INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Mechanisms of regulation of prostaglandin synthesis by cytokines in the bovine corpus luteum

Townson, David Harrison, Ph.D.

The Ohio State University, 1993
MECHANISMS OF REGULATION OF PROSTAGLANDIN SYNTHESIS BY CYTOKINES IN THE BOVINE CORPUS LUTEUM

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By

David Harrison Townson, B.S., M.S.

****

The Ohio State University

1993

Dissertation Committee

J.L. Pate
J.S. Ottobre
C.L. Brooks
J.D. Harder

Approved by

Adviser
Department of Dairy Science
To the love of my life, Lisa
ACKNOWLEDGEMENTS

Throughout my graduate program, I have been fortunate to have received the support, encouragement, and love of many people. I wish to express my sincere thanks to the faculty, staff, and students of the Department of Dairy Science. I can say with great honesty, this is the most caring and cohesive department I've been associated with in my college career.

I would like to especially thank my advisor, Dr. Joy Pate, for her guidance, patience, and friendship throughout my doctoral studies. She has challenged me to be a more critical thinker and has instilled in me the importance of creativity in scientific research. Her constant support and understanding of my scientific and personal endeavors is deeply appreciated. I thank Dr. Joe Ottobre and his wife, Ann, for scientific advice and the thought-provoking discussions of family life, computers, sports, etc. I will always cherish your friendship. I am also deeply indebted to Dr. Charles Brooks for his guidance and for allowing me the use of his laboratory throughout my dissertation research. I always felt welcomed and, at times, I truly felt as if I was one of his graduate students. Gratitude is
also expressed to Dr. John Harder for his service on my doctoral committees and his attempts to broaden my scope of knowledge beyond that of "farm animal" reproduction -- I hope I've made some progress.

The technical and secretarial contributions of Sandy Jones, Michelle Milligan, and Debbie Gallagher made the studies described in this dissertation possible. Many thanks to all of you.

My most heartfelt thanks belong to my family. I am grateful to my parents, my brother and two sisters, and my in-laws for providing the love, support, and encouragement throughout my graduate studies. I am thankful to my children, Kyle and Clint, who have given unwaivering love, joy, and a sense of perspective to my life. Finally, I offer my deepest thanks to my wife, Lisa. Her love and faith in me through the years has provided the strength to endure difficult times and the joy to appreciate good times. I only hope she allows me the rest of my life to express my gratitude.
VITA
David H. Townson

1961 ....................... Born - Dearborn, Michigan

1984 ....................... B.S., Michigan State University, E. Lansing, Michigan

1985-1988 ..................... M.S., University of Wisconsin, Madison, Wisconsin

1988-1993 ..................... Graduate Research Associate/Fellow, Reproductive Physiology, The Ohio State University, Columbus, Ohio

PUBLICATIONS


FIELDS OF STUDY

Major Field: Dairy Science
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>VITAE</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF PLATES</td>
<td>xvi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER I - Review of the Literature</td>
<td>3</td>
</tr>
<tr>
<td>Statement of Problem</td>
<td>27</td>
</tr>
<tr>
<td>CHAPTER II - Regulation of Prostaglandin Synthesis by Interleukin-1β in Cultured Bovine Luteal Cells</td>
<td>28</td>
</tr>
<tr>
<td>CHAPTER III - Mechanisms of Action of TNF-α-Stimulated Prostaglandin Production in Cultured Bovine Luteal Cells</td>
<td>52</td>
</tr>
<tr>
<td>CHAPTER IV - Characterization of the Eicosanogenic Potential of Cultured Bovine Luteal Cells Stimulated with Interleukin-1-beta and Tumor Necrosis Factor-alpha</td>
<td>74</td>
</tr>
<tr>
<td>CHAPTER V - Conclusions</td>
<td>101</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>110</td>
</tr>
</tbody>
</table>
Table 1. PGF$_2$$_a$ production by cultured bovine luteal cells exposed to vehicle, TNF-α, or IL-1β for 6 hours on Day 1 of culture (Day 1:6 hr) or for 78 hours (Days 0, 1, and 3; Day 3:6 hr).
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1. The effects of interleukin-1β and the protein synthesis inhibitor, cycloheximide, on prostaglandin $F_{2\alpha}$ production by cultured bovine luteal cells.</td>
<td>44</td>
</tr>
<tr>
<td>Figure 2. The effects of interleukin-1β, exogenous arachidonic acid, and the protein synthesis inhibitor, cycloheximide, on prostaglandin $F_{2\alpha}$ production by cultured bovine luteal cells.</td>
<td>45</td>
</tr>
<tr>
<td>Figure 3. The effects of interleukin-1β and the DNA transcription inhibitor, actinomycin D, on prostaglandin $F_{2\alpha}$ production by cultured bovine luteal cells.</td>
<td>46</td>
</tr>
<tr>
<td>Figure 4. The effects of the phospholipase A$<em>2$ inhibitor, aristolochic acid, and phospholipase C inhibitor, compound 48/80, on interleukin-1β-stimulated prostaglandin $F</em>{2\alpha}$ production by cultured bovine luteal cells.</td>
<td>47</td>
</tr>
<tr>
<td>Figure 5. The effects of exogenous arachidonic acid on interleukin-1β-stimulated and phospholipase A$<em>2$-inhibited prostaglandin $F</em>{2\alpha}$ production by cultured bovine luteal cells.</td>
<td>48</td>
</tr>
<tr>
<td>Figure 6. The effects of exogenous arachidonic acid on interleukin-1β-stimulated and phospholipase C-inhibited prostaglandin $F_{2\alpha}$ production by cultured bovine luteal cells.</td>
<td>49</td>
</tr>
<tr>
<td>Figure 7. Chronic versus acute effects of interleukin-1β on prostaglandin $F_{2\alpha}$ production by cultured bovine luteal cells in the presence of increasing concentrations of arachidonic acid.</td>
<td>50</td>
</tr>
</tbody>
</table>
Figure 8. Chronic versus acute effects of interleukin-1β on progesterone production by cultured bovine luteal cells in the presence of increasing concentrations of arachidonic acid.

Figure 9. The effects of murine recombinant tumor necrosis factor-alpha cycloheximide on prostaglandin F$_{2\alpha}$ production by cultured bovine luteal cells.

Figure 10. The effects of murine recombinant tumor necrosis factor-alpha and cycloheximide on progesterone production by cultured bovine luteal cells.

Figure 11. The effects of tumor necrosis factor-alpha, arachidonic acid and cycloheximide on prostaglandin F$_{2\alpha}$ production by cultured bovine luteal cells.

Figure 12. The effects of tumor necrosis factor-alpha and the DNA transcription inhibitor, actinomycin D, on prostaglandin F$_{2\alpha}$ production by cultured bovine luteal cells.

Figure 13. The effects of tumor necrosis factor-alpha, the phospholipase A$_2$ inhibitor, aristolochic acid, and the phospholipase C inhibitor, compound 48/80, on prostaglandin F$_{2\alpha}$ production by cultured bovine luteal cells.

Figure 14. The effects of tumor necrosis factor-alpha, arachidonic acid and phospholipase A$_2$ inhibitor, aristolochic acid, on prostaglandin F$_{2\alpha}$ production by cultured bovine luteal cells.

Figure 15. Effects of exogenous progesterone and estradiol on tumor necrosis factor-alpha, arachidonic acid, and TNF-α+AA-stimulated on luteal prostaglandin F$_{2\alpha}$ production.
Figure 16. Quantification of immunodectable prostaglandin endoperoxide (PGH) synthase in cultured luteal cells based on optical density using a volume integration procedure.

Figure 17. The effect of interleukin-1β on prostaglandin F₂α production by cultured bovine luteal cells.

Figure 18. The effects of phospholipase A₂ inhibitor and/or exogenous arachidonic acid on interleukin-1β-stimulated prostaglandin F₂α production.
## LIST OF PLATES

<table>
<thead>
<tr>
<th>PLATES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plate I.</strong></td>
<td>Validation of prostaglandin endoperoxide (PGH) synthase antibody specificity.</td>
</tr>
<tr>
<td><strong>Plate II.</strong></td>
<td>Immunodetectable prostaglandin endoperoxide PGH synthase expression in various bovine tissue samples.</td>
</tr>
<tr>
<td><strong>Plate III.</strong></td>
<td>The effects of tumor necrosis factor-α and interleukin-1β on prostaglandin endoperoxide (PGH) synthase expression in cultured bovine luteal cells.</td>
</tr>
</tbody>
</table>
INTRODUCTION

The corpus luteum is an endocrine gland that develops within the ovary of the female animal following ovulation and regulates reproductive function. The corpus luteum is unique in that, unlike any other endocrine gland, it is a transitory organ; it forms, functions, and regresses within the span of a single estrous or menstrual cycle. During its lifespan, the corpus luteum influences ovarian function and is essential for the maintenance of pregnancy.

The short-lived nature of the corpus luteum is perhaps its most interesting facet. Many investigators have characterized the histological and hormonal aspects of corpus luteum development and function. Others have attempted to understand its regulatory mechanisms. Despite past efforts, there is relatively little known about the control of luteal function at the time of regression (i.e., luteolysis). Understanding luteolysis and the mechanisms which regulate the process is of critical importance as a means to control reproduction in animals.

The research described in this dissertation was designed to examine the regulation of prostaglandin production in bovine luteal cells because prostaglandins may
be important for the complete elimination of the corpus luteum at the time of luteolysis.
CHAPTER I
Review of the Literature

HISTOLOGICAL ASPECTS OF THE CORPUS LUTEUM

The corpus luteum of most animal species is composed of small (<20μm) and large (>20μm) luteal cells, as well as a number of other nonluteal cell types. Small and large luteal cells are believed to originate from the thecal and granulosal compartments, respectively, of the ovulated follicle. Although the derivation of small and large luteal cells remains somewhat controversial [1], one study, which utilized monoclonal antibodies generated against specific cell antigens, demonstrated that thecal and granulosal cells differentiate into small and large luteal cells, respectively [2]. Small and large luteal cells differ not only in size, but also in steroidogenic capacity [3,4], morphology [5,6], and responsiveness to hormones [7,8,9,10,11,12,13,14,15,16]. These differences between small and large luteal cells most likely constitute a continuation of differentiated function between thecal and granulosal cells [17] from which the luteal cells are derived. However, the physiological relevance of different populations of luteal cells within the corpus luteum is not entirely clear. Several
researchers suggest that some form of cell-to-cell communication may exist between small and large luteal cells within the corpus luteum [8,18,19]. Nonetheless, the presence of mixed luteal cell populations has confounded the characterization of the regulatory mechanisms of corpus luteum function and regression. The identification of luteal control mechanisms is further complicated by changes that take place in the ratio of small:large cells throughout the lifespan of the corpus luteum [20,21] and the presence of nonluteal cells within the tissue.

Nonluteal cells may account for as much as eighty percent of the total number of cells which reside in the corpus luteum [20]. Fibroblasts, endothelial cells, eosinophils, mast cells, macrophages, and T lymphocytes have all been identified in corpora lutea of various species [22,23,24,25,26,27]. Unfortunately, the influence of nonluteal cells on corpus luteum function has not been adequately addressed. Future investigations, especially in vitro studies, should place more emphasis on defining the role(s) of nonluteal cells in luteal tissue.

The vasculature of the corpus luteum is perhaps the most fascinating histological aspect of the gland. It is composed principally of large, sinusoidal capillary networks which lack smooth muscle cells [28]. Interestingly, the endothelial cells which comprise the blood vessels of the corpus luteum are phenotypically diverse and represent all
segments of the microvascular bed [29]. The blood vessels are relatively short-lived compared to those of other tissues (e.g., less than 3 days in hamster corpora lutea; [30]) and, thus, it is thought that the phenotypic diversity of the endothelial cells may contribute to the development and subsequent regression of the vasculature [22,31,32,]. Here again is an example wherein nonluteal cells may profoundly affect corpus luteum function. Nonetheless, many aspects of endothelial function in luteal tissue remain unresolved. Only the phenotypic diversity of the endothelial cells appears to have been adequately addressed in recent years [29,33]. Specifically, endothelial cells of the corpus luteum exhibit broad heterogeneity and represent all microvessel segments of the vascular tree. Spanel-Borowski and van der Bosch [29] identified 5 types of endothelial cells within the bovine corpus luteum based on light and scanning electron microscopy. Types 1 and 2 exhibited morphological characteristics consistent with endothelial cells of arteriole and capillary origin, respectively. In contrast, the diversity of cell processes, specifically filopodia, in Types 3-5 distinguished these endothelial cells as being of postcapillary venule and venule origin.
HORMONAL PRODUCTION AND REGULATORY CONTROL
OF THE CORPUS LUTEUM

The principal hormonal product of corpus luteum function is progesterone. The substrate for progesterone biosynthesis is cholesterol, which in luteal cells, as in most steroidogenic cells, may be derived from de novo synthesis or plasma lipoproteins [34]. Of these two pathways, cholesterol derived from plasma lipoproteins constitutes the major source of substrate for steroidogenesis. The alternative pathway, de novo synthesis, is considered a secondary source of cholesterol, but is essential to such diverse cellular functions as electron transport and glycoprotein synthesis in most steroidogenic cells [35].

In the bovine corpus luteum, luteal cells are capable of utilizing cholesterol from either low or high density lipoproteins (i.e., LDL and HDL) [36,37,38]. The lipoproteins are brought into the cell by receptor-mediated endocytosis [39]. Cholesterol transport to the mitochondria is facilitated by microtubules [40]. Once inside the mitochondria, the cholesterol may be converted to pregnenolone by cytochrome P450 side-chain cleavage enzyme [41,42]. Pregnenolone may be further metabolized to form progesterone by 3β-hydroxysteroid dehydrogenase enzyme in the smooth endoplasmic reticulum of the cell [43,44]. In the primate and the rat, progesterone may be further
metabolized to form estrogen through a series of metabolic steps, including the conversion of androgen to estrogen by aromatase enzyme [45,46,47]. The steroids are processed through the golgi apparatus and either stored within the cell or released by exocytosis into the bloodstream [48].

In most species, the pituitary gonadotropin, luteinizing hormone (LH), is considered the primary stimulus of progesterone production in the corpus luteum [32]. The stimulation of steroidogenesis by LH occurs primarily via the cyclic adenosine monophosphate (cAMP)-mediated, second messenger pathway. However, there is recent evidence to suggest that another second messenger system, the calcium-mediated pathway, may also augment steroidogenesis [49]. In either second messenger system, receptor-effector coupling is regulated by G proteins [50]. Consequently, there is stimulation of lipoprotein uptake [32] and cholesterol metabolism [51]. The latter is achieved primarily through the actions of the cytochrome P-450 side chain cleavage complex [52]. Similar mechanisms of supportive function have been ascribed to estrogen and prolactin in luteal tissue of nonruminant species [46,53,54,55].

Although the evidence of LH as the primary trophic hormone in corpus luteum function is compelling, researchers have been unable to explain why progesterone production increases as LH concentrations decrease during corpus luteum
development [56]. In addition, it is unclear why large luteal cells, which account for most of the progesterone production by the corpus luteum, are relatively insensitive to LH stimulation [3,6,48]. Clearly, there must be other factors, in addition to LH, which modulate corpus luteum function.

