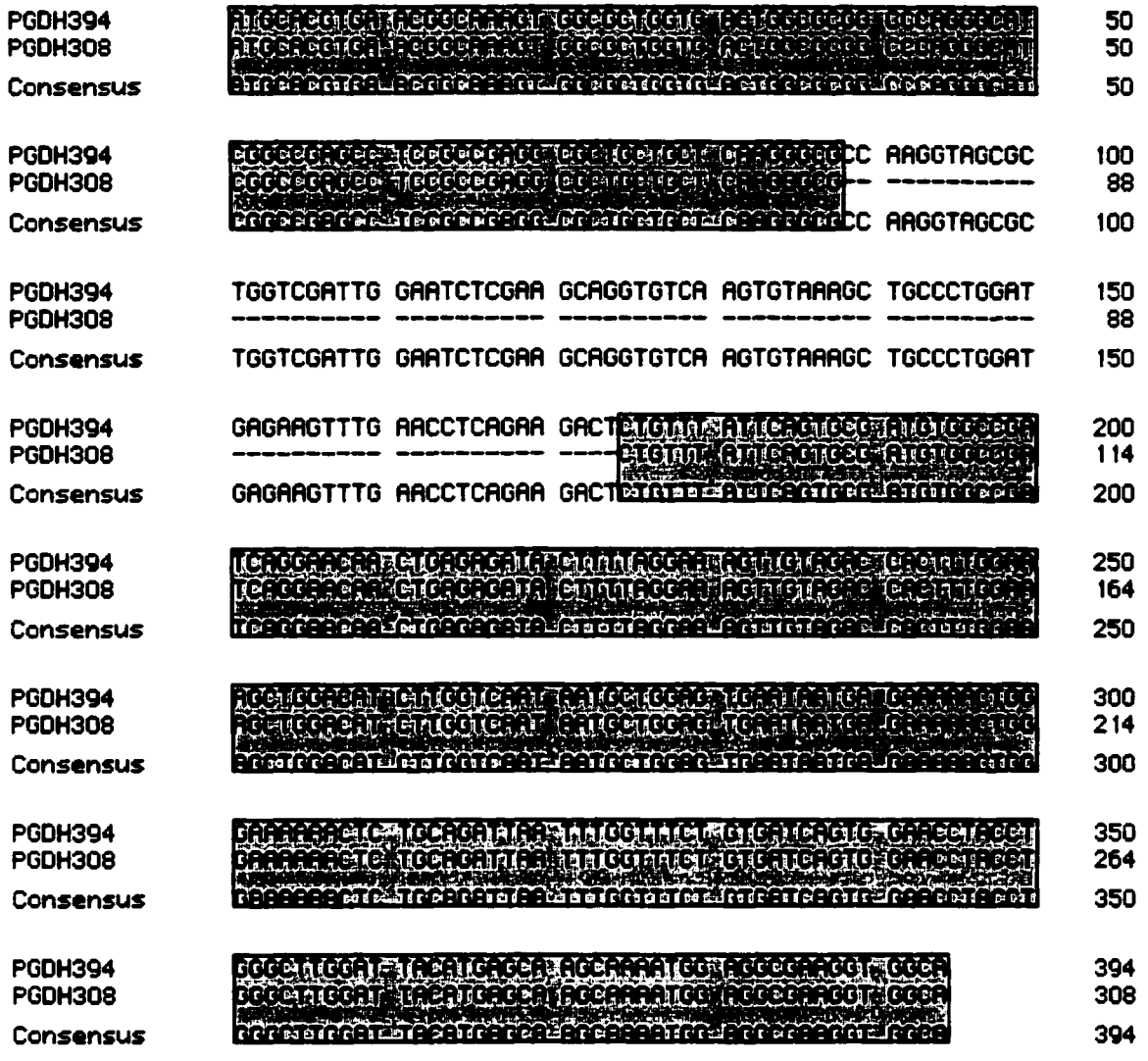

Figure 2.2 Comparison of nucleotide sequence of partial cDNA encoding 15-hydroxyprostaglandin dehydrogenase (PGDH394) and internal competitor (PGDH308). *Bases 88-174 were deleted by digestion with Kas I and Bbs I, overhangs were filled in with Klenow and the construct was gel purified and relegated with T4 ligase.*

The hybridization pattern observed during Northern analysis with the PGDH probe (**Figure 2.1**) and the sequence (**Fig 2.2**) of the probe, suggest that the probe is highly specific for ovine PGDH.

PGDH Enzymatic Activity

The average rate of production of PGFM during the linear portion (0-15 min) of the $PGF_{2\alpha}$ dose response assay appeared to be directly proportional to the dose of $PGF_{2\alpha}$ (data not shown; see **Figure 2.3** for approximation).

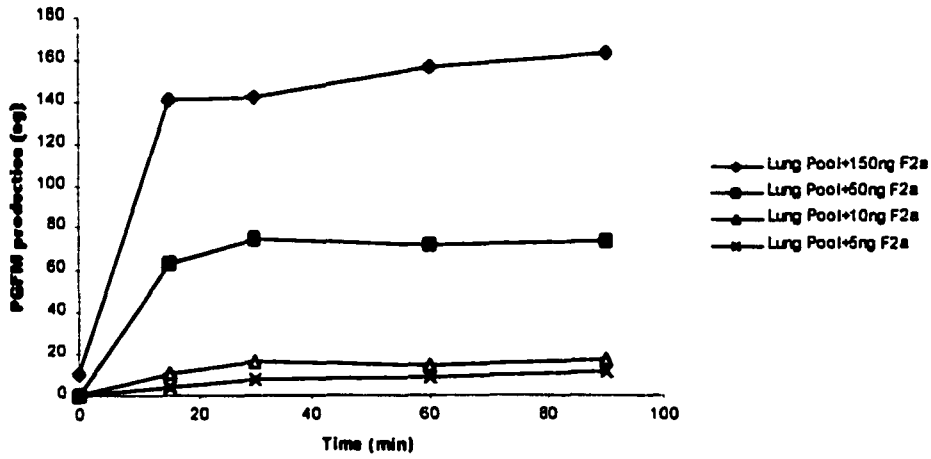

Figure 2.3. Relationship between dose of $PGF_{2\alpha}$ added and PGFM measured. *Lung homogenates (from 50 mg of tissue) were incubated with varying concentrations of $PGF_{2\alpha}$ in 100mM potassium phosphate buffer/2mM nicotinamide adenine dinucleotide (NAD^+) at 37°C. PGFM was measured at various time points by radioimmunoassay. Note that PGFM production was directly proportional to the amount $PGF_{2\alpha}$ added.*

Brain tissue, known to possess no significant PGDH activity, as expected had no significant PGDH activity, lung tissue had the greatest activity and endometrium had moderate PGDH activity (**Figure 2.4**). For logistical reasons, all control samples were sampled at 0, 5, 90, and 180 min only. No tissue had any measurable PGFM at time zero (**Figure 2.4**).

