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An Attempt to Classify the Production and Regulation of Cyclooxygenase-2 During Ovulation and Luteolysis.

James Robert Broussard
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AN ATTEMPT TO CLASSIFY THE PRODUCTION AND REGULATION OF CYCLOOXYGENASE-2 DURING OVULATION AND LUTEOLYSIS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Dairy Science

by

James Robert Broussard
B.S., University of Southwestern Louisiana, 1989
M.S., Louisiana State University, 1992
August, 1996
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ABSTRACT

A series of experiments were designed to determine the cyclooxygenase (COX) isoform responsible for prostaglandin release during ovulation and luteolysis. Initially, a reliable, serum-free culture system was developed to produce large numbers of bovine granulosa cells originally harvested from the small antral follicles of abattoir ovaries. This method allowed adequate numbers of cells to be obtained for the subsequent study involving cyclooxygenase regulation.

In a second experiment, bovine granulosa cells from small antral follicles were isolated and cultured, as in the first experiment for 6 days. Granulosa cells were then exposed to medium alone or medium containing arachidonic acid (10 \( \mu \)M arachidonic acid) or forskolin (10 \( \mu \)M). Bovine granulosa cells were capable of being induced into secreting elevated concentrations of prostaglandins following exposure to forskolin, in a manner similar to preovulatory granulosa cells stimulated in vivo. Prostaglandin formation was inhibited by the addition of hydrocortisosterone to the medium, implicating the COX-2 isoform as being responsible for this prostaglandin formation. Also, data from a third experiment revealed the presence of COX-2 in the granulosa cells of women previously stimulated with FSH.

A fourth experiment was performed to determine if dexamethasone regulates COX formation in the bovine uterus during luteolysis. Plasma estradiol concentrations indicated that daily administration of dexamethasone (days 13-22) during the estrous cycle resulted in an inhibition of follicle development. Although luteal regression occurred at
the normal time in these animals, uterine biopsies on days 16, 19 and 22 of the cycle and western blot analysis revealed that COX-2 was not produced by the uterine endometrium at any of these times.
INTRODUCTION

It is astonishing to reflect upon the accuracy of early reports involving mammalian reproductive processes. It has been over 70 years since the first reports implicated the uterus in luteolysis. After determining that hysterectomizing guinea pigs resulted in an extension of the estrous cycle, Loeb (1927) concluded "it is possible that the uterus, in particular its mucosa, produces an internal secretion which exerts a specific, abbreviating effect on the life of the corpus luteum". Since this earlier report, the uterus has been shown to regulate the functional lifespan of the corpus luteum in common farm animal species including the pig (Moeljono et al., 1976), the sheep (Wiltbank, 1966) and the cow (Malven and Hansel, 1964).

Today, it is accepted by most that the luteolytic hormone in ruminants is prostaglandin F₂α. It is also accepted that luteolysis is a local process, involving the veno arterial transfer of prostaglandin from uterine vein to the ovarian artery (Hixon and Hansel, 1974). The mechanism by which uterine prostaglandin travels to the ovary has been termed the luteolytic counter-current transfer (McCracken et al., 1972). However, the manner in which prostaglandin F₂α induces luteolysis, once traversed to the corpus luteum, remains controversial (Goldberg and Ramwell, 1975; Hansel and Convey, 1983).

The scope of prostaglandin study has broadened from the initial studies on its role as a luteolysin to other reproductive processes. Although not fully understood, the mechanisms by which prostaglandins induce ovulation are currently undergoing intense
Espey (1980) hypothesized a general model for mammalian ovulation that included the accumulation of prostaglandins in the follicular fluid of preovulatory follicles. The process of ovulation was compared to that of inflammation, where prostaglandins were believed to perform many of the same actions. Some of these actions include activating quiescent fibroblasts, serine proteases and collagenase, causing an overt destruction of the follicle wall.

The enzyme responsible for the majority of prostaglandin production is the cyclooxygenase (COX) enzyme (Sigal, 1991). It is a rate limiting step in the formation of all types of prostaglandins, including prostaglandins \( \text{E}_2 \), \( \text{I}_2 \) and \( \text{F}_2\alpha \), or those familiar to scientists in the field of reproductive physiology. Recently, research has unveiled a new isoform of the COX enzyme (Rosen et al., 1989). The original isoform (COX-1) and the newly discovered isoform (COX-2), perform different biological functions that will be discussed in detail later in this review. Also, the COX-2 isozyme is inducible and is regulated differently than the original isoform first isolated from sheep vesicular glands (Miyamoto et al., 1976). The two major classifications of cyclooxygenase regulatory agents are those of nonsteroidal and steroidal origin, with each having distinct actions on either isoform.

The studies reported herein were undertaken at the St. Gabriel Louisiana State University Agricultural Research Station and at The Pennington Biomedical Center in Baton Rouge, Louisiana. The experiments were conducted 1) to determine the feasibility of producing an in vitro bovine granulosa cell serum-free culture system to
better understand COX activity in these cells, 2) to determine the effects of dexamethasone on prostaglandin formation by in vitro-cultured bovine granulosa cells and 3) to determine the origin and function COX-2 during the luteolytic process in cattle.
LITERATURE REVIEW

The discovery of a novel COX isoform raised many questions regarding its function and regulation. The COX-2 isozyme, which is capable of producing large amounts of prostaglandin upon stimulation (Dewitt et al., 1993), has become an object of interest to scientists involved in the field of reproductive physiology. Some of the characteristics of the COX-2 isozyme suggest its involvement in reproductive processes, such as ovulation and luteolysis, both of which require the rapid elevation of prostaglandin formation.

This review will be directed toward understanding prostaglandin synthesis, and the function of prostaglandins in the ovulatory process. The regulation of each COX isoform will also be discussed, with the major emphasis on steroidal regulation of the COX isozymes. Finally, the effects of glucocorticoid administration to laboratory and large animals on estrous cycle events will be reviewed.

Arachidonic Acid Metabolism and Prostaglandin Formation

In the early 1930s, it was noted that human semen and seminal vesicle extracts caused uterine tissue to contract and relax (Hadley, 1992). This active purified substance, prostaglandin, was subsequently isolated from sheep vesicular glands in the mid 1970s (Miyamoto et al., 1976). Prostaglandins belong to the ecosanoid family of chemically related substances that also includes: leukotrienes, prostacyclin and thromboxane (Toh et al., 1992). Since this initial discovery, prostaglandins have been shown to be intricately involved in a number of biological processes. Some of these

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processes include tissue remodeling (Cromwell, 1988; Zurier, 1988), vascular homeostasis (Weksler et al., 1982), immune cell function (Jonat et al., 1990) and reproduction (Hixon and Hansel, 1974; Sirois et al., 1992).

The metabolic process from which prostaglandins are derived has been termed the "arachidonic acid cascade" (Toh et al., 1992). All prostaglandins are variants of a basic 20-carbon, bilayered molecule (arachidonic acid). This 20-carbon fatty acid is made available to the organism as a component of the diet (nonruminants) or synthesized in the liver from linoleic acid (ruminants) and stored in the cells as a component of the phospholipid bilayer of the cell membrane. In order to become available to the cell for metabolism, arachidonic acid must be freed from the cell membrane by the enzyme phospholipase A₂, after which it is then converted to prostaglandins via the COX enzyme (Sigal, 1991).

Although two major routes of arachidonic acid metabolism have been defined, the COX and lipoxygenase pathways (Murdoch et al., 1993), this discussion will be concerned only with the COX pathway that involves prostaglandin formation. Figure 1 illustrates an overview of arachidonic acid metabolism. The COX enzyme performs two functions in the conversion of arachidonic acid to prostaglandin (Sigal, 1991). Once arachidonic acid is liberated from the cell membrane, COX initially performs an oxidative function by introducing two molecules of oxygen to form Prostaglandin G₂. Prostaglandin G₂ then undergoes a reduction, or peroxidase reaction, to form prostaglandin H₃.
Membrane Phospholipids

\[ \xrightarrow{\text{Phospholipase A}_2} \]

\[ \xrightarrow{\text{COX}} \]

\[ \xrightarrow{\text{Oxidation}} \]

\[ \xrightarrow{\text{5-Lipoxygenase}} \]

\[ \xrightarrow{\text{5-HPETE}} \]

\[ \xrightarrow{\text{Leukotriene A}_4} \]

\[ \xrightarrow{\text{Leukotriene B}_4} \]

\[ \text{Prostaglandin E}_2 \]

\[ \text{Prostaglandin F}_2 \]

\[ \text{Prostaglandin I}_2 \]

\[ \text{Prostaglandin G}_2 \]

\[ \text{Prostaglandin H}_2 \]

Figure 1. A schematic drawing of arachidonic acid metabolism to prostaglandin (adapted from Sigal, 1991)
From this point, prostaglandin H\(_2\) is further reduced to other metabolites that are more familiar in the study of reproductive physiology, including prostaglandin E\(_2\), I\(_2\) and F\(_2\alpha\) (Sigal, 1991).

Recent Discoveries in COX Metabolism

Until the late 1980s, it was assumed that there was only one gene encoding the COX enzyme (DeWitt and Smith, 1988). The COX enzyme was first isolated from sheep vesicular glands, and has been the focus of many of the prostaglandin regulatory studies conducted during the past 15 years (Miyamoto et al., 1976; Hedin et al., 1987). This original isoform, termed COX-1 in the scientific literature, is translated from a 2.8 kb mRNA transcript and has a mw of 69,000 and 70,000 in the mouse (Hedin et al., 1987) and bovine species (Sirois, 1994), respectively. Also, the primary structure of the COX-1 enzyme (8,11,14-icosatrienoate, hydrogen-donor:oxygen oxidoreductase, EC 1.14.99.1) has been reported (DeWitt and Smith, 1988). However, recent studies have established the presence of another pharmacologically distinct, but related, isoform of the original COX enzyme (Kujubu et al., 1987; Rosen et al., 1989).

Rosen et al. (1989) described the transcription of a novel cyclooxygenase isoform by in vitro-cultured sheep tracheal mucosal cells. A 70,000 mw cyclooxygenase protein and corresponding 2.8 kb mRNA were coordinately expressed, but the levels did not increase proportionately to the increase in COX activity. However, rehybridization of Northern blots revealed the presence of a new tissue-specific 4.0 kb mRNA that exhibited increased expression during in vitro culture. The authors
suggested that the novel protein's relatedness to COX and its increased parallel enzymatic activity further suggest that the larger mRNA may encode for a second COX protein.

Recent reports have provided direct evidence of the existence of a distinct COX isoform that is derived from a unique mRNA transcript (Kujubu et al., 1991). It has been noted that the gene encoding the COX-2 isoform is the TIS10 primary response gene (Kujubu et al., 1991), which has been cloned (O'Bannion et al., 1992). This gene was the first primary response gene for which cell-type restricted expression has been reported (Kujubu et al., 1987). Cyclooxygenase-2 mRNA production was initially reported to occur following forskolin stimulation of Swiss 3T3 cells that were previously transfected with the TIS10 gene (Kujubu et al., 1991). This second COX isoform is translated from a 4.0 kb mRNA and has a molecular weight of 72,000 to 74,000 (Sirois and Richards, 1992).

Although pharmacologically distinct, the COX-1 and -2 isoforms have a homology of greater than 60% to one another (Kujubu et al., 1991). More importantly, the amino acid residues believed to be important for catalysis are identical, and each isoform has the same affinity for and capacity to convert arachidonic acid to prostaglandin \( H_2 \). One major difference between the two isozymes appears to be their dissimilar expression.

The proposed biological roles of each COX isoform have been compiled (see review by DeWitt et al., 1993) and are presented in Table 1. The COX-1 isoform is
a constitutively expressed enzyme that is present in most mammalian cells, although the level of expression does vary between cell types (Kujubu et al., 1991). In contrast, COX-2 is undetectable in most tissues under normal physiological conditions, but its expression can dramatically increase during periods of inflammation or mitogenic stimulation (Kujubu et al., 1993). Many mammalian cell types can be stimulated to produce the COX-2 isozyme by a variety of compounds including serum (Mitchell and Trautman, 1993), lippopolysaccharide (Lee et al., 1992), human chorionic gonadotropin (hCG; Sirois and Richards, 1992; Sirois et al., 1992; Sirois, 1994), platelet-derived growth factor (O'Bannion et al., 1992) and forskolin and phorbol esters (Kujubu et al., 1991).

Another notable difference in the characterization of the two COX isoforms is their reaction kinetics. Because the COX-1 isoform is constitutively expressed in most tissues, it has been termed a "housekeeping gene product" that regulates normal cellular processes, such as kidney function and vascular homeostasis (Jonat et al., 1990). Conversely, COX-2 has been grouped in the immediate early gene family, by virtue of its ability to rapidly produce high concentrations of prostaglandins during situations such as inflammation (Ryseck et al., 1992) and ovulation (Sirois and Richards, 1992; Sirois, 1994). This characteristic differentiates the COX-2 isoform from the original COX-1 cyclooxygenase enzyme. The COX-2 isoform has been determined to increase prostaglandin formation as much as 10- to 80-fold following stimulation (Simonson et al., 1991).
Table 1. A compilation of characteristics assigned to the COX-1 and -2 isoymes including: homology, expression, range of expression, tissue expression regulation and proposed biological activity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>COX-1</th>
<th>COX-2</th>
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<tr>
<td>Homology</td>
<td>Similar to COX-2 (&gt;60%)</td>
<td>Similar to COX-1 (&gt;60%)</td>
</tr>
<tr>
<td>Expression</td>
<td>Constitutive</td>
<td>Inducible</td>
</tr>
<tr>
<td>Range of expression</td>
<td>Can increase 2 to 4 fold following stimulation</td>
<td>Can increase 10 to 80 fold following stimulation</td>
</tr>
<tr>
<td>Major tissues expressing each isoform</td>
<td>Platelets, endothelial cells, fibroblasts, stomach, kidneys, smooth muscle most tissues</td>
<td>Prostate, brain, activated monocytes or synoviotes during inflammation, follicles preceding ovulation</td>
</tr>
<tr>
<td>Effect of glucocorticoids</td>
<td>Little to none</td>
<td>Inhibits expression</td>
</tr>
<tr>
<td>Proposed role of enzyme</td>
<td>&quot;Housekeeping&quot; gene; produces prostaglandins that regulate normal kidney and gastric function and vascular homeostasis</td>
<td>&quot;Inflammatory response gene&quot;; produces prostaglandins involved in inflammation &quot;Immediate early gene&quot;; controls mitogenesis, produces prostaglandins involved in cell growth and ovulation</td>
</tr>
</tbody>
</table>

(adapted from DeWitt et al., 1993)
The rapid generation and degradation of the COX-2 isozyme within a tissue makes it a logical candidate for being the paracrine factor that allows adjacent cells to relay information to each other (Murdoch et al., 1993). The rapid activation of genes whose products (COX-1 and -2) are responsible for prostaglandin synthesis in endotoxin-stimulated macrophages (Lee et al., 1992), may represent an important mechanism by which stimulated cells signal their neighbors of their impending division. This mechanism lends credence to the hypothesis that prostaglandin accumulation in preovulatory follicles is responsible for the cascade of events that occur following the rapid activation of quiescent fibroblasts. This process ultimately culminates in ovulation, and release of the oocyte from the dominant follicle into the oviduct for fertilization (Espey, 1980).