In addition to progesterone, the corpus luteum is a considerable source of arachidonic acid (AA)[57], the majority of which is sequestered in the membrane phospholipids of the luteal cells. It is not clear how the corpus luteum obtains AA, but one possibility is that AA is derived from lipoproteins utilized during progesterone synthesis. Lipoproteins are a good source of AA and AA precursors [58]. Furthermore, luteal cells can bind AA directly [59]. Thus, it is possible for the corpus luteum to accumulate AA directly or through biosynthetic mechanisms. It is unclear why the corpus luteum accumulates such a vast supply of AA during its lifespan, but it may serve to facilitate corpus luteum regression [7,58,60].

It has also been proposed that AA within the corpus luteum may be metabolized through cyclooxygenase and lipoxygenase pathways to produce factors which affect luteal function [61,62]. Among the many AA metabolites possibly produced by the corpus luteum, prostaglandins (which are products of the cyclooxygenase pathway) have received the most attention [19,61,63].
Prostaglandins have been ascribed supportive and inhibitory roles with regard to corpus luteum function [64, 65, 66, 67, 68, 69]. However, there is a considerable lack of information regarding the regulation of AA metabolism in the corpus luteum. Furthermore, there have been few attempts to address the relevance of AA release and metabolism within the corpus luteum despite the prevalence of AA and AA metabolites in luteal tissue.

Immunoregulatory peptides, also known as cytokines, may regulate corpus luteum function in a paracrine or autocrine fashion. Recent evidence of the cytokine, tumor necrosis factor-alpha (TNF-α), in the corpus luteum is indicative that immunological mechanisms may play a role in luteal function [70]. There is also evidence that other cytokines, such as interleukin-1β (IL-1β), interferon-alpha (IFNα), and interferon-gamma (IFN γ), may be involved in corpus luteum regression [71, 72, 73]. Specifically, it has been proposed that an immunological response is an integral part of luteolysis [26, 74].

Growth factors may constitute an intraovarian source of regulation of corpus luteum function. The recent interest in growth factors stems from evidence that these small peptides possess biological actions beyond that for which they were originally identified [75]. To date, evidence of the following growth factors has been demonstrated in luteal tissue: 1) insulin-like growth factor I (IGF-I) [76, 77, 78],
2) basic fibroblast growth factor (bFGF) [79, 80, 81, 82],
3) transforming growth factor-beta (TGF-β) [80, 83, 84],
4) epidermal growth factor (EGF) [85], and 5) vascular
endothelial growth factor (VEGF) [86].

The definitive actions of growth factors in luteal
tissue requires further examination. Future studies need to
address inconsistencies among early investigations. This is
especially true of *in vitro* studies wherein cell-to-cell
contact and communication may be critical for an accurate
description of the actions of a particular growth factor
[80]. Certain growth factors, like bFGF and VEGF, appear to
stimulate corpus luteum development by enhancing endothelial
cell proliferation and angiogenesis [81, 82, 86]. Others,
like IGF-I and TGF-β, affect steroidogenesis and, thus, may
influence differentiated function of luteal cells [77, 84].
Collectively, growth factors may be viewed as permissive
regulators of corpus luteum function since their paracrine
and/or autocrine actions appear secondary to that of
gonadotropin support.

**CORPUS LUTEUM REGRESSION**

**Functional and Structural Considerations**

Corpus luteum regression is distinguished by two
phases: 1) functional regression, and 2) structural
regression. Functional regression is defined as a
significant decline in progesterone production. In
contrast, structural regression is somewhat equivocally defined. Degenerative changes in endothelial cells [87,88], presence of autophagic vacuoles in the tissue [89], or a decline in mass of the CL [90,91] are all examples of what has been referred to as structural regression.

It is evident from earlier discussions of hormone production and regulatory control of the corpus luteum that establishing a temporal, or cause-effect, relationship between functional and structural regression is difficult. Clearly the characterization of each aspect of regression is dependent upon the endpoint selected in a given experiment. Bearing this in mind, the next several paragraphs are devoted to a discussion of functional and structural regression of the corpus luteum without reference to temporal relationships which may or may not exist.

Initially, functional regression was postulated to result from a decline in trophic support. For example, the suppression of pituitary gonadotropins, specifically LH, results in decreased plasma progesterone concentrations and premature cyclicity [92]. Withdrawal of estrogen in pseudopregnant rabbits attenuates progesterone production [53]. However, when trophic support is re-established (i.e. gonadotropins or estrogen re-administered, respectively), there is resumption of progesterone secretion and normal lifespan of the corpus luteum [53,93]. Thus, although trophic factors sustain CL function, a transient loss of
trophic support does not predispose the corpus luteum to regression. Furthermore, the observation that LH concentrations are adequate to support other aspects of ovarian function during luteolysis (e.g., growth of the preovulatory follicle) suggests that a loss of trophic support is not a mechanism of functional regression.

Functional regression may occur if luteal cells become less responsive to trophic support. This may result from diminished binding of trophic factors to luteal receptors [94] or functional modification of the hormone-receptor complex during luteolysis [49]. Other studies reveal that luteal function is diminished by mechanisms independent of the generation of cAMP, specifically those involving the metabolism of cholesterol to progesterone [36,95,96]. In addition, the calcium-mediated, second messenger pathway is of particular interest because of its disparate effects on luteal progesterone production [11,97,98]. Future research efforts will hopefully resolve some of the complexities of second messenger systems in luteal cells and clarify the role of second messengers in aspects of functional regression.

Degenerative effects to the blood vasculature and its constituents appear to be among the earliest indications of structural regression [87,88]. Originally, blood flow to the corpus luteum was thought to be altered, specifically decreased, at the time of luteolysis. However, recent
evidence suggests that decreases in luteal progesterone production are not accompanied by acute changes in luteal blood flow. [99,100]. Rather, any decrease in luteal blood flow is perceived to be a consequence of degeneration rather than vasoconstriction of blood vessels [56]. The degenerative effects are presumably attributable to ischemic conditions which develop as a result of diminished blood flow [101]. Generally, ischemic injury is characterized by disturbances in plasma membrane function and biochemical alteration of the cell. Specifically, the cell is compromised by accelerated turnover of membrane phospholipids and dramatic influxes of extracellular calcium [101]. In the corpus luteum, initial evidence of ischemia includes condensation and fragmentation of nuclear and cytoplasmic constituents of endothelial cells [87]. Presumably, as the vasculature deteriorates, other cell types, including luteal cells, degenerate as a result of ischemic conditions [102,103].

Additional signs of structural regression of the corpus luteum include: rapid infiltration of macrophages and lymphocytes [26]; increased numbers of lysosomes in luteal cells [104]; and evidence of autophagy and heterophagy within the luteal tissue [89]. The presence of self-destructive elements within regressing luteal tissue (i.e., autophagy) is perhaps the basis of recent studies which suggest that luteolysis may occur by programmed cell death,
also known as apoptosis [105], or by autoimmune-response mechanisms [106].

In summary, the loss of luteal blood flow, the degenerative effects to endothelial cells and luteal cells, and the predominance of autophagic and heterophagic mechanisms within the tissue, all contribute to the structural regression of the corpus luteum. Structural regression of the CL leads to a decline in luteal mass and, ultimately, the elimination of the gland.

Current thinking about the relationship between functional and structural aspects of corpus luteum regression belie the nature of the physiological process. Signals that initiate functional regression are perceived to be of primary importance, yet, as previously shown, the effects of diminished progesterone production alone are reversible [53,93]. Undoubtedly, functional and structural aspects of regression are inextricably linked. Therefore, future studies should address the entire process of luteolysis and focus on mechanism(s) of regression which mediate the inexorable destruction of the gland.

In the following sections, current hypotheses regarding the mechanism(s) of corpus luteum regression are presented, including a discussion of the role of prostaglandins and the immune system in this process.
Role of Prostaglandins

Prostaglandins are hormonal agents synthesized by virtually all mammalian tissues and influence tissue function in both an autocrine and paracrine fashion [107,108]. Classically, prostaglandin biosynthesis is divided into three phases: 1) mobilization of arachidonic acid (AA) from cell membrane phospholipids, 2) conversion of arachidonate to an endoperoxide intermediate, prostaglandin H$_2$ (PGH$_2$), and 3) rearrangement or reduction of PGH$_2$ to yield one of the biologically active prostanoids (PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, PGI$_2$, or TXA$_2$;[107]). Prostaglandin E$_2$, prostacyclin (PGI$_2$), and prostaglandin F$_2$ (PGF$_{2\alpha}$) are among the prostaglandins of primary interest to reproductive biologists. The actions of PGE$_2$ and PGI$_2$ are thought to enhance luteal function [63,109,110], primarily through stimulation of cAMP-mediated second messengers. In essence, the effects of PGE$_2$ and PGI$_2$ augment those of LH. Conversely, the effects of PGF$_{2\alpha}$ are primarily mediated through a calcium-dependent second messenger [11,111,112] and are generally inhibitory to corpus luteum function.

Regulation of prostaglandin production may occur at any of the aforementioned levels of biosynthesis. However, acute regulation is thought to occur at the level of AA mobilization. Activation of phospholipase enzymes, specifically phospholipase A$_2$ (PLA$_2$), are of critical
importance since they cleave AA from membrane phospholipids [113]. However, prostaglandin production is also dependent on enzymatic metabolism of AA. Prostaglandin endoperoxide synthase (PGH synthase), also known as cyclooxygenase, is responsible for the two-step conversion of AA to PGH₂ [114]. Concentrations of PGH synthase vary considerably among cell types and may fluctuate in response to various humoral factors [107]. Thus, regulation of PGH synthase may serve as a chronic form of regulation. At the third level of prostaglandin biosynthesis, the biologically active prostanoids are formed from either the enzymatic (PGI₂ and TXA₂) or nonenzymatic (PGD₂, PGE₂, and PGF₂α) conversion of PGH₂ [107]. Regulation of prostaglandin synthesis at this level, therefore, is dependent upon the prostanoid of interest.

Alternatively, AA may be metabolized through lipoxygenase or epoxyxygenase pathways [108,115,116]. The products of these two metabolic pathways have received little attention in reproductive biology. The exceptions include: 1) Leukotriene B₄, which is postulated as a chemoattractant for immune cells during luteolysis [117,118], and 2) 5-HETE, which is thought to suppress progesterone production by the corpus luteum [61,119]. Both leukotriene B₄ and 5-HETE are products of the lipoxygenase pathway and are produced by luteal tissue. Collectively,
the metabolites of PGH synthase, lipoxygenase, and epoxyxygenase pathways are referred to as eicosanoids.

In the following paragraphs, current knowledge of the role(s) of prostaglandins in luteal function is detailed. Both uterine- and luteal-derived forms of prostaglandins are discussed. In addition, information regarding the regulatory control of prostaglandin synthesis is provided when possible.

Pulsatile secretion of PGF$_{2\alpha}$ from the uterus initiates corpus luteum regression in most domestic livestock [56,120]. Prostaglandin F$_{2\alpha}$ travels from the uterus to the corpus luteum by a mechanism of vascular, countercurrent transfer [121]. The prostanoid diffuses from uterine vein to ovarian artery, reaching the corpus luteum through the ovarian vasculature.

In sheep, pulsatile secretion of PGF$_{2\alpha}$ is believed to develop from positive feedback between the uterus and corpus luteum [122]. A positive feedback loop is initiated by PGF$_{2\alpha}$ secretion from the uterus which, in turn, stimulates oxytocin release from the corpus luteum [123,124]. Oxytocin subsequently triggers additional PGF$_{2\alpha}$ production from the uterus. Collectively, these events are postulated to result in regression of the corpus luteum [125,126].

Pulsatile secretion of PGF$_{2\alpha}$ and oxytocin is established by periodic desensitization of uterine and luteal tissues to stimulus [122]. The refractory period,
however, is short-lived (approximately 6 hours) and, thus, pulsatile release of PGF$_{2\alpha}$ and oxytocin occurs throughout the luteolytic period. The patterns of pulsatile secretion are demonstrable primarily during the late luteal phase [127,128]. Thus, evidence of a uterine-luteal feedback loop during luteolysis is further substantiated.

In cattle, the physiological role of ovarian oxytocin during luteolysis is less certain. In a recent study [129], oxytocin secretion in mature heifers was stimulated by noradrenaline infusions during the mid- and late-luteal phase. Noradrenaline challenge reduced endogenous concentrations of oxytocin in corpora lutea by approximately 75%, but did not trigger spontaneous luteolysis or shorten the duration of the estrous cycle. The authors concluded that ovarian oxytocin may have a permissive, rather than direct, role in luteolysis in cattle. More importantly, however, their observations also argue against the possibility of a PGF$_{2\alpha}$-oxytocin positive feedback loop for initiating luteolysis in cattle.

Uterine PGF$_{2\alpha}$ biosynthesis is at least partially regulated by progesterone and estradiol. These steroids enhance prostaglandin production in the uterus at several levels. Progesterone stimulates phospholipid accumulation [130], phospholipase activity [131], and PGH synthase mRNA expression [132] within the endometrium. Similarly, estradiol stimulates PLA$_2$ activity [133] and PGH synthase
activity [134]. Thus, together, progesterone and estradiol provide an endocrine environment within the uterus conducive to stimulation of prostaglandin production. The signal responsible for initiating PGF$_{2\alpha}$ release from the uterus, however, is not known [122].

Detrimental effects of uterine PGF$_{2\alpha}$ on corpus luteum function remain unclear. One hypothesis holds that uterine PGF$_{2\alpha}$ reduces luteal blood flow since PGF$_{2\alpha}$ is a potent vasoconstrictor [135]. However, decreased blood flow to the corpus luteum is postulated to be an indirect consequence of capillary degeneration rather than PGF$_{2\alpha}$-induced vasoconstriction [56]. This is based on evidence that endothelial cells within the corpus luteum degenerate after PGF$_{2\alpha}$-induced luteolysis [136] and that luteal capillaries lack smooth muscle cells [28]. Direct, cytotoxic effects of PGF$_{2\alpha}$ on endothelial cells of the corpus luteum have not yet been demonstrated.

An alternative action of uterine PGF$_{2\alpha}$ is thought to involve direct effects of PGF$_{2\alpha}$ on luteal cells. Prostaglandin F$_{2\alpha}$ inhibits both LH- and lipoprotein-stimulated P$_4$ production by luteal cells in vitro [36,95,96]. Prostaglandin F$_{2\alpha}$ causes chromatin decondensation in luteal cells [137]. In addition, a direct cytotoxic effect of PGF$_{2\alpha}$ on ovine luteal cells, albeit only large luteal cells, has been observed [138].
Both functional and cytotoxic effects of PGF$_{2\alpha}$ on luteal cells may be mediated by the calcium-dependent, second messenger pathway. Briefly, PGF$_{2\alpha}$ activates phospholipase C which, in turn, cleaves inositol trisphosphate (IP$_3$) and diacylglycerol (DAG) from membrane phospholipids [139,140,141]. Release of IP$_3$ triggers an increase in intracellular free calcium [142], whereas DAG, with calcium as a co-factor, stimulates protein kinase C [143]. Phosphorylation events associated with the activation of protein kinase C are believed to inhibit progesterone production by the luteal cells [98,144]. In contrast, IP$_3$-mediated, sustained elevations of intracellular free calcium are thought to initiate cytotoxic effects [7,10].