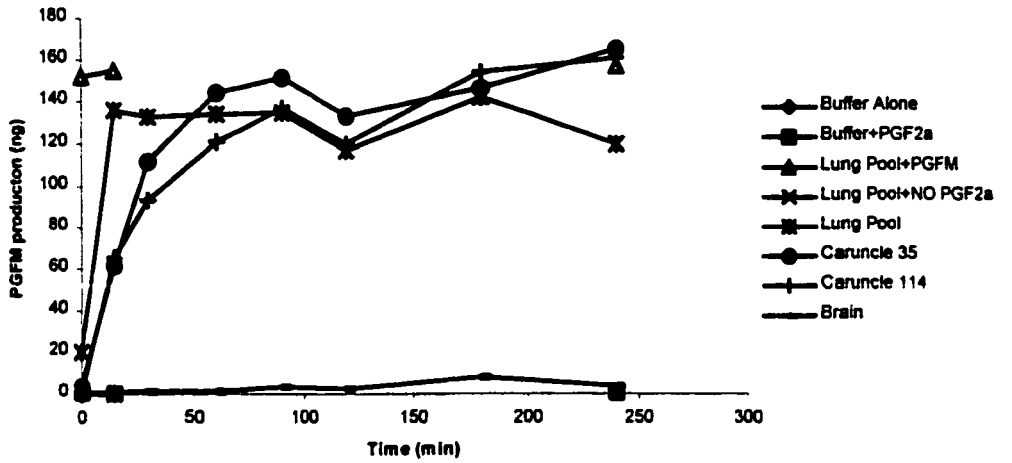

Figure 2.4. PGFM production in various body tissues and controls. *Tissue homogenates (from 500 mg of tissue) were incubated with varying concentrations of $PGF_{2\alpha}$ in 100mM potassium phosphate buffer/2mM nicotinamide adenine dinucleotide (NAD^+) at 37°C. PGFM was measured at various time points by radioimmunoassay. Production was directly proportional to the amount $PGF_{2\alpha}$ added. Note: Buffer alone, Buffer + $PGF_{2\alpha}$ and Lung pool + $PGF_{2\alpha}$ samples all had no PGFM production and values were superimposed on the ame pint on the chart.*

To ensure that comparisons of PGDH activity were made when substrate wasn't limiting enzymatic rate, a dose response experiment was conducted (using increasing doses of tissue homogenate) to determine the times during which the assay was linear. The average rate of conversion of PGFM appeared to be directly proportional to the quantity of tissue homogenate added (**Figure 2.5**). The quantity of PGFM produced at each time point by luteal homogenates appeared to be highly correlated with the quantity of luteal homogenate (**Figure 2.6**).

Enzymatic activity of PGDH, measured by rate of conversion of $PGF_{2\alpha}$ to PGFM, was greater in corpora lutea on both day 4 of the estrous cycle ($p < 0.05$) and day 13 of pregnancy ($p < 0.05$) than on day 13 of the estrous cycle in nonpregnant ewes (**Figure**

2.7, 2.8). The mean rate of conversion of $PGF_{2\alpha}$ into PGFM was 0.29, 0.65, and 0.64 ng PGFM/ mg luteal protein/ min for corpora lutea from day 13 of the estrous cycle, day 4 of the estrous cycle, and day 13 of pregnancy, respectively (**Figure 2.8**).

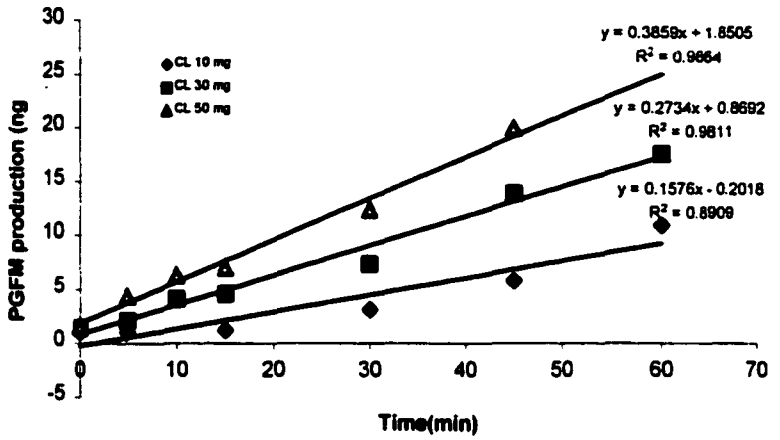

Figure 2.5 *Tissue homogenates were incubated with 50 ng/ml of $PGF_{2\alpha}$ in 100mM potassium phosphate buffer/2mM nicotinamide adenine dinucleotide (NAD^+) at 37°C. PGFM was measured at various time points by radioimmunoassay. Note that PGFM production was directly proportional to the amount of tissue homogenate added. Note: missing point was due to lost samples from those time points.*