In summary, the COX enzyme plays an essential role in the conversion of arachidonic acid to prostaglandin in the process known as the "arachidonic acid cascade". This enzyme is essential to prostaglandin formation, and performs rate limiting actions in the conversion of arachidonic acid to prostaglandin G₂ and H₂. In turn, these prostaglandins are converted to other isoforms. It has been determined that two isoforms of the COX enzyme exist (Kujubu et al., 1991). Both isoforms are produced by a variety of tissues in the body; however, each isoform performs different biological functions. With the general function of each isoform fairly well defined, understanding the regulatory mechanisms of each isoform has now become the focus of research efforts.
Induction of COX Activity During Mammalian Ovulation

Until now, this review has focused on COX-2 formation during inflammation. Recent studies have implicated prostaglandins in mammalian ovulation, which has previously been compared with an inflammatory response (Espey, 1980). This hypothesis has recently been updated (Espey, 1994). This basic model for ovulation in mammals is summarized in Figure 2. Briefly, prostaglandins are responsible for activating quiescent fibroblasts, which then activate serine proteases. Collagenase is then believed to be responsible for the degradation of the collagen fibres in the preovulatory follicle wall, resulting in ovulation. This process was hypothesized to be triggered by the preovulatory surge in LH.

The LH surge has since been shown to induce COX activity in rat preovulatory follicles (Hedin et al., 1987). A 72,000 mw COX protein was induced by mural granulosa cells following human chorionic gonadotropin (hCG) administration. The protein was produced by mural granulosa cells of preovulatory follicles 5 to 7 hours following exogenous administration of 10 IU hCG. Its production was shown to continue in increasing amounts until the time of expected ovulation. However, rat corpora lutea isolated 24 hours following hCG administration did not contain this novel 72,000 mw protein. Although the molecular weight of this COX enzyme was greater than that noted to be the case of the original rat isoform (72,000 vs. 70,000 mw, respectively), no mention of the possibility of this being a novel isoform was made in the latter study (Hedin et al., 1987).
Figure 2. A schematic presentation summarizing prostaglandin activity during mammalian ovulation (adapted from Espey, 1980).
Building on these data, an in vivo and in vitro study performed by Sirois et al. (1992) described the presence of the novel COX mRNA in rat preovulatory follicles. For the in vivo portion of the study, hypophysectomized, immature female rats were administered estradiol-17β (1.5 mg/day for 3 days) and FSH (1 µg for 2 days). Follicles were isolated from donor ovaries each hour for 11 hours following administration of an ovulatory dose of hCG. A similar experiment was performed in vitro, with donor follicles collected immediately following hCG administration, which were later evaluated for COX-2 mRNA and protein production during the same time frame as the in vivo portion of the study.

Results from the Northern blot analysis indicated that the COX-2 isoform is induced by hCG. Production of the 4.4 kb mRNA that translates the COX-2 isoform was noted to be rapid and transient, with concentrations increasing from an undetectable level at 0 hour and reaching maximal concentrations 4 hours post hCG stimulation. Levels of COX-2 mRNA were again undetectable at the 6 through 11 hour sampling periods. Western blot analysis, using an antibody which recognizes both isoforms, demonstrated that the actual COX-2 protein was translated 4 hours following in vivo administration of hCG and was continually produced throughout the sampling period. A similar trend in COX-2 mRNA and protein production was noted in the in vitro study (Sirois et al., 1992).

A study performed by the same authors was designed to determine the follicle cell type responsible for producing the COX-2 isoform (Sirois and Richards, 1992).
Follicles were harvested from hypophysectomized rats that were estrogen primed as previously described (Sirois et al., 1992). The follicles were then dissected to isolate the granulosa cell layer from the rest of the follicle tissue. Western blot analysis indicated that the COX-2 isoform was selectively induced in granulosa cells, while being absent in residual follicle tissue, which included the membrane granulosa and theca cells. Also, a 59,000 mw gel band was reported, and assumed to be a breakdown product of the COX-2 isoform.

The most recently published study pertaining to COX-2 production in preovulatory follicles was performed in cattle (Sirois, 1994). Holstein heifers were administered 25 mg of prostaglandin F$_2$α on day 7 of the estrous cycle to induce corpus luteum regression. An ovulatory dose of hCG (3,000 IU) was administered 36 hours following the prostaglandin injection. Ovaries were collected at 6 hour intervals for 18 hours, then every 2 hours until 26 hours post-hCG injection. The cells of whole preovulatory follicles produced COX-2 beginning at 20 hours post-hCG. Following isolation of granulosa cells and theca interna cells from the follicle, it was found that COX-2 was exclusively produced in granulosa cells. No detectable levels of COX-2 were produced by the theca interna cells. Also, increasing concentrations of prostaglandin F$_2$α were released into the follicular fluid 22 through 26 hours following hCG administration.

The purification and identification of COX-2 in hCG induced rat and bovine follicles represents an important step toward understanding the role of prostaglandins.
in the biological process of ovulation. The selective localization of COX-2 in the granulosa cells of preovulatory follicles and its transient expression following the LH/hCG surge, indicate that the regulation of COX-2 is under stringent transcriptional control. Moreover, the acute induction of COX-2 by gonadotropins in granulosa cells before ovulation is a unique biological event, which may allow molecular mechanisms of COX-2 to be studied in vivo and in vitro.

**Differential Regulation of COX Metabolism**

The understanding of COX regulatory mechanisms has improved since the discovery of the novel COX isoform. It is now known that certain compounds are capable of specifically inhibiting catalysis of arachidonic acid by either or both isoforms. These regulatory agents have been subdivided into two major groups including nonsteroidal and steroidal inhibitors of COX formation (Dewitt et al., 1993). A variety of nonsteroidal anti-inflammatory agents have been researched, while the major steroidal agent investigated to date has been cortisol and related synthetics (Mitchell and Trautman, 1993).

**Nonsteroidal Regulation**

The oxidative function of COX is the target site of aspirin and other nonsteroidal anti-inflammatory drugs (NSAID), which are considered to be competitive inhibitors of COX-1 (Vane, 1971). DeWitt and Smith (1988) have demonstrated that aspirin inhibits the oxidative function of COX via irreversible acetylation of the "active site" serine residue at position 530. Mizuno et al. (1982) compared the effects of
various anti-inflammatory drugs on the oxidative and peroxidase activity of the COX enzyme. It was determined that several NSAID including: aspirin, indomethacin, flurbiprofen and sulindac sulfide inhibited the oxidative activity of COX. However, other nonsteroidal agents, such as phenylbutazone and BW755c have been shown to inhibit the peroxidase activity of COX.

Meade et al. (1993) designed an experiment to determine if NSAID inhibit COX-2 production, and to determine if these agents possess any selectivity towards one specific isozyme. Cos-1 cells were transfected with cDNA for both COX-1 and-2 and incubated in medium with or without indomethacin or aspirin. Results of this study indicated that both aspirin and indomethacin specifically inhibited COX-1 activity, without affecting the production of COX-2.

Further investigation into NSAID has revealed preferential selectivity between the COX-1 and -2 isozymes (Meade, 1992). As earlier reported (Meade et al., 1993), sulindac sulfide specifically inhibited COX-1 production by Cos-1 transfected cells. However, the NSAID 6-methoxy-2-naphthylacetic acid, selectively inhibited COX-2 production. Another NSAID ibuprofen was found to inhibit both isoforms equally.

In summary, NSAID are capable of selectively inhibiting either or both of the COX isoforms. In the future, it is possible that in vivo administration of NSAID that are capable of selectively inhibiting COX-2 production, may be found to spare the "housekeeping" activity of somatic cells, while reducing inflammation. Since COX-2 is the isozyme responsible for prostaglandin formation in the preovulatory follicle, it
is also interesting to speculate the possible role these NSAID could play as a method of birth control.

Steroidal Regulation

Since initial reports focused on the original COX isoform, hormonal regulatory studies involving glucocorticoids were often contradictory. Dexamethasone (9-flouro-11, 17, 21-trihydroxy-16 -methylpregna-1, 4-diene-3, 20-dione), is a synthetic corticosteroid that has been utilized in many COX regulatory studies, provides very potent glucocorticoid effects in vivo and in vitro. Dexamethasone is similar in structure to endogenously produced glucocorticoids and, in fact, is produced by modification of cortisol (Asdell, 1978).

Prior to the characterization of the COX-2 isoform, in vitro studies indicated that dexamethasone restricted COX formation by inhibiting phospholipase A₂, resulting in a decrease in amount of available arachidonic acid (DiRosa et al., 1984). This idea was supported by Nasjletti et al. (1984) who noted a decrease in prostaglandin secretion by rat brain medullary slices that were cultured in medium supplemented with dexamethasone. In contrast, when arachidonic acid was added to dexamethasone-supplemented culture medium, prostaglandin secretion was not decreased. These results led the authors to believe that dexamethasone had decreased prostaglandin formation by inhibiting phospholipase A₂ activity, and subsequent arachidonic acid release from the cell membrane. However, later studies have challenged the idea that dexamethasone
inhibits prostaglandin formation at the phospholipase A₂ level (Davidson et al., 1987; Bronnegard et al., 1988).

Although prostaglandins and corticosteroids are known to be active participants in the inflammatory response, in vivo studies indicated that dexamethasone had no in vivo inhibitory effects on systemic prostaglandin synthesis (Lewis and Piper, 1975; Nara-Fejes-Toth et al., 1984). Nasjletti et al. (1984) designed an in vivo experiment to determine the effects of dexamethasone on urinary excretion and plasma concentrations of prostaglandins. It was noted that rats administered subcutaneous injections of dexamethasone did not have reduced concentrations of prostaglandins in either plasma or urinary excretions. Some experimental animals actually excreted higher concentrations of prostaglandins in plasma when compared with saline-treated controls; however, this phenomenon was attributed to a decrease in metabolism rather than a physiological effect on prostaglandin metabolism.

Nara-Fejes-Toth et al. (1984) reported total body and renal prostaglandin content following dexamethasone administration by measuring excretory prostaglandin metabolites. Under normal physiological restraints, rabbits were fed either water alone or water containing dexamethasone (1 mg/kg per day) for 7 days, and the urinary excretions were collected. The administration of dexamethasone failed to cause a decrease in urinary prostaglandin metabolite production when compared with animals in the control group. The authors hypothesized this lack in response may be due to the
fact that the dose of dexamethasone administered was not high enough to provide a physiological affect.

Interestingly, the authors did note in their discussion that the majority of studies that were performed to this date that implicated dexamethasone in prostaglandin inhibition all utilized cells that were stimulated by endotoxins (Blackwell et al., 1980; Hirata et al., 1980) or injury (Danon and Assouline, 1978). The hypothesis that dexamethasone regulates prostaglandin production only in stimulated cells launched a series of experiments involving COX regulation by dexamethasone during the inflammatory process.

In retrospect, Masferrer et al. (1990) may have unknowingly published one of the first reports in the scientific literature involving dexamethasone inhibition of COX-2 catalysis. Male Balb-C mice were divided into one of four groups receiving intravenous saline, endotoxin (lipopolysacharride, LPS), endotoxin plus dexamethasone or dexamethasone alone. Peritoneal macrophages were harvested from the mice and allowed to attach to a 24-well tissue culture plate. It was noted that macrophages harvested from mice treated with LPS secreted increased levels of prostaglandin E₂ and the prostaglandin I₂ metabolite (6-keto PGF₁α) when compared with control animals that received only saline. When LPS and dexamethasone were injected simultaneously, prostaglandin secretion decreased to control levels. Dexamethasone administration alone did not affect prostaglandin secretion above or below that of the control concentrations.
Still with the knowledge of the existence of only one COX isoform, Masferrer et al. (1992) performed a classic endocrine study in an attempt to determine the mechanics of COX regulation by dexamethasone. In order to remove any endogenous glucocorticoid effect, peritoneal macrophages of adrenalectomized (ADX) mice were cultured for 2 hours following in vivo administration of saline, LPS, dexamethasone or LPS plus dexamethasone. Prostaglandin production (6-keto-PGF1α and PGE2) by the macrophages of ADX mice increased when LPS was administered. As previously described by Masferrer et al. (1990), in intact mice dexamethasone decreased prostaglandin formation to control levels. Prostaglandin secretion was also decreased to control levels following in vitro culture of macrophages simultaneously stimulated with LPS and dexamethasone.

A similar trend was noted in the production of a protein (COX) with an approximate molecular weight of 70,000 to 72,000 by LPS-stimulated mouse macrophages (Masferrer et al., 1990, 1992). Western blot analysis of macrophages harvested from LPS stimulated intact (Masferrer et al., 1990) or ADX (Masferrer et al., 1992) mice indicated the production of COX to be greater than from macrophages retrieved from control (unstimulated) mice. As with prostaglandin production itself, dexamethasone decreased this LPS stimulation of COX to levels similar to macrophages harvested from control mice. Since the initial report by Masferrer et al. (1990), dexamethasone has been shown to inhibit COX activity in other biological tissues including human dermal cells (Seibert et al., 1990), bronchial alveolar macrophages.
(O'Sullivan et al., 1993), bovine placenta (Izhar et al., 1992) and mouse uterine stroma (Jacobs et al., 1994).

Although the COX-2 isoform was not specifically characterized as such in the studies reviewed so far, it may now be extrapolated from existing data (Masferrer et al., 1990, 1992) that the decrease in prostaglandin secretion by stimulated mouse peritoneal macrophages may have actually been a result of the inhibition of COX-2 catalysis. This conclusion may be reached on the basis of the differing kinetics of the two isoforms. It is now realized that the COX-2 isoform is activated only upon stimulation, and is able to produce levels of prostaglandins 10 to 80 times greater than the COX-1 isoform. Also, these studies indicated that the molecular weight of the COX isozyme noted in stimulated cells of all species evaluated to date is larger than the original COX-1 isozyme, which is characteristic of the COX-2 isozyme (Sirois et al., 1992).

It was not until the early 1990s that the dispute over COX regulation by dexamethasone was resolved. With identification of the amino acid sequence of COX-2 (Kujubu et al., 1991), Lee et al., 1992 prepared antibody specific to COX-2 by injecting rabbits with a conjugated amino acid sequence specific to the mouse COX-2 carboxyl terminus. A second polyclonal antibody was prepared against purified ram seminal vesicle cyclooxygenase. This polyclonal antibody recognized both the COX-1 and COX-2 isoforms, but possessed a preferential affinity for COX-1.
The antibody specific for COX-2 did not detect COX in ram seminal vesicles extracts, indicating the majority of COX present was COX-1 (Lee et al., 1992). An in vitro comparison of COX-1 and COX-2 by western blot analysis revealed that COX-2 was present only in rat macrophages previously stimulated with LPS (10 µg/ml). This COX-2 expression was specifically inhibited when dexamethasone (2 µM) was introduced into the incubation medium. These results indicate that endogenous COX present in cells prior to LPS stimulation consists mainly of COX-1. Furthermore, enhanced activity of COX and the subsequent increase in prostaglandin secretion induced by LPS was due mainly to the selective expression of COX-2 and not due to increased COX-1 catalysis.