Interestingly, Hansel and co-workers [7] noticed that many of the effects of PGF$_{2\alpha}$ are mimicked by the addition of arachidonic acid to luteal cells. Arachidonic acid injected directly into the corpus luteum [59] or infused into a branch of the ovarian artery of heifers in which all vascular connections between the ovary and uterus have been severed [145] results in regression of the corpus luteum. These results raise the possibility that arachidonic acid and/or its metabolites may influence corpus luteum function and indirectly indicate that the corpus luteum of the cow is capable of perpetuating its own regression [7].
The observation that the corpus luteum contains a tremendous amount of arachidonic acid (approximately 3 mg/g of tissue; [57]) is suggestive that prostaglandins of luteal rather than uterine origin may contribute to luteal function. Luteal tissue is capable of metabolizing AA [146,147,148] and binding various eicosanoids [149,150]. Luteal function in cattle is postulated to be guided by the delicate balance of luteotrophic (PGE₂ and PGI₂) and luteolytic (PGF₂α and 5-HETE) eicosanoids produced within the corpus luteum [14,61,65]. Similar mechanisms of AA metabolism may control luteal function in primates [148,151,152], particularly because in primates the uterus does not initiate luteolysis [153]. Also, recent studies in the rat [154,155] indicate luteolysis may be an intrinsic property of the corpus luteum, controlled by endogenously derived PGF₂α, whereas the uterus mediates aspects of maternal recognition of pregnancy and subsequent luteal function. Collectively, there is compelling evidence for involvement of endogenously-derived AA and AA metabolites in corpus luteum function, including luteal regression: 1) the corpus luteum is a tremendous source of AA, 2) the corpus luteum is capable of metabolizing AA, 3) in most tissues, AA and AA metabolites are derived from, and act upon, the same tissue (suggestive of autocrine/paracrine, rather than endocrine, mechanisms of action), 4) luteal cells are capable of binding AA metabolites and have high affinity.
receptors specific for PGF$_2$α, PGI$_2$, and PGE$_2$, 5) AA and AA metabolites directly affect luteal cell function in vitro, and 6) endogenously-derived AA metabolites are believed to initiate regression of the corpus luteum in some animals (particularly those species in which the uterus, and thus uterine-derived PGF$_2$α, is not responsible for luteolysis).

There is little information regarding the regulatory control of AA release and metabolism in luteal tissue. Recent studies indicate, however, that secretory products of immune cells, or cytokines, may modulate AA metabolism, primarily prostaglandin biosynthesis, within the ovary [71,73,156]. Within the corpus luteum, secretion of cytokines and AA metabolites may constitute an important mechanism for the progression of luteolysis. This and other immunological aspects of corpus luteum regression will be discussed in subsequent sections.

Role of the Immune System

Immunological events are hypothesized to control regression of the corpus luteum [26,74,157]. This hypothesis is substantiated by several critical studies. Although macrophages and/or lymphocytes are present in corpora lutea throughout the luteal phase [26,158,159,160,161], there is a considerable increase in the number of T lymphocytes and macrophages prior to the onset of luteolysis [26]. Immune cell activation is facilitated by cell surface proteins known as major
histocompatibility complex (MHC) molecules [162]. In the corpus luteum, expression of MHC molecules on luteal cells is increased coincident with the time of regression [106]. As macrophages and T lymphocytes become activated within a tissue they secrete peptides known as cytokines which potentiate the immune response. Luteal cells are susceptible to the cytotoxic effects of cytokines in vitro [71]. In addition, cytokines inhibit luteal cell steroidogenesis [71,74] and dramatically stimulate luteal prostaglandin production [71,73,74]. Thus, immune cells and the cytokines they secrete may play an integral role in both functional and structural aspects of luteolysis.

A predominant role of macrophages during regression of the corpus luteum is to phagocytize cells and cell remnants. Paavola [160], in studies utilizing electron microscopy, noted that macrophages were capable of engulfing luteal cells in their entirety. Interestingly, the luteal cells showed no visible signs of deterioration, but were considered functionally senescent because they lacked steroidogenic activity. Debris-laden macrophages have been observed in regressing corpora lutea of sheep as well [163]. Thus, by virtue of their heterophagic activity, macrophages constitute an integral part of structural regression of the corpus luteum.

Understanding the role of MHC protein expression on luteal cells may offer some insight into the mechanisms of
initiation and specificity of regression. The predominant form of MHC molecule expressed by luteal cells may dictate the nature of the immunological response. For example, expression of Class I MHC molecules on tumor cells mediates recognition by T-cytotoxic lymphocytes and, thus, facilitates tumor regression [164]. Similarly, cytokine-induced expression of Class I MHC molecules on luteal cells may target the corpus luteum for destruction [71]. Endothelial cells may also be susceptible to this form of attack from immune cells [165]. Alternatively, aberrant expression of Class II MHC molecules is thought to be responsible for a number of autoimmune disorders [166,167]. Since Class II MHC protein expression on luteal cells is increased at the time of luteolysis, Benyo and co-workers [106] hypothesized that the demise of the corpus luteum may occur as a consequence of autoimmune-response mechanisms.

As previously noted, many of the luteolytic effects of immune cells may be mediated through the actions of cytokines. The ability of certain cytokines to profoundly stimulate luteal prostaglandin production may be of particular significance [71,73,74]. The cytotoxic effects of cytokines [168,169,170,171] are thought to be mediated by mechanisms of arachidonic acid release and/or metabolism [168,172,173,174,175]. Specifically, cytokines are known to upregulate phospholipases [176,177,178] and PGH synthase [179,180,181]. Similar mechanisms of cytokine-induced
phospholipase and/or PGH synthase stimulation may take place in luteal cells to augment luteolysis. Recent evidence of increased phospholipase A2 activity in luteal tissue at the time of corpus luteum regression [182,183,184] tends to substantiate such speculation. In addition, the generation of reactive oxygen species (i.e., hydrogen peroxide, superoxide anions, or hydroxyl radicals) in luteal cells is accompanied by stimulation of phospholipase A2 activity [182]. In this context, cytokine-stimulated luteal cells, in addition to macrophages, may be responsible for the generation of reactive oxygen species postulated to mediate luteolysis [185].

Of final note is the observation that eicosanoids modulate activation of immune cells and cytokine secretion [186,187,188]. In this context, luteal-derived prostaglandins may modulate the extent of an immunological response during luteolysis. Luteolysis may be envisioned as a process wherein cytokine-induced cytotoxicity of luteal cells perpetuates luteal regression and is accompanied by an upregulation of prostaglandin biosynthesis. Luteal prostaglandins, in turn, may enhance or prohibit further activation of immune cells and cytokine secretion, and thus modulate luteolytic, immune-response mechanisms. In essence, a feedback loop between immune cells and luteal cells may be established in which cytokines and prostaglandins participate as paracrine/autocrine
regulators. Thus, endogenous cytokines and prostaglandins may facilitate luteolysis in those species in which the role of the uterus is questionable. In others, the cytokine-prostaglandin feedback loop may serve as a highly responsive mechanism to control the progression of luteolysis.
STATEMENT OF PROBLEM

Many aspects of regression of the corpus luteum remain poorly understood, particularly those which entail the role of prostaglandins and cytokines in this process. The studies presented herein were designed to identify mechanisms by which cytokines, specifically interleukin-1-beta and tumor necrosis factor-alpha, influence luteal prostaglandin production. The aforementioned cytokines were selected primarily because synthesis and secretion of these two cytokines within ovarian tissue has been previously demonstrated. In addition, these cytokines are cytotoxic in endocrine tissues [172,189] and, to some extent, the cytotoxic effects are directly or indirectly related to prostaglandin production [174,175,177]. Prostaglandins may stimulate and/or attenuate further secretion of cytokines by immune cells and, thus, serve as immunomodulators. In this context, prostaglandin production and cytokine production within the corpus luteum may collectively mediate communication between luteal cells and immune cells to ensure complete and irreversible regression. The present studies explore one side of this proposed communication loop, namely, the cellular actions of cytokines on prostaglandin production by luteal cells.
CHAPTER II
Regulation of Prostaglandin Synthesis by Interleukin-1β in Cultured Bovine Luteal Cells

SUMMARY

Prostaglandins produced within the corpus luteum may serve as local modulators of corpus luteum function. The present study was designed to characterize the cellular mechanisms by which the cytokine, interleukin-1β (IL-1β), stimulates prostaglandin production in cultured luteal cells. Cycloheximide and actinomycin D did not affect basal, but completely inhibited IL-1β-stimulated prostaglandin F$_{2α}$ (PGF$_{2α}$) production (p<.05). The phospholipase A$_2$ inhibitor, aristolochic acid (PLA$_2X$), and phospholipase C inhibitor, compound 48/80 (PLCX), suppressed IL-1β-stimulated (p<.05), but not basal, PGF$_{2α}$ production. The addition of exogenous AA to phospholipase-inhibited cultures circumvented the inhibitory effect of PLCX, but not PLA$_2X$, suggesting that phospholipase A$_2$ is a key regulatory point of IL-1β action. Chronic exposure of the luteal cells to IL-1β resulted in stimulatory effects beyond that of increasing AA availability, presumably by upregulation of prostaglandin endoperoxide (PGH) synthase. Chronic exposure
of luteal cells to IL-1β also inhibited progesterone production, but this effect appeared to be independent of endogenous PGF$_{2\alpha}$ production. The ability of IL-1β to comprehensively stimulate luteal PGF$_{2\alpha}$ production while inhibiting luteal progesterone production is suggestive that IL-1β may facilitate regression of the corpus luteum.

**INTRODUCTION**

Pulsatile secretion of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) from the uterus is believed to initiate regression of the corpus luteum in most domestic animals [120,122]. However, the mechanism(s) by which uterine PGF$_{2\alpha}$ affects luteal function remains unresolved. In addition, there is no indication that PGF$_{2\alpha}$ secretion from the uterus initiates luteal regression in primates [153]. Based on these observations, it is possible that factors within the ovary contribute to the process of luteolysis.

Prostaglandins produced within the luteal tissue may be involved in regression of the corpus luteum. A role for luteal prostaglandin secretion and its effect on corpus luteum function has been suggested in cattle [61,63,65,190], rabbits [191], and monkeys [64,67,192]. However, little is known regarding the factors which regulate prostaglandin production within the corpus luteum. Gonadotropins stimulate prostaglandin biosynthesis in preovulatory follicles [193], and steroids modulate prostaglandin
production in uterine tissue [122]. However, other than an inhibitory effect of progesterone on luteal prostaglandin production in vitro [194], there is little information available regarding the factors which regulate prostaglandin biosynthesis in the corpus luteum.

Recently, the cytokine, interleukin-1β (IL-1β), has been implicated as a paracrine regulator of ovarian prostaglandin biosynthesis [156]. IL-1β is expressed in ovarian tissue [195], and is known to stimulate ovarian [156] and luteal [73] prostaglandins. Endothelial cells, fibroblasts, and macrophages secrete IL-1β [170], and all of these cell types have been identified in luteal tissue [24,26]. Thus, intraovarian secretion of IL-1β may constitute a mechanism of corpus luteum regression in certain species by augmenting luteal prostaglandin production. However, the cellular actions of IL-1β and the extent to which IL-1β potentiates prostaglandin biosynthesis in luteal cells has not been examined.

In the present study, the importance of gene expression, protein synthesis, and phospholipase activity for IL-1β-stimulated PGF$_{2\alpha}$ production in bovine luteal cells was determined. The present findings reveal that gene activation and protein synthesis are necessary for IL-1β-stimulated PGF$_{2\alpha}$ production. These events appear to be linked with the stimulation of phospholipases, specifically phospholipase A$_2$ (PLA$_2$), or a protein required for
phospholipase activation. In addition, evidence is presented which indicates that prostaglandin endoperoxide (PGH) synthase may be stimulated by chronic exposure to IL-1β.

**MATERIALS AND METHODS**

**Materials**

Powdered Ham’s F-12 culture medium was obtained from Mediatech (Washington, D.C.). Dialyzed calf serum was purchased from Hazelton Research Products (Lenexa, KS) and gentamicin was obtained from Gibco Labs (Grand Island, NY). Collagenase (Type I) was acquired from Worthington Systems (Freehold, NJ) and an insulin-transferrin-selenium premix (ITS) was purchased from Collaborative Research Products (Lexington, MA). BSA (Fraction V), HEPES, and Tris salt were obtained from Sigma Chemical Co. (St. Louis, MO). Twenty-four-well tissue culture plates and centrifuge tubes were purchased from Corning Glass Works (Corning, NY). Arachidonic acid was obtained from Cayman Chemical Co. (Ann Arbor, MI). Aristolochic acid and Compound 48/80 were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). The bovine LH (NIAMMD-bLH-4) was supplied by the National Hormone and Pituitary Program (Baltimore, MD). (1,2-3H) progesterone was purchased from New England Nuclear (Boston, MA); and [3,6,9,11,12,13,15-3H(N)] prostaglandin F₂α was obtained from Amersham (Arlington Heights, IL). The
antiprostaglandin F$_{2\alpha}$-keyhole limpet hemocyanin was kindly provided by Dr. Richard Fertel (The Ohio State University, Columbus, Ohio); Dr. Gordon Niswender (Colorado State University, Fort Collins, CO) donated the antiprogesterone-11-bovine serum albumin (GDN-337). Recombinant bovine interleukin-1β (IL-1β) was a generous gift from American Cyanamid Co. (Princeton, NJ) and had biological activity of $12.5 \times 10^6$ U/mg.

**Methods**

Corpora lutea (CL) were obtained from regularly cycling, non-lactating dairy cows. Each CL was removed between days 9-12 of the estrous cycle (day 0 = standing estrus) using a transvaginal procedure previously described [38]. Once removed, the CL were placed in HEPES-buffered, Ham's F-12 culture medium (culture medium) and transported to the laboratory on ice for further processing.

Under aseptic conditions, the luteal tissue was dissociated into cell suspensions using techniques previously described [38]. Briefly, the luteal tissue was minced into cubes (~1 mm$^3$) and enzymatically dispersed by two, one-hour dissociations in which culture medium containing collagenase (2000 Units/gram of tissue), 0.5% bovine serum albumin (BSA), and gentamicin (20μg/ml) was used. The dispersed cells were washed 3-4 times to remove residual BSA and collagenase. The washing procedure entailed repeated centrifugation and resuspension of the
cell pellet with serum-free culture medium containing gentamicin. Washed cells were subsequently counted in a hemocytometer; viability was determined by trypan blue exclusion.

Approximately $3 \times 10^5$ luteal cells/well were placed in serum-coated 24-well plates containing a total volume of 1 ml culture medium supplemented with insulin (5\(\mu\)g/ml), transferrin (5\(\mu\)g/ml), selenium (5 ng/ml), gentamicin (20 \(\mu\)g/ml), and LH (2.5 ng/ml). The cultures were incubated at 37°C in 5% CO\(_2\) and 95% air for a period of 30 hours. The culture medium was replenished after 24 hours and treatments, performed in duplicate, were added for an additional 6 hours. In the last experiment, cells were cultured and treated for a period of up to three days to examine chronic versus acute effects of IL-1\(\beta\). During this 3-day period, fresh medium and treatments were added after 24 and 48 hours. The conditioned medium from all experiments was collected six hours after the final treatment and stored at -20°C until radioimmunoassay (RIA). Cells were counted on the last day of culture using a microscope equipped with an ocular micrometer grid. All hormone data were corrected for live cell number and normalized to $5 \times 10^4$ cells. Each experiment was repeated 5 to 7 times. The luteal cells used for each repetition were derived from an individual CL (i.e., n=total number of CL per experiment; shown in figure legends).
The first experiment was designed to evaluate the importance of protein synthesis for IL-1β action. Luteal cell cultures were treated with/without IL-1β (2.5 ng/ml) and with/without a protein synthesis inhibitor, cycloheximide (CHX; 10μg/ml). In addition, exogenous arachidonic acid (AA; 1μg/ml) was added to cell cultures, in combination with the aforementioned treatments, to determine the role of prostaglandin substrate availability in the context of CHX and IL-1β action.