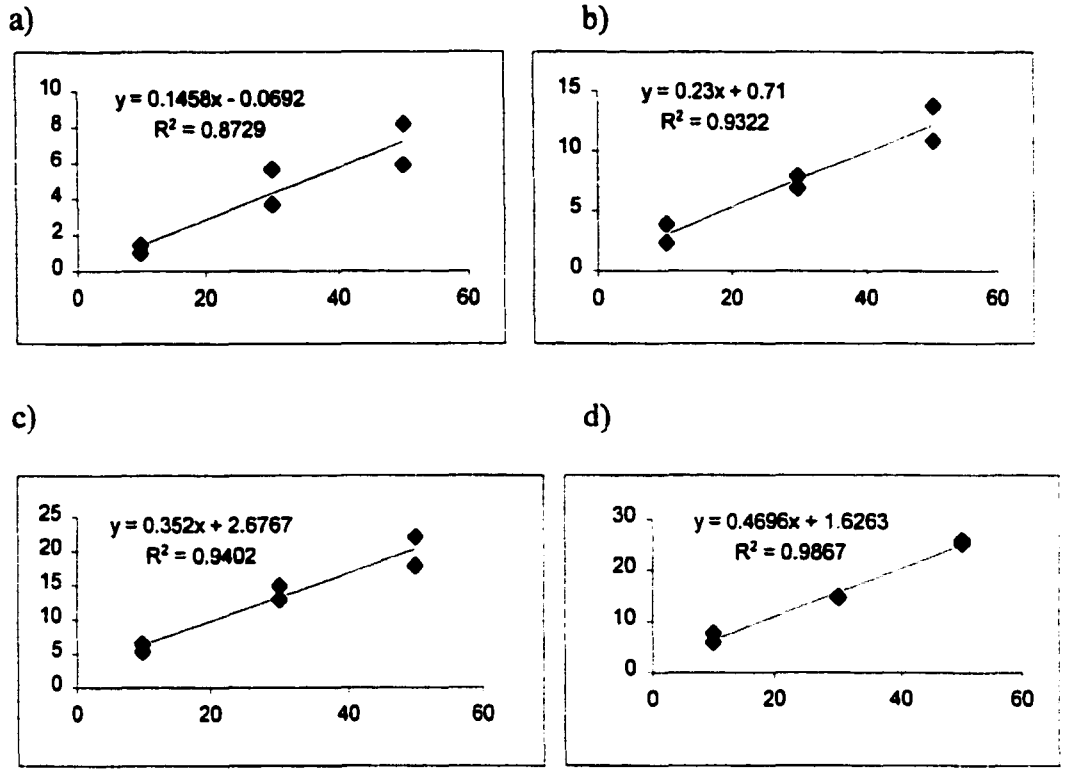

Figure 2.6. Correlation of PGFM appearance with tissue dose at various times. *a) 15 min b) 30 min c) 45 min d) 90 min Luteal homogenates were incubated with 50 ng/ml of $PGF_{2\alpha}$ in 100mM potassium phosphate buffer/2mM nicotinamide adenine dinucleotide (NAD^+) at 37°C. PGFM was measured at various time points by radioimmunoassay. X- axis represents dose of tissue (mg). Y- axis- represents PGFM production (ng). Note that PGFM production was directly proportional to the amount of tissue homogenate added. Based on these results, it was concluded that the enzyme assay was linear through 90 minutes.*

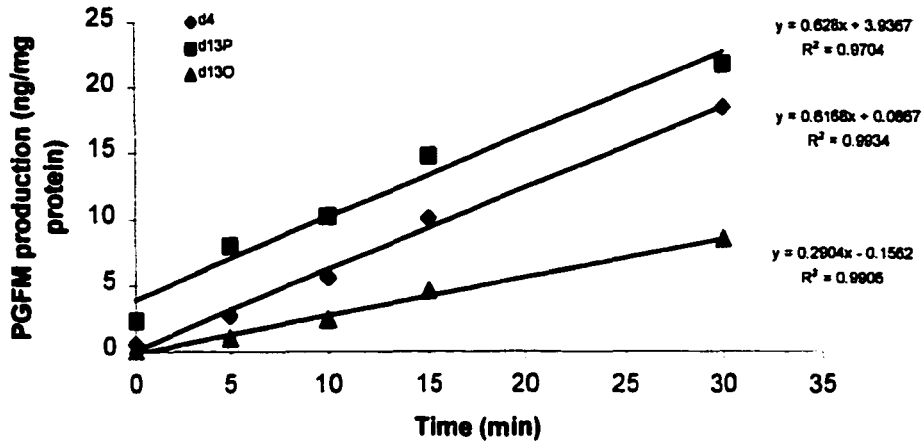

Figure 2.7. PGFM production by corpora lutea on days 4 and 13 postovulation in nonpregnant ewes and day 13 in pregnant ewes. *Luteal homogenates were incubated with 50 ng/ml of $PGF_{2\alpha}$ in 100mM potassium phosphate buffer/2mM nicotinamide adenine dinucleotide (NAD^+) at 37°C. PGFM was measured at the designated time points by radioimmunoassay.*

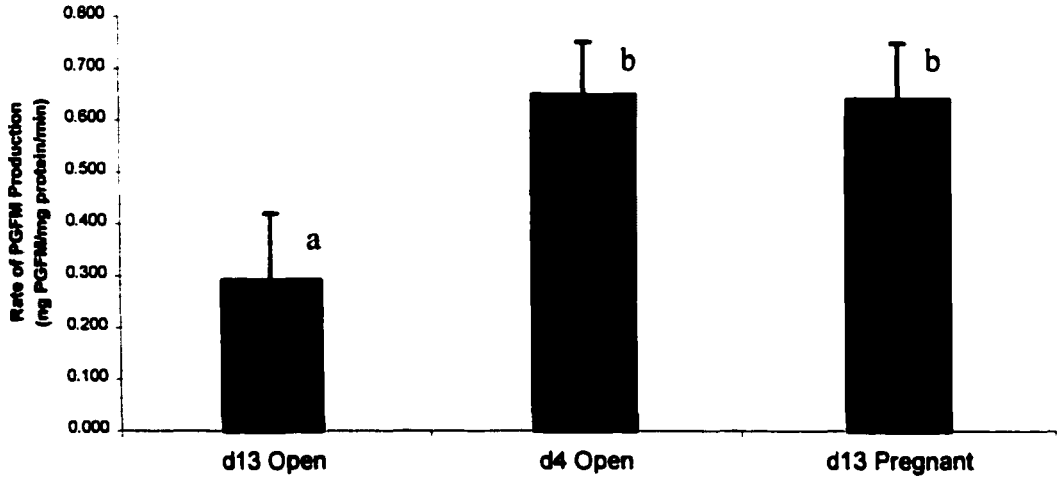

Figure 2.8. Rates of PGFM production by corpora lutea on days 4 and 13 postovulation in nonpregnant ewes and day 13 in pregnant ewes. *Average rates of PGFM production were determined for individual animals for 0-30 minutes of the enzyme assay. Error bars indicate standard error. Bars with different letters are different ($p<0.05$).*

Discussion

This study provides the first direct evidence that the corpus luteum can convert $PGF_{2\alpha}$ to PGFM. This novel biological mechanism may explain, to some extent, the mechanism of luteal resistance to $PGF_{2\alpha}$ during early pregnancy and during the early portion of the estrous cycle. Catabolism of $PGF_{2\alpha}$ locally in the corpus luteum might prevent $PGF_2\alpha$, of uterine origin from reaching receptors on large luteal cells. Alternatively, PGDH activity might oppose the biosynthetic function of COX-2 in the corpus luteum, preventing local accumulation of $PGF_{2\alpha}$ in the corpus luteum.

The conversion of $PGF_{2\alpha}$ to PGFM in ovine corpus luteum is most likely due to the activity of type I PGDH and not carbonyl reductase. The presence of carbonyl reductase would allow conversion of 13, 14-dihydro-15-keto $PGF_{2\alpha}$ into 9-keto-13, 14-dihydro-15-keto PG (13, 14-dihydro-15-keto PGE_2). If this had occurred, complete conversion of $PGF_{2\alpha}$ to PGFM would not have occurred. The radioimmunoassay used to measure PGFM crossreacts with 13, 14-dihydro-15-keto PGE_2 at 100-fold lower sensitivity. Therefore every 100 molecules of PGE_2 catabolized to 13, 14-dihydro-15-keto PGE_2, would have resulted in the apparent measurement of 1 molecule of PGFM. However, when high concentrations of luteal homogenate and $PGF_{2\alpha}$ were used in the validation studies, and the reaction was allowed to proceed to completion, addition of $PGF_{2\alpha}$ resulted in appearance of an equimolar amount of PGFM (**Figure 2.3**). In other words, all starting $PGF_{2\alpha}$ could be accounted for as PGFM; this would not have been possible in the presence of carbonyl reductase activity because some of the PGFM would have been converted to PGEM and consequently not measured by the radioimmunoassay

for PGFM. Furthermore, the demonstration of the presence of mRNA encoding PGDH in luteal tissue (**Figure 2.1**) provides more evidence that the conversion of $PGF_{2\alpha}$ to PGFM is catalyzed by PGDH.