In the same study (Lee et al., 1992), a time course for COX-1 and -2 mRNA transcription was also determined following LPS stimulation. It was noted that the majority of mRNA contained in unstimulated rat macrophages was COX-1. However, when macrophages were stimulated with LPS, the majority of mRNA transcribed was that of COX-2. It was also noted that the kinetics of the COX-2 mRNA was fairly rapid, with maximal production occurring 6 hours following LPS stimulation. This high production of COX-2 mRNA was noted to continue for 24 hours, or the remainder of the time frame analyzed in this study. These data support those of Kujubu and Herschman (1992), who reported that dexamethasone directly inhibits TIS10 mRNA accumulation in transfected Swiss 3T3 cells.
These results were further investigated to determine dexamethasone regulatory actions on interleukin-1-induced COX production in vitro (Feng et al., 1993). In this study, antibodies utilized for western blot analysis were produced in a manner similar to that reported by Lee et al. (1992), and their specificity determined using cos-7 cells previously transfected with recombinant rat DNA that transcribed both COX-1 and COX-2. Western blot analysis indicated that COX-2 is induced by interleukin-1 in vascular smooth muscle cells; whereas, COX-1 production is not affected. Also, dexamethasone was noted to inhibit the transcription of COX-2 mRNA and the translation of COX-2 itself. Dexamethasone had no effect on COX-1 translation.

Taken together, the studies reported herein and those of others (Raz et al., 1989; Jacobs et al., 1994), indicate that COX metabolism and regulation is dependant upon the level of stress imposed on the animal system being evaluated. Stress, either natural or induced, appears to encourage the production of the COX-2 isozyme. This isozyme is rapidly induced upon stimulation, and is capable of increasing prostaglandin production anywhere from 10 to 80 fold (Simonson et al., 1991). It is these characteristics that have intrigued scientists in reproductive physiology. Since prostaglandins have been implicated in reproductive processes, such as ovulation and luteolysis, investigations into the role of COX-2 in these processes have been initiated.

Effect of Dexamethasone Administration on Reproductive Cyclic Events

The discovery of a novel, inducible isoform of the cyclooxygenase enzyme has increased the interest in reproductive processes of large and small animals that require the
presence of prostaglandin. However, this isoform is stimulated in several biological tissues by a variety of compounds, including serum constituents (Mitchell and Trautman, 1993), lippopolysaccharide (Lee et al., 1992), hCG (Sirois and Richards, 1992; Sirois et al., 1992; Sirois, 1994), platelet derived growth factor (O'Bannion et al., 1992) and forskolin and phorbol esters (Kujubu et al., 1991). Once stimulated, the COX-2 isoform is capable of producing prostaglandins at a rate that is 80 times greater than COX-1 (Dewitt et al., 1993)

The majority of research involving COX catalysis in reproduction has focused on prostaglandin formation during the ovulatory process. It has been determined that the COX-2 isoform is responsible for the majority of prostaglandin production by rat (Sirois and Richards, 1992; Sirois et al., 1992) and bovine (Sirois, 1994) granulosa cells immediately prior to ovulation. For each of these species, it was noted that COX-2 is stimulated by the preovulatory LH surge. Time course analyses performed in these studies have indicated that the production of COX-2 mRNA and protein are necessary for prostaglandin synthesis in preovulatory follicles. Differences in COX-2 kinetics may be responsible for the disimilar intervals from LH peak to ovulation observed among species.

Early studies have provided evidence that the luteolytic hormone, prostaglandin F$_2$α, is responsible for luteolytic process in ruminants. Uterine prostaglandin secretion has been theorized to be involved in the luteolytic process of sheep (McCracken et al., 1972) and cattle (Hixon and Hansel, 1974). McCracken et al. (1971) demonstrated that prostaglandin F$_2$α of uterine origin is transferred from the uterine vein to the ovarian
artery, where it was then believed to be responsible for corpus luteum regression. This model of luteal regression has been termed the counter current mechanism in the scientific literature. These data were supported by Hixon and Hansel (1974) who reported that sectioning the broad ligament ipsilateral to the corpus luteum resulted in an extension of the bovine estrous cycle. The fact that exogenous prostaglandin F$_2$α administration to cows in the luteal phase of the estrous cycle causes luteal regression also supports the idea that prostaglandins are necessary for luteolysis in ruminants (see review by Hansel et al., 1973).

These findings lead one to consider that luteolysis in ruminants is a local mechanism involving the uterus and ipsilateral corpus luteum. However, additional observations have resulted in contradictory results. Milvae and Hansel (1980) reported that there is no significant increase in ovarian prostaglandin F$_2$α concentration during periods when concentrations are high in the uterine vein. Questions also arise due to the fact that luteal cells themselves are capable of secreting prostaglandins during in vitro culture, indicating that luteal regression may not require prostaglandin F$_2$α of uterine origin (Shemesh and Hansel, 1975; Milvae and Hansel, 1983). These findings suggest that arachidonic acid may be transferred from the uterus in a counter current exchange rather than prostaglandin F$_2$α.

Various corticosteroids have been shown to adversely affect reproductive performance in farm animals. Cummings et al. (1980) reported that cattle injected daily with dexamethasone (30 mg) from the day of estrus (day 0) through day 2 of the
estrous cycle had no effect on ovulation of estrous cycle length. This phenomenon has also been reported in cyclic mares (Ellsworth et al., 1983). However, when dexamethasone (30 mg) was administered during the luteal phase of the estrous cycle (days 13 through 17), the length of the estrous cycle was extended in this study (Abdalla et al., 1980). Moreover, although estrous cycle length was extended, fertility of the subsequent estrous cycles were not impaired.

Liptrap and McNally (1976) reported the effects of hydrocortisone on the ovulatory mechanism of Holstein heifers. The experimental animals were administered hydrocortisone (1,000 mg/day from days 16 to day 4 of the subsequent estrous cycle) exhibited delayed ovulation (by 2 days) when compared with saline injected animals. Ovulation was completely inhibited when ACTH was administered during the same time period. This decrease in ovulation rate appeared to be caused by an abatement of LH secretion, since an ovulatory injection of hCG (10,000 IU) restored ovulation rates to those observed in control animals.

The adverse effects of stress on bovine reproduction have been directly linked to an inhibition of gonadotropin release from the pituitary. Physical restraint for 15 minutes, twice daily during the follicular phase in cattle prevented the preovulatory surge in LH (Stoebel and Moberg, 1982b). No delay in the onset of estrus was observed when ewes were restrained following estradiol administration; however, when animals were primed with estradiol during restraint, the LH surge was delayed 3 hours. Confinement induced stress has also been determined to decrease the
amplitude and frequency of LH secretion in ewes (Rasmussen and Malven, 1983) and monkeys (Norman and Smith, 1992).

The administration of exogenous cortisol has also been shown to affect pituitary release of LH. The administration of cortisol to preovulatory staged Holstein heifers resulted in a prevention of the LH surge in three of four animals (Stoebel and Moberg, 1982a). Similarly, the amplitude and frequency of LH secretion was decreased in postpartum suckled and nonsuckled beef cows (Dunlap et al., 1981).

Hydrocortisone and dexamethasone have also been demonstrated to affect the developing corpus luteum. Hereford heifers injected with ACTH (100 IU/day) on days 2 through 8 of the estrous cycle had lower weight corpora lutea at slaughter (day 9) than did animals administered the vehicle control treatment (Wagner et al., 1972). In a subsequent study, Wagner et al. (1977) demonstrated that ovarian perfusion of ACTH or hydrocortisone did not affect luteal function. However, carotid infusion on days 2 to 9 of the estrous cycle resulted in a prolonged rise in progesterone production during the infusion period. No differences were noted in plasma progesterone concentrations on days 9 to 11 between the treated and control animals.

More recent studies have suggested that the lifespan of the bovine corpus luteum is extended following ACTH or dexamethasone administration. Pool et al. (1983) allotted crossbred beef cows to either free pasture or pen confinement and subdivided these groups into those receiving ACTH (200 IU/day) or vehicle on days 17 through 21 of the estrous cycle. Animals confined to pens exhibited an extended estrous cycle
length of approximately 2 days when compared with their pasture-housed counterparts. This extension of the estrous cycle was attributed to increased corpus luteum function as determined by plasma progesterone. When the data from pen and pasture housed animals were combined, the interval from progesterone decline to subsequent estrus was extended an average of 5.5 days. It appears that physical stress and induced stress (i.e., ACTH injections) have different physiological effects: physical stress causing increased corpus luteum lifespan and ACTH stress causing abnormal follicle development.

The same research group reported the effects of dexamethasone administration on reproductive performance in cattle (Ingraham et al., 1984). Animals were again assigned to pen or pasture confinement and dexamethasone was administered to cows allotted to each group on day 16 of the estrous cycle (20 mg/day) and days 17 through 20 (30 mg/day). Interestingly, plasma progesterone and rectal palpation indicated that half (8 of 16) of the animals receiving dexamethasone had prolonged luteal function. Plasma progesterone profiles of individual animals indicated that the decline in progesterone concentration to below 1 ng/ml occurred later in some dexamethasone treated animals when compared with the saline injected controls. Results from the endocrine profiles were substantiated by rectal palpation of functional corpora lutea.

Vighio and Liptrap (1990) observed some of the same trends of luteal extension following dexamethasone (2 mg) administration to cattle on days 13 through 17 of the bovine estrous cycle. Mean progesterone secretion above 1 ng/ml was extended an average of 10 days. It was also reported by the authors that dexamethasone lessened
both estrogen and LH secretion. The estrogen surge was abated by dexamethasone, and the pulsatile release of LH was decreased in amplitude and frequency. These results suggest that dexamethasone may decrease estrogen secretion; thereby, decreasing estrogens biphasic effect on gonadotropin release from the pituitary. This decrease may be adequate to slow follicle development, but maintain corpus luteum function.

Cortisol has also been shown to act directly on bovine granulosa cells of small follicles (Kawate et al., 1993). Granulosa cells cultured in vitro in medium alone secreted significantly more estradiol than those cultured in medium containing cortisol (10 μM). Similarly, granulosa cells cultured in medium alone possessed a higher number of LH receptors on the cell membrane than did those cultured in medium containing cortisol (.1 or 10 μM). These data suggest that cortisol suppresses estradiol production by bovine granulosa cells by decreasing the number of LH receptors available for recognizing circulating LH.

A further study suggests that dexamethasone has direct effects on the brain (Pool, 1980). The administration of dexamethasone to cows on days 2 through 5 of the estrous cycle (20 mg) resulted in a lower number (25%) exhibiting standing estrus following exogenous estrogen administration. However, 100% of the control animals that were administered physiological saline exhibited standing estrus. This is in contrast to a standing estrus response noted in ovariectomized dairy heifers injected with estrogen (Melampy et al., 1957), which suggests dexamethasone can also act directly on the central nervous system by altering estrogen sensitivity.
Summary

It is now apparent that prostaglandins play a vital role in many biological processes. The literature pertaining to prostaglandin function in reproductive processes has continued to expand in recent years with the discovery of the novel, inducible form of COX. However, the role of prostaglandins in reproductive processes requires a separation of their possible physiological actions from their pharmacological effects. Although pharmacological effects of prostaglandins are clinically significant, they may not reflect their true involvement in normal physiological processes.

It appears that stress, either natural or induced, has deleterious effects on reproduction. Its adverse effects on mammalian ovulation may be considered to be two-fold. Gonadotropin release is inhibited by chronic stress induction, resulting in abnormal or slowed follicle development. However, acute stress that occurs just prior to or during estrus may inhibit ovulation by suppressing prostaglandin formation in the preovulatory follicle. This mechanism of suppression may be related to cyclooxygenase activity, more specifically, COX-2 activity (Sirois, 1994).

One of the most notable observations as a result of dexamethasone administration is prolongation of the duration of the estrous cycle due to extended luteal function (Abdalla et al., 1980; Ingraham et al., 1984; Vighio and Liptrap, 1990).

It is not clear why only some animals exhibit extended luteal function following dexamethasone administration; however, it has been suggested that animals undergoing normal luteal regression, do so because the luteolytic mechanism was initiated prior to
Dexamethasone administration (Ingraham et al., 1984; Vighio and Liptrap, 1990).
Dexamethasone may suppress luteolysis in those cows having extended luteal function by inhibiting cyclooxygenase activity in the uterus (Ingraham et al., 1984; Vighio and Liptrap, 1990), thereby disrupting the counter current exchange of prostaglandin F₂α from the uterine vein to the ovary artery (Hixon and Hansel, 1974).

To date, the use of live animals for determining the effects of certain drugs remains the optimal method of obtaining true-to-fact biological conclusions. However, the use of current in vitro culture techniques may also be employed to gain further understanding of molecular events that occur during the microprocesses of reproduction. Tissue culture techniques allow researchers to isolate specific cell types and determine their function in processes such as ovulation. However, the medium used in these culture systems should be defined, and should not contain biological serum as a protein source. This is especially true, since serum alone has been shown to induce P450 side chain cleavage enzymatic activity in bovine granulosa cells (Skinner and Osteen, 1988) and bovine granulosa-luteal cells (Meidan et al., 1990). Similarly, serum constituents have been reported to induce prostaglandin formation by activation of the COX enzyme (Lee et al., 1992; Feng et al., 1993), thereby masking the effects of any added regulatory agents.

Therefore, it is of the utmost importance to produce a somatic cell culture system that would allow for cultivation of small numbers of granulosa cells, without stimulating catalysis of the enzyme being studied. This type of in vitro culture system
would aid researchers working with small amounts of tissue by allowing for adequate numbers of cells to be produced for proper enzymatic studies to be performed. Also, a more defined culture system would preferably provide a more repeatable method of cultivation of different cell types, thereby allowing more consistent results to be obtained over time.

Based on the current knowledge from the scientific literature, a hypothesis was formulated. A hypothesis that involved the COX-2 isofom, which rapidly and transiently produces prostaglandins in large amounts, in reproductive processes. Such processes as ovulation and luteolysis are believed to require large amounts of prostaglandin for completion; Therefore, in vitro and in vivo studies were designed to determine the role and origin of prostaglandin formation during these reproductive processes.

References


CHAPTER I

EFFECT OF MEDIA SUBSTITUTES ON BOVINE MURAL GRANULOSA CELL FUNCTION AND PROLIFERATION DURING IN VITRO CULTURE

Introduction

In vitro studies involving the culture of granulosa cells have expanded our knowledge of hormonally regulated follicular enzymes (Orly et al., 1980; Savion et al., 1981b). To date, the most efficient method of increasing cell numbers in vitro involves use of serum-supplemented culture medium. However, the interpretation of results for hormonally-regulated enzymes is often unpredictable or complicated by the addition of serum to the culture medium (Orly et al., 1980; Erickson, 1983).

Serum contains an undefined composite of hormones, nutrients and growth and attachment factors necessary for cell maintenance and proliferation (Savion et al. 1981a). Studies involving the effects of serum supplementation on granulosa cell function demonstrate that serum constituents alone are capable of inducing steroidogenesis, while masking the effects of added regulatory agents, such as forskolin and LH (Savion et al., 1981a; Skinner and Osteen, 1988; Meidan et al., 1990).