A DNA transcription inhibitor, actinomycin D (Act D; 5 μg/ml), was used in the second experiment to determine the importance of gene activation for IL-1β action. Again, cell cultures were treated with/without IL-1β and with/without Act D for a period of six hours.

In the third experiment, IL-1β, AA, and phospholipase inhibitors were used to examine the role of phospholipases, and thus substrate availability, in IL-1β-stimulated PGF₂α production. A phospholipase A₂ inhibitor, aristolochic acid (PLA₂X, 7.5 μg/ml) and a phospholipase C inhibitor, Compound 48/80 (PLCX, 4 μg/ml), were used to inhibit the release of AA from membrane phospholipids. Each inhibitor was added to cell cultures alone or in combination with IL-1β and/or AA.

The last experiment was designed to examine chronic versus acute effects of IL-1β on PGF₂α production by cultured bovine luteal cells. The ability of the luteal cells to respond to increasing concentrations of exogenous
AA in the presence of IL-1β was also studied. Treatment groups consisted of cell cultures treated with IL-1β on days 0, 1, and 3 of culture (chronic group) or on day 3 only (acute group). The culture medium was replenished in both groups on days 1 and 3. In addition, both groups were administered 0, .05, .5, 5.0, 50, or 100 μg/ml AA during the last two hours of culture.

Radioimmunoassay

Prostaglandin F$_{2a}$ and P$_4$ were quantified by RIA of unextracted samples of conditioned medium. Prostaglandin E$_2$ and 6-Keto-PGF$_{1a}$ (the stable metabolite of prostacyclin) were not measured in the present study because the profiles of these prostaglandins have been previously reported to parallel that of PGF$_{2a}$ in IL-1β-stimulated luteal cells [73]. The cross-reactivity of the antisera with other prostaglandins or steroids has been previously reported [196,197]. Intra- and interassay coefficients of variation for PGF$_{2a}$ were 11.5% and 16.3%, respectively; for P$_4$, 16.9% and 23.0%, respectively. The limits of sensitivity for each assay were 2.5 pg/ml (PGF$_{2a}$) and .1 ng/ml (P$_4$).

Statistical Analysis

Data were analyzed by analysis of variance in which variation due to CL was separated from that of treatment. Significant treatment effects (i.e., p<.05) were further analyzed by comparing means of treatments using the Student-Neuman-Keuls multiple-range test.
RESULTS

The Effects of Cycloheximide and Actinomycin D on IL-1β-Stimulated Prostaglandin F$_{2\alpha}$ Production.

The protein synthesis inhibitor, cycloheximide (CHX), completely blocked IL-1β-stimulated PGF$_{2\alpha}$ production (Figure 1). The inhibitory effect of CHX was specific to IL-1β action since CHX did not affect basal PGF$_{2\alpha}$ production (p>.05; Figure 1) or P$_4$ production (results not shown).

Exogenous substrate, arachidonic acid (AA), was added to IL-1β+CHX-treated cultures in an attempt to circumvent the protein synthesis requirement of IL-1β-stimulated prostaglandin production (Figure 2). Prostaglandin F$_{2\alpha}$ production was greater in the presence of AA than in controls. Cycloheximide did not affect AA-stimulated PGF$_{2\alpha}$ production (AA vs. CHX+AA, p>.05), but did inhibit IL-1β+AA-stimulated PGF$_{2\alpha}$ production (IL-1β+AA vs. IL-1β+CHX+AA, p<.05). This demonstrated the specificity of CHX toward IL-1β-stimulated cellular mechanisms. Thus, under acute conditions, IL-1β-stimulated prostaglandin production in luteal cells is entirely dependent upon protein synthesis.

In the next experiment, actinomycin D (Act D) was used to determine whether gene transcription is required for IL-1β action. The protein synthetic requirement of IL-1β-stimulated PGF$_{2\alpha}$ production observed in the previous experiment may have resulted from the activation of specific genes and/or the translation of existing mRNA. There was no
effect of Act D on basal PGF$_{2\alpha}$ production (p>.05; Figure 3) or P$_4$ production (results not shown). However, Act D prevented stimulation of PGF$_{2\alpha}$ production by IL-1β (Figure 3).

The Effects of Phospholipase A$_2$ and C Inhibitors on IL-1β-Stimulated Prostaglandin F$_{2\alpha}$ Production.

The phospholipase A$_2$ inhibitor, aristolochic acid (PLA$_2$X), and the phospholipase C inhibitor, Compound 48/80 (PLCX), completely blocked IL-1β-stimulated PGF$_{2\alpha}$ production (Figure 4). There was no effect of either inhibitor on basal PGF$_{2\alpha}$ production (p>.05).

The presence of exogenous AA significantly enhanced PGF$_{2\alpha}$ production in IL-1β-treated cultures, but the response in PLA$_2$X- and PLCX-treated cultures differed (Figure 5 and Figure 6, respectively). Exogenous AA was incapable of overcoming the inhibitory effect of PLA$_2$X on IL-1β-stimulated PGF$_{2\alpha}$ production (Figure 5). The inhibitory effect of PLA$_2$X was considered to be specific to IL-1β action since, in a separate experiment, PLA$_2$X did not inhibit basal or AA-stimulated PGF$_{2\alpha}$ production (results not shown). The addition of AA to IL-1β+PLCX-treated cultures, however, circumvented the inhibitory effect of PLCX such that PGF$_{2\alpha}$ production by IL-1β+AA and IL-1β+PLCX+AA-treated cultures did not differ (p>.05; Figure 6).
Chronic versus Acute Effects of IL-1β in Arachidonic Acid - Stimulated Luteal Cell Cultures.

PGF$_{2\alpha}$ production was greater in cultures of bovine luteal cells chronically exposed to IL-1β (day 0, 1, and 3 of culture) than in those that were acutely exposed to the cytokine (day 3 only; p<.05; Figure 7). There was a significant effect of increasing concentrations of exogenous arachidonic acid (p<.05), and the pattern of response was similar in both acute and chronic groups. In addition, luteal cells chronically exposed to IL-1β produced less P$_4$ than cells acutely exposed (p<.05; Figure 8). There was no effect of exogenous arachidonic acid on P$_4$ production (p>.05), nor was there any difference in the pattern of response for the two treatment groups (p>.05).

DISCUSSION

IL-1β significantly stimulated PGF$_{2\alpha}$ production by bovine luteal cells in the current study. This result is in agreement with a previous study from this laboratory [73]. In the present study, luteal cells were exposed to IL-1β (2.5 ng/ml) for as little as 6 hours, yet the cells were sufficiently stimulated to produce significant amounts of PGF$_{2\alpha}$. This relatively short period of time facilitated the elucidation of cellular mechanisms of IL-1β action, without detrimental effects due to inhibitors, in freshly cultured luteal cells.
The observation that IL-1β did not alter luteal progesterone production in the initial experiments of this study is consistent with a previous report from this laboratory [73]. In this and the previous study, the cells received relatively low concentrations of LH (2.5 ng/ml) and were not exposed to exogenous lipoproteins. Typically these conditions result in what may be considered basal, rather than stimulated, progesterone production. In addition, the abbreviated exposure of luteal cells to IL-1β (6 hrs) and the absence of other cytokines in the medium (i.e., other cytokines which typically act in concert with IL-1β in vivo) may account for the lack of effect on progesterone production. It is interesting to note, however, that chronic exposure of luteal cells to IL-1β (78 hrs) inhibited luteal P₄ production in the present study. The addition of exogenous AA to IL-1β-stimulated cultures further enhanced PGF₂α production, but did not affect P₄ production. It appears, therefore, that the inhibition of P₄ synthesis by IL-1β occurred by a mechanism that was independent of endogenous PGF₂α production. This interpretation is consistent with a previous study from our laboratory [72] in which another cytokine, interferon-gamma, inhibited LH-stimulated progesterone production in the presence of the prostaglandin inhibitor, indomethacin. Thus, although it appears that the effects of IL-1β are directed toward eicosanogenic, rather than steroidogenic, mechanisms in
luteal cells, the possibility that IL-1β may alter LH- or lipoprotein-stimulated progesterone production warrants further study.

Phospholipase inhibitors were used to determine the importance of PLA₂ and PLC in IL-1β stimulation of PGF₂α production by luteal cells. Both PLA₂X and PLCX inhibited IL-1β-stimulated PGF₂α production without affecting basal PGF₂α or P₄ production. This indicates that IL-1β stimulation of luteal cells is mediated by PLA₂ and PLC. However, the inability of exogenous AA to circumvent the effect of PLA₂X, but not PLCX, suggests that PLA₂ is a key regulatory point of IL-1β action. The concentration of AA provided to the luteal cells (1 μg/ml) did not maximally stimulate prostaglandin production, but apparently mimicked the conditions of IL-1β stimulation because treatment of luteal cells with AA or IL-1β alone resulted in similar PGF₂α production (results not shown). Therefore, under the conditions imposed by the aforementioned experiments, the primary action of IL-1β (i.e., to upregulate PLA₂) was ablated by PLA₂X and replaced by exogenous AA. This interpretation of IL-1β action is consistent with previous studies of other non-endocrine cell types [176,178,198]. Increases in concentrations of PLA₂ mRNA, PLA₂ activity, and PLA₂ protein have been observed in IL-1β-stimulated cells [176,178,181]. Stimulation of PLA₂, rather than PLC, by IL-1β is consistent with the reported importance of these two
phospholipases in the liberation of AA from cell membrane phospholipids. Phospholipase A$_2$ cleaves AA directly from phospholipids, whereas PLC does so indirectly via an inositol triphosphate/diacylglycerol mechanism [199]. Therefore, the stimulation of phospholipases, particularly PLA$_2$, is an acute effect of IL-1$\beta$ in luteal cells. The stimulation of PLC appears secondary to that of PLA$_2$.

Actinomycin D and cycloheximide were used in this study to determine the importance of gene activation and protein synthesis for IL-1$\beta$-stimulated PGF$_{2\alpha}$ production in luteal cells. IL-1$\beta$ stimulates the synthesis of PLA$_2$ protein in chondrocytes [178] and PGH synthase in endothelial cells [114]. Similar enzymes may be upregulated in bovine luteal cells since Act D and CHX completely inhibited IL-1$\beta$-stimulated PGF$_{2\alpha}$ production. The results indicate that both RNA synthesis and protein synthesis are activated by IL-1$\beta$. Similar cellular actions of IL-1$\beta$ have been reported in other non-endocrine cell types [114]. However, in the current study, the presence of exogenous AA did not circumvent the inhibitory effect of CHX, confirming that protein synthesis is an absolute requirement of IL-1$\beta$-regulated prostaglandin production in luteal cells. The observation that Act D and CHX did not diminish basal PGF$_{2\alpha}$/P$_4$ production, or cell numbers indicated there were no deleterious effects of these inhibitors on the viability of the cells.
The purpose of the final experiment was to examine whether the duration of exposure of luteal cells to IL-1β influenced the ability of the cells to metabolize AA, presumably through the activation of PGH synthase enzyme. Luteal cells chronically exposed to IL-1β exhibited an enhanced capacity to metabolize AA and, thus, produce more PGF$_{2\alpha}$. Both IL-1β treatment groups, however, had similar patterns of response to exogenous AA. In addition, there was apparently little depletion of endogenous stores of AA as a result of three days of culture since the minimal effective concentration of exogenous AA (5.0 μg/ml) did not differ between the two treatment groups. Chronic exposure of the luteal cells to IL-1β resulted in stimulatory effects of prostaglandin synthesis beyond that of merely increasing substrate availability. Thus, it is plausible that upregulation of PGH synthase occurs in IL-1β-stimulated luteal cells, particularly if the cells are exposed to the cytokine for an extended period of time (> 48 hours).

Recently it has been suggested that the immune system may be involved in corpus luteum regression [23,71]. In this context, luteal prostaglandins may regulate aspects of the luteolytic process since, in other tissues, prostaglandins modulate cytokine production and the destructive effects of immune cells [186,187,200]. A similar role for luteal prostaglandins may exist in the ovary since the demise of the corpus luteum is associated
with a considerable amount of tissue destruction. Alternatively, luteal prostaglandins may constitute an indirect measure of other eicosanogenic metabolites (e.g., hydroxyl radicals, lipoxygenase products, etc.) thought to impact corpus luteum function [62,63,185].

The present study is the first to report specific cellular mechanisms by which IL-1β stimulates PGF$_{2\alpha}$ production in luteal cells. Collectively, these results support the possibility of regulation of luteal prostaglandin production by IL-1β. The ability of IL-1β to stimulate PGF$_{2\alpha}$ production comprehensively and profoundly in luteal cells is consistent with a role of perpetuating prostaglandin production in luteal tissue. Similar actions of IL-1β may take place within the corpus luteum in order to sustain the process of luteolysis.
Figure 1. The effects of interleukin-1β (IL-1β; 2.5 ng/ml) and the protein synthesis inhibitor, cycloheximide (CHX; 10 μg/ml), on prostaglandin F$_{2α}$ production by cultured bovine luteal cells. Treatments were initiated in fresh culture medium during the last 6 hours of a 30-hour culture period. Values are expressed as mean +/- SEM (n=7); different superscripts are significantly different (p<.05).
Figure 2. The effects of interleukin-1β (IL-1β; 2.5 ng/ml), exogenous arachidonic acid (AA; 1 μg/ml), and the protein synthesis inhibitor, cycloheximide (CHX; 10 μg/ml), on prostaglandin F$_{2α}$ production by cultured bovine luteal cells. Treatments were initiated in fresh culture medium during the last 6 hours of a 30-hour culture period. Values are expressed as mean +/- SEM (n=6); different superscripts are significantly different (p<.05).
Figure 3. The effects of interleukin-1β (IL-1β; 2.5 ng/ml), and the DNA transcription inhibitor, actinomycin D (Act D; 5 μg/ml), on prostaglandin F$_{2a}$ production by cultured bovine luteal cells. Treatments were initiated in fresh culture medium during the last 6 hours of a 30-hour culture period. Values are expressed as mean +/- SEM (n=5); different superscripts are significantly different (p<.05).
Figure 4. The effects of the phospholipase A₂ inhibitor, aristolochic acid (PLA₂X; 7.5 μg/ml), and the phospholipase C inhibitor, compound 48/80 (PLCX; 4 μg/ml), on interleukin-1β (IL-1β; 2.5 ng/ml)-stimulated prostaglandin F₂α production by cultured bovine luteal cells. Treatments were initiated in fresh culture medium during the last 6 hours of a 30-hour culture period. Values are expressed as mean +/- SEM (n=7); different superscripts are significantly different (p<.05).
Figure 5. The effects of exogenous arachidonic acid (AA; 1 μg/ml) on interleukin-1β-stimulated (IL-1β; 2.5 ng/ml) and phospholipase A₂-inhibited (PLA₂X; 7.5 μg/ml of aristolochic acid) prostaglandin F₂α production by cultured bovine luteal cells. Treatments were initiated in fresh culture medium during the last 6 hours of a 30-hour culture period. Values are expressed as mean +/- SEM (n=7); different superscripts are significantly different (p<.05).
Figure 6. The effects of exogenous arachidonic acid (AA; 1 μg/ml) on interleukin-1β-stimulated (IL-1β; 2.5 ng/ml) and phospholipase C-inhibited (PLCX; 4 μg/ml of compound 48/80) prostaglandin F$_{2a}$ production by cultured bovine luteal cells. Treatments were initiated in fresh culture medium during the last 6 hours of a 30-hour culture period. Values are expressed as mean +/- SEM (n=7); different superscripts are significantly different (p<.05).
Figure 7. Chronic versus acute effects of interleukin-1\(\beta\) (IL-1\(\beta\); 2.5 ng/ml) on prostaglandin \(F_2\alpha\) production by cultured bovine luteal cells in the presence of increasing concentrations of arachidonic acid. Values are expressed as mean +/- SEM (n=5). There was an effect of IL-1\(\beta\) exposure (p<.05) and arachidonic acid (AA) treatment (p<.05). The pattern of response to AA, however, was similar for the two IL-1\(\beta\) treatment regimes (IL-1\(\beta\) X AA interaction, p>.05).
Figure 8. Chronic versus acute effects of interleukin-1β (IL-1β; 2.5 ng/ml) on progesterone (P₄) production by cultured bovine luteal cells in the presence of increasing concentrations of arachidonic acid. Values are expressed as mean +/- SEM (n=5). There was an effect of IL-1β exposure (p<.05), but not arachidonic acid (AA) treatment (p>.05). The pattern of response to AA was similar for the two IL-1β treatment regimes (IL-1β X AA interaction, p>.05).
CHAPTER III
Mechanisms of Action of TNF-α-Stimulated Prostaglandin Production in Cultured Bovine Luteal Cells