There is indirect evidence that $PGF_{2\alpha}$ of luteal origin is required for luteolysis in rats. First, removal of immune cell-derived cytokines by splenectomy (Matsuyama et al., 1987) results in delayed luteolysis. This effect is reversed by injection of isolated splenocytes. Furthermore, immunosuppressive doses of dexamethasone also prevent luteolysis (Wang et al., 1993). Thus, it appears that the presence of immune cell-derived cytokines (IFN-γ, TNF-α, and IL-1), that stimulate luteal biosynthesis of $PGF_{2\alpha}$ (reviewed in Pate, 1995), are a necessary component of the luteolytic machinery.

Indeed, there is compelling evidence that a threshold dose of $PGF_{2\alpha}$ is required before the corpus luteum is committed to luteolysis. The secretion of progesterone can recover in corpora lutea exposed to a subluteolytic dose of $PGF_{2\alpha}$ (Silvia et al., 1986; Juengel et al., 1999). Furthermore, the percentage of luteal cells that are apoptotic after $PGF_{2\alpha}$, administration appears to be related to the dose of $PGF_{2\alpha}$ administered (Juengel et al., 1999). Finally, in ewes treated with a subluteolytic dose of $PGF_{2\alpha}$, the occurrence of apoptotic cells wanes, while in ewes treated with a luteolytic dose of $PGF_{2\alpha}$, the occurrence of apoptotic cells persists (Juengel et al., 1999). Thus, a major biological function of PGDH in the corpus luteum could be to prevent luteal concentrations of $PGF_{2\alpha}$ from reaching threshold levels and initiating luteolysis at an inopportune time. The observation that the occurrence of elevated PGDH enzymatic activity is temporally correlated with luteal resistance to $PGF_{2\alpha}$ supports the existence of such a mechanism.

The mechanisms responsible for regulation of PGDH activity in the corpus luteum remain to be described. It is interesting that PGDH activity is elevated in the corpora lutea of pregnant ewes as well as nonpregnant ewes. It is possible that the hormonal cues inducing PGDH enzymatic activity are different in pregnant and nonpregnant ewes. Estradiol inhibits expression of PGDH in rats (Chang and Tai, 1985; Chang, 1987; Erman et al., 1987), so it is conceivable that estradiol secreted from the developing dominant follicle of the ewe could downregulate PGDH and promote luteal sensitivity to $PGF_{2\alpha}$.

Conclusions and Implications

This study provides insight into a possible mechanism of luteal resistance to the luteolytic actions of $PGF_{2\alpha}$ in the ovine corpus luteum. Resistance of the corpus luteum to $PGF_{2\alpha}$ on day 4 of the estrous cycle is a major barrier to synchronizationof estrus in ruminants with $PGF_{2\alpha}$. Enzymatic activity of PGDH appears to be a major factor in this physiological state. Thus, PGDH might be a potential pharmacological target to enhance the efficacy of $PGF_{2\alpha}$ as a luteolytic agent in ruminant females early in the estrous cycle. Simultaneous administration of PGDH inhibitors and $PGF_{2\alpha}$ might result in an increased luteolytic potency of $PGF_{2\alpha}$. In conclusion, a better understanding of these mechanisms might ultimately foster development of more effective methods to synchronize estrus in ruminants.

During early pregnancy, inadequate secretion of progesterone is involved in the loss of 13 (Macmillan et al., 1991) to 30 percent (Wiltbank, 1956) of bovine embryos. The role of $PGF_{2\alpha}$-inhibition of luteal progesterone secretion at this time has not been documented. Inhibition of the biological activity of $PGF_{2\alpha}$ by PGDH at this time could

potentially decrease the incidence of embryonic loss by fostering an adequate level of secretion of progesterone. A better understanding of these mechanisms might ultimately foster development of refined management practices to reduce embryonic loss due to premature luteal regression or inadequate secretion of progesterone.

Chapter 3

Messenger ribonucleic acid encoding inducible cyclooxygenase-2 in the ovine corpus luteum on days 4 and 13 of the estrous cycle and day 13 of pregnancy

Abstract

To gain insight into the mechanisms by which the corpus luteum of early pregnancy in the ewe becomes resistant to the luteolytic actions of $PGF_{2\alpha}$, steady-state concentrations of mRNA encoding COX-2 were determined in corpora lutea of ewes during periods of known luteal resistance to $PGF_{2\alpha}$. A quanitative-competitive reverse transcription polymerase chain reaction (QC-RT-PCR) was used to determine levels of mRNA encoding COX-2 on days 4 (n=11) and 13 (n=7) of the estrous cycle in nonpregnant ewes and on day 13 of pregnancy (n=10). Messenger RNA encoding COX-2 was negligable (<1.875 amol/ μg poly A^+ RNA) in corpora lutea from ewes on day 13 of the estrous cycle. In corpora lutea from ewes on day 4 of the estrous cycle, levels of mRNA encoding COX-2 were 43.77± 27.75 amol/μg poly A^+ RNA. In corpora lutea from ewes on day 13 of pregnancy, levels of mRNA encoding COX-2 were 10.57± 4.41 amol/μg poly A^+ RNA. From these data, it can be concluded that decreased basal expression of mRNA encoding COX-2 is not a mechanism of luteal resistance to $PGF_{2\alpha}$.

Introduction

Results from recent studies provide evidence that luteolysis in domestic ruminants requires $PGF_{2\alpha}$ of luteal origin in addition to $PGF_{2\alpha}$ of uterine origin. Cytokines (IFN-γ, TNF-α, and IL-1) secreted from immune cells stimulate luteal $PGF_{2\alpha}$ synthesis (Nothnick and Pate, 1990; Fairchild-Benyo and Pate, 1992; reviewed in Pate, 1995). Removal of immune cells in rats, and consequently these cytokines, by splenectomy (Matsuyama et al., 1987) or administration of immunosuppressive levels of dexamethasone (Wang et al., 1993) results in delayed luteolysis. Recent evidence suggests a role for COX-2 in luteolysis. For example, in cows, levels of luteal mRNA encoding COX-2 are enhanced by $PGF_{2\alpha}$ on day 11 of the estrous cycle, a time when the corpus luteum is sensitive to the luteolytic effects of $PGF_{2\alpha}$ (Tsai and Wiltbank, 1997). However, on day 4 of the estrous cycle, when the corpus luteum is resistant to the luteolytic effects of $PGF_{2\alpha}$ (Rowson et al., 1972), COX-2 expression is downregulated in response to $PGF_{2\alpha}$ (Tsai and Wiltbank, 1997). Thus, the resistance of the day 4 corpus luteum to $PGF_{2\alpha}$ might involve attenuation of the local $PGF_{2\alpha}$ levels by attenuation of luteal COX-2 expression. Therefore, the objective of this study was to compare expression of mRNA encoding COX-2 in ovine luteal tissue during periods of luteal responsiveness and resistance to $PGF_{2\alpha}$. Levels of mRNA encoding COX-2 were measured on days 4 and 13 postovulation in nonpregnant ewes and on day 13 postcoitus in pregnant ewes to test the hypothesis that COX-2 expression in the corpus luteum is suppressed on day 4 of the estrous cycle and day 13 of pregnancy in ewes relative to day 13 of the estrous cycle.