A recent attempt to propagate bovine granulosa cells using serum substitutes met with only limited success (Roberts and Echternkamp, 1994). However, it has been determined that the addition of insulin (Channing et al., 1976; Veldhuis et al., 1985; Langhout et al., 1991), epidermal growth factor, fibroblastic growth factor, and high-
density lipids (Savion et al., 1981a) alone, or in combination can maintain cellular viability or support proliferation at a lower rate compared with serum-supplemented cultures.

Two experiments were designed to determine the effects of commercially-prepared serum replacers on in vitro proliferation and progesterone secretion by bovine granulosa cells during in vitro culture. The primary objective of this study was to evaluate the ability of serum-free culture systems to promote proliferation of granulosa cells without inducing steroidogenesis.

**Materials and Methods**

**Culture Medium and Supplements**

The basal medium, Dulbecco's modified Eagle medium/Hams F-12 nutrient mixture (DMEM/F-12; 1:1 vol/vol), and GMS-X* (GMSX; media supplement containing 1.0 g/L insulin, .67 mg/L sodium selenite, .55 g/L transferrin and .2 g/L ethanolamine), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco Laboratories (Grand Island, NY). The SerXtend* (SerXtend; containing FGF, EGF, insulin, BSA, transferrin (pasteurized human), ethanolamine, thiocetic acid, selenium and hydrocortisone (<50 mg/ml protein) was provided by Irvine Scientific (Santa Ana, CA). The fatty acid-free BSA (FAF-BSA) was purchased from Sigma Chemical Co. (St. Louis, MO). A photograph of the medium substitutes used in this study as received by the manufacturer are represented in Figure 1.1.
Figure 1.1. A photograph of the medium substitutes utilized in Experiment 1, which include from left to right (back) SerXtend, FAF-BSA, GMS-X and (front) FBS.
Granulosa Cell Isolation

For each experiment in Chapter I, ovaries from mixed breed beef cows were collected from a local abattoir in warm PBS containing 100 units penicillin and 100 \( \mu g/ml \) streptomycin (antibiotics) and transferred to the laboratory within 7 hours of collection. A single cell suspension of granulosa cells was obtained by aspirating 2- to 8-mm follicles using a 20-gauge needle attached to a 10-ml syringe (Langhout et al., 1991), and follicular aspirates were dispensed into 15-ml polypropylene conical centrifuge tubes (Figure 1.2). Approximately 10 to 15 ovaries were required to perform each replicate, with each ovary possessing 5 to 10 follicles. Tubes containing the granulosa cells were allowed to settle at room temperature for 15 minutes to allow sedimentation of cumulus oocyte complexes and aggregated granulosa cells.

The supernatant was aspirated and the granulosa cells (primarily mural granulosa) were centrifuged at 200 \( \times \) g for 10 minutes at 20 °C. Granulosa cells were then resuspended in 10 ml of basal medium (DMEM/F-12) containing 10% fetal bovine serum, and antibiotics (seeding medium). The cells were washed by centrifugation three times to remove follicular fluid and any residual contaminants. Following the final granulosa cell wash, the cells were resuspended in 10 ml of seeding medium, and the number of viable (non-stained) cells was determined manually by the use of a hemocytometer and the trypan blue exclusion method that was adapted from the previous work of Freshney (1987).
Figure 1.2. A photograph of the tools utilized to isolate and culture bovine granulosa cells including the needle and syringe, bovine ovary, tissue culture flasks and conical tube.
Experiment 1

The objective of Experiment 1 was to develop a defined culture system that would allow granulosa cell proliferation under serum-free conditions. The efficacies of two commercially-prepared medium additions to grow granulosa cells were compared with common medium supplements, FBS and FAF-BSA. For the initial seeding, $5 \times 10^5$ viable cells were placed into duplicate sets of 25-cm$^2$ tissue culture flasks (5 flasks/treatment/replicate; Costar; Cambridge, MA) containing 5 ml of seeding medium. Cells were allowed to attach to the substratum for 48 hours in seeding medium and then washed once with basal medium. Culture flasks containing granulosa cells were randomly allotted to the following treatments: basal medium alone (1), basal medium containing 1 % vol/vol FBS (2), 1 % vol/vol GMS-X (3), 4 mg/ml FAF-BSA (4), or 5 % vol/vol SerXtend (5). All cultures were maintained at 39 °C in an atmosphere of 95% air and 5% CO$_2$.

On culture days 1, 2, 4, 6 and 8 of treatment culture, the number of granulosa cells/flask was determined and the culture medium was harvested. At each count, except days 1 and 8, media were aspirated and replaced with fresh treatment media in the remaining flasks.

Experiment 2

Based on the results of Experiment 1, two dose response trials were conducted to evaluate different concentrations of SerXtend and different seeding procedures on the
rate of proliferation of granulosa cells. In the first trial, granulosa cells were initially seeded in 25-cm² flasks with 5 x 10⁵ cells in seeding medium for 48 hours. Following the seeding period, treatment media were allotted to duplicate sets of flasks (5 flasks/treatment/replicate). The treatments consisted of basal medium alone (1), or basal medium containing 5% (2), 2.5% (3), 1.25% (4) or .625% SerXtend (5).

A second dose response trial was carried out to determine if reducing the initial seeding rate and period would allow adequate cell growth. Following the initial viability assessment and count, cells were seeded at 2.5 x 10⁵ cells/25 cm² flask and cultured in medium containing 10% serum for 24 hours prior to treatment culture. Treatment cultures consisted of basal medium (1), or basal medium containing 2.5% (2), 1.25% (3), .625% (4), .3125% (5) or .078% (6) SerXtend. Granulosa cell counts were determined on days 1, 2, 4, 6 and 8 of treatment culture for each trial and medium was replaced in the remaining flasks every other day of treatment culture, except on days 1 and 8.

Determination of Granulosa Cell Numbers and Medium Harvest

At designated intervals for each experiment, the number of granulosa cells in each flask was determined by removing the cells from the bottom of the culture vessel using a .05% trypsin solution containing .2 mg/ml EDTA (Sigma). During the trypsinization procedure, flasks were incubated on an incubator/shaker at 39°C for 5 to 10 minutes, or until the cells became rounded and lifted off the substratum. The cell
suspension was then diluted with seeding medium (1:2 vol/vol) to inactivate the trypsin. The number of granulosa cells/treatment was determined using a hemocytometer and cell viability determined by the trypan blue dye exclusion method (Freshney, 1987). In Experiment 1 and 2, medium was harvested from flasks at each cell count, and centrifuged at 200 x g for 20 minutes at 20°C for later progesterone assay.

Progesterone Concentration

Progesterone was analyzed in Experiments 1 and 2 using a previously validated RIA (Thompson et al., 1983). The RIA was performed in duplicate with appropriate standards. Standards were extracted with petroleum ether prior to RIA analysis. The progesterone antibody cross reacts with pregnenolone (0.5%), estrone (<0.01%), testosterone (<0.01%) and androstenedione (0.04%). Media samples were diluted 50-fold prior to the assay, with a 50-μl volume of sample added to each tube. All values were obtained in a single assay, with an intra-assay coefficient of ≤12.5 % with a percent recovery rate and binding rate of [3H] progesterone of 75% and 38%, respectively.

Statistical Analysis

For Experiment 1, granulosa cells from each of two groups of ovaries were randomly divided into two replicates for each treatment and sampling time, with a third batch of ovaries providing cells for a single replicate (n=5 replicates/treatment/time). For Experiment 2, two replicates were performed from each of two ovary batches (n=4
replicates/treatment/time) for each response curve. Cell counts and progesterone concentrations were analyzed using least square analysis of variance and the PROC GLM procedure of SAS (1985). If the differences between treatments were significant, the PDIFF procedure of SAS was used to compare means. Data were analyzed using the model components of ovary batch, treatment, ovary x treatment, replicate(ovary batch x treatment), time, ovary batch x time, treatment x time, ovary batch x treatment x time and residual. The effect of ovary batch and ovary batch x treatment was tested using replicate(ovary batch x treatment) as the error term. The effect of treatment was tested using ovary batch x treatment and the effect of time was tested using ovary batch x time. The effect of treatment x time was tested using ovary batch x treatment x time, and the remaining terms were tested with the residual.

Results

Experiment 1

Photomicrographs (40x) of bovine granulosa cells following 8-days of in vitro culture in medium supplemented with different substitutes are depicted in Figures 1.3 through 1.5. Morphological characteristics of cells cultured in this study were similar to those reported by Savion et al. (1981a). In the present study, cells cultured in basal medium alone tended to become flattened and enlarged. Trypan blue exclusion staining performed on cells at each count also indicated low cell viability by day 6 of culture (data not shown). Those cells cultured in either FBS, FAF-BSA or SerXtend appeared
Figure 1.3. A photomicrograph (40X) of bovine granulosa cells that were initially seeded ($5 \times 10^5$ cells/flask) in medium containing 10% FBS for 48 hours. The photograph was made following 8 days of treatment culture in medium alone.
Figure 1.4. A photomicrograph (40X) of bovine granulosa cells that were initially seeded (5 x 10^5 cells/flask) in medium containing 10% FBS for 48 hours. Pictures were taken following 8 days of treatment culture in medium containing 1% FBS (top) or 1% GMSX (below).
Figure 1.5. A photomicrograph (40X) of bovine granulosa cells following 8-days of serum-free culture in medium containing either 4 mg/ml FAF-BSA (top) or 5% SerXtend (below).
healthy, maintained an "epithelial-like" appearance and were > 85% viable on day 8 of culture. Cells cultured in these treatments formed small, tightly bound colonies from which they continued to proliferate until confluency. However, the cells cultured in GMSX appeared to take on a "fibroblast-like" appearance, exhibiting signs of elongation and a low nuclear to cytoplasmic ratio.

The initial seeding of bovine granulosa cells in medium supplemented with 10% FBS provided attachment and subsequent proliferation for all treatment media except the control. Cell populations during days 1 to 8 of culture in different treatment media are demonstrated in Figure 1.6. The basal mixture of DMEM/F-12 (Control) did not enhance cell proliferation. The addition of calf serum or FAF-BSA to the medium provided an increase (P < .01) in cell numbers through day 4 when compared with granulosa cells cultured in the control medium, while a significant decline (P < .05) in cell numbers followed confluency at day 4.

Treatment 3 (GMSX) also produced higher (P < .001) cell numbers compared with the control group through confluency (day 4 of treatment culture), followed by a rapid decline by day 6 and a slight, nonsignificant increase on day 8 of culture. The number of cells/flask was greater in Treatment 3 on day 2 and 4 of the culture period when compared with both the Treatment 2 (P < .01) and Treatment 4 (P < .01) and was greater (P < .001) than the control treatment on day 8. The SerXtend (Treatment 5)
Figure 1.6. Proliferation (±SEM) by granulosa cells cultured in basal medium alone or medium containing FBS (1%), GMSX (1%), FAF-BSA (4 mg/ml) or SerXtend (5%) following an initial seeding period of 48 hours and an initial seeding rate of $5 \times 10^5$. 

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produced the highest proliferation rate. Cell numbers in this group were greater
(P < .001) than all other treatments and there was no decline following confluency.

The steroidogenic capability of bovine granulosa cells in extended serum-free
culture was evaluated by determining the amount of progesterone present in the media
at 2-day intervals (Figure 1.7). Progesterone concentrations are reported on a
normalized basis as ng of progesterone/100,000 viable cells. On days 1 and 2 of
treatment culture, Treatments 1, 2, 3 and 4 produced greater (P < .001) concentrations
of progesterone when compared with those of Treatment 5. On day 4 of treatment
culture, granulosa cells in Treatment 4 produced more (P < .05) progesterone than
Treatment 1, 3 and 5 but was not different from Treatment 2.

The bovine granulosa cells in Treatments 1 and 5 produced the least amount of
progesterone. The ranking trend in progesterone production was similar for days 6 and
8, with Treatments 2, 3 and 4 producing more (P < .001) progesterone than Treatments
1 and 5. Overall, progesterone production by cells in all treatments was elevated
beginning on day 4 of culture; however, progesterone secretion by granulosa cells
cultured in Treatments 2, 3 and 4 remained significantly higher (P < .01) than
Treatments 1 and 5 throughout the remaining culture period.

Experiment 2

Experimental conditions for Experiment 2 also provided for cell proliferation
for each SerXtend medium concentration evaluated (Figure 1.8). There were no
Figure 1.7. Progesterone production (±SEM) by granulosa cells cultured in different treatment media in Experiment 1. Progesterone concentrations were normalized on a number of viable cells/ng of progesterone (10^3) basis. Bars within days with different letters differ as indicated in the Results text section.
Figure 1.8. Proliferation (±SEM) by granulosa cells cultured in basal medium or basal medium containing 5%, 2.5%, 1.25% or .625% SerXtend following an initial seeding period of 48 hours and an initial seeding rate of $5 \times 10^5$. 

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difference in cell proliferation rates between SerXtend treatments at each evaluation time. Bovine granulosa cell proliferation was greater ($P < .01$) in the SerXtend treatments (2 through 5) when compared with the controls on days 1, 2, 4 and 6, but proliferation of these groups was not significantly different from the control group on day 8 of culture. For each treatment, cell confluency was reached at approximately day 4 of treatment culture, after which cell numbers declined. Cell numbers remained consistently low in the control treatment during the 8-day evaluation period, indicating that the culture medium alone was not capable of stimulating the granulosa cells into mitotic division.

On days 1 and 2 of culture, the cells in the control group produced a higher ($P < .05$) concentration of progesterone when compared with the SerXtend treatments (Figure 1.9). However, on day 6 of culture, progesterone secretion was significantly higher ($P < .01$) in the SerXtend Treatments 3, 4 and 5 (2.5 %, 1.25% and .625 % SerXtend, respectively). Also, progesterone production by SerXtend-treated cells gradually increased during the culture period, and was significantly greater for each SerXtend treatment when compared with the control treatment (medium alone) on day 8 of treatment culture.

Figure 1.10 represents the proliferation of cells that were seeded at a rate of 2.5 x $10^5$ cells/25-cm$^2$ flask and cultured in medium containing 10% serum during the initial 24 hour seeding period. As expected, cells cultured in the control medium alone

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Figure 1.9. Progesterone production (±SEM) by granulosa cells cultured in different concentrations of SerXtend in Experiment 2. Progesterone concentrations were normalized on a number of viable cells/ng of progesterone (10^3) basis. Bars within days with different letters differ as indicated in the Results text section.
Figure 1.10. Proliferation ($\pm$SEM) by granulosa cells cultured in basal medium, or basal medium containing 2.5%, 1.25%, .625%, .3125% or .078% SerXtend following an initial seeding period of 24 hours and an initial seeding rate of $2.5 \times 10^5$. 

