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The effects of intraluteal infusion of prostaglandin-synthesis inhibitors on the function of the primate corpus luteum

Sargent, Eva Lee, Ph.D.

The University of Arizona, 1988
THE EFFECTS OF INTRALUTEAL INFUSION OF
PROSTAGLANDIN-SYNTHESIS INHIBITORS ON THE FUNCTION
OF THE PRIMATE CORPUS LUTEUM

by
Eva Lee Sargent

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF PHYSIOLOGY
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For the Degree of
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THE UNIVERSITY OF ARIZONA

1988
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Eva Lee Sargent entitled The Effects of Intraluteal Infusion of Prostaglandin-Synthesis Inhibitors on the Function of the Primate Corpus Luteum and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Patricia B. Hoeyer 4/18/88
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Exogenous prostaglandins (PGs) have been reported to suppress or to promote the function of the primate corpus luteum in vitro and in vivo, but the role of endogenous ovarian prostaglandins in regulating luteal function during the menstrual cycle is unknown. Infusion (via osmotic pump) of the prostaglandin-synthesis inhibitor sodium meclofenamate into the corpus luteum, but not via the jugular vein, during the midluteal phase of the menstrual cycle resulted in a decline in progesterone levels and premature menses in rhesus monkeys (Macaca mulatta). These results suggest that meclofenamate suppresses the production of an obligatory luteotropic prostaglandin or other metabolite of arachidonic acid. We were unable to confirm that ovarian prostaglandin synthesis was diminished during treatment, since we could not consistently measure a gradient in PGE or \( \text{PGF}_2 \alpha \) across the ovary.

Dispersed cells from the macaque corpus luteum produced \( \text{PGF}_2 \alpha \) in vitro. Production was stimulated by exposure to arachidonic acid and was inhibited by meclofenamate and another prostaglandin-synthesis inhibitor, flurbiprofen. Although the two drugs were potent inhibitors of prostaglandin synthesis in vitro,
intraluteal infusion of flurbiprofen in monkeys did not mimic the luteolytic effects of meclofenamate.

These studies provide the first evidence of an obligatory luteotropic role for a metabolite of arachidonic acid during the primate luteal phase. However, the data suggest that the luteolytic effect of meclofenamate in vivo is not mediated entirely by the inhibition of local prostaglandin synthesis. Further studies are needed to determine the mechanism(s) of meclofenamate-induced luteolysis and to identify the putative obligatory luteotropin.
CHAPTER 1

INTRODUCTION

The primate corpus luteum is a transient endocrine gland which develops, secretes progesterone, and regresses during the luteal phase of the menstrual cycle. Its ephemeral nature belies its importance; progesterone creates the necessary uterine environment for successful implantation and pregnancy, and timely luteolysis allows for renewed gametogenesis if pregnancy does not occur. The search for regulators of luteal function and lifespan has kept scores of investigators busy, but the story is incomplete. A confounding issue is the diversity of luteal control mechanisms among species. Factors which promote luteal structure and function in one species may oppose them in another. Such is the case with estrogen, which is luteotropic in the rabbit but luteolytic in the primate. Another interspecies quandary is the role of prostaglandins in luteal function and lifespan; that is the subject of these investigations.

In many domestic ungulates and laboratory animals, prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$) is produced by the uterus and acts on the corpus luteum to cause luteolysis. Removal of the uterus therefore prolongs luteal lifespan. Prostaglandin $E_2$ (PGE$_2$) is also produced by the uterus and
exerts a luteotropic influence, often countering the effects of PGF$_{2\alpha}$. In the primate however, removal of the uterus does not affect the corpus luteum. Numerous investigators have nonetheless treated primates with exogenous PGF$_{2\alpha}$ and have reported everything from no effect to transient falls in progesterone levels to premature menses. Studies in vitro have been less at odds; treatment of primate luteal cells with PGF$_{2\alpha}$ seems to thwart progesterone production, while PGE$_2$ may promote it.

Very few studies have addressed the physiological importance of prostaglandins in primate luteal function. Such a physiological role is suggested by the fact that the corpus luteum produces prostaglandins and has prostaglandin receptors. These findings have fostered the theory that the corpus luteum may control its own function via production of, and reaction to, luteotropic or luteolytic prostaglandins. The few experiments intended to test this hypothesis are inconclusive due to shortcomings in design or interpretation. No attention has been given to a possible role for the more recently discovered thromboxanes, leukotrienes, or similar compounds, which are related to the prostaglandins by a common biochemical precursor, arachidonic acid.
The experiments described here were designed to test the hypothesis that prostaglandins or other metabolites of arachidonic acid are physiological regulators of primate luteal function or lifespan. The experimental approach is novel; the studies are based on the in vivo infusion of inhibitors of arachidonic acid metabolism into the ovary of the rhesus monkey (*Macaca mulatta*), and the investigation of subsequent changes in the function and lifespan of the corpus luteum of the menstrual cycle.
CHAPTER 2

REVIEW OF THE LITERATURE

Overview of the Menstrual Cycle

The menstrual cycle of primate species, like the estrous cycles of other mammals, is characterized by a recurring series of changes in ovarian structure and function. The menstrual cycle is unusual in that it can be divided into temporally distinct follicular and luteal phases. Ovulation, the release of the oocyte from the mature ovarian follicle, separates the two phases. Structural and functional changes in the primate ovary are regulated by the pituitary gonadotropic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH are released from the pituitary in response to gonadotropin releasing hormone (GnRH) from the hypothalamus. The ovarian steroids, estrogen and progesterone, exert both positive and negative feedback on FSH, LH and GnRH secretion; thus ovarian cyclicity is at least in part the result of endocrine interactions involving the ovary, the pituitary, and the hypothalamus (Knobil, 1973).

The follicular phase occupies approximately the first thirteen to fourteen days of the twenty-eight day menstrual cycle. Follicular recruitment results in the
emergence of a cohort of small growing follicles, drawn from a larger pool of dormant primordial follicles (diZerega and Hodgen, 1981). The recruited follicles enlarge and develop additional layers of granulosa cells surrounding the oocyte. Many follicles begin to develop but most undergo atresia (degeneration) as the follicular phase progresses; generally in higher primates only one follicle matures and ovulates during each menstrual cycle.

The factors which determine the selection of the particular follicle which is destined to ovulate are not completely understood. In monkeys removal of the largest follicle on days eight through twelve of the cycle delays ovulation for twelve days, apparently because no other follicle is able to assume the dominant role, and a new cohort of follicles must enter the maturing pool (Goodman et al., 1977). This suggests that the dominant follicle itself suppresses the development of the others, possibly by secreting a locally-active inhibitor (see Goodman and Hodgen, 1983 for review of the control of follicular development).

The dominant follicle, under the influence of FSH and LH, secretes increasing amounts of the steroid hormone estradiol. Estradiol primes the reproductive tract for the transport of gametes and fertilization. The high level of estradiol (often called the preovulatory estradiol
surge) produced by the fully mature follicle is able through positive feedback to elicit the release of a surge of FSH and LH from the pituitary. It is this midcycle gonadotropin surge, and particularly its LH component, which stimulates ovulation. The release of the oocyte from the ruptured follicle signals the end of the follicular phase and the beginning of the luteal phase.

The corpus luteum, the dominant structure of the luteal phase, is formed from the wall of the ruptured follicle in a process known as luteinization. Both the granulosa cells, which undergo hypertrophy, and the surrounding theca interna cells become part of the corpus luteum. Neo-vascularization completes the luteinization process (Stouffer, 1988a). The steroid hormone progesterone, which prepares the uterus for implantation of the conceptus, is the primary compound produced by the corpus luteum. In the nonfertile menstrual cycle, the corpus luteum is functional for about sixteen days, during which plasma progesterone levels show a characteristic rising, plateau, and falling pattern. Falling progesterone levels signal the functional and structural demise of the corpus luteum (luteolysis). Menses will then occur due to loss of progesterone support for the uterine endometrium. The first day of menstrual bleeding marks the beginning of a new follicular phase. If
pregnancy occurs, the syncytiotrophoblast of the developing placenta secretes a gonadotropic hormone, chorionic gonadotropin (CG), which prevents the regression of the corpus luteum and assures continued progesterone support for the uterine endometrium (Stouffer, 1988b). Ovarian cyclicity is thus suspended for the duration of pregnancy.

Control of Luteal Function and Lifespan

The regulation of luteal lifespan is crucial to primate fertility. If the corpus luteum regresses early in the fertile menstrual cycle, pregnancy is not maintained. Conversely if the corpus luteum regresses late in the nonfertile cycle, follicular development and ovulation are delayed. The ability to therapeutically control human luteal lifespan has important applications in both promoting and arresting fertility. Unfortunately, the physiological factors which control luteal function and lifespan are incompletely understood. In general the primate corpus luteum may be influenced by both positive (luteotropic) and negative (luteolytic) factors. Luteotropins and luteolysins may affect either progesterone production or tissue maintenance, or both.
The Role of Luteinizing Hormone

Luteinizing hormone (LH) binds to specific receptors on luteal cells and stimulates progesterone production through a cyclic AMP (cAMP)-dependent mechanism (Ling and Marsh, 1977). Although rhesus monkey (Macaca mulatta) luteal cells in vitro respond to gonadotropins with a dose-dependent increase in progesterone production (Stouffer et al., 1977a), the degree to which LH controls the corpus luteum in vivo is less clear. Data from one laboratory suggest that once ovulation has occurred, LH is not necessary for normal progesterone secretion and luteal lifespan (Asch et al., 1982; Balmaceda et al., 1983). The majority of evidence however, suggests that when LH is absent (due to removal of the pituitary, ablation of the hypothalamus, administration of LH-antisera, or administration of a GnRH antagonist), plasma progesterone profiles are subnormal and menses is premature (Vande Wiele et al., 1970; Mougdal et al., 1972; Hutchinson and Zeleznik, 1984; Mais et al., 1986; Fraser et al., 1987).

Hutchinson and Zeleznik (1984) used a variation of the "hypothalamic clamp" technique (Knobil, 1980) to control endogenous LH release in monkeys. After abolishing endogenous GnRH secretion via lesions in the hypothalamus, LH secretion was driven by the intermittent administration of synthetic GnRH. They present particularly compelling
evidence that when LH release is stopped, progesterone production declines markedly. Although premature menses occurred, the authors did not, as was done in previous studies, assume that this was synonymous with structural luteolysis. In a later study they demonstrated that during the premature menstrual bleeding induced by four days of LH deprivation, the corpus luteum was still able to respond to restored LH release with normal progesterone production, and luteal lifespan was not altered (Hutchinson and Zeleznik, 1985). This elegant separation of effects reinforces LH's essential role in luteal progesterone production, but suggests that LH may be uninvolved in controlling the functional lifespan of the corpus luteum.

Early studies measuring immunoreactive LH levels in monkeys throughout the luteal phase described a low unchanging pattern of LH secretion (Monroe et al., 1970; Kirton et al., 1970a; Niswender and Spies, 1973). With use of the more sensitive Leydig cell bioassay, a decline in LH levels during the luteal phase was observed (Ellinwood and Resko, 1983). Luteolysis, however, does not appear to be simply the result of LH withdrawal. Neither continued administration of exogenous LH (Vande Weile, 1970) or continued stimulation of LH secretion in the hypothalamic clamp model (Knobil, 1980; Hutchinson and
Zeleznik (1984) extends the natural lifespan of the corpus luteum.

Several alternative mechanisms have been proposed whereby LH could control luteal lifespan. LH is released from the pituitary in a pulsatile manner in women (Yen et al., 1972; Santen and Bardin, 1973) and monkeys (Dierschke et al., 1970; Norman et al., 1984). Numerous investigators have reported that in women (Filicori et al., 1986) and monkeys (Norman et al., 1984; Ellinwood et al., 1984) the frequency of pulsatile LH secretion declines during the late luteal phase of the cycle. It is uncertain whether spontaneous luteal regression is a result of this decrease in the frequency of LH release. Luteal phases of normal length can be obtained in monkeys in the hypothalamic clamp model with an unvarying pattern of one LH pulse/hour (Knobil, 1980; Hutchinson and Zeleznik, 1984) or in women with one LH pulse/90 min (Soules et al., 1987) — frequencies which mimic the LH pattern of the early luteal phase (Ellinwood et al., 1984) or early follicular phase (Soules et al., 1987). These results suggest that a change in pulse frequency is not necessary to cause timely luteolysis, but do not address the possibility that a lower pulse frequency may not provide adequate gonadotropin support during the late luteal phase. Hutchinson and coworkers recently investigated the ability
of their standard dose of GnRH to support luteal function when given less frequently (Hutchinson et al., 1986). When a GnRH regimen which mimicked the LH pattern of the late luteal phase (1 pulse/8 h) was begun during the early luteal phase, there was no change in progesterone patterns or the length of the luteal phase in three of four monkeys. The data remain equivocal, because the lower GnRH pulse frequency resulted in a greater amplitude LH pulse, which may have contributed to luteal maintenance.

The ability of macaque luteal cells in vitro to respond to gonadotropins decreases around the time of luteolysis (Stouffer et al., 1977a). This lack of response led to two hypotheses: that either a decrease in the number or affinity of LH receptors on the corpus luteum (Cameron, 1981) or a decrease in the responsiveness of adenylate cyclase to LH stimulation (Eyster, 1984) might be involved in luteolysis. Support for the first hypothesis was not forthcoming. The affinity of LH receptors in the monkey corpus luteum remains constant throughout the luteal phase. Although the number of LH receptors decreases as menses approaches, this appears to be an effect rather than a cause of luteolysis (Cameron, 1981). Changes in the cAMP response to LH are indeed seen in the late luteal phase, and this decrease in the ability of adenylate cyclase to respond to LH may be an
important luteolytic mechanism (Eyster, 1984). The factor(s) mediating this decrease in gonadotropin sensitivity is unknown.

The Role of Estrogen

Estrogen has also been implicated in the control of luteal lifespan. In the rabbit, estrogen produced by the follicles is luteotropic (Keyes and Nalbandov, 1967). In contrast, exogenous estrogens are luteolytic in the primate. Orally administered estrogens, particularly diethylstilbestrol (DES), are effective postcoital contraceptives (Morris and van Wagenen, 1966; Kuchera, 1971). Systemic administration of DES or estradiol, either orally (Auletta et al., 1972; Gore et al., 1973; Auletta et al., 1976), intravenously (Auletta et al., 1978), or subcutaneously in silastic capsules (Karsch et al., 1973), lowers progesterone levels and shortens the luteal phase in women and monkeys. Intra-corpus luteum implantation of silastic capsules containing estradiol also induced premature menses (Schoonmaker et al., 1982).

The site of action of estrogen's luteolytic effect has been widely disputed. Hoffman (1960) observed premature menses in women after implantation of crystalline estradiol into the corpus luteum-bearing ovary, but not with identical treatment of the contralateral ovary; this suggests a local site of action.
In addition, estrogens lower basal and gonadotropin-stimulated progesterone production by human and macaque luteal cells in vitro (Stouffer et al., 1977b; Williams et al., 1979; Thibier et al., 1980). The mechanism of this inhibition is particularly unclear in view of a recent report that the macaque corpus luteum does not contain receptors for estrogen (Hild-Petito et al., 1987).

Estrogen's ability to lower pituitary LH secretion through negative feedback suggests a possible central mechanism of action for estrogen-induced luteolysis. One study reported no change in LH levels during estrogen treatment (Auletta et al., 1978). Other studies, however, show a decrease in LH levels when estrogen is administered (Karsch and Sutton, 1976; Schoonmaker et al., 1982; Westfahl and Kling, 1982), suggesting that a fall in LH mediates estrogen's effect. Furthermore, the luteolytic effect of estrogen in cycling monkeys is absent in monkeys whose luteal phases are supported by exogenous pulsatile GnRH administration (Hutchinson et al., 1987). Thus the hypothalamus appears to be a major site of action of estrogen-induced luteolysis. In a recent study, subcutaneous implantation of either estradiol or progesterone capsules in cycling rhesus monkeys was without sustained effect, but a combination of the two steroids resulted in premature luteolysis (Hutchinson et
al., 1987). The authors speculate that estrogen is luteolytic only in the presence of a progesterone-induced decrease in the frequency of LH release.