Materials and Methods

Unless otherwise indicated, all reagents and materials were obtained from Sigma Chemical Co.(St. Louis, MO) or Fisher Scientific (Denver, CO). Messenger RNA encoding COX-2 was quantitated using a quantitative, competitive reverse transcription polymerase chain reaction (QC-RT-PCR) assay that has been validated for measurement of COX-2 mRNA in bovine (Tsai et al., 1996; Tsai and Wiltbank, 1996) and ovine Tsai and Wiltbank, 1997; Burns et al., 1997) tissue.

Cloning of COX-2

Plasmids containing native and competitor cDNAs for COX-2 were kindly provided by Dr. Milo Wiltbank (University of Wisconsin, Madison). Briefly, standard and competitor cDNAs encoding bovine COX-2 were generated from bovine endometrial RNA utilizing RT-PCR. Primers were based on the highly homologous areas of sequence of human, mouse, and rat sequence. The PCR primers were as follows: upstream primer: 5'- AGGTGTATGTATGAGTGTAGGA- 3'; and downstream primer: 5'- GTGCTGGGCAAAGAATGCAA- 3'. A 484 bp DNA was cloned into pCR II vector (Invitrogen; Carlsbad, CA) and an internal competitor (378 bp) generated by digestion with *Alu I* restriction endonucleases. The cDNAs were oriented in the reverse direction in pCR II. The native COX-2 was found to be 97.5% identical in nucleotide sequence to ovine COX-2 in the corresponding region of the gene (Burns et al., 1997).

QC-RT-PCR for COX-2

To generate RNA standards, plasmids containing native and competitor COX-2 cDNAs were linearized with *BamH I* restriction endonucleases and reverse transcribed using a Maxiscript T7 kit (Ambion; Austin, TX) per manufacturer instructions.

Standards were generated by diluting known amounts of RNA standard in the following manner: the first point on the standard curve contained 240 amol of COX-2 RNA and each subsequent point contained a 0.5 dilution for a total of nine standards. Each tube containing standard or sample in the assay contained an equal amount of competitor (15 amol; equimolar with point 5 on the standard curve). Standards and samples were reverse transcribed for 90 minutes at 42°C (1 X Promega MLV reverse transcriptase buffer, 5 μM random hexamer primers, 200μM dNTPs, 80 U Promega MLV reverse transcriptase), then cDNAs were amplified as follows: 95°C, 30 seconds; 57°C, 30 seconds; 72°C, 30 seconds; 35 cycles, followed by 95°C, 30 seconds; 57°C, 30 seconds; 72°C, 10 minutes; one cycle (1 X Promega PCR buffer, 200 μM dNTPs, 0.4 μM each of upstream and downstream primers, 1 U *Taq* polymerase). The PCR products were subjected to electrophoresis on 6% polyacrylamide gels and stained with ethidium bromide. The gels were visualized on a UV transilluminator and the image captured with a CCD camera and analyzed using GelExpert software (Nucleotech; San Mateo, CA). The concentration of mRNA encoding COX-2 in samples was determined by comparison to the standard curve which was expressed as the ratio of the log of the intensity of the native band to the log of the intensity of the competitor band. Concentrations of mRNA encoding COX-2 were expressed as amol COX-2 transcript/μg poly A^+ RNA. Every assay was run with triplicate pooled luteal RNA samples as a quality control measure. The intra-assay coefficient of variation was 19%.

Semiquanitative RT-PCR for Glyceraldehyde-3-phosphate Dehydrogenase

To ensure the integrity of poly A RNA used for measurement of mRNA encoding COX-2, levels of mRNA encoding glyceraldehyde-3-phosphate dehydrogenase

(GAP3DH) were measured utilizing a previously described and validated semiquanitative PCR assay (Tsai et al., 1996; the native and competitor partial cDNAs encoding GAP3DH were graciously provided by Dr. Milo Wiltbank, University of Wisconsin, Madison). The competitor GAP3DH cDNA was 850 bp in length.

Briefly, a pair of primers (upstream 5'-TGTGTCCAGTATGGATTCCACCC–3' and downstream 5'-TCCACCACCCTGTTGCTGTA-3') were used to amplify the cDNA which was reverse transcribed for COX-2 analysis. This amplification was carried out in the presence of an approximately equimolar amount of GAP3DH competitor cDNA. Amplification conditions were identical those used for PCR of COX-2.

Statistical Analysis

The preplanned comparisons were: day 13 of the estrous cycle vs. day 4 of the estrous cycle, and day 13 of the estrous cycle vs. day 13 of pregnancy. However, statistical analyses were not possible on the data from COX-2 measurements in this experiment. Since mRNA encoding COX-2 was undetectable in luteal tissue of ewes on day 13 of the estrous cycle, there were no means and variances to use for ANOVA. Differences in mean levels of mRNA encoding GAP3DH between days of the estrous cycle were tested using Fisher's F-protected least significant difference test.

Results

An example of a standard curve as described in materials and methods is included (Figure 3.1).

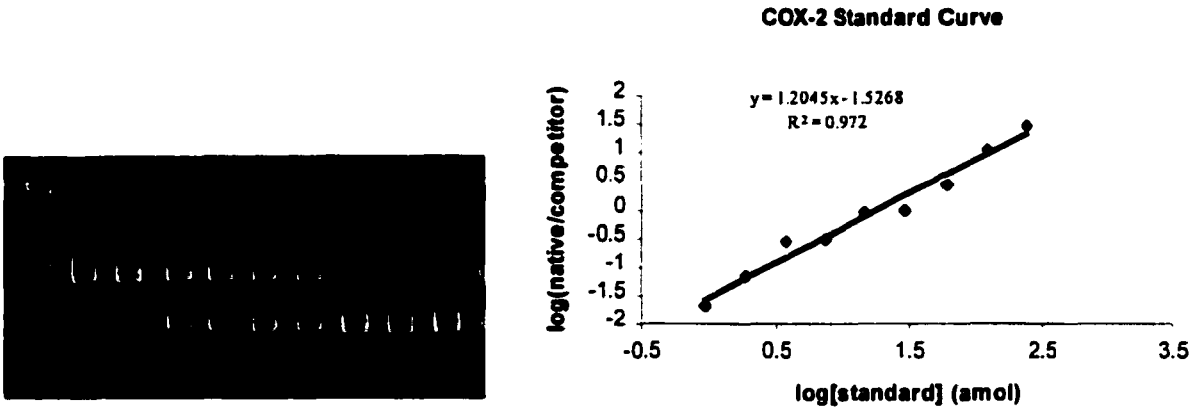

Figure 3.1- Typical QC-RT-PCR standard curve. *Left- Photograph of a typical standard curve using 240 amol and 0.5 dilution for each subsequent standard, and 15 amol competitor. Right- Plot of standard curve depicted on the left*

Levels of mRNA encoding COX-2 were 43.77± 27.75 amol/μg poly (A^+) RNA in luteal tissue of ewes on day 4 of the estrous cycle (**Fig 3.2**) and 10.57± 4.41 amol/μg poly (A^+) RNA in luteal tissue of ewes on day 13 pregnancy. Levels of mRNA encoding COX-2 were undetectable in luteal tissue of ewes on day 13 of the estrous cycle (<1.875 amol/μg poly (A^+) RNA; **Fig 3.3**).