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did not increase in number. However, when cultured in medium containing .3125% or greater SerXtend, granulosa cell populations gradually increased beginning on day 4 and continuing through day 8 of treatment culture. Treatments 2 and 3 produced a significantly higher (P < .001) number of cells when compared with Treatments 4, 5 and 6 on day 6 of treatment culture. On day 8 of treatment culture, Treatment 2 produced a higher (P < .001) number of cells than Treatment 5 but was not greater than Treatments 3 and 4. Proliferation rates of granulosa cells cultured in Treatment 6 medium did not differ from the control group (medium alone).

Discussion

These experiments demonstrate that granulosa cells can be cultivated in a serum-free culture system. The basal medium was able to maintain the majority of cells that had attached during the initial serum seeding period for up to 8 days in culture, as was previously reported for granulosa cells cultured in Hams F-12 medium (Skinner and Osteen, 1988). In contrast, both the FBS and FAF-BSA induced granulosa cell proliferation at approximately the same rate. These data are in agreement with those of Borano and Hammond (1985) who noted similar proliferations rates of bovine granulosa cells between FBS and FAF-BSA.

Removal of serum from granulosa cell cultures (control) resulted in a significant decline in progesterone production over the culture interval (Figures 1.7 and 1.9). In comparison, progesterone production by cells cultured in FBS- or FAF-BSA-
supplemented media increased from days 4 to 6 of culture and was maintained at day 8. Progesterone concentrations for the FBS- and FAF-BSA-supplemented treatments were significantly greater than in control culture at these sampling periods. Therefore, serum and its albumin constituent, may mask the effects of luteotropic agents added to the medium in nonproliferative cells (control) during the 6 to 8 day culture period following serum removal. However, the high proliferative capacity of granulosa cells grown in medium containing SerXtend appears to override the ability of the cells to produce progesterone immediately following serum removal. Therefore, if granulosa cells are grown to confluency using medium containing the SerXtend, regulatory agents may be added following a relatively short equilibration period in a maintenance medium.

Differences in cell proliferation rates may offer a possible explanation for the high progesterone production observed in the control group versus the low production by SerXtend treated cells. Progesterone production by cells incubated in the SerXtend treatment (Figure 1.7) was inversely related to cell numbers (Figure 1.6). When mitogenic activity was high (days 1 through 4), progesterone production was low, and when proliferation rates were low (days 6 and 8), progesterone production was increased. This inverse relationship between proliferation and steroidogenesis has been reported for bovine granulosa cells (Orly et al., 1980; Erickson, 1983; Meidan et al., 1990). These data, along with other results from our laboratory, indicate that granulosa
cells cultured in extended serum-free environments for up to 8 days are capable of secreting progesterone upon subsequent stimulation.

The stimulation of progesterone production by nonproliferating cells in the SerXtend treatment, during the latter stages of culture, may be a function of the insulin contained in the SerXtend preparation. Mitogenic and steroidogenic effects of insulin have been reported for bovine (Savion et al., 1981a; Langhout et al., 1991; Spicer et al., 1993), porcine (Borano and Hammond, 1985; May et al., 1988) and primate (Woodruff et al., 1993) granulosa cells in serum-free culture systems. Langhout et al. (1991) reported an increase in cell proliferation and steroidogenesis by bovine granulosa cells collected from both small and large follicles. The mitogenic effects of insulin have been associated with its affinity for the IGF1 receptors, as opposed to direct action through its own receptor (May and Schomberg, 1981; Savion et al., 1981a).

In our study, the medium supplement (GMS-X, consisting of insulin, selenium, transferrin and ethanolamine) adequately supported granulosa cell proliferation but produced levels of progesterone similar to that of FBS. This phenomenon has been reported by other investigators (Veldhuis et al., 1985; Langhout et al., 1991; Spicer et al., 1993), and may be considered a problem in studies involving hormonal regulation of follicular enzymes. Progesterone secretion stimulated by serum-supplemented culture media may mask some effects of added regulatory agents, such as forskolin or cAMP, when studying the regulation of the P450scc enzyme (Skinner and Osteen,
Similarly, serum constituents have been reported to induce prostaglandin formation by activation of the COX enzyme (Mitchell and Trautman, 1993).

The SerXtend used in this study is classified as a serum substitute containing less than 50 mg/ml of protein. According to the manufacturers recommendations, 12.5 ml of SerXtend should be added to 500 ml of serum, which in turn reduces the amount of serum needed in the culture medium. However, in the present study, SerXtend alone was added to the media without the addition of serum. SerXtend is reported to contain FGF, EGF, insulin, BSA, transferrin, ethanolamine, thiocytic acid, selenium and hydrocortisone. This substitute was studied because of the observation by Savion et al. (1981a) that EGF and FGF alone did not produce a mitogenic effect in vitro, but when combined with insulin, these growth factors increased cell proliferation over that of insulin alone. In our study, the SerXtend milieu also produced a mitogenic effect greater than the insulin treatment (GMS-X).

When cells were seeded at an initial rate of $5 \times 10^5$, cell confluency was reached between days 2 and 4 of the culture interval for the SerXtend treatment. In contrast, when granulosa cells were seeded at a lower rate ($2.5 \times 10^5$), they tended to proliferate in a moderate rate during the first 4 days of treatment culture, and were slower to reach confluency; however, their cell number increased by the 6th and 8th day of culture. In a previous report, granulosa cells originally seeded in FBS and then cultured on a
plastic substratum in serum-free medium containing FGF and high density lipids did not proliferate (Savion et al., 1981a). However, in this study, insulin-containing medium was able to induce proliferation when cells were seeded at low concentrations. Apparently, cell-to-cell contact is the limiting factor in the proliferation of granulosa cells in serum-free media when no collagen matrix is utilized.

Currently, the most effective methods to culture bovine granulosa cells require the addition of serum to the culture medium, which often produces variable responses. A commercially-prepared serum substitute (SerXtend) was able to stimulate bovine granulosa cell proliferation in culture for up to 8 days without significant loss of cellular integrity as determined by the trypan blue dye exclusion method. Moreover, when performing hormonal regulation studies, it may be beneficial to use SerXtend to avoid triggering steroid synthesis during the proliferative stages of culture. This ability to grow granulosa cells from small follicles may, in the future, enhance our understanding of granulosa cell growth and differentiation. The use of this serum substitute may allow for the determination of the causative factors involved in granulosa cell proliferation and steroidogenesis and their specific mechanisms during folliculogenesis.

References


CHAPTER II

CORTICOSTERONE REGULATES PROSTAGLANDIN FORMATION BY FORSKOLIN STIMULATED BOVINE MURAL GRANULOSA CELLS FOLLOWING EXTENDED SERUM-FREE CULTURE

Introduction

The most efficient methods of increasing cell numbers in vitro involves the use of serum-supplemented culture medium. However, the interpretation of results from experiments dealing with hormonally-regulated enzymes is complicated by the addition of serum to the culture medium (Orly et al., 1980; Erickson, 1983). This is due to an undefined mixture of hormones, nutrients and growth and attachment factors that serum contains (Savion et al., 1981).

Studies involving the effects of serum supplementation on granulosa cell function demonstrate that serum constituents alone are capable of inducing steroidogenesis, while masking the effects of exogenous regulatory agents, such as forskolin and LH (Savion et al., 1981; Skinner and Osteen, 1988; Meidan et al., 1990). However, Broussard et al. (1995) noted that a commercially-prepared serum substitute was able to promote bovine granulosa cell proliferation, while minimizing steroidogenesis. The mitogenic potential of this serum substitute enabled the production of large number of cells through in vitro culture, allowing low numbers of granulosa cells to be increased prior to the addition of regulatory agents, such as forskolin or corticosterone.
Espey (1980) proposed a general model for mammalian ovulation, which included the accumulation of prostaglandins in the liquor folliculi near the time of ovulation. The exact mechanism of prostaglandin-induced ovulation is not known; however, the ecosinoids are thought to have a variety of local functions in the preovulatory follicle, including fibroblast proliferation, chemotaxis, vasodilation and destruction of connective tissue (for review see Espey, 1980, 1994). Several reports implicate COX-2 (the inducible isoform of the cyclooxygenase enzyme) in the rapid formation of the cyclooxygenase prostaglandins in the rat preovulatory follicle (Sirois and Richards, 1992; Sirois et al., 1992) following stimulation by hCG near the time of ovulation. More recently, the COX-2 isozyme was found to be present in the granulosa cells of hCG stimulated preovulatory follicles of the cow (Sirois, 1994).

Therefore, a preliminary and two primary experiments were designed to determine if bovine granulosa cells cultured in a serum-free in vitro environment were able to respond to the regulatory agent, forskolin, an adenylate cyclase activator that has been reported to mirror in vivo LH activity (Meidan et al., 1992), by producing progesterone and prostaglandins. The primary objective of the study was to establish if the COX isozyme is responsible for prostaglandin formation by forskolin stimulated, in vitro cultured granulosa cells. Corticosterone, which specifically inhibits COX-2 prostaglandin formation (Lee et al., 1992), was added to the culture medium to distinguish between the function of COX-1 and COX-2 in this prostaglandin formation.
Materials and Methods

Culture Medium and Supplements

The basal medium, Dulbecco's modified Eagle medium/Hams F-12 nutrient mixture (DMEM/F-12; 1:1 v/v), and GMS-X* (GMS-X; a medium supplement containing 1 g/L insulin, .67 mg/L sodium selenite, .55 g/L transferrin and .2 g/L ethanolamine), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco Laboratories (Grand Island, NY). Arachidonic acid and hydrocortisone were purchased from Sigma Chemical (St. Louis, MO). The SerXtend* (SerXtend; a serum substitute reported by the manufacturers to contain fibroblast growth factor (FGF; bovine), epidermal growth factor (EGF; murine), insulin, BSA, transferrin (pasteurized human), ethanolamine, thioctic acid, selenium and hydrocortisone (<50 mg/ml protein) was supplied by Irvine Scientific (Santa Ana, CA).

Granulosa Cell Isolation

Ovaries from mixed breed beef cows of unknown origin were collected from a local abattoir in warm PBS containing antibiotics and transferred to the laboratory within 5 hours. A single cell suspension of granulosa cells was obtained by aspirating 2- to 8-mm diameter follicles, as previously described by Broussard et al. (1995). Briefly, the follicular aspirates were dispensed into 15-ml polypropylene conical centrifuge tubes that were incubated at room temperature for 15 minutes to allow for sedimentation of cumulus oocyte complexes and aggregated granulosa cells. The
follicular fluid was aspirated and the cells washed three times with 10 ml of DMEM/F-12 containing 10% FBS, 100 units penicillin and 100 μg/ml of streptomycin (seeding medium) by centrifugation at 200 x g for 10 minutes at 20°C. The cells were then resuspended in 10 ml of seeding medium, and the number of viable cells determined (Freshney, 1987).

Culture Phases

For each experiment described herein, the granulosa cell culture was split into three separate phases including a seeding, a proliferative, and an experimental phase (see Figure 2.1). The seeding and proliferative phases were utilized to produce large cell numbers for the experimental phase and were performed as previously described (Broussard et al., 1995) in a similar manner for all experiments. For the initial seeding phase, 2.5 x 10^5 viable cells were pipetted into duplicate sets of 25-cm^2 tissue culture flasks (Costar; Cambridge, MA) containing 5 ml of seeding medium. Cells were allowed to attach to the substratum for 24 hours in seeding medium and then washed once with 2 ml of basal medium. Once seeded, the granulosa cells were washed once with 2 ml of basal medium, and then 5 ml of basal medium containing SerXtend (1 % v/v) was dispensed into the duplicate sets of flasks. This proliferative phase consisted of a 6 day granulosa cell culture in SerXtend to allow for granulosa cell proliferation to confluence, followed by a wash with 2 ml of basal medium and a 1-day equilibration period with basal medium containing 1% v/v GMSX (GMSX medium; five ml/flask).
Figure 2.1. A schematic drawing of the experimental design for Experiment 1. The abbreviations "FBS" represents fetal bovine serum, "GMSX" represents GMSX medium, "Forsk" represents forskolin, "h" represents hour and "AA" represents arachidonic acid.
Medium was changed every other day during the proliferation phase, and all cultures were maintained at 39°C in an atmosphere of 95% air and 5% CO₂.

Preliminary Experiment

The objective of the Preliminary Experiment was to determine if bovine granulosa cells respond to the regulatory agent, forskolin, following 6 days of serum-free culture by secreting progesterone into the medium. Flasks (n = 72, 6 treatments x 4 sampling periods x 3 replicates) containing a confluent monolayer of granulosa cells were randomly allotted to one of six treatment media consisting of 5 ml of basal medium alone (Treatment 1, negative control) or basal medium containing forskolin (Treatment 2), 1% FBS (Treatment 3; positive control), 1% FBS plus forskolin (Treatment 4), GMSX medium (Treatment 5) and GMSX medium plus forskolin (Treatment 6). All treatment media that contained forskolin (Treatments 2, 4 and 6) did so at a 10 μM concentration. The treatment culture persisted for 6 days, with medium changes and sampling times occurring on days 1, 2, 4 and 6 of treatment culture.

Experiment 1

The specific objective of Experiment 1 was to determine if in vitro cultured bovine granulosa cells were capable of producing prostaglandin F₂α in response to forskolin stimulation at the time previously determined to occur in in vivo preovulatory follicles (Sirois, 1994). A duplicate set of 25 cm² flasks (n = 36; 3 treatments x 4
sampling periods x 3 replicates) were grown to confluency as described in the Culture Phase section. The experimental phase began immediately following the 1-day equilibration period, and was designated as Time 0 (hour) for the experimental phase (see Figure 2.1).

Granulosa cells cultured in the control treatment (Treatment 1; see Figure 2.1A) remained in GMSX medium for the remainder of the culture period (24 hours). Granulosa cells cultured in Treatment 2 medium (see Figure 2.1B) were incubated in GMSX medium 16 hours (beginning from Time 0), at which time the flasks were washed once with 2 ml of basal medium and replaced with 5-ml of GMSX medium plus arachidonic acid (10 μM) was added. For Treatment 3 (see Figure 2.1C), granulosa cells were exposed to a 5 hour incubation period (beginning at Time 0) with GMSX medium plus forskolin (10 μM). Flasks were then washed once with 2 ml of basal medium and the granulosa cells were then cultured for 11 hours in GMSX medium. At hour 16, flasks in Treatment 3 were again washed once with 2 ml basal medium that was replaced with GMSX medium plus arachidonic acid (10 μM). For Treatments 1 and 2, GMSX medium was removed from the flasks 5 hours following the initiation of the experimental phase and replaced with 5 ml of fresh GMSX medium to remain consistent with medium changes in Treatment 3.

The medium was harvested at 2-hour intervals during the sampling period (18, 20, 22 and 24 hours post-initiation of the experimental phase) and frozen at -80°C until
later assay for prostaglandin $F_2\alpha$. During the experimental phase, all flasks contained 5 ml of treatment medium, which was removed from each treatment flask at each sampling period and replaced with fresh culture medium appropriate to that treatment. Culture medium was not replaced in treatment flasks on days 1 and 8 of treatment culture.