These studies with exogenous estrogens suggest a possible physiologic role for estrogen as a luteolysin. Unlike corpora lutea of other mammals, the primate corpus luteum produces substantial amounts of estradiol and estrone throughout its lifespan (Butler et al., 1975; Stouffer et al., 1980). Many authors have suggested that this estrogen production is a self-destructive mechanism by which the corpus luteum regulates its lifespan. Butler and coworkers (1975) found that estrogen levels were higher in late versus early luteal phase macaque corpora lutea. In contrast, Stouffer and coworkers (1980) found that the estrogen-synthetic ability of macaque luteal cells in vitro decreases in the late luteal phase. A role for estrogen as a physiological luteolysin has become particularly doubtful in view of the studies of Ellinwood and Resko (1983). They used an aromatase inhibitor to suppress both systemic and intraluteal estrogen levels by eighty to ninety percent. Despite this suppression of luteal estrogen production, the luteal phase was not lengthened. The evidence to date does not support estrogen as the long-sought physiological regulator of primate luteal function and lifespan.
The Role of Metabolites of Arachidonic Acid

In many domestic and laboratory animals, the uterus plays a primary role in the regulation of luteal lifespan. When the uterus is removed during the estrous cycle, the luteal phase is prolonged. This effect prompted the search for a substance secreted by the uterus which could affect luteal function. In 1969, Pharriss and Wyngarden published their finding that infusions of prostaglandin F\(_{2\alpha}\) (PGF\(_{2\alpha}\)), a metabolite of arachidonic acid, into the uterus or heart of pseudopregnant rats lowered the progesterone content of the ovaries. Luteal lifespan, as assessed by the maintenance of pseudopregnancy, was shortened by subcutaneous injections of PGF\(_{2\alpha}\) (Pharriss and Wyngarden, 1969). PGF\(_{2\alpha}\) is produced by the uterus, and has since been confirmed as the "uterine luteolytic hormone" (Horton and Poyser, 1976) in the rat, ewe, guinea pig, rabbit, hamster, mouse, cow, mare, sow and Mongolian gerbil (see Horton and Poyser, 1976 for review). It is proposed that uterine PGF\(_{2\alpha}\) reaches the ovary via a countercurrent transfer from the utero-ovarian vein to the ovarian artery (McCracken et al., 1972).

Following the elucidation of PGF\(_{2\alpha}\) as the uterine luteolytic factor, other prostaglandins were studied for possible effects upon the corpus luteum. Prostaglandin E\(_2\)
(PGE$_2$), which is also produced by the uterus, seems in many systems to directly oppose the actions of PGF$_{2\alpha}$. Intrauterine infusion of PGE$_2$ prolongs the natural lifespan of the corpus luteum in ewes (Pratt et al., 1979), and concomitant treatment with PGE$_2$ blocks some of the luteolytic effects of exogenous PGF$_{2\alpha}$ (Reynolds et al., 1981). Exogenous prostaglandin I$_2$ (PGI$_2$, prostacyclin) has a luteotropic effect in bovine corpora lutea, promoting progesterone production in vivo and in vitro. The in vivo effect is not due to a change in circulating levels of LH (Milvae and Hansel, 1980). PGI$_2$ is produced by caprine (Band et al., 1985) and bovine (Wallach, 1978; Milvae and Hansel, 1983) corpora lutea. In cows, luteal levels of PGI$_2$ decline with increasing luteal age. This decline is correlated with the fall in progesterone production (Milvae and Hansel, 1983). Prostaglandins, such as PGI$_2$, may be physiologically important bovine luteotropins. Infusion of the prostaglandin-synthesis inhibitor indomethacin into the uterine lumen of the cow on days four to six of the estrous cycle lowered plasma progesterone levels and shortened the luteal phase (Milvae and Hansel, 1985).

In nonprimate species the luteolytic role of PGF$_{2\alpha}$ is well-established, and physiologic roles for various luteotropic prostaglandins (PGE$_2$ and PGI$_2$) are postulated.
In primates, the role of metabolites of arachidonic acid in luteal function and lifespan is less clear. Although the primate uterus produces PGF$_{2\alpha}$, hysterectomy in women (Beling et al., 1970) and monkeys (Neill et al., 1969; Castracane et al., 1979) does not affect luteal lifespan. Uterine PGF$_{2\alpha}$ is therefore not involved in primate luteolysis.

The effects of exogenous PGF$_{2\alpha}$ on the primate corpus luteum in vivo have been widely studied, with varying results. Most of these studies have involved systemic infusion or injection of PGF$_{2\alpha}$. This approach is hindered by the difficulty of delivering an adequate dose of prostaglandin to the ovary. The half-life of PGF$_{2\alpha}$ in the circulation is about one minute and its metabolites are without activity (Oliw et al., 1983). Many of the early studies on women were done as an adjunct to inducing therapeutic abortion with PGF$_{2\alpha}$; thus experimental design was hindered by practicality. One of the first studies was done by Lehmann and coworkers (1972). Injection of 25 mg of PGF$_{2\alpha}$ on day twenty-one of the menstrual cycle resulted in a fall in progesterone levels and premature menses; however the sample number in this experiment was one. Wentz and Jones (1973) undertook a similar study in which thirteen women were infused with 25 mg of PGF$_{2\alpha}$ over eight hours. This treatment resulted in a fall in
progesterone levels in all of the women, and premature menses in some. This last finding is difficult to interpret due to variability in individual cycle lengths among these women. The day of treatment also varied between days three and twelve of the luteal phase. LeMaire and Shapiro (1972) used about half of the above dose, 12 mg i.v. over eight hours (although the treatment varied among subjects), in treating six women between days nineteen and thirty-one of the menstrual cycle. They reported no changes in progesterone levels or luteal phase length. Similarly Kojonja and coworkers (1978) infused only 25 \( \mu \text{g/min} \) PGF\(_2\alpha\) for five hours (7.5 mg) into ten women at midluteal phase and found no change in progesterone levels. Whether these findings merely suggest that higher doses are needed is unclear; Jewelwicz and coworkers (1972) treated three women on days five or nine of the luteal phase with 48 mg PGF\(_2\alpha\) i.v. over eight hours, yet observed no changes in either progesterone levels or luteal phase length. Dose may in fact be very important. Auletta and coworkers (1973) treated monkeys with various doses of PGF\(_2\alpha\) i.v. and found that delivery of 20 \( \mu \text{g} \) over twenty minutes resulted in a fall in progesterone, while lower doses raised progesterone levels. Another problem in interpreting the above results is the varied times of treatment; several studies suggest
that the corpus luteum may be susceptible to the
luteolytic effects of PGF$_{2\alpha}$ only at certain times during
its lifespan. For example, in a study in rhesus monkeys,
a five-day regimen of PGF$_{2\alpha}$ (30 mg per day) resulted in
premature menses only when begun during the midluteal
phase of the menstrual cycle (Kirton et al., 1970b).

Hypothesizing that the corpus luteum is the target
of exogenous PGF$_{2\alpha}$, two groups studied the effect of
intraluteal delivery of the prostaglandin. In one of
these studies (Korda et al., 1975) various doses of PGF$_{2\alpha}$
were injected into either the corpus luteum or ovarian
stroma of women. Either 500 or 1000 $\mu$g of PGF$_{2\alpha}$ injected
into the corpus luteum (on day five or eight of the luteal
phase) lowered progesterone levels and shortened luteal
lifespan. The same doses injected into the ovarian stroma
were without effect. Only one woman was treated with each
regimen, so again low sample number is a problem. These
workers made the interesting observation that progesterone
levels, which fell during the treatment, rose to normal
luteal phase levels during the premature menstrual
bleeding. This suggests that the corpus luteum suffered
only a transient functional decline and not structural
luteolysis.

A study by Auletta and coworkers (1984a) also
relied upon intraluteal delivery of PGF$_{2\alpha}$; osmotic mini-
pumps implanted in rhesus monkeys infused PGF$_{2\alpha}$ into the corpus luteum at the rate of 10 ng/hour. The treatment was begun on days five through seven of the luteal phase and continued until menses occurred. In all six of the animals studied, this treatment lowered progesterone levels and shortened the luteal phase. Infusion of the same amount of PGF$_{2\alpha}$ into the stroma of the other ovary or subcutaneously, or infusion of the vehicle into the corpus luteum, was without effect. It can be concluded from this work that exogenous PGF$_{2\alpha}$ exerts its effects directly upon the corpus luteum.

Several synthetic prostaglandin analogs have been investigated for effects upon the corpus luteum. The PGF$_{2\alpha}$ analog 15-methyl-PGF$_{2\alpha}$ methyl ester, when given in three 500 µg injections to rhesus monkeys on day twenty-two of the menstrual cycle, reduced plasma progesterone levels by fifty percent. The same dose terminated pregnancy in three monkeys treated on day twenty-eight of the pregnant menstrual cycle (Wilks, 1980). In a later study (Wilks, 1983) four synthetic prostaglandins (PGF$_{1\alpha}$ methyl ester, PG-analog-amide, PGF$_{2\alpha}$-1,15-lactone, and PGE-analog) were examined for effects upon the corpus luteum. PGF$_{1\alpha}$ methyl ester caused an eighty percent reduction in progesterone levels in nonpregnant monkeys. PGF-analog was only one third as active in this respect.
PGF$_2\alpha$-1,15-lactone and PGE-analog (which stimulated uterine contractions) had no effect upon progesterone levels. Combinations of the four analogs were highly effective in terminating pregnancy, presumably because of their combined actions on the corpus luteum and uterus.

The PGF$_2\alpha$ analog cloprostenol has been tested for luteolytic properties in the marmoset monkey. A single i.m. injection of 0.5 µg was effective in lowering progesterone levels in all of the monkeys treated between days ten and seventeen of the luteal phase, but was less effective earlier (Summers et al., 1985). The same analog, when infused into the corpus luteum of early pregnancy at 0.035 µg/min for thirty minutes, resulted in a fall in progesterone and termination of pregnancy in marmoset monkeys (Hearn and Webley, 1987).

The above studies are the basis of what is almost a folklore about PGF$_2\alpha$ and primate luteolysis. The effects of exogenous PGF$_2\alpha$ have been termed "functional" or "transient" luteolysis and reviewers of the subject have concluded that PGF$_2\alpha$ is all-important in primate luteolysis or that it is uninvolved. A physiological role for PGF$_2\alpha$ in luteal function is strongly suggested by these findings: the primate corpus luteum contains PGF$_2\alpha$ (Challis et al., 1976; Shutt et al., 1976; Swanston et al., 1977; Patwardhan and Lanthier, 1985; Vijayakumar and
Walters, 1987), produces PGF$_{2\alpha}$ in vitro (Challis et al., 1976; Balmaceda et al., 1979; Patwardhan and Lanthier, 1985; Johnson and Ottobre, 1987), and has putative receptors (binding sites) for PGF$_{2\alpha}$ (Powell et al., 1974; Rao et al., 1977). It is thus possible, as has been suggested by numerous authors, that locally-produced PGF$_{2\alpha}$ acts on the corpus luteum to cause luteolysis. This PGF$_{2\alpha}$ might be the primate luteolysin controlling the lifespan of the corpus luteum, or may be one of several factors interacting to exert this control.

The physiological importance of PGF$_{2\alpha}$ in primate luteal function has seldom been studied. An experiment by VanOrden and coworkers (1977) found no cyclic changes in circulating levels of PGF$_{2\alpha}$ (or PGE$_2$) during the menstrual cycle. Although originally interpreted to suggest that prostaglandins are not regulators of the menstrual cycle, these findings are in fact more the result of the limited amount of prostaglandins produced by the ovary (as compared to whole-body levels) and rapid prostaglandin metabolism. These problems were overcome in a study by Auletta and coworkers (1984b). They measured the levels of a PGF$_{2\alpha}$ metabolite (13,14-dihydro-15-keto PGF$_{2\alpha}$, or PGFM) in ovarian venous blood, and found that levels of PGFM increased significantly in the venous output from the corpus luteum-containing ovary during the late luteal
phase. This increase in PGFM was significantly correlated with falling progesterone levels. The essential determination missing from this work is that the increase in PGFM is a result of increased intra-luteal PGF$_{2\alpha}$ production, rather than increased degradation of preexisting PGF$_{2\alpha}$.

Prostaglandin production does appear to change with the developmental stage of the corpus luteum. Many investigators have considered the ratio of PGF$_2$ to PGE$_2$ to be of primary importance. According to one study (Challis et al., 1976) the human corpus luteum in vitro produces more PGE$_2$ than PGF$_{2\alpha}$, and in corpora lutea collected in the late luteal phase there is a fall in PGF$_{2\alpha}$ production. In contrast Balmaceda and coworkers (1979) found that PGF production by the macaque corpus luteum in vitro is significantly higher in tissues collected during the late luteal phase, while PGE production is significantly lower, than in tissues collected earlier in the luteal phase. Ratios of PGF to PGE production (4:1) were significantly greater in corpora lutea from the mid- and late, as opposed to early, luteal phase. Similarly, Patwardhan and Lanthier (1985) found a significantly greater level of PGF$_{2\alpha}$ versus PGE$_2$ production (5:1) by human corpora lutea collected during the late luteal phase. There were no significant differences in production of these two
prostaglandins by corpora lutea collected in the mid- or early luteal phase. These findings parallel endogenous prostaglandin levels measured in excised corpora lutea in the same study. Endogenous levels of PGF\(_{2\alpha}\) were highest in the midluteal phase, as were concentrations of PGE\(_2\). PGF\(_{2\alpha}\) levels were significantly higher than PGE\(_2\) levels only in tissues collected during the late luteal phase. An earlier study by Swanston and coworkers, in which corpora lutea were removed from women during the early, mid- or late luteal phase, reported that the mean PGF\(_{2\alpha}\) level was highest in corpora lutea from the late luteal phase, although differences between the three groups were not statistically significant (Swantson et al., 1977).

Another experiment addressing the physiological role of PGF\(_{2\alpha}\) in luteal lifespan is that of Manuagh and Novy (1976). The authors treated rhesus monkeys systemically with indomethacin, an inhibitor of prostaglandin synthesis. Two animals were treated in the early luteal phase, one and three days after the preovulatory estradiol peak, and two were treated in the midluteal phase, six and seven days after the preovulatory estradiol peak. This treatment did not alter progesterone levels or luteal phase length. The dose of indomethacin used (28-31 mg/kg/day) was adequate to delay parturition in two pregnant monkeys tested, presumably by interfering
with uterine PGF$_{2\alpha}$ production. Unfortunately, whether the treatment actually lowered intra-ovarian or intraluteal prostaglandin levels in the nonpregnant monkeys was not determined. Without this information, the authors' conclusions about the lack of a physiological role for PGF$_{2\alpha}$ are speculative. Furthermore, indomethacin interferes with the production of all of the prostaglandins, including suspected luteotropins PGE$_2$ and PGI$_2$. Thus the use of a drug such as indomethacin might not be expected to provide answers about the specific role of PGF$_{2\alpha}$.

A similar study was recently undertaken by Gibson and Auletta (1986). Five women were treated late in the luteal phase (two to five days before menses) with 600 mg of the prostaglandin-synthesis inhibitor ibuprofen. Blood samples were collected every thirty minutes for an hour before treatment and five hours after treatment for the measurement of progesterone and LH. Each women was studied in two consecutive cycles, in which either ibuprofen or a placebo was given. There were no significant differences in progesterone levels over time or between the two treatment groups. When the data were expressed as the ratio of progesterone to LH, this ratio declined significantly with placebo, but not with ibuprofen, treatment. The authors believe that the
attenuated decline in this ratio in the ibuprofen-treated group suggests a "luteal sparing" effect of ibuprofen, i.e. the suppression of a luteolytic prostaglandin. Although prostaglandin-synthesis inhibition has been reported to inhibit LH release in monkeys (Carlson et al., 1977), the authors ignored the potentially interesting LH decline seen in this study, attempting to negate it by expressing the data as the ratio of progesterone to LH. The site of action of ibuprofen is thus unknown, as is whether the dose used was adequate to suppress prostaglandin levels within the ovary.

The role of luteotropic prostaglandins in the primate has not received the attention given PGF$_{2\alpha}$. Primate corpora lutea contain PGE$_2$ (Challis et al., 1976; Patwardhan and Lanthier, 1985; Vijayakumar and Walters, 1987), produce it in vitro (Balmeceda et al., 1979; Patwardhan and Lanthier, 1985; Johnson et al., 1987), and have PGE$_2$ receptors (Tanaka et al., 1981). Studies involving actions of exogenous PGE$_2$ on primate luteal cells in vitro are described in the following pages. A recent abstract reported that macaque luteal cells in vitro also produce PGI$_2$ (Johnson et al., 1987). Studies of PGI$_2$ actions in vitro are described later, as is a study of the potentially luteotropic action of PGD$_2$. 
In addition to serving as the precursor for prostaglandins, arachidonic acid is metabolized to form a plethora of other compounds, including epoxyeicosatrienoid acids (EET's), leukotrienes (LT's), lipoxins (LX's), thromboxanes (TX's), and hydroperoxy- and hydroxyeicosatetraenoic acids (HPETE's and HETE's). There have been few studies on the potential luteotropic or luteolytic actions of these non-prostaglandin metabolites, most of which were discovered quite recently. Several epoxygenated derivatives of arachidonic acid, which are formed by cytochrome P-450 oxidation, stimulate the release of LH from pituitary cells in vitro (Snyder et al., 1983). The epoxyeicosatrienoic acid 5,6 EET is also a potent vasodilator (Carroll et al., 1987). The leukotrienes are produced by white blood cells and have important pathophysiological actions (Gerrard, 1985). Leukotriene B₄ (LTB₄) induces leukocyte accumulation, modulates pain responses, and mediates changes in vascular permeability and blood flow (Ford-Hutchinson, 1985). Such actions could be involved in structural luteolysis. Exogenous leukotriene C₄ (LTC₄) causes a dose-dependent release of LH from pituitary cells in vitro (Hulting et al., 1985), a finding which suggests a wider physiologic role for the leukotrienes. The recently discovered lipoxins are also formed by leukocytes, and lipoxin A is a
potent stimulator of protein kinase C (Hansson et al., 1986), a mediator of hormone action in many tissues. The thromboxanes are produced by many tissues, including most types of white blood cells, and stimulate smooth muscle contraction and the aggregation of platlets. The corpus luteum is richly vascularized and it is possible that luteal cells are influenced by the secretions of blood cells, or by secretions of capillary endothelial cells. The ability of macrophages, when co-cultured with luteinized granulosa cells, to stimulate progesterone production has been reported in women (Halme et al., 1985) and mice (Kirsch et al., 1983).