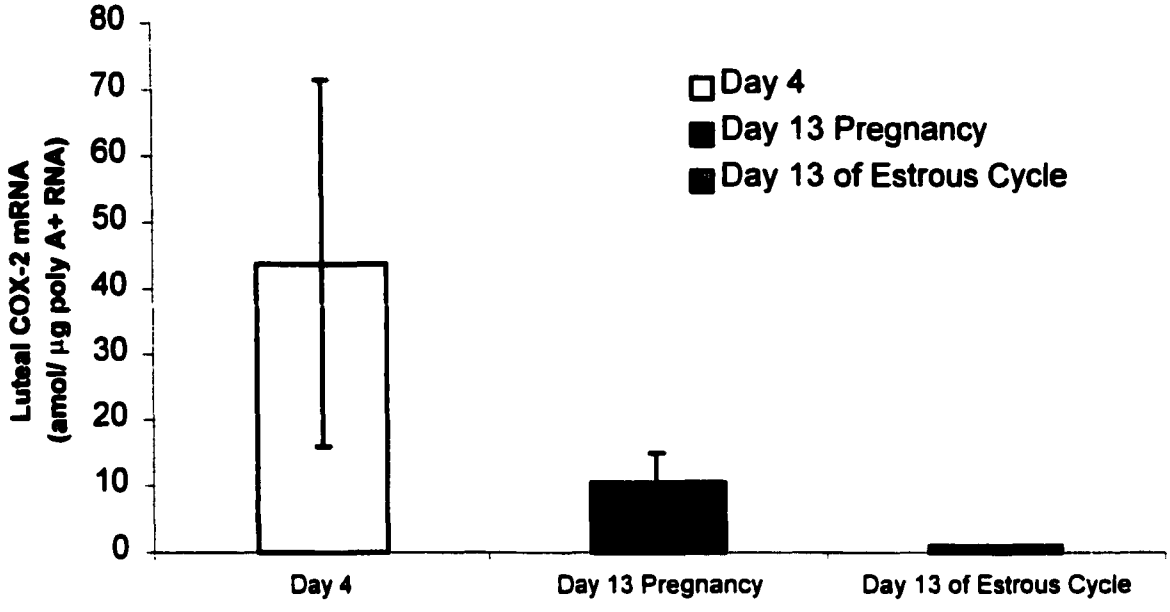

Figure 3.2- Levels of mRNA encoding COX-2 in the ovine corpus luteum on days 4 and 13 of the estrous cycle and day 13 of pregnancy. *Expressed in amol mRNA/ µg poly (A^+) RNA. Error bars represent standard deiation.*

The inability to measure COX-2 in corpora lutea of ewes on day 13 of the estrous cycle was not due to degradation of the RNA. To confirm this, mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAP3DH; a constitutively expressed, unregulated gene in the corpus luteum) was measured by semiquanitative QC-RT-PCR (Tsai et al., 1996). Expression of GAP3DH was not different across treatments ($p = 0.77$).

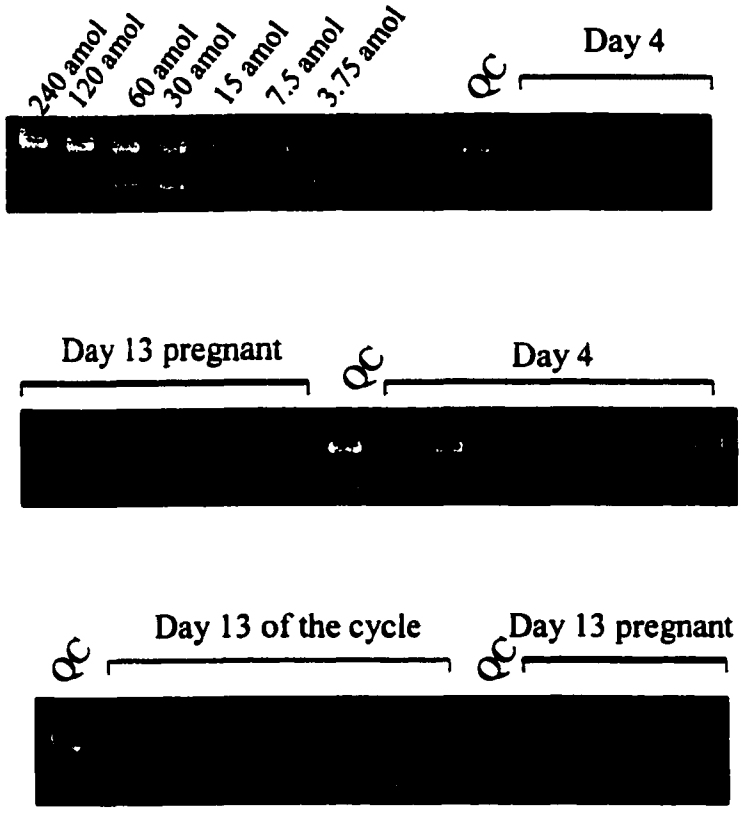

Figure 3.3- *Numbers above bands in upper gel represent mass of standard. In this assay, 15 amol of competitor RNA was used in all standard and sample reactions.* QC- *pool for intrassay CV determination. All samples were analyzed by QC-RT-PCR of one microgram of luteal poly (A^+) RNA. Intraassay coefficient of variation was 19%.*

Discussion

The ability of the corpus luteum to regress is likely dependent on its capacity to synthesize and secrete $PGF_{2\alpha}$. The ability of the corpus luteum to synthesize $PGF_{2\alpha}$ is related to expression of COX-2. In ewes treated with oxytocin, a two-fold induction of mRNA encoding COX-2 in the caruncular endometrium was associated with a 14-fold increase in concentrations of PGFM in the jugular vein (Burns et al., 1997). If levels of PGFM in the jugular vein accurately represent uterine secretion of $PGF_{2\alpha}$, then

these observations support a high degree of correlation between mRNA encoding COX-2 and biosynthesis of $PGF_{2\alpha}$. If the relationship between expression of mRNA encoding COX-2 and biosynthesis of $PGF_{2\alpha}$ is similar in the corpus luteum, then one can reasonably speculate that corpora lutea on day 4 of the estrous cycle should have a much greater capacity to synthesize $PGF_{2\alpha}$ than corpora lutea on day 13 of the estrous cycle, based on our measurements of mRNA encoding COX-2.