SUMMARY

Tumor necrosis factor-alpha (TNF-α) has been implicated as a modulatory agent of ovarian function, including regression of the corpus luteum. The purpose of the present study was to examine the cellular mechanism(s) of TNF-α-stimulated prostaglandin F₂α (PGF₂α) production by luteal cells. Luteal cells from midcycle bovine corpora lutea (Day 9-12 of the estrous cycle) were placed in serum-free culture for a period of 30 hours. Treatment with the transcription inhibitor, actinomycin D (5 μg/ml), or the protein synthesis inhibitory, cycloheximide (10 μg/ml), completely inhibited TNF-α (50 ng/ml)-stimulated PGF₂α production. The addition of the phospholipase A₂ inhibitor, aristolochic acid (7.5 μg/ml), to luteal cultures inhibited TNF-α-stimulated, but not basal, PGF₂α production. In contrast, the phospholipase C inhibitor, compound 48/80 (4 μg/ml), did not affect either basal or TNF-α-stimulated PGF₂α secretion. Exogenous arachidonic acid (AA; 1 μg/ml) stimulated PGF₂α production
and exerted an additive effect in combination with TNF-α. Arachidonic acid was incapable, however, of counteracting the inhibitory effects of cycloheximide or aristolochic acid in TNF-α-treated cultures. Pretreatment of luteal cells with progesterone (1.75 μg/ml) or estradiol (1 μg/ml) reduced TNF-α-, AA-, and TNF-α+AA-stimulated, but not basal, PGF$_{2\alpha}$ production. In conclusion: 1) TNF-α-stimulated PGF$_{2\alpha}$ production in bovine luteal cells requires RNA and protein synthesis, 2) activation and/or synthesis of phospholipase A$_2$ mediates TNF-α action, 3) steroids modulate TNF-α action, and 4) the provision of substrate, namely arachidonic acid, is a key regulatory step of TNF-α-stimulated PGF$_{2\alpha}$ production.

INTRODUCTION

The cytokine, tumor necrosis factor-alpha (TNF-α), has been implicated as a regulatory agent of folliculogenesis and luteal function [23,71,201,202,203]. The appeal of TNF-α as a paracrine, and possibly autocrine, regulator of ovarian function stems from evidence that: 1) TNF-α modulates hormone biosynthesis in thecal, granulosal, and luteal cells [71,201,204,205], and 2) TNF-α mRNA, protein, and bioactivity are detectable within ovarian tissue [26,70,90,206].

The observation that TNF-α alters thecal and granulosal steroidogenesis suggests that it may influence follicular
development. TNF-α enhances follicular progesterone production [205], but inhibits FSH-induced aromatase enzyme [204]. Based on this evidence, Roby and Terranova [203] speculated that TNF-α may control estrogen secretion of developing follicles and thus, by inference, may modulate selection of preovulatory follicles.

In the corpus luteum, TNF-α may facilitate the process of luteolysis. TNF-α suppresses LH-stimulated progesterone production and is cytotoxic to luteal cells when combined with the cytokine, interferon-gamma [71]. In addition, luteal cells constitute an endogenous source of TNF-α [205] and secretion of TNF-α in the corpus luteum is most evident at the time of luteolysis [26,90].

In some cell types, the cytotoxic effects of TNF-α require the stimulation of phospholipases and possibly the metabolism of arachidonic acid (AA)[172,175,177,207,208]. TNF-α-induced cytotoxicity in luteal cells may occur by similar mechanisms since cytolysis is accompanied by synergistic increases in prostaglandin production (AA metabolites) [71]. The purpose of the present study was to characterize the cellular actions of TNF-α-stimulated prostaglandin production in bovine luteal cells and to provide insight into the possible underlying, cytotoxic effects of TNF-α.
MATERIALS AND METHODS

Materials

Powdered Ham's F-12 culture medium was obtained from Mediatech (Washington, D.C.). Dialyzed calf serum was purchased from Hazelton Research Products (Lenexa, KS) and gentamicin was obtained from Gibco Labs (Grand Island, NY). Collagenase (Type I) was acquired from Worthington Systems (Freehold, NJ) and an insulin-transferrin-selenium premix (ITS) was purchased from Collaborative Research Products (Lexington, MA). BSA (Fraction V), HEPES, and Tris salt were obtained from Sigma Chemical Co. (St. Louis, MO). Twenty-four-well tissue culture plates and centrifuge tubes were purchased from Corning Glass Works (Corning, NY). Arachidonic acid was obtained from Cayman Chemical Co. (Ann Arbor, MI). Aristolochic acid and Compound 48/80 were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). The bovine LH (NIAMMD-bLH-4) was supplied by the National Hormone and Pituitary Program (Baltimore, MD). [1,2-^3H] progesterone was purchased from New England Nuclear (Boston, MA); and [3,6,9,11,12,13,15-^3H(N)] prostaglandin F_2\, was obtained from Amersham (Arlington Heights, IL). The antiprostaglandin F_2\,-keyhole limpet hemocyanin was kindly provided by Dr. Richard Fertel (The Ohio State University, Columbus, Ohio); Dr. Gordon Niswender (Colorado State University, Fort Collins, CO) donated the antiprogestosterone-
11-bovine serum albumin (GDN-337). Recombinant murine tumor necrosis factor-alpha (TNF-α) was purchased from Gibco BRL (Grand Island, NY). This preparation of TNF-α is reported to be ≥ 95% pure by SDS-PAGE and has an endotoxin content of ≤ 0.1 EU/μg.

Methods

Midcycle, bovine corpora lutea (CL) were obtained from regularly cycling, non-lactating dairy cows using a transvaginal approach [38]. Each CL was placed in HEPES-buffered, Ham's F-12 culture medium supplemented with antibiotics and transported to the laboratory on ice for further processing.

Using aseptic technique, the luteal tissue was enzymatically dissociated into cell suspensions as previously described [38]. The tissue was minced into small cubes (approx. 1 mm³) and enzymatically dispersed by two, one-hour dissociations. The dissociation medium contained collagenase (2000 Units/gram of tissue), 0.5% bovine serum albumin (BSA), and gentamicin (20 μg/ml). Following dissociation, the dispersed cells were washed 3-4 times to remove residual BSA and collagenase. This was accomplished by repeated centrifugation and resuspension of the cell pellet with serum-free medium. The speed of centrifugation at each step was gradually decreased to facilitate the removal of red blood cells and other nonluteal cell
contaminants. The luteal cells were subsequently counted in a hemocytometer to estimate concentration. Luteal cell viability was estimated by trypan blue exclusion.

Luteal cells were placed in serum-coated 24-well plates at a concentration of $3 \times 10^5$ cells/well. The culture medium was supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), gentamicin (20 µg/ml), and LH (2.5 ng/ml). The luteal cells were cultured at 37°C in 5% CO$_2$ and 95% air for a period of 30 hours. Unless otherwise noted, treatments were initiated in fresh culture medium during the last 6 hours of the 30-hour experimental period. The conditioned medium from all experiments was collected 6 hours after treatment, stored at -20°C, and assayed by RIA. Cells were counted at the end of culture using a microscope equipped with an ocular micrometer grid. Hormone data were corrected for cell number and normalized to $5 \times 10^5$ cells. Individual CL were used in each repetition of an experiment (i.e., n=number of CL/experiment; shown in figure legends).

Cycloheximide (CHX; 10 µg/ml) was used in the first experiment to determine the importance of protein synthesis for TNF-α-stimulated (50 ng/ml) prostaglandin production by luteal cells. Luteal cells were treated with/without TNF-α and with/without CHX. In addition, exogenous arachidonic acid (AA; 1 µg/ml) was added to cell cultures, in combination with the above treatments, to determine whether
the presence of TNF-α or CHX alters the ability of luteal cells to metabolize AA.

Actinomycin D (Act D; 5 μg/ml), an inhibitor of RNA synthesis, was used in the next experiment to assess the importance of gene activation for TNF-α action. Cell cultures were treated with/without TNF-α and with/without Act D.

Phospholipase inhibitors were used to examine the role of phospholipases in TNFα-stimulated PG production. The phospholipase A2 inhibitor, aristolochic acid (PLAX; 7.5 μg/ml), was added to luteal cells with/without TNF-α, as was the phospholipase C inhibitor, compound 48/80 (PLCX; 4 μg/ml). Exogenous AA was added to cultures, in combination with TNF-α and a phospholipase inhibitor, to determine whether AA circumvents the inhibitory effects of the phospholipase inhibitor.

In the last experiment, luteal cells were exposed to progesterone (P₄; 1.75 μg/ml) or estradiol benzoate (E₂; 1 μg/ml) during the first 24 hours of culture and prior to TNF-α treatment. After steroid pretreatment, the culture medium was removed and fresh medium, containing the steroids, TNF-α, and/or exogenous AA was added to the cultures (i.e., total culture period = 30 hours).
RESULTS

Luteal PGF$_{2\alpha}$ production was greater in TNF-α-treated than control (C) cultures (P<.05; Figure 9). Cycloheximide completely inhibited TNF-α-stimulated PGF$_{2\alpha}$ production, but there were no differences in luteal PGF$_{2\alpha}$ production among C, CHX, or TNF-α+CHX-treated cultures (Figure 9). In addition, luteal P$_4$ production was slightly, but significantly, increased in TNF-α, CHX, and TNF-α+CHX-treated cultures compared to controls (Figure 10). The addition of arachidonic acid alone, or in combination with TNF-α, to cultures significantly stimulated luteal PGF$_{2\alpha}$ production (P<.05; Figure 11). However, when CHX was added to these treatments, PGF$_{2\alpha}$ concentrations did not differ from controls (Figure 11). Actinomycin D (Act D) also blocked TNF-α-stimulated luteal PGF$_{2\alpha}$ production (P<.05; Figure 12), but did not affect basal PGF$_{2\alpha}$ (Figure 12) or P$_4$ production (results not shown).

The addition of phospholipase A$_2$ inhibitor (PLA$_2$X) to luteal cells inhibited TNF-α-stimulated, but not basal PGF$_{2\alpha}$ production (Figure 13). In contrast, the phospholipase C inhibitor (PLCX) did not affect either basal or TNF-α-stimulated PGF$_{2\alpha}$ secretion (Figure 13). When 1μg/ml of AA was added to luteal cells to circumvent the effect of phospholipase A$_2$ inhibitor, PGF$_{2\alpha}$ production was comparable to that of cells treated with TNF-α alone (Figure 14). However, based on previous experiments, 1 μg/ml of AA alone
is known to stimulate luteal PGF$_{2\alpha}$ production to the same extent as TNF-α (i.e., approximately 350 pg/50,000 cells), and AA stimulation is relatively unaffected by PLA$_2$X (results not shown). Thus, in the present study, PLA$_2$X inhibited TNF-α-stimulated luteal PGF$_{2\alpha}$ even in the presence of exogenous AA.

Pretreatment of luteal cells with estradiol or progesterone for 24 hours suppressed TNF-α-, AA-, and TNF-α+AA-stimulated PGF$_{2\alpha}$ production (Figure 15). Steroid pretreatment did not, however, affect basal PGF$_{2\alpha}$ production.

**DISCUSSION**

Recent studies indicate there is immunoreactive [70,205] and bioactive TNF-α within the CL [23,26]. Furthermore, TNF-α bioactivity increases at the time of luteolysis [23], but apparently after the decline in progesterone production [90]. *In vitro*, TNF-α: 1) inhibits LH-stimulated luteal P$_{4}$ production, 2) stimulates luteal prostaglandin production, and 3) is markedly cytotoxic to luteal cells when combined with the cytokine, interferon-gamma [71]. These observations strongly suggest involvement of TNF-α in the process of luteolysis, but a definitive role for TNF-α has not been characterized.

TNF-α-induced cytotoxicity in luteal cells requires the presence of interferon-gamma and is temporally associated
with synergistic increases in prostaglandin production [71]. In other endocrine cells, the cytotoxic effects of TNF-α and interferon-gamma are attributable to the metabolism of AA [169,172,208,209]. Specifically, inhibitors of prostaglandin endoperoxide synthase (PGH synthase, also known as cyclooxygenase) and lipoxygenase block cytokine-induced cytotoxic effects [172,173,174]. Other studies suggest that activation of phospholipases, rather than the metabolism of AA, is directly responsible for cytokine-induced cytoxicity [177,210].

The current study is the first to characterize the relative importance of activation of phospholipases and, indirectly, PGH synthase in TNF-α-stimulated, luteal prostaglandin production. Phospholipase inhibitors were used to ascertain the role of phospholipase A2 (PLA2) and phospholipase C (PLC) in TNF-α-stimulated luteal cells. PLA2X completely inhibited TNF-α-stimulated PGF2α production, whereas PLCX did not. In a previous study, PLA2X and PLCX (used at the same concentrations as the current study) effectively inhibited PGF2α production in interleukin-1β-stimulated luteal cells [211]. Thus, the results indicate that TNF-α stimulation of luteal cells is mediated by PLA2 enzyme rather than PLC enzyme. The observation that PLA2X inhibited TNF-α-stimulated PGF2α production in the presence of exogenous AA suggests that PLA2, rather than PGH synthase, is a key regulatory point of TNF-α action. The
concentration of AA used in these experiments (1 μg/ml) enhanced luteal prostaglandin production to a similar extent as TNF-α alone, but was not maximally stimulatory (results not shown). Therefore, the stimulatory action of TNF-α on luteal prostaglandin production (via upregulation of PLA₂) was essentially ablated by PLA₂X, but circumvented by the provision of exogenous AA. This interpretation is consistent with the demonstrated actions of TNF-α in nonluteal cell types [168,212] and the proposed mechanism of TNF-α cytotoxicity in a TNF-α-sensitive cell line [177].