The first evidence of a role for a non-prostaglandin metabolite of arachidonic acid in the regulation of the corpus luteum is that of Hansel and coworkers (see Milvae, 1986 for review). The synthesis of luteotropic $\text{PGI}_2$ by bovine luteal cells may be controlled by local levels of the hydroxyeicosatetraenoic acid 5-HETE (Milvae et al., 1986). In vitro, 5-HETE caused a decrease in progesterone and $\text{PGI}_2$ production by bovine luteal cells; $\text{PGF}_{2\alpha}$ production was unchanged. Bovine corpora lutea were found to contain high endogenous levels of 5-HETE (36-46 ng/10^6 cells). 5-HETE may play an important physiological role by suppressing luteal production of $\text{PGI}_2$. In addition intrauterine infusion of
the lipoxygenase inhibitor nordihydroguaiaretic acid lengthens the luteal phase of the estrous cycle in the cow, suggesting a physiological, luteolytic role for a lipoxygenase-derived metabolite of arachidonic acid (Milvae et al., 1986).

**Mechanisms of Action of Prostaglandins in the Corpus Luteum**

**Effects on Blood Flow**

PGF$_{2\alpha}$ is a potent vasoconstrictor. The first mechanism of action suggested for the luteolytic effect of PGF$_{2\alpha}$ was a limitation of blood flow to the ovary or corpus luteum (Pharriss and Wyngarden, 1969). Experiments to test this idea yielded somewhat conflicting results. Studies in the ewe, rat and rabbit demonstrated a decrease in blood flow to the corpus luteum coincident with luteolysis (see Behrman et al., 1979 for review). This drop in blood flow may however be an effect rather than a cause of luteolysis. In the rabbit for example, systemic treatment with exogenous PGF$_{2\alpha}$ resulted in a significant decrease in progesterone levels within two hours, but there was no decline in luteal blood flow until eight hours (Bruce and Hillier, 1974). In the sheep, intrauterine administration of PGF$_{2\alpha}$ or analogs having ten-fold more and fifty-fold less vasoconstrictive activity than PGF$_{2\alpha}$, resulted in identical decreases in progesterone
levels (Nett et al., 1976). In rhesus and patas monkeys, infusion of up to 50 μg/min PGF$_2$α for two and a half hours had no effect on ovarian blood flow, although progesterone levels fell (Auletta et al., 1973).

Despite the fact that functional luteolysis (a decrease in progesterone production) can apparently occur without a change in luteal or ovarian blood flow, the possibility remains that a later decrease in blood flow promotes structural luteolysis. In any case changes in blood flow are at best only one of several mechanisms whereby prostaglandins affect the corpus luteum.

Gonadotropin-like and Anti-gonadotropic Actions

Prostaglandins possess both gonadotropin-like and gonadotropin-antagonistic actions. Some of the anti-gonadotropic actions of PGF$_2$α can be attributed to a loss of LH receptors on luteal cells. PGF$_2$α, when given to rats in vivo, or when added to rat luteal cells in vitro, causes a decrease in the number of gonadotropin receptors (Hitchens et al., 1974; Behrman et al., 1979). This loss, measured by the ability of isolated cells to bind LH or CG, is not evident until eight hours after PGF$_2$α administration, and thus does not appear to be the cause of decreased progesterone production, which occurs earlier. PGF$_2$α treatment elicits a similar effect in sheep; a decrease in the number of LH receptors is
detectable at 22.5 hours, following an earlier decline in circulating progesterone levels at 7.5 hours (Diekman et al., 1978). In rhesus monkeys intraluteal injection of 500 μg of PGF$_2$α resulted in a decline in progesterone levels, and a binding study five hours later revealed a decrease in gonadotropin binding capacity with no change in affinity (Sotrel et al., 1981). In addition, co-incubation of macaque luteal particulates with PGF$_2$α lowers LH binding capacity (Cameron, 1981). PGF$_2$α may mediate alterations in gonadotropin binding via changes in membrane fluidity. Administration of a luteolytic dose of PGF$_{α}$ in the cow (Goodsaid-Zalduondo et al., 1982) and the rat (Carlson et al., 1984) resulted in a decrease in membrane fluidity, accompanied by an increase in gel-phase lipids which persisted at physiological temperature. A decrease in the fluidity of rat luteal membranes may be responsible for the PGF$_2$α-induced reduction in aggregation of occupied LH receptors, a process reportedly necessary for activation of adenylate cyclase (Luborsky et al., 1984). The effect of PGE$_2$ on gonadotropin binding has been studied in the ewe; no changes in binding were seen at forty-eight or seventy-two hours after intra-ovarian PGE$_2$ treatment (Weems et al., 1985).

In vitro, prostaglandins acutely alter luteal cell responses to gonadotropins, and to a lesser extent may
affect basal cyclic AMP (cAMP) or progesterone production. The pioneering study of these actions in primate luteal cells was conducted by Marsh and LeMaire (1974). Incubation of human luteal slices with PGE$_2$ increased the activity of adenylate cyclase, and stimulated cAMP and progesterone production. These results were confirmed in similar studies by Maeyama and coworkers in 1976.

A study by Stouffer and coworkers (1979) examined the in vitro effects of both PGE$_2$ and PGF$_{2\alpha}$ on macaque luteal cells collected during the mid- (four to seven days postovulation) and late (eight to ten days postovulation) luteal phase of the menstrual cycle. At midluteal phase, cells responded to 5 µg/ml of either PGE$_2$ or PGF$_{2\alpha}$ with increased progesterone production. PGE$_2$ was approximately ten-fold more potent than PGF$_{2\alpha}$ in this respect. The addition of human chorionic gonadotropin (hCG) also stimulated progesterone production, and neither prostaglandin altered the steroidogenic response to hCG. In the late luteal phase, neither prostaglandin affected basal progesterone production, but both PGF$_{2\alpha}$ and PGE$_2$ abolished hCG stimulation of luteal cells. Stimulation of progesterone production by dibutyryl cAMP was not abolished by either prostaglandin -- suggesting that the abolition of hCG's effect takes place at a step prior to cAMP generation.
The concept that PGF$_{2\alpha}$ interferes with LH/CG action in primate luteal cells by the midluteal phase (seven to eight days postovulation) of the cycle was extended to human luteal cells in a study by Hamberger and coworkers (1979). The addition of 1 µg/ml PGF$_{2\alpha}$ in combination with hCG decreased cAMP production by fifty percent, as compared to that elicited by hCG alone. Dennefors and coworkers (1982) examined further the effects of PGE$_2$ on cAMP production and the effect of PGF$_{2\alpha}$ on both cAMP and progesterone production. In human corpora lutea obtained one to three days postovulation, 1 µg/ml of PGE$_2$ caused a six-fold increase in cAMP; the same amount of PGF$_{2\alpha}$ had no effect on cAMP or progesterone production. Whereas hCG stimulated cAMP production three-fold, the combination of hCG and PGE$_2$ was synergistic — causing a thirty-fold increase in cAMP levels. PGF$_{2\alpha}$, in contrast, had no effect on hCG stimulation in corpora lutea collected during the early luteal phase. In corpora lutea from the midluteal phase (seven to eleven days postovulation), PGF$_{2\alpha}$ significantly blunted the stimulatory effect of hCG on cAMP and progesterone production, but had no effect on basal production of either substance. PGE$_2$ had no effect on corpora lutea of this age, either alone or in combination with hCG. In corpora lutea collected during the late luteal phase
(twelve to fourteen days postovulation) PGF$_{2\alpha}$ had no effect on hCG-stimulated cAMP or progesterone production. Recently, PGI$_2$ was reported to stimulate cAMP production by human corpora lutea collected during the early luteal phase (Hamberger, 1988).

The above studies agree on several points. First, PGE$_2$ and PGI$_2$ mimic the actions of gonadotropins. In contrast, PGF$_{2\alpha}$ decreases hCG stimulation of cAMP and/or progesterone production. Second, these effects depend upon the age of the corpus luteum; PGF$_{2\alpha}$ is effectively anti-gonadotropic only during a period which extends from approximately seven to eleven days after ovulation. Third, the actions of prostaglandins on gonadotropin-stimulated progesterone production appear to be mediated by changes in the activity of adenylate cyclase. A recent study by Molskness and coworkers examined in detail the effects of prostaglandins and arachidonic acid on the activation of adenylate cyclase in macaque luteal tissue (1987). Homogenates were prepared from corpora lutea obtained during the midluteal phase. Prostaglandins E$_2$, I$_2$, D$_2$ and to a lesser extent F$_{2\alpha}$ were able to stimulate adenylate cyclase. This effect was not additive to, nor inhibitory of, the stimulatory effect of hCG. In contrast PGA$_2$ and arachidonic acid decreased hCG-stimulated adenylate cyclase activity, but had no effect on basal activity.
PGF$_2\alpha$'s ability to inhibit hCG-stimulated cAMP production appears to depend upon the presence of intact luteal cells; this is the case in rats (Dorflinger et al., 1983) as well as primates. This suggests that the anti-gonadotropic effect of PGF$_2\alpha$ is mediated by a second messenger which is lost in disrupted cells. Evidence from studies of rat corpora lutea suggests a role for calcium in the mechanism of action of PGF$_2\alpha$. Calcium directly inhibits LH-stimulated adenylate cyclase in rat luteal membranes (Dorflinger et al., 1984). Treatment of rat luteal cells with the calcium ionophore A23187 decreases LH-stimulated cAMP production. This effect is dependent upon extracellular calcium, and A23187 has no direct effect on adenylate cyclase or phosphodiesterase activity (Dorflinger et al., 1984). Extracellular calcium is however not required for the anti-gonadotopic effect of PGF$_2\alpha$, and the effect is not attenuated by treatment with verapamil, a calcium channel blocker (Gore and Behrman, 1984). Thus intracellular, rather than extracellular calcium appears to be involved in the response to PGF$_2\alpha$. The ability of PGF$_2\alpha$ to lower Ca$^{++}$-ATPase activity in luteal microsomes, but not in plasma membranes, suggests a mechanism whereby PGF$_2\alpha$ might increase levels of free intracellular calcium (Albert et al., 1984). PGF$_2\alpha$ was recently shown to increase intracellular calcium levels in
bovine luteal cells (Davis et al., 1987). Evidence of increased turnover of phosphotidylinositides in response to PGF$_{2\alpha}$ in rat (Leung et al., 1986; Raymond et al., 1983) and bovine (Davis et al., 1987) luteal cells suggests the involvement of inositol-triphosphate, a mobilizer of intracellular calcium, as a mediator of PGF$_{2\alpha}$-induced luteolysis (see Berridge, 1984 for review of the role of phosphotidylinositides as mediators of cellular response).

**Effects on Tissue Maintenance**

Luteolysis can be considered a two step process— a loss of progesterone secretion followed by tissue deterioration. There are relatively few studies on the direct effects of prostaglandins on luteal structure. In the ewe, a decrease in the number of secretory granules and an increase in lipid accumulation are observed in luteal cells collected twelve hours after treatment with PGF$_{2\alpha}$. By twenty-four to forty-eight hours more drastic degenerative changes are seen, including swelling of the smooth endoplasmic reticulum and granulation of mitochondria (Nett et al., 1976). A similar study noted, six hours after PGF$_{2\alpha}$ treatment, an increase in lysosome formation (McClellan et al., 1977). In domestic ungulates two types of steroidogenic luteal cells, "large" and "small", which differ functionally and morphologically have been identified (see Niswender et al., 1985 for
review). Reportedly, in the ewe, the large luteal cells contain the majority of receptors for PGF$_{2\alpha}$ and PGE$_2$ (Fitz et al., 1982). Examination of ovine large luteal cells six hours after PGF$_{2\alpha}$ treatment revealed several changes in morphology as compared to untreated controls. Large luteal cells had smoother plasma membranes surrounded by extruded cytoplasmic contents (Fitz et al., 1984). A recent study in the ewe (Braden et al., 1988) revealed both a decrease in the size and number of large luteal cells by thirty-six hours after PGF$_{2\alpha}$ treatment. A decrease in the number of small luteal cells, with no change in size, was noted at twenty-four hours.

A study in primates suggests an effect of PGF$_{2\alpha}$ on luteal cell morphology. The infusion of 45 µg of PGF$_{2\alpha}$ in rhesus monkeys over twenty-four hours on days twenty-two to twenty-five of the menstrual cycle resulted in corpora lutea whose appearance was different from controls. The corpora lutea from treated monkeys were less homogeneous in cell population, had abnormally-shaped lipid droplets and fewer organelles. These features mirror morphological changes seen in the authors' laboratory during spontaneous luteolysis. The PGF$_{2\alpha}$ treatment also resulted in a fall in progesterone levels (Kirton and Koering, 1973).
The Biochemistry of Arachidonic Acid Metabolism

Overview of the Biosynthesis of Major Metabolites

Arachidonic acid is a ubiquitous component of cell membranes (Irvine, 1982) and its metabolism by a variety of enzyme systems is equally widespread. The formation of prostaglandins, thromboxanes, hydroperoxy- and hydroxyeicosatetraenoic acids, leukotrienes, lipoxins, and epoxyeicosatrienoic acids from arachidonic acid will be discussed here. An overview of the biosynthetic pathways involved is given in Figure 1.

The source of arachidonic acid is membrane phospholipids, in which arachidonate is stored in the fatty acyl chains, primarily in the two position (Irvine, 1982). Upon appropriate stimulation arachidonic acid is released by the action of phospholipases, particularly phospholipase A₂ (van den Bosch, 1980).

Prostaglandins are found in a wide variety of tissues (Newton and Roberts, 1982); indeed the enzyme fatty acid cyclooxygenase, which catalyzes the initial reaction in the synthesis of prostaglandins and thromboxanes, has been found in almost all animal tissues (Christ and van Dorp, 1972). Tissue-specific differences in the major prostaglandin(s) produced seem to be related to substrate availability, degree and type of enzyme activities present and rates of biosynthesis and
Figure 1. Overview of the metabolism of arachidonic acid.
metabolism (Gibson, 1982). The biosynthesis of the 1-series prostaglandins (PGF₁α, PGE₁, etc) from dihomo-γ-linolenic acid, of the 2-series from arachidonic acid, and of the 3-series from 5,8,11,14,17-eicosapentaenoic acid proceed by essentially analogous pathways (Gibson, 1982). Because of the comparatively small amounts of dihomo-γ-linolenic acid and eicosapentaenoic acid in mammalian cells (Gerrard, 1985) only those reactions leading to the 2-series prostaglandins are discussed here.

The first step in the production of prostaglandins and thromboxanes is the conversion of arachidonic acid to the endoperoxide prostaglandin G₂ (PGG₂). This reaction is catalyzed by the enzyme fatty acid cyclooxygenase. The proposed reaction mechanism (Hamberg and Samuelson, 1967) is shown in Figure 2. The reaction begins with the stereospecific removal of a hydrogen at carbon 13 and the addition of a molecule of oxygen at carbon 11, with a shift of the 11-cis double bond to the 12-trans position. The peroxide group at carbon 11 then attacks carbon 9 and a peroxide is formed between these two positions. Simultaneous ring closure between carbons 8 and 12 forms the cyclopentane ring. The shift of the 14-cis double bond to the 13-trans position, and the introduction of a hydroperoxy group at carbon 15 completes the formation of PGG₂. Prostaglandin H₂ (PGH₂), another endoperoxide
Figure 2. Biosynthesis of PGG$_2$ and PGH$_2$
intermediate, is formed from $\text{PGG}_2$ by cleavage of the 15-hydroperoxy group to form a hydroxyl group, a reaction catalyzed by prostaglandin hydroperoxidase. The cyclooxygenase and hydroperoxidase activities have not been separated (Ueno et al., 1982; Mizuno et al., 1982) and can be collectively called prostaglandin endoperoxide synthetase, or prostaglandin synthase. The endoperoxide intermediates $\text{PGG}_2$ and $\text{PGH}_2$ are the common precursors of the 2-series prostaglandins and the thromboxanes. They are unstable and have a half-life of four to six minutes in aqueous environments (Oliw et al., 1983).