Experiment 2

The objective of Experiment 2 was to determine if corticosterone, which selectively inhibits prostaglandin formation by the COX-2 isozyme was capable of inhibiting prostaglandin secretion by forskolin-stimulated granulosa cells. The experimental phase ($n = 36$ flasks; 4 treatments x 3 sample periods x 3 replicates) for Treatment 1 (control) and Treatment 2 in Experiment 2 were identical to Treatments 1 and 3 in Experiment 1, respectively. The experimental phase for Treatments 3 and 4 in Experiment 2 was identical to Treatment 3 in Experiment 1, except corticosterone (1 $\mu$M and 10 $\mu$M, respectively) was added 16 hours into the experimental phase, along with arachidonic acid.

Media were harvested at 2-hour intervals during the sampling period (18, 20 and 22 hours of the experimental phase) and frozen at -80°C until assayed for prostaglandin $F_2\alpha$. During the experimental phase, all flasks contained 5 ml of treatment medium, which was removed from each treatment flask at each sampling period and replaced with fresh medium appropriate to that treatment.
Progesterone and Prostaglandin F₂α Determination

Progesterone values for the Preliminary Experiment were analyzed using a radiouimmunoassay previously validated by Thompson et al. (1983). The RIA was performed in duplicate with appropriate standards. Standards were extracted with petroleum ether prior to RIA analysis. The progesterone antibody used cross reacts with pregnenolone (.5%), estrone (< .01%), testosterone (< .01%) and androstenedione (.04%). Each medium sample was diluted 50-fold prior to the assay, with a 50-μl volume of sample added to each tube. The percent recovery and binding of [³H] progesterone was 75% and 38%, respectively.

Prostaglandin F₂α values were obtained using an enzyme immunoassay kit originating from Cayman Chemical (Ann Arbor, MI). The assay was performed in accordance with the protocols that accompanied the kits. According to the manufacturer the prostaglandin F₂α antibody has a cross reactivity of 100 % with prostaglandin F₂α, F₁α and F₃α; 7% with 6-keto prostaglandin F₁α; < .5% with prostaglandin A₃; .3% with 2,3-dinor-6-keto prostaglandin F₁α and < .1% with prostaglandin E₂, 6,15-dideto-13,14-dihydro prostaglandin F₁α, thromboxane B₂ and prostaglandin E₃. Media samples were added to the 96-well plates precoated with murine monoclonal antibody at a volume of 50 μl, and the plates read at 412 nm. Values for both progesterone and prostaglandin F₂α were obtained in a single assay, with an intra-assay coefficient of ≤13 %.
Statistical Analysis

For the Preliminary Experiment and Experiments 1 and 2, granulosa cells from one group of ovaries were randomly divided into three replicates for each treatment and sampling time (n=3 replicates/treatment/time period). Progesterone and prostaglandin F$_2$alpha concentrations in the medium were analyzed using least square analysis of variance and the PROC GLM procedure of SAS (1985). If the differences between treatments were significant, the PDIFF procedure of SAS was used for mean comparisons. Data were analyzed using the model components of treatment, replicate (treatment), time, treatment x time and residual. The effect of treatment was tested using treatment x time and the effect of time was tested using replicate (treatment), and the remaining terms were tested with the residual.

Results

Preliminary Experiment

Results from the Preliminary Experiment indicate that bovine granulosa cells were capable of responding to forskolin by secreting progesterone in vitro (Figure 2.2). Granulosa cells cultured in the control and control plus forskolin treatments did not differ in mean progesterone production on days 1, 2 and 4 of incubation. Mean progesterone production was significantly increased (P<.01) from cells cultured in FBS plus forskolin when compared with cells cultured in FBS alone at all sample periods evaluated. A similar significant increase was noted for cells cultured in GMSX medium.
Figure 2.2. Mean progesterone production (±SEM) by bovine granulosa cells following a seven day serum-free culture period. Letters within each treatment day represent a significant difference of at least $P < .001$. 

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when compared with GMSX medium plus forskolin treatment on days 2 and 4 of culture. However, progesterone production was not different between the two GMSX medium treatments on days 1 and 6 of culture. Progesterone production was not different among the control treatment (Treatment 1) and the FBS and GMSX treatments (Treatments 3 and 5) at all sampling periods.

Experiment 1

Granulosa cells cultured in the control medium secreted the least amount of prostaglandin $F_2\alpha$ into the culture medium (Figure 2.3). The addition of arachidonic acid to the medium during the experimental phase (Treatment 2) resulted in a numerically higher ($P > .05$) secretion of prostaglandin $F_2\alpha$ than cells cultured in the control medium. However, those cells treated with forskolin and arachidonic acid (Treatment 3) secreted much larger amounts ($P < .0001$) of prostaglandin $F_2\alpha$ into the medium than the control or the arachidonic acid treated cells at the 18, 20, 22 hour sampling periods. Cells in Treatment 3 also secreted significantly more ($P < .05$) prostaglandin $F_2\alpha$ at the 24 hour sampling period when compared with control and Treatment 2 concentrations.

Experiment 2

Granulosa cells in the control (Treatment 1) and forskolin plus arachidonic acid (Treatment 2) treatments secreted concentrations of prostaglandin $F_2\alpha$ into the medium at a rate similar to their respective secretions in Experiment 1 (Figure 2.4). Granulosa
Figure 2.3. Mean Prostaglandin F₂α concentrations 18, 20, 22 and 24 hours following initiation of the experimental phase (Pooled SEM = 6.71). Different letters within each hour of the experimental phase represents a significant difference of at least P < .0001.
Figure 2.4. Mean prostaglandin F$_{2\alpha}$ concentrations 18, 20 and 22 hours following the initiation of the experimental phase (Pooled SEM = 7.14). The abbreviation "Cort" represents corticosterone. Different letters within each hour of the experimental phase represent a significant difference of at least $P < .0001$. 

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cells in Treatment 2 secreted significantly more (P < .0001) prostaglandin F$_2$α than the control group at each sampling period evaluated. The addition of 1 μM of corticosterone to the culture medium decreased prostaglandin F$_2$α secretion at 18 (P < .001) and 20 (P < .05) hours after the cells were exposed to forskolin treatment. There was no difference (P = .06) in prostaglandin F$_2$α secretion between Treatments 2 and 3 at the 22 hour sampling period. However, when 10 μM of corticosterone (Treatment 4) was added to the medium, prostaglandin F$_2$α secretion by the granulosa cells was significantly decreased (P < .0001) to levels similar to the control group (Treatment 1) at all time periods.

**Discussion**

It would be of great benefit to be able to cultivate bovine granulosa cells in a serum-free environment. This is especially true since FBS alone has been noted to stimulate hormonally regulated enzymes, such as the P450 side chain cleavage enzyme in the production of progesterone (Savion et al., 1981; Skinner and Osteen, 1988) and the COX enzyme in the production of prostaglandins (Lee et al., 1992; Mitchell and Trautman, 1993). In a Preliminary Experiment, forskolin significantly increased progesterone production by granulosa cells that had previously been cultivated in a serum-free environment. Progesterone secretion by granulosa cells was significantly greater when stimulated by forskolin in the FBS- and GMSX-based medium when compared with nonstimulated counterparts (Treatments 3 and 5, respectively) on days 2 and 4 of treatment culture. These data indicate that cultivation in a serum-free
environment does not adversely affect the ability of bovine granulosa cells to respond to a potent regulatory agent, forskolin.

Forskolin was utilized in the current study because it has been reported to mimic gonadotropin activity in bovine granulosa cells (Meidan et al., 1990; 1992). Results of Experiment 1 indicated that forskolin-stimulated prostaglandin F₂α secretion from granulosa cells cultivated in a manner similar to those in the Preliminary Experiment was increased when compared with the control treatment. The prostaglandin F₂α precursor (arachidonic acid), added to ensure its availability to the cells, did not stimulate prostaglandin F₂α secretion above control levels, and was significantly less than the forskolin-stimulated cells.

These results are in agreement with in vivo studies performed in the rat (Sirois and Richards, 1992; Sirois et al., 1992) and indicate that compounds having luteinizing hormone-like activity stimulate prostaglandin F₂α formation in bovine granulosa cells. More specifically, bovine granulosa cells harvested from small follicles and grown in a serum-free, in vitro environment were able to produce prostaglandin F₂α at time periods similar to those noted to occur in in vivo preovulatory follicles (Sirois, 1994). These previous findings, along with the results from the current study, lend credence to the hypothesis that prostaglandin F₂α plays a significant role in the process of mammalian ovulation during the critical time period just preceding the release of the oocyte into the reproductive tract (see reviews by Espey, 1980; Murdoch et al., 1993).
Results from Experiment 2 indirectly implicate COX-2 as being the isoform that is responsible for the majority of prostaglandin $F_2\alpha$ secretion by in vivo cultured, forskolin-stimulated, bovine granulosa cells. Prior to the characterization of COX-2, studies indicated that glucocorticoids and/or dexamethasone suppress prostaglandin formation in endotoxin stimulated human mononuclear cells (Seibert et al., 1990) and interleukin 1 stimulated human dermal cells (Raz et al., 1989). More specifically, Lee et al. (1992) determined that dexamethasone specifically inhibits COX-2 mRNA production of prostaglandin in endotoxin-stimulated macrophages of the mouse, without affecting COX-1 mRNA production. Therefore, the results of the current study suggest that prostaglandin secretion by bovine granulosa cells is a function of COX-2 enzymatic activity.

It is apparent that serum-free culture is a viable alternative to FBS supplemented culture, at least in-so-far as the enzymes that regulate progesterone and prostaglandin $F_2\alpha$ are concerned. Also, the 7-day serum-free culture apparently allows for granulosa cell maturation. Richards and Bogovich (1982) previously reported that granulosa cells harvested from small antral follicles of the rat are incapable of producing prostaglandins following LH stimulation. The ability to grow steroidogenically functional bovine granulosa cells from small antral follicles may enhance our understanding of granulosa cell growth and differentiation during the process of folliculogenesis. It also appears that translation of COX-2 is the rate limiting process in determining the interval from
the preovulatory surge in LH to ovulation, which is conserved across species. In the future, a similar culture system may provide answers to other regulatory mechanisms in different cell types.

References


CHAPTER III
THE PRESENCE OF A NOVEL CYCLOOXYGENASE ISOFORM IN THE MURAL FOLLICULAR GRANULOSA CELLS OF FSH STIMULATED WOMEN

Introduction

Cyclooxygenase is a rate limiting enzyme that catalyzes the conversion of arachidonic acid to various prostaglandins (Sigal, 1991). The original COX isoform has an approximate MW of 69,000 and 70,000 in the mouse (Hedin et al., 1987) and bovine species (Sirois, 1994), respectively. Both proteins are translated from a 2.8 kb mRNA transcript. There are reports in the literature describing the presence of a novel COX (COX-2) isoform produced by in vitro cultured tracheal mucosal cells (Rosen et al., 1989). This isoform has a MW of 72,000 and is translated from a 4.0 kb mRNA (Rosen et al., 1989). A similar COX isoform has also been isolated in the bovine (Sirois et al., 1994) and humans (Hla et al., 1994).

Cyclooxygenase has been shown to play an important role in prostaglandin formation during mammalian ovulation, which has been compared to an inflammatory response (Espey, 1980). The accumulation of prostaglandins in the follicular fluid in humans is inhibited by the administration of the NSAID indomethacin (Killick and Elstein, 1987), and has since been reported to inhibit production of both the COX-1 and -2 isoforms (Meade et al., 1993). This inactivation of the cyclooxygenase enzyme occurs by an irreversible acetylation of the Ser-530 position (DeWitt et al., 1990).

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The cDNA encoding this protein, has been cloned in human vascular endothelial cells using polymerase chain reaction primers from the murine TSI10 sequence (Jones et al., 1993). Human COX-2 is translated from a 4.3 kb mRNA and has an approximate MW of 72,000 (Jones et al., 1993). Hla and Neilson (1992) reported the stimulation of prostaglandin secretion by cos-7 cells previously transfected with human COX-2 cDNA. This increase was appreciably reduced following a 2 hour incubation in medium containing dexamethasone.

Recent reports have demonstrated the presence of COX-2 in the granulosa cells of preovulatory follicles of the rat (Sirois and Richards, 1992; Sirois et al., 1992). Production of the COX-2 isoform was noted to occur 2 to 5 hours following the administration of an ovulatory dose of hCG (Sirois and Richards, 1992). Also, the mural granulosa cells of preovulatory follicles have been stimulated to transcribe COX-2 mRNA and translate COX-2 protein following hCG administration in prostaglandin F$_2$α short-cycled cows (Sirois, 1994).

The current study was undertaken to determine if the COX-2 isoform is present in human preovulatory follicles. This would be the first report implicating this novel isoform in human ovulation. Granulosa cells were harvested from the ovaries of FSH stimulated females by the use of transvaginal ultrasound guided aspiration, and the COX cellular content determined by western blot analysis using a polyclonal antibody that recognizes both the COX-1 and -2 isoforms.
Materials and Methods

Materials

For the Stimulation protocol, the Pergonal* and Metrodin* used in this study were purchased from Serono Laboratories, Randolf, MA, the leuprolide acetate (Lupron*) from TAP Pharmaceuticals Inc., Deerfield, IL and the Pregnyl (hCG) was purchased from Organon Inc., West Orange, NJ. The oocyte flushing medium, Human Tubal Fluid Medium (HTF-HEPES) was purchased from Irvine Scientific, Inc., Santa Ana, CA and the medium supplement Plasmanate* purchased from Miles, Inc., Elkhart, ID.

FSH Stimulation

Female patients (n=16) ranging from 29 to 41 years of age (average age ±SEM = 35±.9) with nonendocrine reproductive deficiencies were utilized in the study. The reproductive anomalies persisting in these patients, or their spouses included ovarian adhesions, tubal blockage and unexplained male factor. Patients were advised to notify the clinic as to the initial day of menses (day 1 of the menstrual cycle), and plasma estradiol concentrations were determined and an ultrasound examination performed to ensure that there was no ovarian cystic condition.

Subjects were GnRH downregulated with a GnRH agonist (Lupron; 5 mg/ml) prior to follicle stimulation. Females were administered daily injections of .1 ml of Lupron, beginning on day 15 of the menstrual cycle, for 15 days followed by 9 days of
.5 ml injections of Lupron. Subjects were administered (i.m.) an average of 3 ampules of Metrodin (75 IU FSH per ampule) and 2 ampules of Pergonal (75 IU FSH and 75 IU LH per ampule) per day for 9 days, beginning on the 20th day of Lupron therapy. On day 9 of FSH stimulation, patients were administered a 10,000 IU i.m. injection of hCG to initiate in vivo oocyte maturation.

Granulosa Cell Collection and Isolation

Ultrasonic examinations and follicle aspirations were conducted using an Ultrasound System 4 (Advanced Technology Laboratories, Tempe, AZ) ultrasound attached to a 7.5 MHz probe. Follicles with a diameter greater than 1 mm were aspirated from individual patients under general anesthesia using a 16 guage needle (O'Neil Aspiration and Irrigation Needle, Go Medical Industries Ltd, Subiaco, Western Australia) to obtain oocytes for in vitro fertilization (IVF).