It was unexpected to see the lowest levels of mRNA encoding COX-2 on day 13 of the estrous cycle. However, it has recently been revealed that COX-2 expression in the corpus luteum is essentially absent in the absence of stimulation by $PGF_{2\alpha}$ (Milo Wiltbank, personal communication). This, at first, appears to be in contradiction with apparent $PGF_{2\alpha}$-resistance in the corpus luteum on day 4 of the estrous cycle. However, the greater ability of the day 4 corpus luteum to catabolize $PGF_{2\alpha}$ could prevent the biological consequences of a greater level of mRNA encoding COX-2. Thus, the apparent greater capacity of the corpus luteum on day 4 of the estrous cycle to synthesize $PGF_{2\alpha}$ does not necessarily result in an actual increase in luteal output of $PGF_{2\alpha}$.

The ability of the corpus luteum to synthesize $PGF_{2\alpha}$ is not exclusively dependent upon expression of COX-2, the constitutively expressed COX-1 is also capable of supporting prostanoid biosynthesis. Thus, the absence of detectable mRNA encoding COX-2 in corpora lutea from day 13 of the estrous cycle does not prove that the corpus luteum cannot produce $PGF_{2\alpha}$. Because COX-1 is a constitutively expressed enzyme and is not regulated to any significant extent in the bovine corpus luteum (Tsai et al., 1997), this seems an unlikely possibility.

It is also possible that basal expression of COX-2 is not key in mediating luteolysis, rather the degree to which COX-2 can be upregulated by $PGF_{2\alpha}$ could be the key mechanism. Since COX-2 is an inducible, tightly regulated gene (Vane et al., 1998), and may have been measured in corpora lutea to exposure to increased levels of $PGF_{2\alpha}$, it is possible that COX-2 was measured outside of a meaningful biological context in this experiment. In other words, it may have been more prudent to measure mRNA encoding COX-2 in the corpora lutea of ewes treated with $PGF_{2\alpha}$ on days 4 and 13 of the estrous cycle and on day 13 of pregnancy. Thus, it seems that the most reasonable explanation for the absence of expression of mRNA encoding COX-2 on day 13 of the estrous cycle is a lack of exposure of those corpora lutea to $PGF_{2\alpha}$.

In light of these possibilities, it might be prudent to measure COX-2 expression in ovine corpora lutea from days 4 and 13 of the estrous cycle and day 13 of pregnancy *in response to a $PGF_{2\alpha}$ challenge*. I hypothesize that corpora lutea from day 13 of the estrous cycle would increase expression of mRNA encoding COX-2, in response to exogenous $PGF_{2\alpha}$, to a greater degree than corpora lutea from day 4 of the estrous cycle and day 13 of pregnancy. The rationale supporting this contention is derived from data that show a lack of increase in levels of mRNA encoding COX-2 in the corpora lutea of cows administered $PGF_{2\alpha}$ on day 4 of the estrous cycle (Tsai and Wiltbank, 1998).

In summary, the observation that decreased basal expression of mRNA encoding COX-2 was not associated with the occurrence of luteal $PGF_{2\alpha}$-resistance was unexpected but, in retrospect, not very surprising. The possibility for a role of modulation of COX-2 expression, and consequent luteal $PGF_{2\alpha}$ synthesis, as a means of luteal $PGF_{2\alpha}$-resistance, still cannot be ignored and merits further investigation.

Conclusions and Implications

This study provides insight into a possible mechanism for luteal resistance to the luteolytic actions of $PGF_2\alpha$ in the ovine corpus luteum. Resistance of the corpus luteum to $PGF_{2\alpha}$ on day 4 of the estrous cycle is a major barrier to the efficacy of estrous synchronization in ruminants with $PGF_2\alpha$. It has been documented that $PGF_{2\alpha}$ is unable to induce upregulation of COX-2 in the corpora lutea of ewes on day 4 of the estrous cycle, in contrast to $PGF_{2\alpha}$ induced upregulation of COX-2 on day 11of the estrous cycle (Tsai and Wiltbank, 1998).

A better understanding of the role of COX-2 in luteolysis and luteal resistance to $PGF_{2\alpha}$, should ultimately result in a refined understanding of both physiological processes. Further study might reveal the reason for elevated COX-2 during luteal $PGF_{2\alpha}$ resistance and result in a method to induce luteolysis in cows, prior to day 5 of the estrous cycle, by administration of $PGF_{2\alpha}$. Furthermore, a better understanding of these mechanisms might ultimately foster development of refined management practices, which affect these physiological processes, to reduce embryonic loss due to premature luteal regression or inadequate secretion of progesterone. Based on the results of this experiment, it appears that PGDH might be a far more interesting candidate than COX-2, for a target to facilitate manipulating luteal resistance to $PGF_{2\alpha}$.

Chapter 4

Summary and a Refined Biological Model for Maternal Recognition of Pregnancy and Luteolysis in Ruminants

Forward

Chapter four is written to communicate *my ideas, philosophies, and opinions* on some biological phenomena and how they relate to luteolysis and maternal recognition of pregnancy. These ideas, in some instances are supported by published data, but in general are my opinions, and should be considered with this in mind.

PGDH, COX-2, luteolysis, and luteal resistance to prostaglandin $F_{2\alpha}$

The results of these experiments provide convincing evidence that modulation of intraluteal concentrations of $PGF_{2\alpha}$ could be a mechanism for timing luteolysis. Catabolism of $PGF_{2\alpha}$ appears to be of primary importance in luteal $PGF_{2\alpha}$ resistance, while the role of luteal synthesis of $PGF_{2\alpha}$ remains questionable. During periods of luteal resistance to the luteolytic activity of $PGF_{2\alpha}$, the corpus luteum is refractory to an ill-timed luteolytic signal. In a biological context, it is reasonable and logical that the corpus luteum is resistant to $PGF_{2\alpha}$ early in the estrous cycle and during early pregnancy. If pregnancy occurs, such mechanisms prevent the corpus luteum from regressing prematurely and compromising the embryo. The exact biochemical mechanism of luteal

resistance to $PGF_{2\alpha}$ that occurs early in the estrous cycle and early pregnancy but not during the mid-cycle is unknown. Nonetheless, I would like to propose a possible explanation.

Early in the estrous cycle (days 1-5) and during early pregnancy (days 13-16; reviewed in Silvia et al., 1991), the corpus luteum is exposed to greater levels of $PGF_{2\alpha}$ than the mid-luteal phase of the estrous cycle (~day10). During the early estrous cycle in ewes and cows (day 0-5; Rowson et al., 1972), the corpus luteum is exposed to $PGF_{2\alpha}$ of intraovarian origin. During the preovulatory LH surge and afterward, the corpus luteum is rapidly developing and characterized by a high degree of tissue remodeling as the ruptured follicle luteinizes. During early luteal development, the vascular structure of the follicle proliferates dramatically (reviewed in Grazul-Bilska et al., 1997). The occurrence of tissue remodeling is facilitated by $PGF_{2\alpha}$. One of the primary biological functions of $PGF_{2\alpha}$ is removal of vascular tissue by inducing apoptosis in endothelial cells. Production of $PGF_{2\alpha}$ by the ovary during this time would be detrimental to the function of the corpus luteum if there were no mechanisms in place to transiently prevent $PGF_{2\alpha}$ action in the developing corpus luteum. One potential mechanism that would result in this physiological state is catabolism of $PGF_{2\alpha}$. Data from this dissertation certainly support this assertion.