Prostaglandin $D_2$ ($\text{PGD}_2$) can be formed from either $\text{PGG}_2$ or $\text{PGH}_2$, i.e., the $\text{PGD}_2$ isomerase catalyzed reaction, the loss of a proton at carbon 11 and the protonation of carbon 9, can occur before or after the hydroperoxidase step (Gibson, 1982). In Figure 1, $\text{PGD}_2$ is derived directly from $\text{PGH}_2$.

The formations of $\text{PGE}_2$ and $\text{PGF}_{2\alpha}$ from $\text{PGG}_2$ (or $\text{PGH}_2$ -- analogous to the situation above) are interrelated as shown in Figure 3. The formation of $\text{PGE}_2$ is catalyzed by $\text{PGE}_2$ isomerase. The isomerization of the endoperoxide to a $\beta$-hydroxyketone is accomplished by the loss of a proton from carbon 9 and the protonation of carbon 11. $\text{PGF}_{2\alpha}$ can be formed directly from $\text{PGE}_2$ by reduction of the ketone group at carbon 9; this reaction is catalyzed
Figure 3. Biosynthesis of PGE$_2$ and PGF$_{2\alpha}$. 
by prostaglandin-9-keto-reductase, which has been found in a limited number of tissues (Hensby, 1975; Leslie and Levine, 1973) including the human corpus luteum (Watson et al., 1979). The human corpus luteum appears also to be able to convert \( \text{PGF}_2\alpha \) to \( \text{PGE}_2 \) (Watson et al., 1979). The rat kidney (Gibson, 1982) and the rabbit kidney and stomach (Yasamoto, 1983) contain a prostaglandin-9-hydroxydehydrogenase activity which catalyzes the conversion of \( \text{PGF}_2\alpha \) to \( \text{PGE}_2 \). \( \text{PGF}_2\alpha \) can also be formed from \( \text{PGD}_2 \), a reaction catalyzed by an 11-keto reductase. This activity has been found in the liver of the rabbit, but not in the other organs (Reingold et al., 1981), and the liver enzyme has been purified (Wong, 1981). \( \text{PGF}_2\alpha \) can also be formed directly from \( \text{PGH}_2 \) by enzymatic or non-enzymatic reduction. The relative importance of enzymatic versus non-enzymatic \( \text{PGF}_2\alpha \) formation in various tissues remains unclear (see Yasamoto, 1983 for review).

Prostaglandin \( \text{I}_2 \) (\( \text{PGI}_2 \) or prostacyclin) is formed from \( \text{PGH}_2 \) in a reaction catalyzed by prostacyclin synthase. In the reaction mechanism proposed by Fried and Just (1976) a carbonium ion is formed on one of the endoperoxide oxygens followed by opening of the endoperoxide through the carbon 5 double bond.

Prostaglandins \( \text{A}_2 \) and \( \text{B}_2 \) (\( \text{PGA}_2 \), \( \text{PGB}_2 \)) are derivatives of \( \text{PGE}_2 \), under acidic and basic conditions
respectively, which have not been found in vivo (Gerrard, 1985). Prostaglandin C₂ (PGC₂) is likewise an apparently unnatural isomer of PGA₂, although enzymes which can catalyze these isomerizations have been reported in the plasma of various species (Yamamoto, 1983).

The thromboxanes are synthesized from PGG₂ (or again directly from PGH₂) in a reaction catalyzed by thromboxane synthetase. The details of this mechanism are not entirely clear; the subject has been reviewed (Granstrom, et al., 1983). The PGI₂ formation mechanism proposed above (Fried and Just, 1976) was also extended to thromboxane A₂ (TXA₂), with the polarization of the endoperoxide being in the opposite sense of that required for PGI₂. TXA₂ has a half-life of thirty-six seconds in water at 37°C (Gibson, 1982) and spontaneously hydrolyzes to thromboxane B₂ (TXB₂), an inactive metabolite.

Hydroperoxyeicosatetraenoic acids (HPETE's) are formed via the lipoxygenase-catalyzed incorporation of one molecule of oxygen into arachidonic acid (Needleman et al., 1986). The actions of 12- 15- and 5-lipoxygenases result in hydroperoxy groups on carbons 12, 15, and five respectively. The 5- and 15-lipoxygenase pathways are shown in Figure 1. The HPETE's are reduced by peroxidases (or nonenzymatically) to form corresponding hydroxyeicosatetraenoic acids (HETE's). Repeated
lipoxygenase action on the same substrate fatty acid reduces the hydroperoxide groups to form di- and trihydroxy acids (not shown) (Hansson et al., 1983).

The leukotrienes are derived from 5-HPETE, which is dehydrated to form leukotriene A₄ (LTA₄). This reaction is probably enzymatic (Hansson et al., 1983) and the presumptive enzyme has been termed leukotriene A₄ synthetase. LTA₄ can be converted to leukotriene B₄ (LTB₄) by leukotriene B₄ synthetase or to leukotriene C₄ (LTC₄) by glutathione s-transferase. LTC₄ gives rise to leukotriene D₄ (LTD₄) via a reaction catalyzed by a glutamyltranspeptidase, and LTD₄ can be converted to leukotriene E₄ (LTE₄) via an aminopeptidase (Hansson et al., 1983). These pathways are shown in Figure 1.

The lipoxins, as shown in Figure 1, are formed by human leukocytes upon incubation with 15-HPETE (Serhan et al., 1984). According to Fitzsimmons and coworkers (1986), 15-HPETE undergoes a second lipoxygenation to form 5, 15-diHPETE which is enzymatically dehydrated (as in the formation of LTA₄), and hydrolyzed to form lipoxins A₄ and B₄ (LXA₄ and LXB₄).

Oxidation of arachidonic acid by cytochrome P-450 leads to a number of products, including monohydroxy acids (Capdevila et al., 1981) and, as shown in Figure 1, epoxyeicosatrienoic acids (EET's) (Chacos et al., 1982;
Capdevila et al., 1983; Synder et al., 1983). These reactions, in which the double bonds of arachidonic acid are epoxidated, have been demonstrated in both the liver and kidney (see Needleman et al., 1986 for review).

The Control of Arachidonic Acid Metabolism

Control of free arachidonic acid levels

The level of free arachidonic acid in cytoplasm is usually low (Bills, et al., 1977), and the rate-limiting step in the biosynthesis of prostaglandins and related substances is the release of arachidonic acid from membrane phospholipids (Gerrard, 1985; Irvine, 1982; Vonkeman and van Dorp, 1968; van Dorp et al., 1964). In general the balance between release of arachidonic acid by phospholipases and its reesterification by acyl transferases determines the level of free arachidonic acid in the cytoplasm. It is an increase in phospholipase activity rather than a decrease in acyl transferase activity which provides the arachidonic acid for the production of active metabolites (reviewed by Irvine, 1982). Phospholipase A₂ activity is particularly important as it catalyzes the hydrolysis of the 2-acyl ester bond, where almost all arachidonate is held (Irvine, 1982), of phosphotidylcholine and phosphotidylethanolamine. There is no clear agreement as to which phospholipids provide the bulk of free
arachidonic acid in most tissues (Irvine, 1982). Some authors (Gerrard, 1985; Gibson, 1982) favor a major contribution by phosphotidylcholine, except in platelets, in which phosphotidylethanolamine is acknowledged as the major source (Gerrard, 1985; Irvine, 1982). Phosphotidylinositol can also serve as a source of arachidonic acid, in which case the phospholipid is hydrolysed by phospholipase C to yield diacylglycerol which can then be deacylated to liberate arachidonic acid (Irvine, et al., 1982).

A myriad of factors influence the activity of the A$_2$ phospholipases; these have been extensively reviewed (van den Bosch, 1980; Dennis, 1987; Chang et al., 1987). Over forty phospholipase A$_2$ enzymes, most derived from snake venoms, have been characterized (Chang et al., 1987). Study of the A$_2$ phospholipases is complicated by their not following classical Michaelis-Menton kinetics; enzyme activity increases exponentially at substrate concentrations above the critical micelle concentration (Dennis, 1987). The work of Dennis and coworkers with an extracellular water-soluble PLA$_2$ suggests that the A$_2$ phospholipases require both phosphotidylcholine binding to an activator site and the substrate lipid binding to a catalytic site for activity (Dennis, 1987).
On the physiological level, several hormones, stretch or deformation, and tissue injury increase phospholipase $A_2$ activity (van den Bosch, 1980). Suggested mechanisms of action for these factors are many. In various tissues there is evidence for inactive zymogen forms of $A_2$ phospholipases, which may be activated by unknown enzymes (van den Bosch, 1980). Although phospholipase $A_2$ has an absolute requirement for calcium (Gerrard, 1985; Irvine, 1982; van den Bosch, 1980), a role for intracellular calcium as a physiological mediator of arachidonic acid release remains speculative (Irvine, 1982; van den Bosch, 1980; Chang et al., 1987). Protein kinase C, inositol triphosphate, diacylglycerol, guanine nucleotide regulatory proteins, and cAMP reportedly influence phospholipase $A_2$ activity (Chang et al., 1987).

Glucocorticoids and their pharmacological analogs are useful experimentally for inhibiting phospholipase $A_2$, and clinically for reducing prostaglandin or leukotriene-mediated inflammation (Irvine, 1982; Flower, 1981; van den Bosch, 1980). The anti-inflammatory effect of steroids was first tied to the inhibition of arachidonic acid release (Gryglewski, 1975) and later to unequivocal inhibition of phospholipase $A_2$ (Hong and Levine, 1976). It was then shown that a steroid-induced protein (lipocortin) was responsible for inhibiting phospholipase $A_2$ in the lung.
(Flower and Blackwell, 1979), in macrophages (Carnuccio et al., 1980), and in neutrophils (Hirata et al., 1980). The corticosteroid analog dexamethasone may also lower free arachidonic acid by its effect on the production of arachidonate from linoleate, on phospholipid methyltransferases and acyl transferases, or on arachidonate esterification (reviewed by Irvine, 1982). Thus corticosteroids and their analogs can be used to lower the level of free arachidonic acid available for biosynthesis of active metabolites. Conversely the addition of exogenous arachidonic acid has been routinely used to increase biosynthesis of these compounds in vitro.

Control of prostaglandin endoperoxide synthetase

Assuming an adequate supply of free arachidonic acid, the next possible point of biosynthesis control, in the case of prostaglandins and thromboxanes, is prostaglandin endoperoxide synthetase, which contains both fatty acid cyclooxygenase and prostaglandin hydroperoxidase activities. The enzyme has a subunit molecular weight of about 70,000 daltons, with two identical subunits each containing a heme group (Gerrard, 1985). It is localized in the microsomal fraction of cell homogenates and more precisely in the endoplasmic reticulum (Rollins and Smith, 1980). The proposed scheme of prostaglandin endoperoxide synthetase action which best
fits activation/inactivation and kinetic data from a variety of systems (Lands and Hanel, 1983) is that proposed by Hemler and Lands (1980). Their work pioneered the view of the reaction as a "lipid hydroperoxide-initiated free radical chain reaction in which the positive feedback aspects of product activation are countered with negative feedback features of self-catalyzed inactivation" (Lands and Hanel, 1983). Unlike most enzymatic reactions, then, the prostaglandin endoperoxide synthetase reaction shows an initial lag phase, then rapidly accelerates as hydroperoxide (PGG$_2$) builds up ("product activation"), reaches a maximum velocity and then decelerates and finally stops due to the enzyme's self-inactivation (Hemler and Lands, 1980). The inactivation of the enzyme could be due to a number of factors. Hemler and Lands (1980) ruled out destruction of the necessary heme (heme loss was slower than inactivation), destruction by peroxides (although the enzyme is inactivated by peroxides, phenol, which protects against this effect, does not protect against self-inactivation), and destruction by radicals produced by hydroperoxides (other cyclooxygenases undergo inactivation with no hydroperoxides present). The possibility of inactivation by an unknown intermediate remains in favor (Hemler and Lands, 1980; Lands and Hanel, 1983). Thus the
interplay of \( \text{PGG}_2 \) positive feedback and self-catalyzed inactivation ultimately controls prostaglandin endoperoxide synthetase. Other physiological factors may also be important.

One of these factors is heme. Heme is an essential cofactor for both the cyclooxygenase and hydroperoxidase activities (Lands and Hanel, 1983; Mizuno et al., 1982). Whether the availability of heme could be a physiological regulator is unclear. The primary site of the enzyme, endoplasmic reticulum, is heme-rich. Nonetheless in cells with limited heme production this may be an important regulatory mechanism (Lands and Hanel, 1983). Because hydroperoxides are necessary to initiate the prostaglandin endoperoxide synthetase reaction, the level of these compounds could easily control enzyme activity. In general cells have high peroxidase activity and consequently very low levels of hydroperoxides (Lands and Hanel, 1983; Lands and Kulmacz, 1986). The recruitment of peroxide-forming white blood cells into areas of injury may therefore explain increased prostaglandin synthesis and resultant inflammation (Lands and Hanel, 1983; Simon and Mills, 1980). The report that the binding of platelets to monkey corpora lutea increases their prostaglandin production (Valenzuela et al., 1983) may be a manifestation of this type of mechanism. The
level of prostaglandin endoperoxide synthetase activity in a tissue reflects primarily the availability of the arachidonic acid precursor and the hydroperoxide initiator (Gerrard, 1985; Lands and Kulmacz, 1986). The use of exogenous peroxides to stimulate prostaglandin endoperoxide synthetase is limited by their rapid inactivation by peroxidases (Pace-Asciak and Gryglewski, 1983).

On a proximate, physiological level, recent evidence suggests that ovarian prostaglandin endoperoxide synthetase may be regulated hormonally. In the rat, treatment with hCG in vivo results in a three-fold increase in the amount of immunologically-detectable prostaglandin endoperoxide synthetase (Huslig et al., 1987) in the ovary. The authors suggest that de-novo synthesis of enzyme would be a particularly important regulatory mechanism in the face of self-inactivation.

Clinical and experimental attempts to thwart the synthesis of prostaglandins have been widespread and successful. One group of pharmacological inhibitors, found in numerous over-the-counter preparations, is phenolic compounds (Dewhirst, 1980). Phenolic inhibitors are thought to act by modifying "peroxide tone" (Gerrard, 1985). One such compound, acetaminophen, is an effective inhibitor in tissues with low endogenous peroxide levels,
but is ineffective in tissues in which its slight peroxide-lowering hardly affects high background peroxide levels (Gerrard, 1985). In fact the phenolic agents seems to act in a biphasic manner, stimulating prostaglandin endoperoxide synthetase at low concentrations but inhibiting it at higher concentrations (Dewhirst, 1980). This may be due to the ability of these agents to stimulate hydroperoxidase activity while inhibiting cyclooxygenase activity (Lands and Hanel, 1983). This duality of effect, and the dependence of successful inhibition on endogenous peroxide levels, make these agents somewhat undependable for experimental use as prostaglandin synthesis inhibitors.

The most celebrated prostaglandin synthesis inhibitors are the nonsteroidal anti-inflammatory drugs (NSAID's). This group includes aspirin and the chemically-diverse "aspirin-like" drugs, such as indomethacin, ibuprofen, mfenamic acid, flurbiprofen and meclofenamic acid (Simon and Mills, 1980). These drugs specifically inhibit cyclooxygenase, not hydroperoxidase (Yamamoto, 1983; Mizuno et al., 1982). Nonetheless aspirin, indomethacin and flurbiprofen may have some effect on hydroperoxidase activity, as they protect it from inactivation by alkaline pH, heat and heme (Mizuno et al., 1982). The NSAID's as a group show both competitive
(reversible) and non-competitive (irreversible) inhibition; some have both effects. Aspirin is a competitive inhibitor; it competes with arachidonic acid for the active site on the enzyme, as do indomethacin, ibuprofen and mefenamic acid (Rome and Lands, 1975). The two latter compounds are thought to attach to the heme portion of the enzyme (Gerrard, 1985). Aspirin also demonstrates a non-competitive inhibition of cyclooxygenase; the degree of inhibition is proportional to the amount of time that aspirin is in contact with the enzyme (Rome and Lands, 1975). This time-dependent irreversible inhibition is also seen with indomethacin, flurbiprofen and meclofenamic acid (Rome and Lands, 1975). In the case of aspirin this irreversible inhibition is the result of acetylation of the cyclooxygenase (Roth et al., 1975). The mechanism of non-competitive inhibition by the other drugs is uncertain, but this activity may depend upon the presence of an aryl halogen (chlorine or fluorine) (Rome and Lands, 1975; Gerrard, 1985). The agents which exhibit both types of inhibition may be more effective in clinical and experimental use; Rome and Lands (1975) cite the example that due to its irreversible action low concentrations of flurbiprofen (0.3 \( \mu M \)) can rapidly become more inhibitory than higher (50 \( \mu M \)) concentrations of ibuprofen.
Based on a compilation of data from numerous in vitro systems, the relative potencies of the NSAID's in inhibiting prostaglandin biosynthesis are meclofenamic acid > indomethacin > mefenamic acid > aspirin or ibuprofen (Flower, 1974). Some of these drugs have additional actions. The fenemates have been shown in several systems to block prostaglandin receptors (see Lindner et al., 1979 for review). At high doses meclofenamic acid and flurbiprofen inhibit phospholipase A₂ activity (Chang et al., 1987). Meclofenamic acid also has limited ability to inhibit lipoxygenase activity (Boctor et al., 1986). Indomethacin is an inhibitor of cAMP phosphodiesterase (Flower, 1974; Beatty et al., 1976).