The stimulation of PGF₂α production by TNF-α was inhibited by both actinomycin D and cycloheximide. These results indicate that both transcriptional and translational events of protein synthesis are necessary for TNF-α action, presumably at the level of PLA₂ enzyme. Transcriptional and translational mechanisms mediate TNF-α effects in endothelial cells as well [212]. However, in the case of endothelial cells, RNA and protein synthesis is associated with a phospholipase A₂-activating protein rather than PLA₂ enzyme specifically. Similar mechanisms may exist in TNF-α-stimulated luteal cells, but were not directly addressed by the design of the present study. Furthermore, there was no evidence that Act D and CHX enhance TNF-α-mediated cytotoxicity, although this is reported to occur in other cell types [213].
The observation that CHX inhibited TNF-α-stimulated PGF$_{2\alpha}$ production in luteal cells even in the presence of AA precludes the possibility that TNF-α action is independent of protein synthesis. If TNF-α modulated PGH synthase enzyme (i.e., in addition to PLA$_2$ enzyme), this also apparently required some form of new protein synthesis. Future studies should address whether new protein synthesis entails the synthesis of phospholipases, PGH synthase, or other proteins which may activate these enzymes of prostaglandin biosynthesis.

Previous studies indicate that phospholipase A$_2$ enzyme may be involved in several aspects of luteolysis in luteal cells. Activation of phospholipase A$_2$ is thought to alter membrane fluidity [182,184] and gonadotropin binding of luteal cells [94]. In the aforementioned studies, PGF$_{2\alpha}$ is thought to initiate the stimulation of PLA$_2$, possibly through a calcium-dependent second messenger. Such speculation is consistent with the known cellular actions of PGF$_{2\alpha}$ (stimulates PLC pathway; [141]) and the knowledge that activation of cytosolic PLA$_2$ is a calcium-dependent process [214]. The findings of the present study indicate, however, that TNF-α is capable of stimulating PLA$_2$ at several levels (i.e., RNA synthesis, protein synthesis, and possibly through endogenous AA metabolites) and, therefore, may initiate activation of PLA$_2$ more effectively than PGF$_{2\alpha}$. 
In sheep, TNF-α is thought to modulate secondary, rather than primary, events of luteolysis since concentrations of progesterone decline before TNF-α bioactivity is detectable [90]. However, conclusions extrapolated from the results of a TNF-α bioassay may be confounded by such things as the susceptibility of the cell type to cytotoxicity and subtle effects of TNF-α, such as DNA fragmentation. DNA fragmentation occurs as a result of cytokine-stimulated endonuclease activity [215] and may precede cell death by several hours [213]. Interestingly, the patterns of cytokine-induced DNA fragmentation are strikingly similar to the recently described apoptotic events of luteolysis [105]. In this context, TNF-α secretion within the CL may initiate luteal apoptosis. Juengel and co-workers [105] hypothesized, however, that apoptosis is involved in structural (secondary), rather than functional (primary) regression of the CL. This hypothesis is consistent with the conclusions of Ji and colleagues [90]. Perhaps a cell culture system, similar to the one used in our laboratory, would be a suitable and sufficiently sensitive way to further test the involvement of TNF-α in apoptotic events as well as functional and structural aspects of luteolysis. As stated previously, this system has been used successfully to demonstrate certain cytotoxic effects of cytokines [71].
The observation that exogenous $P_4$ inhibited agonist-stimulated PGF$_{2\alpha}$ production by luteal cells was not entirely unexpected. In previous studies [73,194], $P_4$ suppression of luteal prostaglandin production has been observed. In one study [73], exogenous $P_4$ suppressed interleukin-1$\beta$-(IL-1$\beta$) stimulated PGF$_{2\alpha}$ production. This led to the suggestion that IL-1$\beta$ may influence luteal function most effectively during periods of the estrous cycle when $P_4$ production is characteristically low, such as during luteal development or regression. Similar characterizations of TNF-$\alpha$ action may be hypothesized based on the results of the present study. In addition, since TNF-$\alpha$- and AA-stimulated PGF$_{2\alpha}$ production was suppressed by $P_4$, this indicates that $P_4$ has the capacity to regulate the metabolism and perhaps the release of AA. This interpretation is consistent with the reported mechanisms by which $P_4$ inhibits uterine PGF$_{2\alpha}$ secretion at the time of luteolysis [122].

The inhibitory effect of exogenous $E_2$ on TNF-$\alpha$- and AA-stimulated PGF$_{2\alpha}$ production was not entirely surprising. In uterine tissue, the effects of $E_2$ on uterine PGF$_{2\alpha}$ production are complex and appear to be time- and concentration-dependent. For example, in one study [133], $E_2$ enhanced the rate of turnover of AA from membrane phospholipids, suggestive of an upregulation of phospholipase activity. Recently, however, Raw and Silvia [131] demonstrated that $E_2$ inhibits phospholipase C activity in uterine tissue. With
regard to regulation of uterine PGH synthase, it has been demonstrated that E₂ may increase [134] or decrease [131] PGH synthase activity. In the present study, E₂ suppressed the metabolism, and possibly the release, of AA. Indeed, the inhibitory effect was selective in that agonist-stimulated PGF₂α production was blocked, but basal PGF₂α remained unaffected. Nonetheless, further study is needed to explore the possible disparate effects of E₂ on luteal cells and to clarify the role of E₂ in luteal function.

In conclusion: 1) TNF-α-stimulated PGF₂α production in bovine luteal cells requires RNA and protein synthesis, 2) activation and/or synthesis of phospholipase A₂ mediates TNF-α action, 3) steroids (i.e., P₄ and E₂) modulate TNF-α action, and 4) the provision of substrate, namely arachidonic acid, is a key regulatory step of TNF-α-stimulated PGF₂α production.
Figure 9. The effects of murine recombinant tumor necrosis factor-alpha (TNF; 50 ng/ml) and cycloheximide (CHX; 10 μg/ml) on prostaglandin F$_{2α}$ production by cultured bovine luteal cells. Treatments were initiated in fresh culture medium during the last 6 hours of a 30-hour culture period. Values are expressed as mean +/- SEM (n=4); different superscripts are significantly different (p<.05).
Figure 10. The effects of murine recombinant tumor necrosis factor-alpha (TNF; 50 ng/ml) and cycloheximide (CHX; 10 µg/ml) on progesterone production by cultured bovine luteal cells. Treatments were initiated in fresh culture medium during the last 6 hours of a 30-hour culture period. Values are expressed as mean +/- SEM (n=4); different superscripts are significantly different (p<.05).
Figure 11. The effects of tumor necrosis factor-alpha (TNF; 50 ng/ml), arachidonic acid (AA; 1 µg/ml) and cycloheximide (CHX; 10 µg/ml) or prostaglandin F$_{2\alpha}$ production by cultured bovine luteal cells. Treatments were initiated in fresh culture medium during the last 6 hours of a 30-hour culture period. Values are expressed as mean +/- SEM (n=4); different superscripts are significantly different (p<.05).
Figure 12. The effects of tumor necrosis factor-alpha (TNF; 50 ng/ml) and the DNA transcription inhibitor, actinomycin D (Act D; 5 μg/ml), on prostaglandin F$_{2\alpha}$ production by cultured bovine luteal cells. Treatments were initiated in fresh culture medium during the last 6 hours of a 30-hour culture period. Values are expressed as mean +/- SEM (n=4); different superscripts are significantly different (p<.05).
Figure 13. The effects of tumor necrosis factor-alpha (TNF; 50 ng/ml), the phospholipase A2 inhibitor, aristolochic acid (PLA2X: 7.5 µg/ml), and the phospholipase C inhibitor, compound 48/80 (PLA2X; 4 µg/ml), on prostaglandin F2α production by cultured bovine luteal cells. Treatments were initiated in fresh culture medium during the last 6 hours of a 30-hour culture period. Values are expressed as mean +/- SEM (n=4); different superscripts are significantly different (p<.05).
Figure 14. The effects of tumor necrosis factor-alpha (TNF; 50 ng/ml), arachidonic acid (AA; 1 μg/ml) and phospholipase A₂ inhibitor, aristolochic acid (PLA₂X; 7.5 μg/ml), on prostaglandin F₂α production by cultured bovine luteal cells. Treatments were initiated in fresh culture medium during the last 6 hours of a 30-hour culture period. Values are expressed as mean +/- SEM (n=4); different superscripts are significantly different (p<.05).
Figure 15. Effects of exogenous progesterone (P₄; 1.75 µg/ml) and estradiol E₂; 1 µg/ml) on TNF-, AA-, and TNF+AA-stimulated on luteal PGF₂α production. Luteal cells were exposed to P₄ or E₂ during the first 24 hours of culture. After steroid pretreatment, the culture medium was exchanged with fresh medium and the cells were exposed to P₄ or E₂, TNF and/or AA for an additional 6 hours. Values are expressed as mean +/- SEM (n=4); different superscripts are significantly different (p<.05).
CHAPTER IV

Characterization of the Eicosanogenic Potential of Cultured Bovine Luteal Cells Stimulated with Interleukin-1-beta and Tumor Necrosis Factor-alpha

SUMMARY

Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) stimulate prostaglandin production by cultured bovine luteal cells. The purpose of the present study was to further examine aspects of the prostaglandin biosynthetic pathway in luteal cells potentially regulated by IL-1β and TNF-α. Specifically, experiments were designed to determine whether induction of prostaglandin endoperoxide (PGH) synthase plays a role in IL-1β- and TNF-α-stimulated prostaglandin production. The results indicate that IL-1β upregulates, or at least maintains, PGH synthase expression in luteal cells. Induction of PGH synthase occurred between 6 and 78 hours after IL-1β treatment. In addition, luteal cells exposed to IL-1β for at least 72 hours were sensitive to phospholipase A₂ inhibition, suggesting that IL-1β regulates arachidonate mobilization as well. In contrast, very little, if any, PGH synthase was upregulated in luteal cells as a result of TNF-α stimulation, despite evidence of
increased prostaglandin production. In conclusion, both phospholipase A₂ and PGH synthase enzymes appear to mediate IL-1β-stimulated prostaglandin production in cultured bovine luteal cells. In contrast, stimulation of luteal prostaglandin production in TNF-α-treated cells is not accompanied by an increase of PGH synthase, but may occur by other mechanisms, possibly the upregulation of phospholipases. This is the first study to report a possible divergence in the mechanism of stimulation of prostaglandin production in IL-1β- and TNF-α-treated luteal cells.

INTRODUCTION

Interleukin-1β (IL-1β) and tumor necrosis factor-alfa (TNF-α) are cytokines synthesized and secreted by macrophages and a variety of other cells, including ovarian cells [70,195,206,216]. Cytokine secretion within the ovary may be an important facet of luteal regression. Cytokines influence ovarian steroidogenesis [71,204,205,217,218,219], promote ovarian prostaglandin production [71,73,156], and exert cytotoxic effects [71]. The observation that cytokines potently stimulate prostaglandin production and that prostaglandins, in turn, modulate cytokine secretion [186,187,188], suggests that cytokines and prostaglandins within the corpus luteum (CL) may influence luteal function, particularly at the time of luteolysis.
Prostaglandin production is regulated primarily by arachidonate release (via phospholipase \(A_2\)) and metabolism (via prostaglandin endoperoxide synthase; PGH synthase) \[108,220\]. In luteal cells, IL-1\(\beta\)- and TNF-\(\alpha\)-stimulated prostaglandin production is acutely regulated by phospholipase \(A_2\) enzyme and requires both RNA and protein synthesis \[211,221\]. In addition, IL-1\(\beta\) and TNF-\(\alpha\) apparently do not upregulate PGH synthase under acute conditions (i.e. 6 hours of cytokine treatment). However, in other cell types, upregulation of PGH synthase occurs 12 to 24 hours after cytokine treatment \[179,222\]. Thus, luteal cells may require a similar lag period for PGH synthase to be appreciably increased.

The purpose of the present study was to further examine aspects of the prostaglandin biosynthetic pathway in luteal cells potentially regulated by IL-1\(\beta\) and TNF-\(\alpha\). Specifically, experiments were designed to determine whether induction of PGH synthase plays a role in IL-1\(\beta\)- and TNF-\(\alpha\)-stimulated prostaglandin production by cultured bovine luteal cells. The results indicate that IL-1\(\beta\) upregulates PGH synthase expression in luteal cells more effectively than TNF-\(\alpha\). Induction of PGH synthase occurred between 6 and 78 hours after IL-1\(\beta\) treatment. In addition, luteal cells exposed to IL-1\(\beta\) for at least 72 hours were sensitive to phospholipase \(A_2\) inhibition, suggesting that IL-1\(\beta\) regulates arachidonate mobilization. However, curtailed
exposure of luteal cells to IL-1β during the last 6 hours of a 78-hour culture period affected overall prostaglandin production, presumably because stimulation of phospholipase A₂ is primarily an acute effect of IL-1β.

MATERIALS AND METHODS

Cell Culture Materials

Powdered Ham’s F-12 culture medium was obtained from Mediatech (Washington, D.C.). Dialyzed calf serum was purchased from Hazelton Research Products (Lenexa, KS) and gentamicin was obtained from Gibco Labs (Grand Island, NY). Collagenase (Type I) was acquired from Worthington Systems (Freehold, NJ) and an insulin-transferrin-selenium premix (ITS) was purchased from Collaborative Research Products (Lexington, MA). BSA (Fraction V), HEPES, and Tris salt were obtained from Sigma Chemical Co. (St. Louis, MO). Cell culture vessels and centrifuge tubes were purchased from Corning Glass Works (Corning, NY). Arachidonic acid was obtained from Cayman Chemical Co. (Ann Arbor, MI). Aristolochic acid was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). The bovine LH (NIAMMD-bLH-4) was supplied by the National Hormone and Pituitary Program (Baltimore, MD). [1,2-³H] progesterone was purchased from New England Nuclear (Boston, MA); and [3,6,9,11,12,13,15-³H(N)] prostaglandin F₂α was obtained from Amersham (Arlington Heights, IL). The antiprostaglandin
F₂₅-keyhole limpet hemocyanin was kindly provided by Dr. Richard Fertel (The Ohio State University, Columbus, Ohio); Dr. Gordon Niswender (Colorado State University, Fort Collins, CO) donated the antiprogesterone-11-bovine serum albumin (GDN-337). Recombinant murine tumor necrosis factor-alpha (TNF-α) was purchased from Gibco BRL (Grand Island, NY). This preparation of TNF-α is reported to be ≥ 95% pure by SDS-PAGE and had an endotoxin content of ≤ 0.1 EU/μg. Recombinant bovine interleukin-1β (IL-1β) was a generous gift from American Cyanamid Co. (Princeton, NJ) and had biological activity of 12.5 X 10⁶ U/mg.

Immunoblot Materials

All electrophoretic supplies were purchased from Bio-Rad (Richmond, CA). Electrophoresis reagents were obtained from Sigma Chemical Co. (St. Louis, MO). The Trans-Blot Electrophoretic Transfer Unit was purchased from Hoefer (San Francisco, CA) and PVDF membrane was obtained from Millipore (Bedford, MA). Purified ovine PGH synthase was acquired from Cayman Chemical (Ann Arbor, MI). The BCIP/NBT alkaline phosphatase substrate system was purchased from US Biochemical (Cleveland, OH). Goat anti-rabbit IgG alkaline phosphate conjugate was obtained from Cappel, a division of Organon Teknika (West Chester, PA). The affinity purified rabbit anti-sheep PGH synthase IgG (#9179) was kindly
provided by Dr. JoAnne Richards (Baylor University, Houston, TX).