During early pregnancy, the corpus luteum is exposed to elevated basal levels of $PGF_{2\alpha}$ (Silvia et al., 1991), similar to the corpus luteum of the early estrous cycle. However the source of $PGF_{2\alpha}$ during early pregnancy is embryonic, not ovarian. The primary difference in the pattern of secretion of $PGF_{2\alpha}$ on day 13 of pregnancy compared to day 13 of the estrous cycle is that the amplitude of $PGF_{2\alpha}$ pulses are greater on day 13

of the estrous cycle. In other words, the pattern of secretion during early pregnancy is more sustained and chronic while secretion of $PGF_{2\alpha}$ during luteolysis is more pulsatile. The pattern of secretion and levels of $PGF_{2\alpha}$ in ovarian arterial blood might not be sufficient to cause luteolysis but could still inhibit progesterone secretion sufficiently to result in embryonic death. Thus, the role of PGDH in the corpus luteum of pregnancy could be more subtle; blockage of the inhibitory action of $PGF_{2\alpha}$ on luteal biosynthesis of progesterone.

When one considers some of the molecular and cellular aspects of COX-2 and PGDH biology, some serious questions are generated about the manner in which COX-2 and PGDH are incorporated into a comprehensive biological model. First, "What is the pattern of localization of PGDH?". Based on cell fractionation studies (reviewed in Okita and Okita, 1996), PGDH appears to be localized intracellularly. Analysis of the cDNA sequence of PGDH in several species reveals no evidence of a secretory domain. Second, "What cell types express PGDH?". I hypothesize that PGDH is expressed at highest levels in the large luteal cells. The large luteal cells express the greatest levels of mRNA encoding COX-2 (Tsai and Wiltbank, 1997) and consequently, likely produce a majority of luteal $PGF_{2\alpha}$. If it is true that luteal $PGF_{2\alpha}$ is requisite for luteolysis, then this hypothesis is reasonable. To elaborate, $PGF_{2\alpha}$ of uterine origin would still have access to $PGF_{2\alpha}$ receptors on large luteal cells; however, stimulation of increased luteal output of $PGF_{2\alpha}$, by $PGF_{2\alpha}$ of uterine origin, could not commence because synthesized $PGF_{2\alpha}$ would immediately be catabolized. This seems inefficient and a waste of cellular energy, but so does concurrent expression of PGDH and COX-2 in luteal tissue. However, there is sufficient data in this dissertation to indicate that the corpus luteum

does indeed concurrently express PGDH and COX-2. Based on the hypotheses I have formulated, the general contention is that PGDH serves primarily to prevent intraluteal accumulation of, not protect the corpus luteum from, $PGF_{2\alpha}$ of uterine origin. If this were true, one would expect a greater degree of luteal resistance to $PGF_{2\alpha}$ early in the estrous cycle (ovarian origin) than during early pregnancy (embryonic/uterine origin). The actual relative degrees of luteal resistance to $PGF_{2\alpha}$ early in the estrous cycle and during early pregnancy support this hypothesis.

Hormonal Regulation of COX-2 and PGDH

The hormonal mechanisms by which PGDH activity is regulated in the ovine corpus luteum have not been investigated. However, some discussion about regulation of PGDH in other tissues and in other species (reviewed in Okita and Okita, 1996) might reveal some possibilities.

Enzymatic activity of PGDH is 20-fold greater in the lungs of pregnant rabbits than in the lungs of nonpregnant rabbits (Sun and Armour, 1974; Edgerton-Vernon and Bedwani, 1975). In the corpora lutea of pregnant rabbits and pseudopregnant rabbits, PGDH activity is 15-fold greater than in nonpregnant rabbits (Okita et al., 1992). When injected into male rabbits, progesterone or progesterone and estradiol caused an increase in PGE_2 clearance in the pulmonary vasculature (Bedwani and Marley 1975). Thus it is apparent the progesterone dramatically upregulates PGDH activity in rabbits.

Furthermore, progesterone is known to induce PGDH expression in human myometrium (Thornburn and Challis, 1979). The progesterone antagonist, RU486,

attenuates PGDH in endometrial stromal tissue of women (Cheng et al., 1993). So an apparent role of progesterone in upregulating PGDH is also evident in humans.

Based on the role of progesterone in regulation of PGDH in rabbits and humans, it is reasonable that progesterone can upregulate PGDH in the ewe as well. Coincidentally, the ovine corpus luteum becomes resistant to the luteolytic effects of $PGF_{2\alpha}$ after administration of hCG (Bolt, 1979). Luteolysis is delayed in dairy heifers (Howard et al., 1990) and sows (Guthrie and Rexroad, 1991; Guthrie and Bolt, 1983) treated with hCG. It is possible that the increase in progesterone biosynthesis elicited by hCG could upregulate PGDH activity and result in luteal resistance to $PGF_{2\alpha}$.

The occurrence of luteal resistance to $PGF_{2\alpha}$ during early pregnancy (Silvia et al., 1991) coincides with the window in which the embryonic trophoblast secretes IFN-τ (Farin et al., 1989). It is interesting to speculate that IFN-τ could upregulate PGDH at the level of the corpus luteum or in an indirect manner. Although there is no evidence that INF-τ can leave the uterus and reach the corpus luteum, there is not sufficient evidence to refute this possibility either. Detection of IFN-τ in ovarian arterial blood has been attempted but IFN-τ has never been detected. However, the limit of sensitivity of the assay used to measure IFN-τ was 2 ng/ml (Gordon Niswender, personal communication). Therefore, the possibility exists that INF-τ reaches the ovary in concentrations less than 2 ng/ml. It is also possible that an ovine or bovine chorionic gonadotropin, similar in biological activity to hCG, could regulate PGDH during early pregnancy, although such a hormone has not been described.

Practical Implications

In conclusion, two primary problems in management of ruminant reproduction are 1) luteal resistance to $PGF_{2\alpha}$ prior to day 5 of the estrous cycle in ewes and cows and, 2) early embryonic death. Modulation of intraluteal $PGF_{2\alpha}$ appears to be a key mechanism in controlling the capacity of $PGF_{2\alpha}$ to induce luteolysis. A better understanding of luteal resistance to $PGF_{2\alpha}$ could ultimately result in methods to induce luteolysis in ewes and cows prior to day 5 of the estrous cycle. In addition, the importance of luteal resistance to $PGF_{2\alpha}$ in the prevention of embryonic death merits further study. The results and concepts presented in this dissertation provide insight into biological processes that are related to these two management problems.

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