Control of prostacyclin synthase

Prostacyclin synthase, which catalyzes the conversion of PGH₂ to PGI₂, has a molecular weight of 52,000 daltons and is associated with the microsomal fraction (Smith et al., 1983). Like prostaglandin endoperoxide synthetase, prostacyclin synthase contains heme (Pace-Asciak and Gryglewski, 1983). The enzyme is sensitive to lipid peroxide levels (Pace-Asciak and Gryglewski, 1983) which may act as physiological mediators. The hydroperoxide 15-HPETE is an inhibitor of prostacyclin synthase activity in vitro (Moncada et al.,
1976) but it also increases arachidonic acid release (Hong et al., 1980). Its use as an in vivo inhibitor of prostacyclin synthesis is ruled out by its rapid destruction by peroxidases, as is the use of other inhibitory fatty acid hydroperoxides (Pace-Asciak and Gryglewski, 1983). Tranoylcypromine, a monoamine oxidase inhibitor and anti-.pa depressant, inhibits prostacyclin synthesis in vitro (Pace-Asciak and Gryglewski, 1983; Hong et al., 1980) but has also been shown to lower arachidonic acid release and to effect PGF$_{2\alpha}$ levels (Hong et al., 1980) and PGD$_2$ levels (Ellis et al., 1982). These non-specific actions decrease tranoylcypromine's desirability as a prostacyclin synthesis inhibitor.

9,11,-Diaza-prosta 5,13 dienoic acid, usually cited as a thromboxane synthesis inhibitor (Granstrom et al., 1983) caused in one study a profound inhibition of prostacyclin synthase in the bovine corpus luteum (Sun et al., 1977). Vitamin C is a stimulator of PGI$_2$ synthesis in vitro, at least in part due to direct stimulation of prostacyclin synthase activity (Beetens et al., 1985).

Control of prostaglandin isomerases

The major prostaglandin(s) produced by a tissue depend in part (see Gibson, 1982) on the particular prostaglandin isomerases present (Yamamoto, 1983). PGD$_2$ isomerase is a cytosolic enzyme that in some but not all
tissues examined can be stimulated by the addition of glutathione in vitro (Yasamoto, 1983). PGE$_2$ isomerase appears to be a microsomal enzyme, as does an activity catalyzing the reduction of PGH$_2$ to PGF$_{2\alpha}$ (Yasamoto, 1983). Specific inhibitors of these enzymes, useful to control the synthesis of a selected prostaglandin without affecting the others, have not been forthcoming.

Control of thromboxane synthetase

Many tissues, including (in various species) lung, spleen, heart, bone marrow, gastrointestinal tract, endometrium, uterine decidua and various white blood cells have the ability to produce thromboxanes (Granstrom et al., 1983). In platelets, thromboxane synthetase activity was shown to have a similar subcellular distribution to prostaglandin endoperoxide synthetase (Granstrom et al., 1983). Thromboxanes are smooth muscle constrictors whose primary actions are probably on airway and vascular constriction (Granstrom et al., 1983). Because these processes are important in many pathophysiological states, the search for thromboxane synthesis inhibitors has been vigorous. Synthesis inhibitors fall into three general classes: endoperoxide or TXA$_2$ structural analogs, imidazole and its analogs, and various modifications of pyridine (Gorman, 1983).
Another method for controlling the effects, if not the synthesis, of thromboxanes is the use of receptor antagonists. Several compounds with thromboxane receptor antagonistic actions also affect thromboxane or prostacyclin synthesis (Gerrard, 1985), but 13-azaprostanoic acid is a selective TXA receptor antagonist (Lebreton et al., 1979).

Control of lipoxygenases

5-lipoxygenase, which catalyzes the initial reaction in the formation of most of the leukotrienes, is found primarily in "inflammatory cells" or white blood cells (Hansson et al., 1983), specifically in neutrophils, monocytes and basophils (Gerrard, 1985), and also in the brain, heart, lung and spleen (Malle et al., 1987). The enzyme has a molecular weight of approximately 73,000 and is stimulated by calcium (Malle et al., 1987). Related enzymes which catalyze the formation of HPETE's and HETE's are found in platlets (12-lipoxygenase) and in eosinophils, immature erythrocytes and lymphocytes (15-lipoxygenase) (Gerrard, 1985). Like fatty acid cyclooxygenase, lipoxygenases show product activation by peroxides and later inactivation (Gerrard, 1985). In a proximate sense, ovarian lipoxygenase activity in the rat has been reported to be stimulated by hCG (Reich et al., 1985).
Relatively few inhibitors of lipoxygenase activity have been identified. Retinoids such as vitamin A are selective inhibitors of 5-lipoxygenase. Nordihydroguaiaretic acid (NDGA) is a widely-used inhibitor of both 12- and 5-lipoxygenases (Malle et al., 1987) which also has some cyclooxygenase inhibiting action (Gerrard, 1985). Benoxaprofen is a combined cyclooxygenase and lipoxygenase inhibitor. Vitamin E is a stimulator of 5-lipoxygenase at physiological (plasma) concentrations, but is suppressive at higher concentrations. (Malle et al., 1987). The arachidonic acid analog 5,8,11,14 eicosatetraynoic acid is a competitive inhibitor of arachidonic acid oxidation via all pathways (reviewed by Gerrard, 1985).
CHAPTER 3

OBJECTIVES OF THE PRESENT STUDY

The present study was designed to investigate potential physiologic roles for prostaglandins or other metabolites of arachidonic acid in the regulation of the corpus luteum of the rhesus monkey. The guiding hypothesis was this: If locally-produced metabolites of arachidonic acid were important regulators of the corpus luteum, then intraluteal infusion of an inhibitor of arachidonic acid metabolism should result in changes in luteal function or lifespan.

Highly specific inhibitors, which suppress the production of a particular metabolite without affecting a myriad of others, are not available. Given this limitation, and the data suggesting a potential role for prostaglandins in luteal regulation, two potent inhibitors of cyclooxygenase, sodium meclofenamate and flurbiprofen, were chosen. They are not structurally related, and meclofenamate reportedly has the additional, potentially-advantageous effect of antagonizing prostaglandin actions.

Experiments were performed 1) to determine the effects of sodium meclofenamate and flurbiprofen on progesterone production and the length of the luteal phase; 2) to examine the effects of meclofenamate
treatment on aortic minus utero-ovarian venous levels of PGE and PGF$_{2\alpha}$ and 3) to compare the abilities of meclofenamate and flurbiprofen to alter basal and arachidonic acid-stimulated PGF$_{2\alpha}$ production by macaque luteal cells in vitro.
CHAPTER 4

INTRALUTEAL INFUSION OF A PROSTAGLANDIN-SYNTHESIS INHIBITOR, SODIUM MECLOFENAMATE, CAUSES PREMATURE LUTEOLYSIS IN RHESUS MONKEYS

Abstract

The physiological significance of locally-produced prostaglandins in the regulation of the functional lifespan of the primate corpus luteum is unknown. In the current study, the prostaglandin-synthesis inhibitor sodium meclofenamate was administered to adult female rhesus monkeys beginning in the midluteal phase of the menstrual cycle. Meclofenamate was infused continuously for seven days into the corpus luteum (100 μg/h, n=6) or the jugular vein (100 μg/h, n=3; 1000 μg/h, n=3) via osmotic minipump. As controls, phosphate-buffered saline (PBS) was infused into the corpus luteum (n=7) or jugular vein (n=5). In some of the monkeys receiving intraluteal infusions, chronic aortic and utero-ovarian venous catheters were implanted, and blood samples were collected on alternate days for the measurement of PGE and PGF2α by RIA. Saphenous venous blood was collected daily and progesterone and cortisol levels were determined by RIA. LH levels were determined by the mouse Leydig cell bioassay. Progesterone levels over five days
preceding treatment were not different among groups. A decline in progesterone levels on day one following surgery was observed in all treatment groups, and was accompanied by a one day elevation in cortisol levels. Thereafter, five of seven monkeys who received intraluteal infusions of PBS displayed normal progesterone patterns during treatment and normal luteal phase lengths of $15.4 \pm 1.2$ days (mean $\pm$ standard error of the mean, SEM). In six monkeys who received intraluteal infusions of meclofenamate, progesterone levels typically fell to less than $1 \text{ ng/ml}$ within seventy-two hours after initiation of infusion; progesterone levels during seven days of intraluteal infusion were significantly lower ($p < 0.01$) in meclofenamate- versus PBS-treated monkeys. Meclofenamate infusion into the corpus luteum significantly shortened ($p < 0.01$) the luteal phase to $10.5 \pm 1.0$ days. In contrast, progesterone levels during seven days of meclofenamate infusion into the jugular vein did not differ from those of PBS-treated monkeys, and the length of the luteal phase was unaltered. LH levels, measured daily, did not differ among groups either before or during treatment. Although a venous/arterial gradient in PGE was detected at the time of surgery, we were unable to detect a significant gradient across the ovary in PGE or $\text{PGF}_2\alpha$ at any time after surgery in monkeys treated with
either PBS or meclofenamate. The present data suggest an obligatory luteotropic role for locally-produced metabolites of arachidonic acid, but a physiological role for either PGE or PGF$_{2\alpha}$ in regulating the primate corpus luteum remains equivocal.

**Introduction**

Numerous prostaglandins either stimulate or inhibit cAMP and progesterone production when added to primate luteal tissue in vitro. Following the pioneering work of Marsh and LeMaire (1974), the stimulatory actions of prostaglandin (PG) E$_2$ on preparations of human and monkey luteal tissue were well recognized (Stouffer et al., 1979; Denefors et al., 1982; Molskness et al., 1987). Subsequently, PGI$_2$ (Molskness et al., 1987; Hamberger et al., 1988) and PGD$_2$ (Molskness et al., 1987) were also reported to stimulate cAMP and/or progesterone production. In contrast, Hamberger and coworkers (Hamberger et al., 1979; Denefors et al., 1982) and Stouffer and colleagues (1979) demonstrated that PGF$_{2\alpha}$ suppressed gonadotropin-stimulated cAMP production and steroidogenesis in primate luteal tissue. Most recently, Molskness and coworkers (1987) observed that PGA$_2$ also inhibited gonadotropin-stimulated adenylate cyclase activity in macaque luteal
homogenates. Thus prostaglandins may act in vitro either to promote or to suppress primate luteal function.

In vivo studies on the actions of prostaglandins on primate luteal function have focused on the inhibitory potential of \( \text{PGF}_2\alpha \). Early studies employing systemic administration of \( \text{PGF}_2\alpha \) were hindered by the short half-life of injected prostaglandin and yielded conflicting results (Karim and Hillier, 1975; Kirton, 1975). More recently, the problem of delivering an adequate dose of prostaglandin to the ovary was overcome. Either systemic treatment with \( \text{PGF}_2\alpha \) analogs (Wilks, 1980; 1983) or infusion of \( \text{PGF}_2\alpha \) directly into the corpus luteum (Auletta et al., 1984b) resulted in a decline in progesterone levels and premature menses in monkeys. Since other prostaglandins were not tested (Auletta et al., 1984b), the specificity of the response to \( \text{PGF}_2\alpha \) is unknown.

The relevance of these in vitro and in vivo studies to the roles of endogenous prostaglandins in regulating the primate corpus luteum remains unclear. In contrast to the situation in non-primate species, in which \( \text{PGF}_2\alpha \) of uterine origin is the physiological luteolysin (Horton and Poyser, 1976), the primate corpus luteum is not regulated by uterine prostaglandins. Luteal function in women and monkeys is unchanged following hysterectomy (Neill et al., 1969; Beling et al., 1970; Castracane, et al., 1979).
There is evidence, however, that the primate corpus luteum produces prostaglandins, notably PGF$_{2\alpha}$, PGE$_2$, and PGI$_2$ (Challis et al., 1976; Patwardhan and Lanthier, 1985; Johnson et al., 1987) and has binding sites for PGF$_{2\alpha}$ (Powell et al., 1974; Rao et al., 1977) and PGE$_2$ (Tanaka et al., 1981). Thus the primate corpus luteum may control its own function or lifespan via local production of luteotropic and/or luteolytic prostaglandins.

This study was designed to investigate possible physiologic roles for ovarian prostaglandins during the midluteal phase of the menstrual cycle. We hypothesized that if locally-produced prostaglandins were important regulators of the primate corpus luteum, then intraluteal infusion of an inhibitor of prostaglandin synthesis should result in changes in luteal function and/or lifespan.

**Materials and Methods**

**Experimental Design**

The housing and general care of rhesus monkeys at the Oregon Regional Primate Research Center was described previously (Molskness et al., 1987). Monkeys were checked daily for menses and menstrual records were maintained. Adult females exhibiting normal menstrual cycles of approximately 28 days were used in the current study.
The monkeys were divided among four treatment groups: Intraluteal infusion of 0.05M phosphate-buffered saline (PBS; 0.03M Na$_2$HPO$_4$, 0.019M KH$_2$PO$_4$, 0.15 M NaCl, pH 7.4) at 10 µl/h (n=7); intraluteal infusion of the prostaglandin synthesis inhibitor sodium meclofenamate (Warner-Lambert, Ann Arbor, MI), at 100 µg/10 µl distilled water/h (n=6); jugular infusion of PBS at 10 µl/h (n=5); and jugular infusion of meclofenamate at 100 (n=3) and 1000 µg/10 µl/h (n=3). The dose of meclofenamate infused to obtain an effective intraluteal concentration was extrapolated from doses used in treating arthritis in clinical trials (McEvoy, 1985) and estimates of ovarian blood flow (Bourne, 1975). In preliminary experiments a ten-fold lesser dose was not consistently effective (data not shown).

Alzet osmotic minipumps (Alza, Palo Alto, CA), model 2ML1, which deliver 10 µl/h continuously for seven days, were used for all infusions. Pumps were implanted in the midluteal phase of the menstrual cycle, five to eight days (6.5 ± 1.1, mean ± SEM) after the LH surge. The mean day of pump implantation did not differ significantly among groups.

Anesthesia

Monkeys undergoing pump implantation for intraluteal infusions received 0.2-0.3 mg of atropine
sulfate (Eli Lily and Co., Indianapolis, IN) i.m. and 20 mg of succinylcholine chloride (Quelicin, Abbott Labs, Chicago, IL) i.v. prior to intubation. Halothane (0.8-1.5%, Halocarbon Labs Inc., Hackensack, N.J.) was then administered by vaporization with 25% nitrous oxide and 75% oxygen.

Monkeys undergoing pump implantation for infusions into the jugular vein received 5mg/kg body weight of ketamine HCl (Vetalar, Park Davis, Morris Plains, N.J.) and atropine sulfate i.m. prior to being transported to the surgical suite. An anesthetic dose of ketamine HCl (10mg/kg) was then administered. This anesthesia protocol was also employed during removal of pumps and vascular catheters.

Surgical Procedures

Intraluteal infusions

Figure 4 illustrates the position of the osmotic pump, with the attached needle-catheter inserted into the corpus luteum, and the vascular catheters. The placement of the osmotic pump and catheterization of the corpus luteum were similar to that used by Auletta and coworkers (1984a). A right paramedian pelvic laparotomy was performed. The osmotic pump was buried in the subcutaneous space below the right ribcage. A PV-4 needle-catheter (Catheter and Medical Supply Fabricators, Beaverton, OR)
Figure 4. Placement of the osmotic pump with attached catheter and needle for intraluteal infusions in rhesus monkeys. -- Positions of the utero-ovarian venous and aortic catheters, and subcutaneous needle portals are also shown.
was filled with PBS or meclofenamate solution, connected to the pump and secured with a 3-0 silk ligature, and then tunneled into the peritoneal cavity. In vitro studies using $^{14}$C-labeled meclofenamate (Warner-Lambert) demonstrated that meclofenamate was unable to pass through the walls of the PV-4 catheter during a seven day incubation at 37C. The amount of meclofenamate adhering to the inside of the tubing was also negligible (data not shown). A 21-gauge stainless steel needle was inserted into the ovarian ligament and through the ovarian stroma into the corpus luteum. The needle was secured by a 4-0 silk taper needle suture at the catheter/needle interface to the ovarian ligament.