**Cell Culture (General)**

Bovine corpora lutea (CL) were obtained from regularly cycling, non-lactating dairy cows using a transvaginal approach [38]. Each CL was placed in HEPES-buffered, Ham’s F-12 culture medium supplemented with antibiotics and transported to the laboratory on ice for further processing.

Using aseptic technique, the luteal tissue was enzymatically dissociated into cell suspensions as previously described [38]. The tissue was minced into small cubes (approx. 1 mm³) and enzymatically dispersed by two, one-hour dissociations. The dissociation medium contained collagenase (2000 Units/gram of tissue), 0.5% bovine serum albumin (BSA), and gentamicin (20 μg/ml). Following dissociation, the dispersed cells were washed 3-4 times to remove residual BSA and collagenase. This was accomplished by repeated centrifugation and resuspension of the cell pellet with serum-free medium. The speed of centrifugation at each step was gradually decreased to facilitate the removal of red blood cells and other nonluteal cell contaminants. The luteal cells were subsequently counted in a hemocytometer to estimate concentration. Luteal cell viability was estimated by trypan blue exclusion.
Luteal cells were placed in serum-coated, 25 cm² culture flasks (Experiment I) or 24-well plates (Experiment II). The cells were seeded at a concentration of 2×10^6 cells/flask (Experiment I) or 3×10^5 cells/well (Experiment II). The culture medium was supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), gentamicin (20 µg/ml), and LH (2.5 ng/ml). The luteal cells were cultured at 37°C in 5% CO₂ and 95% air. The conditioned medium from all experiments was collected 6 hours after the final treatment, stored at -20°C, and assayed by RIA. Cells were counted at the end of culture using a microscope equipped with an ocular micrometer grid. Hormone data were corrected for total protein concentration (Experiment I) or cell number and normalized to 5×10^5 cells (Experiment II). Individual CL were used in each repetition of an experiment (i.e., n=number of CL/experiment; shown in figure legends).

**Cell Culture** (Experiment I)

Luteal cells were exposed to vehicle, TNF-α, or IL-1β for 6 and 78 hours. Proteins from whole cell lysates were resolved by SDS-PAGE and prepared for immunoblot analysis. The cells were scraped from culture flasks (3 flasks/treatment) using a rubber policeman and 1 ml solution of 1% SDS, 1mM EDTA. The samples were subsequently aspirated several times with an 18-gauge needle and boiled for 5 minutes in SDS sample buffer (9% SDS, 15% Glycerol, 6%
β-mercaptoethanol, Pyronin-Y for color; mixed 2 parts sample to 1 part SDS sample buffer). Prior to boiling, an aliquot was taken from each sample to determine total protein concentration (BCA assay; Pierce Chemical Co., Rockford, IL).

**Cell Culture (Experiment II)**

Luteal cells were cultured for a total of 78 hours. Treatment groups consisted of cultures exposed to vehicle (control), IL-1β for the first 72 hours of culture (IL-72), or IL-1β for the entire 78 hours of culture (IL-78). The culture medium was exchanged with fresh medium and appropriate treatments at 24 and 72 hours. In addition, cultures within control, IL-72, IL-78 groups were exposed to vehicle (Basal), a phospholipase A₂ inhibitor (PLA₂X), arachidonic acid (AA), or PLA₂X+AA during the last 6 hours of culture.

**Validation of Primary Antibody Specificity**

Specificity of the primary antibody was initially validated by comparing immunodetectable PGH synthase from several bovine tissues with that of purified ovine PGH synthase. Tissue samples were lysates of bovine lung, brain, heart, liver, and corpus luteum. Both microsomal and cytosolic fractions of the lysates were resolved on immunoblots. Each tissue was prepared using techniques
described previously by Hedin et al. [193]. Briefly, the
tissues were homogenized in potassium phosphate buffer (10
mM potassium phosphate, pH 6.8, 1 mM EDTA) and centrifuged
at 30,000 x g for 30 minutes (4°C) to obtain membrane
pellets (microsomal fraction) and soluble supernatants
(cytosolic fraction). Membrane pellets were resuspended in
potassium phosphate buffer containing 10 mM CHAPS and were
sonicated (3X5 sec). The sonicates were centrifuged at
10,000 x g for 5 minutes (4°C). Aliquots of the
supernatants (solubilized microsomal fraction) were either
mixed and boiled in SDS sample buffer (for SDS-PAGE), or
saved for protein analysis. Similarly, aliquots of the
cytosolic fractions were prepared for SDS-PAGE and protein
analysis.

Immunoblot Analysis

Proteins from both tissue samples and cultured luteal
cells were resolved by one-dimensional SDS-PAGE (3.5%
stacking gel and 8% separating gel). Approximately 35 ug of
total protein was loaded per lane.

Sample proteins were transferred from gels to PVDF
membrane using a semi-dry, electrophoretic sandwich
technique previously described by Towbin et al. [223].
Transfers were conducted at 100 mA constant current for
approximately 1 hour, 40 minutes.
Upon completion of transfer, PVDF membranes were placed in a blocking solution of 5% BSA and basic blot buffer (BBB; 10 mM TRIS, pH 7.5, 150 mM NaCl, 0.001% NaN₃) and gently agitated on an oscillating platform for 2 hours (room temperature) to minimize non-specific binding. A 1:10 dilution (2% nonfat dry milk in BBB) of an affinity purified polyclonal antibody generated against ovine PGH synthase (#9179; a gift of Dr. JoAnne Richards, Baylor University) was used as the primary antibody. The PVDF membranes were incubated in primary antibody solution for approximately 12 hours at 4°C. Subsequently, each membrane was rinsed 2x5 min (BBB), 2x5 min (.25% NANODET P-40 in BBB), and 3x5 min (BBB) to remove residual traces of antibody. A 1:500 dilution (5% BSA in BBB) of goat anti-rabbit IgG conjugated to alkaline phosphatase was used as the secondary antibody. The membranes were incubated for 2 hours (room temperature), rinsed again, as previously described, and antigens were visualized using a precipitating substrate solution specific for alkaline phosphatase (BCIP/NBT reaction).

Quantification of PGH Synthase

PVDF membranes containing immunodetectable PGH synthase were immersed in 50% (V/V) methanol to facilitate densitometry. The immunostained membrane was directly scanned while still wet in a computer-controlled, laser densitometer (Molecular Dynamics; Sunnyvale, CA).
Quantitative analysis of the samples involved scanning the PVDF membrane, outlining the bands of interest, calculating background density, and determining band density by using a computer-generated, volume integration procedure. Briefly, the computer calculated the optical density of a band within a designated area of interest and subtracted the background density. For a given gel, the area of interest is outlined by a computer-generated grid. The grid for each band is of uniform size and, thus, eliminates bias of grid size in the calculation of optical density. This approach has been demonstrated previously to be linearly quantitative [224].

Radioimmunoassay

Prostaglandin F$_{2\alpha}$ and P$_4$ were quantified by RIA of unextracted samples of conditioned medium. Prostaglandin E$_2$ and 6-Keto-PGF$_{1\alpha}$ (the stable metabolite of prostacyclin) were not measured in the present study because the profiles of these prostaglandins are similar to that of PGF$_{2\alpha}$ in IL-1β- and TNF-α-stimulated luteal cells [71,73]. The cross-reactivity of the antisera with other prostaglandins or steroids has been previously reported [196,197]. The limits of sensitivity for each assay were 2.5 pg/ml (PGF$_{2\alpha}$) and .1 ng/ml (P$_4$).
Statistical Analysis

Data were analyzed by analysis of variance in which variation due to CL was separated from that of treatment. Significant treatment effects (i.e., p<.05) were further analyzed by comparing means of treatments using the Student-Newman-Keuls multiple-range test.

RESULTS

Experiment I

In all tissue lysates tested, including that of bovine corpus luteum (Plate I), a single band of immunodetectable PGH synthase was found primarily in the microsomal fraction. In contrast, the cytosolic fraction contained very little, if any, PGH synthase and, thus, served as an internal, negative control (Plate I). In addition, this result confirmed the characterization of PGH synthase as an integral membrane protein, found primarily in the endoplasmic reticulum [225,226,227].

The amount of immunodetectable PGH synthase appeared quantitative with tissue sample (i.e., highest concentration of immunodetectable PGH synthase in bovine lung; lowest in brain; Plate II). A difference in optical density of ≥ 12% was selected as the criterion for quantitative changes in immunodetectable PGH synthase among samples based on a 6% coefficient of variation. The relative molecular weight of PGH synthase in the corpus luteum, although not specifically
calculated, was similar, if not slightly lower, than that of purified ovine PGH synthase (Figures 16 and 18).

Collectively, these results indicated the specificity of the primary antibody was adequate and, thus, whole cell lysates, rather than cell fractions, were used to quantify PGH synthase expression in cultured luteal cells (Plate III).

Immunodetectable PGH synthase from cultured luteal cells was characterized by 2-3 strongly reactive bands of similar molecular weight (Plate III). These bands were thought to be differentially glycosylated forms of PGH synthase or, alternatively, forms of PGH synthase breakdown product [193,228]. Nonetheless, rather than attempt to distinguish between individual bands, the entire area of immunodetectable PGH synthase within a given sample (i.e. all 2-3 bands collectively) was outlined and quantified by densitometry.

Expression of PGH synthase was not significantly different among treatment groups after a 6-hour exposure to vehicle, TNF-α, or IL-1β (Day 1:6 hr; Figure 16, p>.05). PGF$_2\alpha$ production by TNF-α- and IL-1β-stimulated cultures was elevated, but not statistically different from control cultures (Day 1:6 hr; Table 1, p>.05).

In contrast, PGH synthase expression and PGF$_2\alpha$ production differed among treatment groups exposed to vehicle, TNF-α, or IL-1β during the 78 hours of culture (Day 3:6 hr; Figure 16 and Table 1, p<.05). Immunodetectable PGH
synthase increased between Day 1:6 hr and Day 3:6 hr in all treatment groups (Figures 18 & 19, p<.05). However, PGF$_{2\alpha}$ production decreased in control cells, but was maintained by TNF-α and IL-1β treatment during the same period of time (Table 1, p<.05).

**Experiment II**

In this experiment, overall PGF$_{2\alpha}$ production differed among control, IL-72, and IL-78 treatment groups (Figure 17, p<.05). Within the control group, there was no difference among the acute treatments (i.e. BASAL vs. PLA$_2$X vs. AA vs. PLA$_2$X+AA, Figure 18, p>.05). However, in the IL-72 group, PGF$_{2\alpha}$ production was inhibited by PLA$_2$X, but stimulated by AA (Figure 18, p<.05). Similarly, PLA$_2$X inhibited, but AA stimulated luteal PGF$_{2\alpha}$ production in the IL-78 groups (Figure 18, p<.05). Even in the presence of exogenous AA, PLA$_2$X inhibited IL-1β-stimulated PGF$_{2\alpha}$ production in both IL-72 and IL-78 treatment groups (Figure 18, p<.05). There was no effect of any treatment on luteal progesterone production (results not shown).

**DISCUSSION**

Cytokines such as IL-1β and TNF-α within the corpus luteum may serve multiple functions to enhance the process of luteolysis. TNF-α, for example, inhibits LH-stimulated progesterone production and is cytotoxic to luteal cells when combined with another cytokine, interferon-gamma [71].
In the present and previous studies [71,73], IL-1β and TNF-α dramatically stimulated luteal PGF$_2\alpha$ production in vitro, indicating that cytokines may also indirectly support the luteolytic effects of uterine PGF$_2\alpha$ in certain species. Cytokine-stimulated prostaglandin production by luteal cells may also be important from the standpoint of structural regression. Cytokine-induced cytolysis has been demonstrated in a variety of cell types [172,208,229], and appears to be associated with mechanisms of arachidonic acid release and/or metabolism [172,175,177]. In light of the results of the present study, similar cytotoxic mechanisms may be ascribed to aspects of luteolysis. Collectively, the stimulatory effects of IL-1β and TNF-α on luteal prostaglandin production may perpetuate regression of the corpus luteum.

The current study is the first to demonstrate a possible divergence in the mechanism of prostaglandin production in IL-1β- and TNF-α-stimulated luteal cells. This divergence was evident from the experiments in which immunodetectable PGH synthase was measured. Although PGH synthase expression was similar among treatment groups on Day 1:6 hr, this dramatically changed as cells were exposed to vehicle or cytokines for an additional 72 hours (Day 3:6 hr). In particular, PGH synthase expression in control cultures increased during this period of time, perhaps
because demand to metabolize arachidonate had diminished (see Table 1).

In IL-1β- and TNF-α-stimulated cells, PGF$_{2\alpha}$ production was similar, but the pattern of PGH synthase expression differed. TNF-α-stimulated luteal cells maintained PGF$_{2\alpha}$ production throughout the 78-hour culture period, but apparently at the expense of PGH synthase expression. In a previous study [221], stimulation of phospholipase A$_2$, rather than PGH synthase, was implicated as the primary regulatory point of TNF-α-stimulated PGF$_{2\alpha}$ production. The results of the current study are consistent with this interpretation and suggest that very little, if any, PGH synthase is upregulated as a result of TNF-α stimulation.

In Experiment I, IL-1β maintained PGH synthase expression in luteal cells such that it was comparable to levels of expression in control cultures. Importantly, PGF$_{2\alpha}$ production declined in control cultures, but remained elevated in IL-1β-treated cultures throughout the 78-hour culture period (see Table 1). Based on the results of the control cultures, it appears that, in luteal cells, PGH synthase expression is inversely related to PGF$_{2\alpha}$ production. This inverse relationship of PGH synthase expression and PGF$_{2\alpha}$ production was unexpected. In mesangial cells, for example, IL-1β and TNF-α increase PGH synthase mass, and induction of PGH synthase parallels prostaglandin production [179]. However, in the mesangial
cell study, prostaglandin production in control cultures did not change throughout the culture period. In the current study, PGF$_{2\alpha}$ production declined over 78 hours in control cultures. It is this observation, therefore, that demand-driven regulation of PGH synthase is postulated.

In TNF-α-treated luteal cells, demand for AA metabolism resulted in maintenance of PGF$_{2\alpha}$ production, but apparently compromised levels of PGH synthase mass. Prostaglandin synthase is a unique enzyme in that it catalyzes its own inactivation in the presence of excess substrate [230]. Thus, increased AA availability (via TNF-α stimulation of phospholipases), without an accompanied increase in PGH synthase synthesis, might deplete endogenous concentrations of PGH synthase. In IL-1β-treated luteal cells, high concentrations of PGF$_{2\alpha}$ production were maintained without a decline in mass of PGH synthase. Both TNF-α and IL-1β have the capacity to stimulate phospholipases [168,176,178]. Therefore, the the ability of these cytokines to upregulate luteal PGF$_{2\alpha}$ production is suggested to diverge at the level of PGH synthase.

The ability of TNF-α and IL-1β to regulate enzymes of prostaglandin biosynthesis may be relevant to the ability of these cytokines to sustain luteal PGF$_{2\alpha}$ production. Based on the results of the current study, IL-1β is perceived to be the more effective cytokine in stimulating PGF$_{2\alpha}$ production since IL-1β, but not TNF-α, influenced both
phospholipase- and PGH synthase-regulated mechanisms. This interpretation of the relative potency of IL-1β and TNF-α to stimulate PGF₂α production in luteal cells is consistent with the reported actions of these cytokines in another cell type [179].