Follicles were lavaged 2 to 3 times with 1 ml of warm flushing medium (HTM-HEPEA containing 10 % plasmanate) to increase the recovery rates of both the follicle enclosed oocyte and the mural granulosa cells, and collected into a 15 ml conical centrifuge tube (Becton Dickinson, Lincoln Park, NJ). The aspirate was then poured into a 60-mm petri dish (Falcon; Becton Dickinson) and searched under a light microscope for oocytes at 37°C in 5% CO₂ using a portable IVF Chamber (IVF Isolette, Hoffman Surgical Equipment Company, Conshocken, PA).
Immediately following isolation of the human oocytes, the remaining granulosa cells were aspirated from the 60-mm petri dish using a pastuer pipette, pooled for each individual patient, and then dispensed into 2 ml epindorf centrifuge tubes (Brinkmann Instrument Company, Westbury, NY). Granulosa cells were washed once by centrifugation at 800 x g for 7 minutes. The supernatant was removed from the centrifuge tubes and discarded, and the granulosa cell pellet stored at -80°C for later assay for COX content.

**Western Blot Analysis**

Protein extraction was performed as previously reported by Sirois and Richards (1992). Granulosa cells were homogenized in a TED buffer (20 mM Tris, pH 8, 10 mM EDTA and .1 mM DEDTC) containing 2 mM octyl glucoside and centrifuged at 30,000 × g for 1 hour at 4 °C. The crude pellets were then sonicated (8 sec/cycle, 3 cycles) in TED buffer containing 45 mM octyl glucoside and the sonicates centrifuged at 100,000 × g for 60 minutes at 4 °C. The supernatant was harvested and stored at -80°C until electrophoretic analysis. Protein concentration was determined by the method of Bradford (1976).

Proteins (50 μg) present in solubilized cell extracts were resolved by one-dimensional sodium dodecyl sulfate-poly acrilamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to nitrocellulose filters as previously described (Sirois and Richards, 1992). Filters were incubated with antibody (9181) that
recognizes both COX-1 and -2 isoforms previously diluted 1:10 in 10 mM Tris buffered saline (pH = 7.5) containing 2% nonfat dry milk. Polyclonal antibodies raised in rabbits against purified ovine COX were affinity purified on a cyanogen bromide-activated sepharose column using ovine COX as the ligand (Sirois and Richards, 1992). [125I] protein A (1 x 10^6 cpm/ml in tris buffered saline containing 2% milk) was used to visualize proteins. Filters were washed in Tris buffered saline containing 5% Tween and exposed to film at -80 °C (Kodak XAR-5, XRP, Eastman Kodak). For quantitative purposes, protein blots for individual patients were separately and arbitrarily rated by two individual technichians on a scale from 1 to 4 (1 = little to no precipitation; 4 = high precipitation).

Plasma Estradiol Concentration

Plasma estradiol concentrations were obtained from the individual patient samples collected prior to ultrasound-guided transvaginal aspiration. Estradiol concentrations were analyzed on a total estradiol and a follicle diameter basis for this study. The four sequential plasma samples harvested and the corresponding ovarian-follicular sonagrams taken from each individual patient immediately prior to ultrasound-guided transvaginal aspiration were used in the analysis. Estradiol concentrations were determined using a fully automated enzyme immunoassay procedure (Immulite*; Los Angeles, CA) that had a reported working range of 20 to 2,000 pg/ml level of detection.
Statistical Analysis

Granulosa cells from 16 patients were harvested and frozen as a single unit. Serum estradiol concentrations and follicle diameters were analyzed using least square analysis of variance and the PROC GLM procedure of SAS (1985). If the differences in estradiol concentration or follicle diameter among COX scores were significant, the PDIF procedure of SAS was used to compare means. Data were analyzed using model components of COX score, patient within COX score, day, COX score x day. The main effect of COX score was tested using patient within COX score mean square as the error term and the effect of day was tested using COX score x day mean square.

Results

To determine whether COX isoforms were present in preovulatory follicles, protein extracts were prepared from granulosa cells isolated approximately 36 hours following administration of an ovulatory dose of hCG. Proteins were analyzed by immuno-blotting using a polyclonal antibody having specificities previously described in rats (Sirois and Richards, 1992) and sheep (antibody No. 9181 recognizes both COX-1 and COX-2 isoforms) (Wimsatt et al., 1993).

The immunoreactive protein appeared as a 52,000 mw band (Figure 3.1), believed to correspond to a breakdown proteolytic fragment of COX-2 as explained by Sirois and Richards (1992) in rat granulosa cells, Hla and Neilson (1992) in human
umbilical vein endothelial cells and in bovine granulosa cells (Sirois et al., 1994). In contrast, the antibody (No. 9181) did not recognize COX-1 in human granulosa cells.

Two of the 16 patient granulosa cell samples were recorded as having a minimal COX score of 1 (Figure 3.1; lanes 1 and 8, from left to right). Two of the patient’s granulosa cells had a score of 2 (lanes 2 and 14), while four of the patient’s granulosa cells were awarded a score of 3 (lanes 3, 7, 11 and 15). There were eight granulosa cell samples that were awarded a score of 4 (lanes 4, 5, 6, 9, 10, 12, 13, and 16).

Total estradiol concentrations, separated by COX score are represented in Figure 3.2. Gel bands classified as having a COX score of 1, 2 or 3 had similar total plasma estradiol concentration on days 5, 4, 3 and 2 before aspiration. The gel bands assigned a COX score of 4 had significantly higher of plasma estradiol 4, 3 and 2 days before transvaginal oocyte aspiration.

Average follicle diameter separated by COX score is represented in Figure 3.3. Those gel bands having a COX score of 4 had significantly larger average follicle diameter when compared with gel bands scoring 2 and 3, 5 days before aspiration. There was no difference in follicle diameter among the different COX scores 4 days prior to transvaginal aspiration. However, average follicle diameter was significantly larger 3 days before transvaginal oocyte aspiration for gel bands rated as 4. There was no difference in mean follicle diameter among the COX scores 2 days before transvaginal aspiration.
Figure 3.1. Western blot analysis for the presence of COX-2. Each lane of the radiogram represents granulosa cells harvested from a single patient. Solubilized extracts were prepared follicle granulosa cells as described in the Materials and Methods section. Proteins (50 μg/lane) were analyzed by one-dimensional SDS-page and immunoblotting, using anti-COX antibody No. 9181 (see Sirois, 1994). Human blood platelets were run as a standard. Markers on the left and right of patient lanes indicate the migration of molecular weight standards.
Figure 3.2. Comparison of total mean plasma estradiol concentration (±SEM) separated by COX score for the 4 plasma samples harvested from individual patients beginning 2 days before ultrasound-guided transvaginal oocyte aspiration. The letter "a" within days represents a significant difference.
Figure 3.3. Average follicle diameter (±SEM) separated by COX score for the 4 ultrasound sessions of individual patient ovaries beginning 2 days before ultrasound-guided transvaginal oocyte aspiration. The letter "a" within days represent a significant difference.
Discussion

Collectively, these results provide convincing evidence that the increased prostaglandin secretion by mammalian follicles before ovulation (Espey, 1980) is associated with the selective induction of COX-2 in granulosa cells. This study is the first to establish that the granulosa cells of human preovulatory follicles are capable of producing COX-2 following the administration of gonadotropins at ovulatory levels, suggesting that the obligatory mechanism regulating the synthesis of prostaglandins is conserved across the rat (Sirois and Richards, 1992), bovine (Sirois, 1994) and human species. It is also important to note that antibody raised in rabbits against ovine COX is capable of recognizing human COX-2 antigen.

Although the molecular weight of the COX-2 isoform differs from conventional reports (Rosen et al., 1989; Xie et al., 1991), this phenomenon has been previously reported. In cattle, Sirois (1994) described the presence of a second, lower mw band of approximately 59,000 isolated from the granulosa cells of hCG stimulated bovine preovulatory follicles. Similarly, Hla and Neilson (1992) noted the presence of a lower mw protein (52,000) isolated from cos-7 cells transfected with cDNA of human COX-2. Hla and Neilson (1992) suggested that the lower molecular weight protein may have been translated from an incomplete mRNA fragment constructed during its amplification via the polymerase chain reaction (PCR) methodology. However, both laboratories also suggested that this lower mw protein is a breakdown product of the
intact COX-2 protein. Since PCR technology was not employed during the study by Sirois (1994) or in the present study, it is assumed this protein is indeed a breakdown product of the intact COX-2 molecule.

Human chorionic gonadotropin has been reported to induce prostaglandin formation in granulosa cells of the cow (Sirois, 1994), rat (Hedin et al., 1987; Sirois and Richards, 1992) and human (Lumsden et al., 1986) preovulatory follicle. However, one important difference in the regulation of rat vs human preovulatory follicles is the distinct time course of COX-2 induction. Rat COX-2 mRNA and protein are curtailed 9 hours after hCG treatment, while the induction of COX-2 protein in human mural granulosa cells occurs later, being detected approximately 36 hours following hCG administration. Although this difference appears surprising at first, it should be expected that granulosa cells of rats and humans at comparable stages of development (preovulatory) would produce prostaglandins at different times following hCG administration due to the differing times of ovulation between the two species.

In the rat, the interval from time of hCG administration to ovulation is approximately 14 to 16 hours (Sirois et al., 1992) compared with 38 to 42 hours reported in humans (Lumsden et al., 1986). These observations may reveal one mechanism regulating the normal duration of the ovulation process. Although it was not possible to measure prostaglandin concentration in the human preovulatory follicles aspirated in the present study, Lumsden et al. (1986) determined that prostaglandin $F_2\alpha$
secretion by granulosa cells increases 36 hours following hCG administration. Also, oocyte maturation appears to coincide with follicle maturation and prostaglandin F\textsubscript{2α} production, since preovulatory follicle prostaglandin F\textsubscript{2α} production increases at the same time the oocyte extrudes the first polar body in vivo (Seibel et al., 1984).

In summary, the identification of COX-2 as a novel, distinct isoform that is present in the granulosa cells of hCG stimulated follicles offers new insight into the regulatory mechanisms involved in human ovulation. This mechanism appears to have been conserved across species, since similar COX-2 stimulation has been reported in the rat (Sirois and Richards, 1992; Sirois et al., 1992) and bovine (Sirois, 1994) species. This mechanism may be the regulatory process by which the length of the ovulatory process is determined across species. These results may lead to in vitro regulatory studies using serum-free culture methods (Broussard et al., 1995) to cultivate human granulosa cells. The understanding of COX-2 production and regulation by NSAIDS may improve our comprehension of the ovulatory process and their potential role as a birth control method in humans (Killick and Elstein, 1987).

References


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CHAPTER IV

EFFECTS OF DEXAMETHASONE ADMINISTRATION TO DIESTRUS COWS ON SYSTEMIC PROGESTERONE, ESTRADIOL AND UTERINE CYCLOOXYGENASE PRODUCTION

Introduction

Prostaglandin H synthetase, also referred to as COX, is the initial rate limiting enzyme in the prostaglandin, lipoxin and leukotriene synthesis pathways (Mitchell and Trautman, 1993). Recent studies involving the regulatory mechanisms of prostaglandin production have demonstrated the presence of two isoforms of the COX enzyme (Rosen et al., 1989). Cyclooxygenase-1 (COX-1; mw ~ 70,000), the original isozyme isolated from sheep vesicular glands (Miyamoto et al., 1976), is translated from a 2.8 kb mRNA. Correspondingly, the COX-2 isozyme is translated from a 4.0 kb mRNA and has a molecular weight of 72,000 (Kujubu et al., 1991; Xie et al., 1991).

Prior to the characterization of the COX-2 enzyme, dexamethasone reportedly inhibited prostaglandin formation in endotoxin stimulated mice, but did not affect its basal production (Masferrer et al., 1990). Similarly, cattle administered dexamethasone intramuscularly exhibited an extended luteal phase that has been reported to be a function of either decreased estrogen and preovulatory LH production (Vighio and Liptrap, 1990) or by an inhibition of the production of enzymes that are necessary to complete the COX pathway of eicosanoid production (Pool et al., 1983; Ingraham et al., 1984). However, more recent studies indicate that dexamethasone selectively
inhibits the inducible form of the COX-2 isozyme in mitogen stimulated macrophages of mice (Masferrer et al., 1992) and rats (Lee et al., 1992).

The objectives of the current experiment were 1) to determine if the COX-2 isozyme is produced by the uterine endometrium during the luteolytic phase of the bovine estrous cycle, and 2) to determine if dexamethasone extends the lifespan of the corpus luteum by inhibiting the production of the COX-2 isozyme by the uterine endometrium during the mid-luteal phase of the estrous cycle.

Materials and Methods

Treatment Allocation

Crossbred beef cows with a body condition score of 6 or greater (0 to 9) were selected from the experimental physiology herd and maintained on native pasture at the St. Gabriel Agricultural Research Station, St. Gabriel, Louisiana. Cows that exhibited two previous estrous cycles ranging between 18.5 and 24 days were allotted to this study. At the initiation of the experiment, cows were randomly assigned to treatments, separated and maintained in .8-ha pastures, with pasture rotation performed as necessary and minerals fed free choice.

Cows in the control group (n=4) were administered an intramuscular injection of the treatment vehicle twice daily (0800 and 2000 h) on days 13 - 22 (estrus = day 0) of the treatment estrous cycle. Cows in the dexamethasone group (n=5) were
similarly administered 8 mg of synthetic dexamethasone (Azium*) twice daily on days 13 through 22 of the treatment estrous cycle.

Blood Sampling

Blood samples were collected via venipuncture daily beginning on day 13 and continuing through day 22 of the treatment cycle. Immediately prior to the morning treatment injection, blood samples were harvested from the tail vein in vaccutainer tubes containing heparin with a 20 gauge vaccutainer needle (Vacutainer®, Becton Dickinson Co., Rutherford, NJ) and placed on ice. The whole blood was then centrifuged at 400 x g for 15 minutes, the plasma aspirated, and stored at -80°C until assayed for progesterone and estradiol.

Hormone Analysis

Progesterone values were obtained using a previously validated radioummunoassay method (Thompson et al., 1983). Estradiol concentrations were determined using commercial estradiol kits (Systems Diagnostic, Webster, TX). All values for each steroid were obtained in a single assay, with an intra-assay coefficient of variation of ≤12.5 %.

Endometrial Sampling

Uterine endometrial biopsies were performed on cows assigned to both treatment groups on days 16, 19 and 22 of the treatment estrous cycle. On the designated days following the morning treatment injection, individual cows were
prepared for uterine biopsy by epidural injection of 5 ml of a 1% lidocaine solution (W.A. Butler Company, Columbus, OH). The external genitalia were cleansed with a betadine solution. A sterile biopsy instrument (Figure 4.1) was introduced through the os cervix and endometrial samples were retrieved in the posterior portion of each uterine horn on alternating collections days. Immediately following retrieval, endometrial tissue was freed from connective tissue, carefully blotted on soft tissue to remove excess blood, dissected into 10 mg segments and frozen at -80°C until subsequent protein assay and western blot analysis.