If the corpus luteum was on the right ovary, catheterization of the right utero-ovarian vein and femoral artery followed (see Figure 1). A PV-5 elbow Silastiport catheter (Catheter and Medical Supply Fabrications) was inserted 15 to 17 cm into the femoral artery and the needle portal was placed subcutaneously in the right lateral periumbilical region. A specially-designed utero-ovarian vein catheter (Catheter and Medical Supply Fabrications) with a "memory U" bend 10 cm from the end was threaded onto a Seldinger catheter guide wire that had a 70-90 degree bend 1.5 cm from its tip. The catheter was inserted into the femoral vein and advanced
to the utero-ovarian vein/vena cava junction and then directed into the utero-ovarian vein. The guide wire was removed and the catheter fully advanced until the "U" was in a natural position within the vena cava and the insertion leg was approximately 10 cm down the utero-ovarian vein. Two silk ligatures, one proximal and one distal to the insertion site in the femoral triangle, secured the catheter in place. The exposed end of the catheter was then attached to the needle portal buried in the subcutaneous space of the right thigh.

After the abdomen was closed, the dead space of the two vascular catheters was filled with sodium heparin (1000 units/ml, Lepho-Med, Inc., Chicago, IL) via the subcutaneous needle portals. Prophylactic antibiotic (Flocillin, 6-9 X 10⁵ units, Bristol Labs, Syracuse, N.Y.) was then administered i.m.

If the corpus luteum was on the left ovary, vascular catheterization was not possible due to variability in the site of insertion of the left utero-ovarian vein into the central venous system. There was no significant difference in luteal phase length between catheterized and noncatheterized animals (data not shown).

The osmotic pump and vascular catheters were removed seven days after placement. The ovarian needle was
removed during a subsequent laparotomy, generally within three months.

Jugular infusions

A midline parapharangeal incision was made and the internal jugular vein identified by blunt dissection. A 2-3 cm segment, near the junction of the omohyoideus and sternocleidomastoid muscles, was dissected free and elevated by two 2-0 silk ligatures. A PV-4 catheter (Catheter and Medical Supply Fabrications) with a silastic elbow 7 cm from the insertion tip was filled with PBS or meclofenamate solution, inserted into the jugular vein, and tied in place with both ligatures. One ligature was placed around the silastic elbow to anchor it. The free end of the catheter was tunneled to the infracostal area and a subcutaneous bed was dissected for placement of the pump. The pump was then inserted into the fossa and the catheter connected and secured with a 3-0 silk ligature. The sternocleimomastoid muscle was attached at two or three places to the sternohyoideus muscle with a 3-0 chromic suture to help protect the catheter and to stabilize the elbow. The infracostal and neck incisions were closed and prophylactic antibiotic (Flocillin, 6-9 X 10^5 units) was administered i.m. The pump and catheter were removed seven days later.
Blood Sample Collection and Hormone Analyses

Saphenous blood samples were drawn daily between 0800 and 0830h from day eight of the menstrual cycle until menses or pump removal, whichever occurred later. Saphenous samples were permitted to clot at 4C and serum was separated from blood cells by 1700 x g centrifugation at 4C for 10 min. Serum samples were stored at -20C prior to hormone analyses.

Estradiol, progesterone, LH and cortisol concentrations in peripheral serum were measured by the Core Hormone Assay Laboratories of the Oregon Regional Primate Research Center. Daily estradiol levels from day eight of the menstrual cycle were used to estimate the day of the LH surge in order to schedule pump placement. The estradiol (Resko et al., 1975), progesterone (Resko et al., 1974) and cortisol (Krey et al., 1985) radioimmunoassays have been previously described. Bioactive LH concentrations were estimated by the mouse Leydig cell bioassay as previously described (Ellinwood and Resko, 1985) except that a different standard, Cynomolgus RP1 (NICHD) was used. Intra- and interassay variabilities were as follows: Estradiol -- 8.3 and 19.8%; progesterone -- 10.7 and 16.5%; LH -- 9.3 and 11.0%; and cortisol -- 3.2 and 11.4%.
Utero-ovarian venous (U-OV) and aortic (A) blood samples (approximately 5 ml) were collected via needle portals into syringes containing 0.1 ml of a 10% EDTA (Sigma, St. Louis, MO), 10mM indomethacin (Sigma) solution. Time zero samples were collected in surgery via the needle portals both before and after insertion of the needle into the corpus luteum. Additional samples were collected on days one, three, five and seven or until the vascular catheters were no longer patent. Syringes were placed immediately into an ice bath for transport to the laboratory. Plasma was separated from blood cells by centrifugation as above. The plasma samples were stored at -150°C prior to prostaglandin analyses.

$\text{PGF}_2\alpha$ and PGE concentrations in U-OV and A plasma were estimated by radioimmunoassay. The $\text{PGF}_2\alpha$ assay was described previously for use in amniotic fluid (Haluska et al., 1987). An additional extraction, into hexane, was performed prior to acidification in order to remove interfering lipids. Samples were assayed in duplicate. Parallelism to the standard curve was confirmed in serially-diluted aliquots of rhesus macaque plasma to which authentic $\text{PGF}_2\alpha$ was added. The slope of the standard curve was $-2.06 \pm 0.2$ (x ± SD) and recovery was $71.6 \pm 4.2\%$ (n=7 assays). Intra- and interassay variabilities for $\text{PGF}_2\alpha$ were, for a "high" plasma pool
(3332 pg/ml) 9% (n=6 replicates) and 19% (n=7 assays), respectively. Intra- and interassay variabilities for a "low" pool (513 pg/ml) were 12% (n=6 replicates) and 21% (n=6 assays), respectively. The PGE assay was performed as previously described for PGF$_{2\alpha}$ (Haluska et al., 1987) with the following modifications. An additional hexane extraction, as noted above, was performed, external recovery standards were used and samples were assayed in duplicate. Iodinated PGE$_2$, [${}^{125}$I]-PGE$_2$ tyrosyl methyl ester, (New England Nuclear, Boston, MA) was used as the labeled ligand. The anti-PGE$_2$ antibody (Institute Pasteur, Dray et al., 1975) displayed 88% cross-reactivity with PGE$_1$; thus the current results are expressed as pg of PGE. The standard curve was linear between 0.5 and 12.5 pg, and the slope was $-3.52 \pm 0.6$ (n=8 assays). Sensitivity was 2 pg. Parallelism to the standard curve was confirmed in aliquots of rhesus monkey plasma to which authentic PGE$_2$ (Upjohn, Kalamazoo, MI) was added. Recovery was $84.0 \pm 3.8\%$ (n=8 assays). Intra- and interassay variabilities for a plasma pool (362pg/ml) were 15% (n=4 replicates) and 17% (n=8 assays), respectively.

Statistical Analyses

Daily progesterone, LH and cortisol levels for five days prior to treatment and seven days during treatment were compared among groups using analysis of variance with
repeated measures over time (Winer, 1971). The progesterone data for infusions into the jugular vein, and the LH data displayed heterogeneity of variance and were log transformed. For ease of presentation, LH levels (as shown in Table 1) were also analyzed utilizing mean values before and during treatment by analysis of variance with repeated measures over time. Comparisons among groups of the interval between pump placement and menses, of luteal phase length, and of the day of pump placement were made using single factor analysis of variance (Winer, 1971). Post-hoc comparisons for the above analyses were made using the Student-Newman-Keuls test (Winer, 1971). Aortic and utero-ovarian venous prostaglandin levels were compared using a three-way analysis of variance with repeated measures over time (NWA Statpak, 1985). The data displayed heterogeneity of variance and were log transformed. Post-hoc comparisons for the prostaglandin analyses were made using Student's t-tests. In all cases differences were considered significant if p < 0.05.

Results

Progesterone patterns in monkeys receiving intraluteal infusions of PBS are shown in Figure 5. Five of the seven PBS-infused controls had normal progesterone levels both before and during the treatment interval.
Figure 5. Progesterone levels (ng/ml serum) in monkeys receiving intraluteal infusions of phosphate-buffered saline (PBS). Data are normalized to the day of pump implantation, day 0. Error bars indicate SEM. Hatched bar on the x-axis indicates the interval of treatment. Shaded area represents the 95% confidence interval for serum progesterone levels during this time in the menstrual cycle, based on untreated monkeys (n=18) in our colony.
Although there was a decline in progesterone during the first 24 hours after surgery, the levels during treatment were within the normal range for this time in the luteal phase. Two of the seven monkeys did not exhibit normal progesterone patterns. Progesterone levels in these two monkeys declined during PBS infusion; levels fell to less than 1 ng/ml within 48 hours and remained low thereafter.

Figure 6 depicts the progesterone pattern of six monkeys receiving intraluteal infusions of meclofenamate. With the onset of treatment, progesterone levels dropped below levels typical of this time in the luteal phase. By the third day of meclofenamate treatment, progesterone levels were below 1 ng/ml. In contrast, progesterone levels in five of seven monkeys receiving intraluteal infusions of PBS did not fall below 1 ng/ml during the seven day treatment period (see Figure 5). Progesterone levels in these two groups (five PBS-infused monkeys vs. six meclofenamate-infused monkeys) were not different before pump placement, but were significantly different (p < 0.01) after intraluteal infusions began.

Progesterone patterns in monkeys receiving either PBS or meclofenamate infusions into the jugular vein are shown in Figure 7. Three monkeys received 100 µg of meclofenamate per hour, the same dose given into the corpus luteum, and three monkeys received ten times that
Figure 6. Progesterone levels in monkeys receiving intraluteal infusions of sodium meclofenamate (MEC).-- Refer to the legend of Figure 5 for details.
Figure 7. Progesterone levels in monkeys receiving infusions of phosphate-buffered saline (PBS) or sodium meclofenamate (MEC) into the jugular vein. -- Refer to the legend of Figure 5 for details.
dose, 1000 µg per hour. The data were combined since neither group was different from vehicle-infused controls. A drop in progesterone levels at 24 hours after surgery was again observed in both PBS- and meclofenamate-treated monkeys. However, progesterone levels typically remained within the normal range throughout the period of meclofenamate or PBS infusion. There were no significant differences in progesterone levels between these two groups, either before or during treatment.

Mean circulating levels of LH over the five days before, and the seven days during intraluteal or jugular infusion of PBS or meclofenamate are shown in Table 1. Samples from four of the five monkeys displaying normal progesterone patterns, and the two monkeys displaying abnormal patterns, during intraluteal infusion of PBS were available for analysis. In all groups, LH levels during treatment were significantly lower ($p < 0.01$) than those before treatment. However, there were no significant differences in LH levels among groups, either before or during treatment. The two PBS-infused monkeys displaying abnormal progesterone patterns had the lowest LH levels throughout the experimental protocol.

Figure 8 depicts the cortisol patterns of monkeys receiving PBS infusions into the corpus luteum or jugular vein. Samples from four of the five monkeys displaying
Table 1. Mean LH Levels (ng/ml ± SEM) Before and During Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Before Days -4 to 0</th>
<th>During Days 1 to 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intraluteal Infusion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal progesterone pattern (n=4)</td>
<td>50.7 ± 10.8</td>
<td>8.3 ± 3.5a</td>
</tr>
<tr>
<td>abnormal progesterone (n=2)</td>
<td>22.2</td>
<td>2.8</td>
</tr>
<tr>
<td>Meclofenamate (n=6)</td>
<td>26.0 ± 6.2</td>
<td>6.1 ± 3.1a</td>
</tr>
<tr>
<td><strong>Jugular Infusion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS (n=5)</td>
<td>28.8 ± 4.9</td>
<td>10.3 ± 6.3a</td>
</tr>
<tr>
<td>Meclofenamate (n=6)</td>
<td>34.5 ± 9.9</td>
<td>7.9 ± 2.0a</td>
</tr>
</tbody>
</table>

a p < 0.01 between columns
Figure 8. Cortisol levels (µg/dl serum) in monkeys receiving infusions of phosphate-buffered saline (PBS) into the corpus luteum (panel A) and jugular vein (panel B). -- Error bars indicate SEM. Data are normalized to the day of pump implantation, day 0. Hatched bar on the x-axis indicates the interval of treatment.
normal progesterone patterns, and the two monkeys displaying abnormal patterns, during intraluteal infusion of PBS were available for analysis. The level of cortisol observed one day after surgery was significantly higher than that observed on any other day (p < 0.05). There were no significant differences in cortisol levels between monkeys receiving PBS infusions into the corpus luteum or jugular vein. Mean cortisol levels in the two PBS-infused monkeys which displayed abnormal progesterone patterns are similar to those of the other monkeys. Cortisol levels in monkeys receiving intraluteal or jugular infusions of meclofenamate were also highest one day after surgery (data not shown).

Table 2 summarizes the time interval between pump placement and menses and the length of the luteal phase in monkeys receiving infusions of either PBS or meclofenamate into the corpus luteum or jugular vein. Normal luteal phase lengths of fourteen to sixteen days were seen in five of seven monkeys receiving infusions of PBS into the corpus luteum, and in animals receiving infusions of either PBS or meclofenamate into the jugular vein. In these groups menses occurred nine to eleven days after pump placement. The two PBS-infused monkeys having abnormal progesterone patterns (see Figure 5) had luteal phases of eleven and twelve days. In those monkeys
Table 2. Interval (mean days ± SEM) Between Pump Placement and Menses and Length of the Luteal Phase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Interval to Menses</th>
<th>Luteal Phase Length&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intraluteal Infusion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal progesterone pattern (n=5)</td>
<td>10.4 ± 1.0</td>
<td>15.4 ± 1.2</td>
</tr>
<tr>
<td>abnormal progesterone (n=2)</td>
<td>4.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Meclofenamate (n=6)</td>
<td>4.5 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.3 ± 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Jugular Infusion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS (n=5)</td>
<td>9.0 ± 1.0</td>
<td>14.4 ± 1.0</td>
</tr>
<tr>
<td>Meclofenamate (n=6)</td>
<td>10.5 ± 0.6</td>
<td>15.5 ± 0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> day of LH surge to onset of menses

<sup>b</sup> p < 0.01 within column

<sup>c</sup> p < 0.01 within column
receiving intraluteal infusions of meclofenamate, the time interval between pump placement and menses and the luteal phase were shortened to five and ten days respectively. The shortening of the interval between pump placement and menses, and the attenuation of the luteal phase, following intraluteal infusion of meclofenamate were significant (p < 0.01) as compared to control monkeys whether or not the two "abnormal" controls were included in the analysis.

Mean PGE levels in the utero-ovarian venous and aortic circulations of animals receiving intraluteal infusions of PBS or meclofenamate are shown in Figure 9. The values from the two utero-ovarian venous and aortic samples obtained in surgery (before and after insertion of the needle into the corpus luteum) are combined on day zero, as no consistent difference between these samples was observed. The data for days one and three and days five and seven are also combined. PGE was non-detectable in all of the aortic samples. In U-OV samples PGE levels ranged from non-detectable to 751 pg/ml. PGE levels were significantly higher (p < 0.01) in the utero-ovarian venous versus aortic circulation at time zero. This gradient decreased or disappeared in later samples, in which no significant differences were observed. There
Figure 9. Utero-ovarian venous and aortic prostaglandin E levels (pg/ml plasma) in monkeys receiving intraluteal infusions of phosphate-buffered saline (PBS; n=4, panel A) and meclofenamate (n=3, panel B).
were no significant differences between PBS- and meclofenamate-treated monkeys.

The pattern of the venous/arterial difference in PGF$_{2\alpha}$ was similar to that of PGE; an apparent, although nonsignificant, positive gradient existed at time zero which was not present in later samples (data not shown). Aortic levels of PGF$_{2\alpha}$ ranged from non-detectable to 875 pg/ml. Utero-ovarian venous levels ranged from 57 to 839 pg/ml. There was no significant difference between PBS- and meclofenamate-treated monkeys or between utero-ovarian venous and aortic PGF$_{2\alpha}$ levels at any time point.

**Discussion**

Infusion of the prostaglandin synthesis inhibitor sodium meclofenamate into the corpus luteum, beginning in the midluteal phase of the menstrual cycle, resulted in a sustained drop in progesterone levels and premature menses in rhesus monkeys. In contrast, peripheral infusion of meclofenamate did not mimic these effects. These results suggest that despite the luteolytic actions of exogenous PGF$_{2\alpha}$ (Wilks, 1980; 1983; Auletta et al., 1984a), the predominant action of locally-produced metabolites of arachidonic acid is to sustain the function and lifespan of the primate corpus luteum.
It is difficult to interpret the acute changes in luteal function observed in the current study since levels of circulating progesterone declined in all treatment groups on the first day following surgery. The relatively minor surgery required for jugular infusions, performed under ketamine anesthesia, provoked a similar decline to that seen with the abdominal surgery, performed under halothane anesthesia. No acute drop in progesterone levels following pump placement for intraluteal infusions was reported by Auletta and coworkers (1984a) but interpretation is difficult because the progesterone data were not normalized to the day of surgery. Progesterone levels in women decline following abdominal surgery (Soules et al., 1980). A more severe effect of "surgical stress" on luteal function was reported by Knobil (1973), who noted that sham hypophysectomies caused premature menses in rhesus monkeys.

The decline in serum progesterone on day one following surgery was accompanied by a transient elevation in serum cortisol levels. The decline in progesterone may be attributable to a direct effect of corticosteroids upon the ovary (Saez et al., 1977; Bambino and Hsueh, 1981). Alternatively, corticosteroids inhibit LH secretion at the level of the hypothalamus or pituitary in monkeys (Obler and Ferin, 1987; Dubey and Plant, 1985). A decline in LH
levels was observed following surgery in all treatment groups. It is not clear whether this decline is related to the postsurgical rise in cortisol, or simply reflects the normal fall in LH during the luteal phase of the menstrual cycle (Ellinwood et al., 1984). Ellinwood and coworkers observed a significant decline in LH levels in untreated rhesus monkeys at midluteal phase of the cycle. If such a decline is a feature of the normal luteal phase, then it is unlikely to cause the post-surgical fall in progesterone levels observed in this study. Despite the acute drop in progesterone following surgery, PBS-treated monkeys typically exhibited progesterone levels within the normal range, and luteal phases of normal length.

Two of the seven monkeys receiving intraluteal PBS infusions displayed abnormal progesterone patterns and short luteal phases. Cortisol patterns in these two monkeys did not appear to differ from those of the other PBS-infused monkeys. This suggests the presence of either an exaggerated stress response, unrelated to the adrenal axis, or an underlying defect in luteal function, in these monkeys. These two monkeys had lower mean LH levels prior to surgery than any other group; whether this is relevant to the deficient luteal phase is unknown. Spontaneous short luteal phases, accompanied by low
progesterone and LH levels, have been observed in rhesus monkeys (Wilks et al., 1976).

The sustained decline in progesterone levels and shortening of the luteal phase seen with intraluteal infusion of meclofenamate could not be duplicated by infusing the same, or a ten-fold higher, dose systemically; thus meclofenamate appears to inhibit corpus luteum function via a local action. A well-established action of meclofenamate is the inhibition of fatty acid cyclooxygenase, the first enzyme in the pathway converting arachidonic acid to the prostaglandins and thromboxanes (Rome and Lands, 1975). Prostaglandins have been implicated in the control of LH secretion (Behrman, 1979), and the prostaglandin synthesis inhibitor indomethacin reportedly blocks estradiol benzoate-induced LH surges in rhesus monkeys (Carlson et al., 1977). As discussed earlier the decline in LH observed after surgery in all groups is probably a normal feature of the luteal phase. In order to accurately assess possible effects of meclofenamate on LH secretion, more frequent sampling would be necessary (Ellinwood et al., 1984). Despite the limitations of the present data, the absence of significant differences in daily LH levels among treatment groups and the fact that premature luteolysis was induced
only when meclofenamate was infused into corpus luteum strongly suggest a local suppression of luteal function.

If the primary action of meclofenamate in the current study was to inhibit the local production of prostaglandins, then our data support a predominant luteotropic role for prostaglandins during the midluteal phase of the menstrual cycle. In preliminary experiments in our laboratory, 7.5 μg/ml of meclofenamate added to dispersed cells from the macaque corpus luteum inhibited arachidonic acid-stimulated PGE and PGF$_{2\alpha}$ production (unpublished observation). We hoped to confirm a similar in vivo action of meclofenamate by detecting a venous/arterial gradient in PGE or PGF$_{2\alpha}$ across the ovary which was diminished by meclofenamate treatment. Although we observed such a U-OV/A gradient for PGE during surgery, it was not detectable in later samples. We were unable to detect a consistent venous/arterial gradient in PGF$_{2\alpha}$ at any time. There were no significant differences between PBS- and meclofenamate-treated animals in this regard. To our knowledge this study is the first attempt to measure a venous/arterial gradient in prostaglandins across the primate ovary. Our findings suggest that the corpus luteum produces little PGE or PGF$_{2\alpha}$ under non-stressed conditions. Alternatively, the uterine venous contribution to, or dilution of, prostaglandin levels in
our utero-ovarian samples cannot be assessed. In addition, our sampling frequency may have been inadequate, particularly if ovarian prostaglandin production, like that of the uterus (Hooper et al., 1986) were pulsatile. We may not have measured the relevant prostaglandin. The ability of PGE$_2$, PGI$_2$, and PGD$_2$ to stimulate adenylate cyclase in primate luteal homogenates (Molskness et al., 1987) suggests these as possible locally-acting luteotropins. Production of prostaglandins other than PGF$_2\alpha$, PGE$_2$ and PGI$_2$ by the primate corpus luteum has not been investigated. Of course levels of prostaglandin which are adequate for paracrine or autocrine regulation of luteal function may be undetectable in venous blood.

Other actions of meclofenamate cannot be ruled out by the current study. Meclofenamate might also affect luteal function by antagonizing the actions of PGE$_2$ or PGF$_2\alpha$ at the level of the prostaglandin receptor, as has been reported in other tissues (Ortmann and Perkins, 1977; Simon and Kather, 1980). Meclofenamate has limited ability to inhibit the lipoxygenase-catalyzed conversion of arachidonic acid to the hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), and leukotrienes (concentration for 50% inhibition = 46.8 $\mu$M for lipoxygenase vs. 0.6 $\mu$M for cyclooxygenase; Doctor et al., 1986). Alternatively, the predominant inhibition of
cyclooxygenase by meclofenamate may shunt arachidonic acid into the lipoxygenase or epoxygenase (Chacos et al., 1982) pathways, increasing the production of luteolytic metabolites. Bovine corpora lutea contain 5-HETE, and treatment of cows with the lipoxygenase inhibitor nordihydroguaiaretic acid delays luteolysis (Milvae et al., 1986). Finally, a non-specific, cytotoxic effect of meclofenamate on luteal cells cannot be ruled out in the present study. However in preliminary experiments in our laboratory, 7.5 μg/ml of meclofenamate added to dispersed cells from the macaque corpus luteum had no cytotoxic effect over eight days of culture (unpublished observation).

The results following jugular infusion of meclofenamate in the current study are consistent with those of Manaugh and Novy (1976), who reported that systemic administration of indomethacin had no effect on progesterone levels or the length of the luteal phase in monkeys. A sufficient amount of indomethacin may not have reached the corpus luteum although the dosage utilized was sufficient to inhibit the timely onset of labor in monkeys. Notably, indomethacin treatment was initiated during the early luteal phase, one and three days after the midcycle estradiol peak in two of four monkeys. The actions of prostaglandins on luteal tissue, and therefore
presumably the results presented here, are dependent on the age or developmental stage of the corpus luteum (Stouffer et al., 1979; Denefors et al., 1982). Production rates of PGF$_{2\alpha}$ and PGE$_2$ also appear to vary with the age of the corpus luteum during the menstrual cycle (Patwardhan and Lanthier, 1985).

In summary, infusion of the prostaglandin synthesis inhibitor sodium meclofenamate into the corpus luteum, but not peripherally, resulted in premature menses in rhesus monkeys. These findings suggest an obligatory luteotropic role for prostaglandins or other arachidonic acid metabolites during the menstrual cycle. However, using the current techniques we were unable to detect significant production of two major prostaglandins, PGE and PGF$_{2\alpha}$, by the ovary bearing the corpus luteum during the second half of the luteal phase. Further studies are needed to determine the pathways and extent of arachidonic acid metabolism by the primate corpus luteum in vivo. Studies using other inhibitors of prostaglandin synthesis are underway to investigate the specificity of the response to meclofenamate.
CHAPTER 5

IN VITRO AND IN VIVO EFFECTS OF TWO PROSTAGLANDIN-SYNTHESIS INHIBITORS, FLURBIPROFEN AND SODIUM MECLOFENAMATE, ON THE CORPUS LUTEUM OF THE RHESUS MONKEY

Abstract

We reported in Chapter 4 that intraluteal infusions of the prostaglandin synthesis inhibitor sodium meclofenamate in rhesus monkeys during the midluteal phase of the menstrual cycle resulted in a decline in progesterone levels and premature menses. In the current studies, the effects of meclofenamate and another prostaglandin-synthesis inhibitor, flurbiprofen, on the primate corpus luteum were compared in vitro and in vivo.

Corpora lutea were collected from normally cycling monkeys (n=5) during the early to midluteal phase and were subjected to collagenase dispersion. PGF$_{2\alpha}$ was measured, by radioimmunoassay, in media from cell incubations performed for six hours in the presence and absence of a range of concentrations of meclofenamate and flurbiprofen from 0.01-100 µM, and in the presence of 10 µM arachidonic acid with and without 100 µM meclofenamate or flurbiprofen. In vivo, flurbiprofen (72 µg/hour) was infused via osmotic pump continuously for seven days beginning in the midluteal phase into the corpus luteum of
rhesus monkeys (n=5). Progesterone levels and the length of the luteal phase were monitored. Comparisons were made among the flurbiprofen-infused monkeys and those receiving phosphate-buffered saline (PBS, n=7) and meclofenamate (n=6), as reported in Chapter 4.

PGF$_{2\alpha}$ production by dispersed cells under control conditions ranged from 35 - 288 pg/ml/5 x 10$^4$ cells/6h. Meclofenamate and flurbiprofen significantly inhibited (p < 0.01) PGF$_{2\alpha}$ production; there were no differences (p > 0.05) between the two drugs or between doses in this regard. Arachidonic acid increased (p < 0.01) PGF$_{2\alpha}$ production three-fold. The stimulatory response to arachidonic acid was abolished by 100 μM meclofenamate or flurbiprofen. In contrast to their similar actions in vitro, intraluteal infusion of flurbiprofen did not mimic the effects of meclofenamate. Progesterone levels over five days before, and seven days during flurbiprofen treatment were not different from those of PBS-infused monkeys. Levels during flurbiprofen treatment were higher (p < 0.01) than those observed during meclofenamate treatment. The length of the luteal phase was not different between flurbiprofen- and PBS-treated monkeys (14.0 ± 1.3 versus 15.4 ± 1.2 days, mean ± SEM).

Our results confirm that dispersed cells from the macaque corpus luteum produce PGF$_{2\alpha}$, and establish the
inhibitory actions of meclofenamate and flurbiprofen in this tissue. The data suggest that the luteolytic effect of meclofenamate may result, at least in part, from actions other than prostaglandin-synthesis inhibition.

Introduction

The physiological significance of prostaglandins or other metabolites of arachidonic acid in the regulation of the functional lifespan of the primate corpus luteum is unknown. Investigators have reported that exogenous PGF$_{2\alpha}$ is luteolytic when infused into the corpus luteum of monkeys (Auletta et al., 1984), and suppresses progesterone and/or cAMP production when added to primate luteal tissue in vitro (Hamberger et al., 1979; Stouffer et al., 1979; Denefors et al., 1982). In contrast, other prostaglandins such as PGE$_2$, PGI$_2$, and PGD$_2$ stimulate progesterone and/or cAMP production when added to primate luteal tissue in vitro (Marsh and LeMaire, 1974; Stouffer et al., 1979; Dennefors et al., 1982; Molskness et al., 1987; Hamberger et al., 1988). The primate corpus luteum produces PGF$_{2\alpha}$, PGE$_2$ and PGI$_2$ (Challis et al., 1976; Patwardhen and Lanthier, 1985; Johnson et al., 1987), and has binding sites for PGF$_{2\alpha}$ (Powell et al., 1974; Rao et al., 1977) and PGE$_2$ (Tanaka et al., 1981). These observations are consistent with the hypothesis that
locally-produced prostaglandins act to promote or suppress the function of the corpus luteum of the menstrual cycle. To date few studies have addressed this possibility.

As reported in Chapter 4, intraluteal infusion of the prostaglandin-synthesis inhibitor sodium meclofenamate during the midluteal phase of the menstrual cycle resulted in a sustained drop in progesterone levels and premature menses in rhesus monkeys. Peripheral infusion of meclofenamate did not mimic these effects; thus meclofenamate appears to act directly upon the corpus luteum. These data suggest that meclofenamate causes luteolysis by suppressing local production of an obligatory, luteotropic metabolite of arachidonic acid. We were unable to confirm that the effects of meclofenamate on luteal function were a result of changes in prostaglandin production, since we could not consistently measure a gradient across the ovary in either PGE\(_2\) or PGE.

The purpose of the current study was two-fold; the first aim was to investigate the ability of two inhibitors of prostaglandin synthesis to suppress prostaglandin production by luteal cells in vitro. Meclofenamate and flurbiprofen were to added incubations of dispersed cells from the monkey corpus luteum, and the effects on basal and arachidonic acid-stimulated PGF\(_{2\alpha}\) production were measured. Our second aim was to
investigate the specificity of the in vivo response to meclofenamic acid reported in Chapter 4. Thus flurbiprofen was infused into the corpus luteum of monkeys, and progesterone levels and the length of the luteal phase were monitored.

**Materials and Methods**

The housing and general care of rhesus monkeys at the Oregon Regional Primate Research Center was described previously (Molskness et al., 1987). Monkeys were checked daily for menses and menstrual records were maintained. Adult females exhibiting normal menstrual cycles of approximately twenty-eight days were used for the current study.

**Studies Utilizing Dispersed Luteal Cells**

The corpus luteum was removed from rhesus monkeys (n=5) during the early to midluteal phase of the cycle, three to seven (5.0 ± 1.4, mean ± SEM) days after the LH surge. The LH surge was estimated to occur the day before the precipitous decline in peak estradiol levels (Molskness et al., 1987).

**Anesthesia and surgical procedures**

The anesthesia was identical to that described in Chapter 4 for the placement of osmotic pumps. Briefly, monkeys received atropine sulfate (0.2-0.3 mg i.m.) and
succinylcholine (20 mg i.v.). Halothane (0.8-1.5%) was then administered by vaporization with 25% nitrous oxide and 75% oxygen. The luteectomy procedure has been described previously (Molksness et al., 1987). Briefly, a paramedian laparotomy was performed and a superficial incision was made in the ovary. The corpus luteum was removed intact via blunt dissection. Flocillin (6-9 x 10^6 units i.m., Bristol Labs, Syracuse, NY) and Nubain (5 mg, i.m., DuPont Pharmaceuticals, NY) were given immediately following surgery.

Preparation and incubation of dispersed luteal cells

The corpus luteum was transported to the laboratory in Ham's F-10 medium (Gibco, Grand Island, NY) at 4°C. The tissue was weighed, minced and dissociated by collagenase treatment as previously described (Stouffer et al., 1976), with the following modifications. The amount of collagenase was decreased from 0.25% to 0.16% due to a change in the activity of our present source of enzyme (Collagenase Type IV, lot number W659233, 200 U/mg activity, Worthington Biochemical, Freehold, N.J.). DNase (0.02%, Sigma, St. Louis, MO) was added to decrease cell clumping. Cell viability was determined by trypan blue exclusion (Seglen, 1976).

Dispersed cells were incubated, at a concentration of 5 x 10^4 viable cells/250 µl Ham's F-10 medium, for six
hours at 37C in a 95% O₂: 5% CO₂ atmosphere, with gentle shaking. Dispersed luteal cells were incubated with various concentrations of sodium meclofenamate (Warner-Lambert, Ann Arbor, MI) and flurbiprofen (The Upjohn Co., Kalamazoo, MI) ranging from 0.01 to 100 μM. Incubations were also performed in the presence of 10 μM arachidonic acid (Cayman Chemical, Ann Arbor, MI) with or without 100 μM meclofenamate or flurbiprofen. Each treatment was applied to two to four replicates of cells.

Media collection and PGF₂α analysis

After incubation, cells were separated from medium by 1700 x g centrifugation at 4C for 10 min. Cells were discarded and media were stored at -20C prior to radioimmunoassay.

The PGF₂α radioimmunoassay was described previously for use in amniotic fluid (Haluska et al., 1987) and plasma (Chapter 4). Unextracted media samples (0.1 ml) were assayed. Intra- and interassay variabilities for a "high" media pool (2211 pg/ml) were 11% (n=4 replicates) and 7% (n=5 assays), respectively. For a "low" media pool (995 pg/ml), intra- and interassay variabilities were 15% (n=4 replicates) and 15% (n=5 assays), respectively. The slope of the standard curve was -2.152 ± 0.2 (mean ± SD). Sensitivity was 3 pg.
Studies Utilizing Intraluteal Infusions

Five monkeys received intraluteal infusions of flurbiprofen at 72 μg/10 μl distilled water/h. An equal weight of Trizma base (Tris[hydroxymethyl]aminomethane, Sigma, St. Louis, MO) was added to the infusate in order to increase the solubility of the flurbiprofen. Flurbiprofen was reported to be more potent than meclofenammatate in inhibiting prostaglandin synthesis (Rome and Lands, 1975). In the present study, the flurbiprofen dose was of equal molarity (0.3 M) to the meclofenammatate dose used for intraluteal infusions in the experiments presented in Chapter 4. Comparisons are made between monkeys receiving intraluteal infusions of flurbiprofen, phosphate-buffered-saline (PBS, n=7), and meclofenammatate (n=6). The two later groups were described in detail in Chapter 4.