Western Blot Analysis

Protein extraction was performed as previously reported by Sirois and Richards, 1992. Uterine endometrial samples were homogenized using a TED buffer (20 mM Tris, pH 8, 10 mM EDTA and .1 mM DEDTC) containing 2 mM octyl glucoside and centrifuged at 30,000 x g for 1 hour at 4°C. The crude pellets were then sonicated (8 sec/cycle, 3 cycles) in TED buffer containing 45 mM octyl glucoside and the sonicates centrifuged at 100,000 x g for 60 minutes at 4°C. The supernatant was harvested and stored at -80°C until electrophoretic analysis. Protein concentration was determined by the method of Bradford (1976).

Western blot analysis using anti-COX antibody No. 9181, was performed as previously described by Sirois (1994). Briefly, 50 μg of total protein present in solubilized cell extracts were resolved by one dimensional SDS-PAGE. Proteins were
Figure 4.1. A photograph of the uterine biopsy instrument utilized to harvest uterine endometrial samples on days 16, 19 and 22 of the treatment cycle.
then electrophoretically transferred to nitrocellulose filters. Filters were incubated with antibody (No. 9181) that recognizes both COX isoforms in the cow. Filters were washed in Tris-buffered saline containing 5 % Tween and exposed to film at -80°C (Kodak XAR-5, XRP, Eastman Kodak). Radiograms were then mounted on cardboard backing and visually analyzed for the presence or absence of either COX.

Statistical Analysis

Progesterone and estradiol data were analyzed using a split-plot design with repeated measures (Gill and Hafs, 1970). The model components included treatment, cow(treatment), day, treatment by day and residual. Means were separated using the PDIFF option of SAS. Days to progesterone decline to < 1ng/ml, days from progesterone decline to estrus and mean treatment cycle length were analyzed using a one-way ANOVA.

Results

Mean estradiol concentrations for cows in the control and dexamethasone treatments are represented in Figure 4.2. A peak in systemic estradiol was noted in the control group on day 15 of the treatment cycle, which was significantly greater (P < .01) than the dexamethasone treated cows. A significant rise (P < .01) in estradiol was also noted in the control cows on days 20 to 22 of the treatment cycle when compared with the levels in the dexamethasone-treated cows. Mean estradiol
Figure 4.2. Mean estradiol concentrations (±SEM) for cows in the dexamethasone and control treatments during the experimental estrous cycle. Animals in the dexamethasone group received 8 mg dexamethasone twice daily between days 13 through 22, while animals in the control group were treated with the vehicle alone. The letter "a" indicates a significant difference between the two treatments (P < .01).
concentrations in the dexamethasone-treated animals remained suppressed during the treatment cycle, never again rising above 3 pg/ml.

Mean progesterone concentrations obtained during the treatment cycle for both treatment groups are represented in Figure 4.3. Mean progesterone concentrations were not different between the control and dexamethasone-treated cows on days 10 to 17 of the treatment cycle. Circulating progesterone concentrations in the dexamethasone treated group were significantly lower ($P < .01$) than the control cows on days 18 to 20 of the cycle. Circulating progesterone levels by the control cows declined on days 21 and 22 of the cycle and were not different from those circulating levels in the dexamethasone treated cows.

There was no difference in the interval from estrus to progesterone decline to $< 1$ ng/ml between the two treatment groups (Table 4.1). Mean intervals from the onset of estrus to the time when progesterone declined to $< 1$ ng/ml in the treatment cycle for control and dexamethasone-treated animals were 21.3 and 19.4 days, respectively ($P < .01$). However, the mean interval from progesterone decline to below 1 ng/ml during the diestrus phase of the treatment cycle to the post-treatment cycle estrus was significantly greater ($P < .01$) for the dexamethasone-treated animals when compared with control cows administered vehicle (11.6 and 2.6 days, respectively). This notable increase in the interval from progesterone decline below 1 ng/ml to estrus.
Figure 4.3. Mean progesterone (±SEM) production by dexamethasone and control treated cows during the experimental estrous cycle. Animals in the dexamethasone group received 8 mg dexamethasone twice daily between days 13 through 22, while animals in the control group were treated with the vehicle alone. The letter "a" indicates a significant difference between the two treatments (P < .01).
Table 4.1. Effect of diestrous dexamethasone injections on the interval from estrus to progesterone decline to < 1 ng/ml, the interval from progesterone decline to < 1 ng/ml to estrus, and mean treatment cycle length.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$P_4$ decline &lt; 1 ng/ml (day)</th>
<th>$P_4$ decline to estrus (days)</th>
<th>Mean treatment cycle length (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.3±.3</td>
<td>2.6±.8$^a$</td>
<td>24±3.5$^a$</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>19.4±.9</td>
<td>11.6±1.6$^b$</td>
<td>31±2$^b$</td>
</tr>
</tbody>
</table>

$^aP_4$ = progesterone.

$^ab$ Means within columns with different superscripts differ (P < .01).
largely contributed to an extension ($P < .01$) in the overall mean treatment cycle length in the dexamethasone-treated cows compared with the control group (31 versus 24 days, respectively).

The western blot analysis indicated that the COX-2 isozyme was not present at any time period evaluated in the uterine endometrial tissue sampled from cows in either treatment group. Detectable levels of the COX-1 isozyme were noted in endometrial tissue samples collected from both treatment groups at various stages of collection (Figure 4.4). However, no discernable pattern was noted between the detection of COX-1 and subsequent time of luteolysis in individual cows in either treatment.

**Discussion**

Parenteral administration of dexamethasone to diestrus cattle resulted in a significant increase in estrous cycle length, as was previously reported (Pool et al., 1983; Ingraham et al., 1984). Importantly, circulating progesterone profiles over the treatment cycle indicate that this lengthening of the estrous cycle was not due to an extension of corpus luteum function. Rather, the absence of an increase in circulating levels of estradiol in dexamethasone treated animals tends to indicate that follicle development was at least, in part, abated. This concept was further supported by the fact that dexamethasone treated animals required an extended 12-day period to reinitiate estrous cycle activity following luteolysis (progesterone $< 1$ ng/ml).
Figure 4.4. Western blot analysis for the presence of the COX-1 isozyme. Numbers within treatment brackets indicate individual cows. Solubilized extracts were prepared from uterine endometrium as described in the Materials and Methods section. Proteins (40 μg/lane) were analyzed by one-dimensional SDS-page and immunoblotting, using anti-COX antibody No. 9181 (see Sirois, 1994). Purified ovine prostaglandin G/H synthase (oPGS-1; 25 ng) was run as a standard. Markers on the left indicate the migration of molecular weight standards.
Previous reports suggest that the administration of dexamethasone to diestrus cattle affects the steroidogenic capabilities of both the growing follicle and the regressing corpus luteum. In vivo, excessive systemic dexamethasone and/or ACTH decreases gonadotropin secretion from the anterior pituitary (Johnson et al., 1982; Li and Wagner, 1983). Vighio and Liptrap (1990) noted that both the frequency and amplitude of LH pulses decrease with dexamethasone administration, thereby inhibiting follicle development and estradiol production. However, basal production of LH was not affected by dexamethasone production (Stoebel and Moberg, 1982; Vighio and Liptrap, 1990).

Ingraham et al. (1984) found that dexamethasone injections to mid-luteal phase heifers resulted in an extended corpus luteum lifespan over saline-treated heifers. It was hypothesized by the authors that dexamethasone acts at the level of the uterus, disallowing formation of the luteolytic substance prostaglandin $F_2\alpha$. These results, in conjunction with those described (Johnson et al., 1982; Li and Wagner, 1983), suggest that the ability of dexamethasone to extend the lifespan of the corpus luteum may be two-fold. The first may be suppression of gonadotropin pulses mediated by the hypothalamus and, in turn follicle development (Johnson et al., 1982), which is necessary for luteolysis to occur (Thatcher et al., 1986). It was suggested that pulsatile secretion of gonadotropins would be adversely affected; however, basal LH secretion would not be influenced, thus allowing for maintenance of the corpus luteum.
A second method by which dexamethasone might increase the lifespan of the corpus luteum is by inhibiting production of uterine Prostaglandin F₂α, as previously proposed in this laboratory (Ingraham et al., 1984). In the current study, it was hypothesized that luteolysis is associated with the induction of uterine COX-2 production, and that dexamethasone treatment would decrease production of the COX-2 isozyme and the subsequent increase in prostaglandin F₂α production that is usually associated with luteal regression. This proved not to be the case.

Different physiological mechanisms have been proposed to regulate the expression of the COX isozymes. The COX-1 isozyme is constitutively produced and believed to be involved in the homeostatic regulation of prostaglandin formation for normal gastric and vascular function (Dewitt et al., 1993). The COX-2 isozyme has been associated with "immediate response" situations, in which prostaglandins must be produced at a rapid rate in instances such as inflammation, ovulation (Dewitt et al., 1993), and possibly luteolysis. Since dexamethasone has been shown to specifically suppress production of the COX-2 isozyme in mitogen stimulated rats (Lee et al., 1992), it was hypothesized that uterine endometrial COX-2 production by cows receiving dexamethasone would be suppressed, while maintaining some level of COX-1 production. Conversely, in the absence of dexamethasone, the uterine endometrium of the control cows was expected to produce some level of COX-2 during the luteolytic phase of the bovine estrous cycle.
The COX-1 isoform was produced sporadically throughout the sampling periods by the uterine endometrium of cows in both the control and dexamethasone treatments. In contrast, the COX-2 isozyme was not detected in either the control or dexamethasone-treated cows. Sirois (1994) has reported the induction of the COX-2 isozyme in the preovulatory follicles of cows treated with human chorionic gonadotropin. However, in the preovulatory follicle, the kinetics of the COX-2 isozyme appear to be quite rapid, with only a short window of production between 18 to 26 hours following the human chorionic gonadotropin injection. In the present study, it is possible that the sampling periods may have been too infrequent to detect production of COX-2 in the uterine endometrium or that uterine COX-2 production is not involved in prostaglandin formation during luteolysis.

In summary, our study suggests that the extension of the bovine estrous cycle by daily injections of dexamethasone (days 13 through 22) is not dependant on an extension of corpus luteum function, but rather on delayed or impaired preovulatory follicle development, as evidenced from decreased circulating estradiol levels. Also, this study does not support the working hypothesis that induction of COX-2 is involved during luteolysis. However, we cannot exclude the possibility that the COX-2 isozyme may be transiently expressed in the uterus at the time of luteolysis and that its lack of detection in the present study could have resulted from infrequent sampling periods. Currently, our laboratory is examining the possibility that the COX-2 isozyme converts
arachidonic acid to prostaglandin in the corpus luteum itself, rather than in the bovine uterine endometrium.

References


CONCLUSIONS

For years there has been disputes on the mechanisms involved in mammalian ovulation and luteolysis. The experiments conducted document the effectiveness of a serum-free culture system as a tool to cultivate large numbers of bovine granulosa cells. This is especially important since it is widely accepted that constituents of serum alone are capable of inducing many cellular processes including steroidogenesis and prostaglandin formation. This serum-free culture system allowed for further study into the effects of Lhlike stimulation on prostaglandin F$_2$α secretion by bovine follicular granulosa cells.

Experiments (Chapter II) that were conducted using this serum-free culture system provided evidence that bovine granulosa cells are capable of producing large amounts of prostaglandin following forskolin stimulation. It is important to note that mural granulosa cells are capable of producing prostaglandin F$_2$α at similar in vivo times. Prostaglandin production was curtailed following the addition of dexamethasone to the medium, implicating induction of the COX-2 enzyme in this process. Finally, the results of in vitro studies (serum-free cultures of bovine granulosa cells with regulatory agents) closely mimicked those observed in vivo, and thereby provide models for the study of the molecular mechanisms of COX-2 gene expression.

By demonstrating that high levels of gonadotropins (hCG) are able to induce COX-2 production by granulosa cells of human preovulatory follicles before ovulation, as in rats and cows, the study performed in Chapter III demonstrates that the expression of COX-2
is a mechanism that has been conserved across species. This mechanism may have been preserved across species to regulate the synthesis of prostaglandins necessary for follicle ovulation to occur.

Interestingly, a marked difference in the time course of induction of COX-2 in rats and humans suggests that the control of COX-2 gene expression could play a pivotal role in defining the normal length of the ovulatory process across species. Moreover, the acute induction of COX-2 by gonadotropin in granulosa cells before ovulation remains a unique physiological event, during which the molecular mechanisms of COX-2 regulation may be readily studied in vivo or in vitro.

The exact mechanisms involved in the process of corpus luteum regression, which occurs towards the latter stages of the bovine estrous cycle, still remains unresolved. The current belief of the mechanisms involved with luteolysis in cattle still lie in the "old school" train of thought that prostaglandin transverses the broad ligament to the ovary and enclosed corpus luteum after being released from an estrogen primed uterus. However, the founding authors of the "counter current mechanism" themselves have since questioned the true physiological relevance of this mechanism. Also, later studies have suggested that there is no counter current exchange of prostaglandin from the uterine vein to the ovarian artery.

Based on the results of the experiment performed in Chapter IV, it appears unlikely that the COX-2 isoform is present in the cells of the uterine endometrium during the period
of luteolysis. Tissue extracts obtained from the bovine uterus during the luteolytic phase of the bovine estrous cycle indicate the absence of the COX-2 isoform at this critical time period (days 16, 19 and 22 of the bovine estrous cycle). Although the COX-1 isoform was noted to be present during this period, no discernable pattern of its production could be correlated with luteal regression in individual animals.

With the COX-2 isoform being recently discovered, it has been largely manipulated in unphysiological situations in attempts to quantify and classify its production. These studies included situations where the COX-2 isozyme was studied in following transfection into Swiss 3T3 and Cos-7 cells. It was the design of the studies included herein to provide a "physiological relevance" of this enzyme to certain reproductive processes. Although such studies have been thought to do so, it is important that further studies continue along these lines to help resolve the mechanisms involved in prostaglandin synthesis and regulation in reproductive and other biological processes.
VITA

James Robert Broussard was born on December 31, 1966, in Lafayette, Louisiana, the son of Dr. Robert Henry and Patricia June Broussard. The family moved to Denver, Colorado, between the years of 1974 and 1976, at which time they moved back to Louisiana and was raised in a suburb of Lafayette, in a home sandwiched between a horse farm and a Brahman ranch. He graduated from Ovey Comeaux High School in May, 1984, and then attended the University of Southwestern Louisiana as an undergraduate in the discipline of Animal Science. He received his Bachelor of Science degree in December of 1989.

In August of 1990 he entered the graduate school at Louisiana State University in the Department of Dairy Science under the direction of Dr. Joseph D. Roussel, and completed the requirements for the degree of Master of Science in field of Reproductive Physiology in May of 1992. On June 9, 1990, he married Devin Ann Briggs, who gave birth to a healthy baby boy named William Sayer Broussard on August 14, 1995.

Currently, he is a research associate, and is a candidate for the degree of Doctor of Philosophy in Reproductive Physiology in the Department of Dairy Science under the supervision of Dr. Joseph D. Roussel.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: James Robert Broussard

Major Field: Animal and Dairy Sciences

Title of Dissertation: An Attempt to Classify the Production and Regulation of Cyclooxygenase-2 During Ovulation and Luteolysis

Approved:

Major Professor and Chairman

Dean of the Graduate School

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March 6, 1996