Alzet osmotic pumps (Alza, Palo Alto, CA), model 2ML1, which deliver 10 μl/h continuously for seven days, were used for all infusions. Pumps were implanted in the midluteal phase of the menstrual cycle, six or seven days (6.2 ± 0.2, mean ± SEM) after the LH surge (estimated as described above). The mean day of pump implantation (6.8 ± 0.75) did not differ significantly among monkeys receiving intraluteal infusions of flurbiprofen, PBS and meclofenammatate.
Anesthesia and surgical procedures

The anesthesia and surgery were identical to that described for intraluteal infusions in Chapter 4. Briefly, a paramedian laparotomy was performed. The osmotic pump was buried in the subcutaneous space below the right ribcage, and the attached needle-catheter was tunnelled into the peritoneal cavity. A 21-gauge stainless steel needle was inserted into the ovarian ligament and through the ovarian stroma into the corpus luteum. Placement of the pump and intraluteal needle are shown in Figure 4. No vascular catheters were used in the current study. The osmotic pump was removed seven days after implantation. The ovarian needle was removed during a subsequent laparotomy, generally within three months.

Blood collection and hormone analyses

Saphenous blood samples were drawn daily between 0800 and 0830h from day eight of the menstrual cycle until menses or pump removal, whichever occurred later. Saphenous samples were allowed to clot at 4C and serum was separated from blood cells by 1700 x g centrifugation at 4C for 10 min. Serum samples were stored at -20C prior to hormone analyses.

Estradiol and progesterone concentrations in peripheral serum were measured by the Core Hormone Assay Laboratories of the Oregon Regional Primate Research
Center. Daily estradiol levels from day eight of the menstrual cycle were used to estimate the day of the LH surge in order to schedule pump placement. The estradiol (Resko et al., 1975) and progesterone (Resko et al., 1974) radioimmunoassays have been described. Intra- and interassay variabilities were as follows: Estradiol -- 8.3 and 19.8%, and progesterone -- 10.7 and 16.5%.

Statistical Analyses

The effects of meclofenamate and flurbiprofen on basal and arachidonic-acid stimulated PGF$_{2\alpha}$ production were analyzed using single factor analysis of variance with repeated measures (Winer, 1971). Dose-responses to the two inhibitors were analyzed by analysis of variance with two repeated measures (NWA Statpak, 1981). The PGF$_{2\alpha}$ data displayed heterogeneity of variance (Bartlett's ChiSquare Analysis, NWA Statpak, 1981) and were log transformed. Daily progesterone levels for five days prior to and seven days during in vivo flurbiprofen treatment were compared among groups using two way analysis of variance with repeated measures over time (Winer, 1971). Comparisons among groups of the interval between pump placement and menses, and of luteal phase length, were made using single factor analysis of variance. Post hoc comparisons were made utilizing Newman-Keuls tests (Winer,
1971). In all cases differences were considered significant if $p < 0.05$.

**Results**

**Studies Utilizing Dispersed Luteal Cells**

In vitro production of PGF$_{2\alpha}$ by dispersed luteal cells under control conditions was detected in all cases. However, the rate of production varied among monkeys, ranging from 35 to 288 pg PGF$_{2\alpha}$/ml/5 x $10^4$ cells/6h ($171 \pm 33$ pg/ml, n=5). Figure 10 depicts PGF$_{2\alpha}$ production by dispersed luteal cells from four monkeys, in the presence of increasing doses of meclofenamate and flurbiprofen. Both drugs, at the three doses tested, significantly inhibited the production of PGF$_{2\alpha}$ as compared to control incubations ($p < 0.01$). There were no significant differences between the effects of meclofenamate or flurbiprofen, or between the doses tested. Preliminary data on lower doses also suggest similar potencies of meclofenamate and flurbiprofen. When tested on luteal cells from two monkeys, 0.01 and 0.1 $\mu$M meclofenamate lowered basal PGF$_{2\alpha}$ production 30 and 64% respectively. The same doses of flurbiprofen resulted respectively in 36 and 78% reductions in PGF$_{2\alpha}$ production.

The effects of 100 $\mu$M meclofenamate and flurbiprofen on basal and arachidonic acid-stimulated
Figure 10. PGF$_{2\alpha}$ production by dispersed cells from the corpus luteum of monkeys (n=4) in response to increasing doses of flurbiprofen and meclofenamate. --- Mean square error among animals within treatment = 0.42; within dose = 0.13.
PGF$_{2\alpha}$ production by luteal cells from five monkeys are depicted in Figure 11. Meclofenamate and flurbiprofen significantly inhibited basal PGF$_{2\alpha}$ production. Addition of 10 $\mu$M arachidonic acid caused a greater than three-fold ($p < 0.01$) increase in PGF$_{2\alpha}$ production above control levels. However, co-incubation with 100 $\mu$M meclofenamate or flurbiprofen abolished the effect of arachidonic acid; PGF$_{2\alpha}$ production did not differ from control levels.

**Studies Utilizing Intraluteal Infusions**

Mean progesterone levels in monkeys receiving intraluteal infusions of flurbiprofen, beginning during the midluteal phase of the cycle, are shown in Figure 12. The monkeys displayed normal progesterone patterns prior to treatment. Although there was a decline in progesterone levels on the day following surgery, mean values returned to the normal range in four of five monkeys, but then declined, on the fifth day of treatment, to levels below the 95% confidence interval for progesterone levels at this time in the luteal phase. One monkey exhibited a markedly different progesterone pattern; levels declined on the first day following surgery to near the limit of detection, and did not recover.

Progesterone patterns prior to treatment did not differ among flurbiprofen-, PBS- (n=5, see Chapter 4, Figure 5) and meclofenamate-treated (see Chapter 4,
Figure 11: Effects of flurbiprofen (Flur), meclofenamate (Mec), arachidonic acid (AA), and arachidonic acid plus flurbiprofen or meclofenamate on the production of PGF$_{2\alpha}$ by dispersed cells from the corpus luteum of monkeys (n=5). -- Asterisks denote significant differences as compared to control (C). Mean square error among animals = 1.36.
Figure 12. Progesterone levels in monkeys receiving intraluteal infusions of flurbiprofen (Flur). For details refer to the legend of Figure 5 (Chapter 4).
Figure 6) monkeys. The decline in progesterone on the fifth day of flurbiprofen treatment was not significant; progesterone levels in flurbiprofen- and PBS-treated monkeys did not differ on any day after pump implantation. In contrast, progesterone levels during flurbiprofen treatment differed \((p < 0.01)\) from those reported during meclofenamate treatment (Chapter 4).

Table 3 summarizes the time interval between pump placement and menses and the length of the luteal phase in monkeys receiving intraluteal infusions of PBS or flurbiprofen. The data are presented separately for monkeys displaying normal and abnormal progesterone patterns (see Figure 12 and Chapter 4, Figure 5). There were no significant differences between PBS- and flurbiprofen-treated monkeys whether or not these "abnormal" monkeys were included in the analysis. In monkeys exhibiting normal progesterone patterns, menses occurred eight to ten days after pump implantation, and normal luteal lifespans of fourteen to fifteen days were observed. In contrast to flurbiprofen, intraluteal infusion of meclofenamate, as reported in Chapter 4, significantly shortened the luteal phase.
Table 3. Interval (mean days ± SEM) Between Pump Placement and Menses and Length of the Luteal Phase

<table>
<thead>
<tr>
<th>Intraluteal Infusion</th>
<th>Interval to Menses</th>
<th>Luteal Phase Length&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PBS</td>
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<tr>
<td>normal progesterone pattern (n=5)</td>
<td>10.4 ± 1.0</td>
<td>15.4 ± 1.2</td>
</tr>
<tr>
<td>abnormal progesterone (n=2)</td>
<td>4.5</td>
<td>11.5</td>
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<tr>
<td>Flurbiprofen</td>
<td></td>
<td></td>
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<tr>
<td>normal progesterone pattern (n=4)</td>
<td>8.8 ± 1.3</td>
<td>14.0 ± 1.3</td>
</tr>
<tr>
<td>abnormal progesterone (n=1)</td>
<td>4.0</td>
<td>9.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> day of LH surge to onset of menses
Discussion

In the current study, dispersed cells from the macaque corpus luteum displayed an ability to produce PGF$_{2\alpha}$ in vitro. That the accumulation of PGF$_{2\alpha}$ in the media represented synthesis as well as secretion of prostaglandin is supported by the observations that a) the levels of PGF$_{2\alpha}$ in the media increased in the presence of precursor (arachidonic acid), and b) PGF$_{2\alpha}$ levels decreased in the presence of prostaglandin synthesis inhibitors. The data confirm the work of others (Challis et al., 1976; Partwarden and Lanthier, 1985; Johnson et al., 1987) who have measured PGF$_{2\alpha}$ in media following the incubation of primate luteal tissue. The source or sources of PGF$_{2\alpha}$ within the cell suspension remain to be established. Both steroidogenic luteal cells and non-luteal cells composing the vascular system are potential producers of prostaglandins. Endothelial cells, for example, produce high levels of prostaglandins (Smith, 1986).

In any case the relationship between the in vitro prostaglandin production observed here and production by the corpus luteum in vivo is unclear, particularly because tissue trauma is a potent stimulator of prostaglandin production (Needleman et al., 1986). We have measured acute production of one prostaglandin over a six hour
period; such production could be in response to the trauma of anesthesia, lutectomy, or cell dispersion. A potential in vivo correlate was reported in Chapter 4. We were able to measure a venous/arterial gradient in PGE across the ovary at the time of surgery, but not thereafter.

Our results establish for the first time the potent actions of two prostaglandin-synthesis inhibitors, meclofenamate and flurbiprofen, on primate luteal cells. The ability of another inhibitor, indomethacin, to suppress prostaglandin production by dispersed cells from the monkey corpus luteum was reported recently (Johnson et al., 1987). We were unable to demonstrate a dose-dependent inhibition of prostaglandin synthesis, because we achieved near-maximal inhibition at the lowest doses used. Our data approximate those reported for dog and rabbit spleen and the rabbit kidney, in which meclofenamate doses from 0.1 to 1.7 μM caused a fifty percent inhibition of prostaglandin synthesis, but are markedly different from the reported I_{50} for bovine seminal vesicle, 15 μM (reviewed by Flower, 1974). Flurbiprofen and meclofenamate exhibit both competitive (reversible) and noncompetitive (time-dependent irreversible) inhibition of cyclooxygenase (Rome and Lands, 1975), the first enzyme in the pathway converting arachidonic acid to the prostaglandins. Flurbiprofen is reportedly four-fold more potent than
meclofenamate as a competitive inhibitor of cyclooxygenase, and three-fold more potent as an irreversible inhibitor (Rome and Lands, 1975). In the current study meclofenamate and flurbiprofen were essentially equipotent at the doses tested.

Despite their comparable abilities to inhibit prostaglandin synthesis in vitro, flurbiprofen did not, when infused into the corpus luteum of monkeys, mimic the luteolytic effect of an equal molar dose of meclofenamate. The effect may have been intermediate. Some suppression of progesterone levels was observed after five days of treatment; however this decline was not statistically significant. The rapid, sustained fall in progesterone levels observed in one flurbiprofen-treated animal may be a manifestation of stress; the monkey was markedly disoriented postsurgically and required sedation. The disparate effects of the two drugs may be a function of dose, although as discussed above flurbiprofen appears to be at least as potent as meclofenamate. Alternatively, our results might be due to different rates of degradation or clearance from the tissue. The half-life of meclofenamate in plasma is eight hours (Glazko et al., 1978), as compared to four hours for flurbiprofen (Adams and Buckler, 1979); however the importance of metabolic clearance in the face of continuous local infusion may be
minimal. We were unable to repeat these studies with a higher dose of flurbiprofen due to its limited solubility.

In addition to their common ability to inhibit prostaglandin synthesis, flurbiprofen and meclofenamate have unshared actions which may explain their different effects on luteal function. Flurbiprofen is a potent inhibitor of leukocyte migration (Adams and Buckler, 1979). This action requires higher doses than are needed to inhibit prostaglandin production (Adams et al., 1977; Ford-Hutchinson et al., 1977). In ewes, treatment with a luteolytic dose of PGF$_2\alpha$ increases local production of a chemoattractant for eosinophils (Murdoch, 1987). The author suggests that the immigration of leukocytes, which secrete several cytotoxic compounds, may be an important luteolytic mechanism. Thus the inhibitory action of flurbiprofen on leukocyte migration may be anti-luteolytic.

Meclofenamate has some ability to inhibit the lipoxygenase-catalyzed metabolism of arachidonic acid (Boctor et al., 1986), an action which has not been reported for flurbiprofen. Thus it is possible that meclofenamate inhibits the production of a lipoxygenase product which is obligatory for normal luteal function.

In addition, meclofenamate is able in many tissues to antagonize the actions of prostaglandins, possibly at
the site of prostaglandin receptors. This receptor-blocking ability is found only among the fenemates (reviewed by Lindner et al., 1976). Preliminary data from our laboratory suggest a direct effect of meclofenamate, but not flurbiprofen, on the prostaglandin-sensitive adenylate cyclase of the rat corpus luteum (Dr. M. Zelenski-Wooten, unpublished observations). In homogenates prepared from corpora lutea of superovulated rats, adenylate cyclase activity stimulated by PGE$_2$ was reduced by 90% in the presence of 50-100 μM meclofenamate. In contrast, flurbiprofen at doses up to 500 μM had no effect on prostaglandin-stimulated activity. The actions of meclofenamate may have been at the level of the prostaglandin receptor, since there was no effect on guanine nucleotide-stimulated activity. Thus the luteolytic effects of meclofenamate may result, at least in part, from a direct inhibition of the prostaglandin-sensitive adenylate cyclase system in the corpus luteum. However, similar studies of meclofenamate effects on prostaglandin action and the adenylate cyclase system in macaque luteal tissue (Molskness et al., 1987) have not yet been performed.

In summary, dispersed cells from the corpus luteum of rhesus monkeys produced PGF$_{2\alpha}$ in vitro, and production was enhanced by the addition of arachidonic acid. Both
basal and arachidonic acid-stimulated PGF$_{2\alpha}$ production were inhibited by flurbiprofen and meclofenamate. However, intraluteal infusion of flurbiprofen into monkeys beginning in the midluteal phase of the menstrual cycle did not mimic the luteolytic effects reported for meclofenamate. The current observations are inconsistent with the hypothesis that the luteolytic effect of meclofenamate is mediated entirely by the suppression of prostaglandin production. Further experiments are needed to elucidate the mechanism by which prostaglandin-synthesis inhibitors alter luteal function, and to determine whether locally-produced prostaglandins influence the function or lifespan of the primate corpus luteum during the menstrual cycle.
CHAPTER 6

SUMMARY

In the planning stages, these studies were simple, even elegant. In determining the physiological relevance of locally-produced prostaglandins in luteal function I took the well-worn and proven path -- ablate the source. Continuous intraluteal infusion of meclofenamate was straightforward; meclofenamate is a potent prostaglandin-synthesis inhibitor with the fortuitous "side effect" of blocking the actions of existing prostaglandins. Meclofenamate treatment resulted in a dramatic shortening of the luteal phase; thus providing the first evidence of an obligatory luteotropic role for prostaglandins in the primate. The best candidates based on reported in vitro effects were PGE$_2$, PGI$_2$, or PGD$_2$. Unfortunately it proved impossible, using our chronic catheter system, to demonstrate a suppression of ovarian prostaglandin production during treatment. Thus the mechanism of meclofenamte's luteolytic effect remained unknown. At the same time it was reported that meclofenamate also blocked lipoxygenase-catalyzed arachidonic acid metabolism. This added the leukotrienes, lipoxins, HPETE's and HETE's to the list of potential luteotropins.
In order to study the specificity of the response to meclofenamate, I compared the effects of meclofenamate and another prostaglandin-synthesis inhibitor, flurbiprofen, on luteal tissue in vitro and in vivo. Both drugs were potent inhibitors of basal and arachidonic acid-stimulated PGF$_{2\alpha}$ production by dispersed cells from the primate corpus luteum. In vivo, however, intraluteal infusion of flurbiprofen did not mimic the effects of meclofenamate. This suggests that unshared actions of the two drugs, for example, inhibition of lipoxygenase activity or antagonism of prostaglandin action, in the case of meclofenamate, or supression of leukocyte migration, in the case of flurbiprofen, may mediate their effects on luteal function.

These studies are the first rigorous attempt to identify a physiologic role for prostaglandins or other metabolites of arachidonic acid in primate luteal function. The dramatic luteolytic effect of meclofenamate suggests that metabolites of arachidonic acid are involved in regulating the functional lifespan of the corpus luteum. The next step, in the classical endocrinologist's approach, is replacement of the ablated compound. Without inhibitors of greater specificity to narrow our choices, finding the important regulators of
luteal function among the plethora of arachidonic acid metabolites is going to require luck, patience, and a bulk discount on osmotic pumps.
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