Raz and colleagues [222] state that total cellular PGH synthase activity may be controlled by either regulating the rates of new enzyme synthesis and degradation or by changing the activity of pre-existing enzyme. It is noteworthy that although PGH synthase expression was inversely related to PGF₂α production in luteal cultures, it was not entirely proportional. These observations indicate that, in luteal cells, changes in PGH synthase expression do not completely explain changes in PGF₂α production, and may represent adjustments in phospholipase and/or PGH synthase activities. Experiment II was designed to indirectly address this issue in IL-1β-treated cells, and to examine whether constant exposure of luteal cells to IL-1β is necessary for the maintenance of elevated PGF₂α production. In previous experiments, luteal cells remained responsive to exogenous AA during the first 30 hours of culture [211]. In the present study (Experiment II), the capacity of luteal cells to metabolize exogenous AA to PGF₂α was diminished by 72 hours (control group), but sustained by continuous (IL-78) or discontinuous (IL-72) treatment with IL-1β. This indicates that IL-1β stimulates PGH synthase such that
luteal cells remain responsive to AA and is consistent with the conclusions of Experiment I. The observation that IL-78 and IL-72 cultures were similarly sensitive to PLA₂X suggests that IL-1β influences PLA₂ enzyme as well. This interpretation is in agreement with the results of a previous study [211]. However, despite the similar patterns of response to AA and PLA₂X in IL-78 and IL-72 cultures, overall PGF₂α production differed between the two groups. This disparity in PGF₂α production between IL-78 and IL-72 groups is attributable to acute effects of IL-1β on luteal cells, effects which diminish within 6 hours of IL-1β removal. Such acute effects may represent a difference in the degree of activation of phospholipase and/or PGH synthase enzymes as previously mentioned.

Of final note is the question of whether cytokines, including IL-1β and TNF-α, induce molecular weight variants of PGH synthase in luteal cells. This question, although not addressed because of the design of the experiments in the present study, may be of particular significance given the recent discovery of antigenically distinct variants of PGH synthase in ovarian cells [228]. These variants appear to be selectively regulated, are possibly encoded by separate genes [231,232] and may influence such events as tissue restructuring during the ovulatory period [228]. Similar variants of PGH synthase may be induced by IL-1β and TNF-α in luteal cells and, thus, may constitute a mechanism
involved in luteolysis. In addition, the possibility of variants of PGH synthase in luteal cells provides an alternative explanation for some of the results observed in the current study (e.g., the 2-3 bands of immunodectable PGH synthase in cultured luteal cells).
Plate I. Validation of PGH synthase antibody specificity. Microsomal (M) and cytosolic (C) fractions of tissue lysates from bovine CL were compared with purified ovine PGH synthase (oPGS).
Plate II. Immunodetectable PGH synthase expression in various bovine tissue samples. Microsomal (M) and cytosolic (C) fractions of tissue lysates from bovine lung (Lanes 2 and 3), heart (Lanes 4 and 5), liver (Lane 6), and brain (Lane 7) were compared with purified ovine PGH synthase (Lanes 1 and 8).
Plate III. The effects of tumor necrosis factor-α and interleukin-1β on prostaglandin endoperoxide synthase (PGH synthase) expression in cultured bovine luteal cells. Luteal cells were exposed to vehicle, TNF-α, or IL-1β for 6 hours on Day 1 of culture (Day 1:6 hr) or for 78 hours (Days 0, 1, and 3; Day 3:6 hr). The two outermost lanes on each side of this example immunoblot are purified ovine PGH synthase (oPGS).
Figure 16. Quantification of immunodectable PGH synthase in cultured lutea cells based on optical density, using a volume integration procedure. Luteal cells were exposed to vehicle, TNF-α, or IL-1β for 6 hours on Day 1 of culture (Day 1:6 hr) or for 78 hours (Days 0, 1, and 3; Day 3:6 hr). Values are expressed as mean +/- SEM (n=3); different superscripts are significantly different (p<.05).
Figure 17. The effect of interleukin-1β on prostaglandin F2α production by cultured bovine luteal cells. Luteal cells were cultured for a total of 78 hours. Treatment groups consisted of luteal cells exposed to vehicle (control), or IL-1β for 72 hours (IL-72) or 78 hours (IL-78). Data represent media collected after a 6-hour incubation (i.e., from hour 72 to 78). Values are expressed as mean +/- SEM (n=5); different superscripts are significantly different (p<.05).
Figure 18. The effects of phospholipase A₂ inhibitor (PLA₂X) and/or exogenous arachidonic acid (AA) on interleukin-1β-stimulated prostaglandin F₂α production. Luteal cells were cultured for a total of 78 hours. Treatment groups consisted of luteal cells exposed to vehicle (control), or IL-1β for 72 hours (IL-72) or 78 hours (IL-78). In addition, the cells were exposed to vehicle, PLA₂X, AA, or PLA₂X+AA during the last 6 hours of culture. Data represent media collected after this 6-hour incubation (i.e., from hour 72 to 78). Values are expressed as mean +/- SEM (n=5); different superscripts are significantly different (p<.05).
Table 1. PGF2α production by cultured bovine luteal cells (pg/mg protein). Luteal cells were exposed to vehicle, TNF-α, or IL-1β for 6 hours on Day 1 of culture (Day 1:6 hr) or for 78 hours (Days 0,1, and 3; Day 3:6 hr). Values are expressed as mean +/- SEM (n=3); different superscripts are significantly different (p<.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1:6 hr.</th>
<th>Day 3:6 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1384 ± 363b</td>
<td>467 ± 108a</td>
</tr>
<tr>
<td>TNFα</td>
<td>1682 ± 249b</td>
<td>1229 ± 403b</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1784 ± 399b</td>
<td>1365 ± 349b</td>
</tr>
</tbody>
</table>
CHAPTER V

CONCLUSIONS

Prostaglandins have been implicated as agents which facilitate ovulation [233] and initiate regression of the corpus luteum [234]. In most domestic animals, secretion of prostaglandins from the uterus, specifically PGF$_{2\alpha}$, is believed to initiate regression of the corpus luteum [120,122]. However, the effect uterine PGF$_{2\alpha}$ secretion and its function in the mechanism of luteolysis within the ovary remains unresolved. In addition, there is no indication that uterine PGF$_{2\alpha}$ secretion initiates luteal regression in primates [153]. Based on these observations, it is possible that factors within the ovary in addition to, or in place of, uterine PGF$_{2\alpha}$ secretion contribute to the process of luteolysis. The studies described and reviewed in this dissertation propose that paracrine communication between immune cells and luteal cells facilitates complete regression of the corpus luteum. Specifically, several hypotheses regarding the effects of cytokine secretion on luteal cells may be formulated and a role for prostaglandins of luteal origin postulated.
The first hypothesis proposed is that cytokines, specifically IL-1β and TNF-α, regulate genetic aspects of luteal prostaglandin production. Studies in this dissertation demonstrated that both cytokines stimulate prostaglandin production through stimulation of RNA and protein synthesis. These results were consistent with those of previous studies in which other cell types were used [114,212]. Such stimulation of genetic and protein synthetic mechanisms of prostaglandin biosynthesis may establish a self-propagating process of prostaglandin formation within the corpus luteum as originally proposed by Rothchild [235]. This perpetuation of prostaglandin production would presumably culminate with the complete regression of the corpus luteum.

The ability of a cell to produce prostaglandins is primarily regulated by: 1) the availability of substrate (arachidonic acid), and 2) the metabolic capacity of PGH synthase. In the current studies, IL-1β and TNF-α upregulated luteal prostaglandin production primarily by the stimulation of phospholipase enzymes.

Inhibitors of phospholipase A₂ and C enzymes effectively suppressed IL-1β-stimulated prostaglandin production by luteal cells. A subsequent experiment revealed, however, that exogenous arachidonic acid overcame the inhibitory effect of phospholipase C inhibitor, but not phospholipase A₂ inhibitor. Therefore, IL-1β is thought to
regulate phospholipase $A_2$, rather than phospholipase $C$, in luteal cells as a primary mechanism of arachidonic acid release. This interpretation is consistent with the reported actions of IL-1β in other cell types [176,178,198] and the relative importance of phospholipases $A_2$ and $C$ in the release of arachidonic acid [199].

In TNF-α-treated luteal cells, phospholipase $A_2$, but not phospholipase $C$, was responsible for the increase in prostaglandin production. There was no effect of phospholipase $C$ inhibitor on TNF-α-stimulated prostaglandin production. Upregulation of phospholipase $A_2$ has been observed in other TNF-α-stimulated cells and has been implicated as a causative mechanism of TNF-α-induced cytolysis [168,177,210]. This is also a plausible explanation for TNF-α-induced cytotoxicity in luteal cells [71].

The upregulation of phospholipase $A_2$ enzyme may be an important facet of luteolysis. The activation of phospholipase $A_2$ perturbs luteal plasma membranes [236] and stimulates superoxide radical formation [237] during luteolysis. In a recent study, changes in superoxide radical formation, cell membrane fluidity, and phospholipase $A_2$ activity occurred concomitantly in PGF$_{2α}$-treated rats [183]. These events developed within 20 minutes of PGF$_{2α}$ injection, but more importantly, they preceded the decline in plasma progesterone concentration. In the context of
luteolysis, stimulation of phospholipase A, by either IL-1β or TNF-α may influence functional aspects of regression (i.e., a decline in progesterone production). The observation that TNF-α suppresses luteal progesterone production more effectively than IL-1β in vitro may have some bearing on this point [71,73]. However, the question of whether IL-1β and TNF-α affect luteal function in vivo remains unresolved.

In the current study, IL-β, but not TNF-α, demonstrated the capacity to stimulate PGH synthase enzyme in cultured luteal cells. Immunodetectable PGH synthase was similar in control- and IL-1β treated groups despite obvious differences in prostaglandin production. In contrast, PGH synthase expression declined over time in TNF-α-treated cultures while prostaglandin production was maintained. Therefore, between IL-1β and TNF-α, IL-1β is hypothesized to be the more efficacious cytokine in stimulating and perpetuating luteal prostaglandin production. The inability of TNF-α to maintain PGH synthase expression and, thus, metabolize arachidonic acid, is viewed as an effect that may eventually compromise the viability of the cell. Differences in the cellular actions of IL-1β and TNF-α may be relevant to the ability of these cytokines to exert cytotoxic effects in corpora lutea.

Cytokines exert cytotoxic effects which are often attributable to the metabolism of arachidonic acid,
including the production of prostaglandins [172,207,208]. Specifically, inhibitors of PGH synthase prevent such cytokine-induced cytotoxic effects [172,173,174]. As previously mentioned, luteal cells are also susceptible to the cytotoxic effects of cytokines, specifically the combination of TNF-α and interferon-gamma [71]. As in other cell types, TNF-α+interferon-gamma-induced cytolysis in luteal cells is accompanied by a synergistic increase in prostaglandin production. However, prostaglandin production is apparently not responsible for the cytolytic effect because, in subsequent experiments, the PGH synthase inhibitor, indomethacin, did not prevent cytokine-induced cytotoxicity (Pate, unpublished results). In other cell types, the role of prostaglandins in cytokine-induced cytotoxicity has, in fact, been disputed.

Suffys et al. [177] demonstrated that the activation of phospholipases, rather than the metabolism of arachidonic acid, is directly responsible for cytokine-induced cytotoxicity in a TNF-α-sensitive cell line. Other studies, using various cell types, seem to substantiate this argument [210,229]. It is noteworthy that, to date, only TNF-α, in conjunction with interferon-gamma, is reported to be cytotoxic to luteal cells [71]. IL-1β, despite its ability to profoundly stimulate luteal prostaglandin production, is apparently unable to exert a similar cytotoxic effect [73]. These observations, along with current evidence that IL-1β
and TNF-α differentially regulate luteal prostaglandin biosynthesis, indirectly suggest a mechanism whereby cytolysis is induced. That is, the activation of phospholipase A₂ is hypothesized to mediate cytokine-induced cytotoxicity. In this context, luteal prostaglandin production may be viewed as a consequence rather than a cause of cytolysis. However, the confirmation of such speculation will require additional study.

If phospholipase A₂ mediates cytokine-induced cytotoxicity, it is important to understand what agent(s) facilitates this process. Several possibilities exist: for example, free fatty acid (e.g., arachidonic acid) may play a critical role, perhaps by stimulating intracellular calcium concentrations [98] or by generating the release of various forms of oxygen radicals [238]. Alternatively, the lysophospholipids formed from phospholipase A₂ activation, if allowed to accumulate, may also exert direct, cytotoxic effects [239]. In IL-1β-stimulated luteal cells, concentrations of intracellular arachidonic acid and lysophospholipids may be regulated such that cytolysis is avoided. The pleiotropic effects of IL-1β are evident from the studies presented in this dissertation. In contrast, upregulation of phospholipase A₂ appears to be the sole mechanism of increased prostaglandin production in TNF-α-exposed luteal cells. Thus, the release and metabolism of arachidonic acid in these cells may not be as tightly
controlled. Ultimately, this may result in TNF-α-induced cytotoxic effects.

The inability of TNF-α to induce luteal cytolysis in the absence of interferon-gamma is notable. It is generally understood that during an immune response, immune cells secrete a multitude of cytokines. The actions of these cytokines are often complementary to ensure an effective response. For example, interferon-gamma enhances the expression of TNF-α receptors on target cells [240] and, likewise, TNF-α upregulates interferon-gamma binding [241]. A similar degree of cooperativity between cytokines may be necessary to instigate cytotoxic effects in luteal cells. Indeed in other cell types, interferon-gamma enhances the cytotoxic effect of TNF-α [189,213]. Based on these observations, the limitations of TNF-α-induced cytolysis in luteal cells is certainly explainable.

Rothchild [235] once stated that in order for luteolysis to occur, a critical event must take place which, "sets in motion the processes that eventually lead to regression, because it will tend to reduce both the suppressing effect of progesterone on PG (i.e., prostaglandin) production and the stimulatory effect of progesterone on its own production."

Some suggest that the release of uterine PGF$_{2α}$ is the critical event of luteolysis, but this explanation does not adequately address the mechanism of luteolysis in all species. Instead, a more plausible 'critical event' would
be one that takes place within the ovary and, quite possibly, within the corpus luteum itself.

From a developmental standpoint, the corpus luteum is really a postovulatory follicle which has temporarily avoided the process of atresia. In many respects, the hormonal and structural changes that take place during follicular atresia and luteal regression are similar. Of particular significance is the observation that cytokine secretion, particularly TNF-α secretion, is a characteristic common to both processes. Luteolysis, therefore, may be envisioned as a process wherein cytokine-induced inhibition of progesterone secretion, and cytolysis of luteal cells, perpetuates regression and is accompanied by an upregulation of prostaglandin biosynthesis. Luteal prostaglandins, in turn, may modulate additional cytokine secretion, thus regulating luteolytic, immune-response mechanisms. In essence, a feedback loop between immune cells and luteal cells may be established in which cytokines and prostaglandins participate as paracrine/autocrine regulators. In this paradigm, intraluteal secretion of cytokines and prostaglandins may facilitate luteolysis in species in which the role of the uterus remains questionable. In others, a feedback loop of cytokine and prostaglandin secretion may serve as a highly responsive mechanism to control the progression of the process.
In summary, IL-1β- and TNF-α-stimulated prostaglandin production in luteal cells may perpetuate the process of luteal regression. Stimulation of luteal prostaglandin synthesis and inhibition of luteal progesterone production by these cytokines may influence functional aspects of regression. In addition, cytokine activation of phospholipases, specifically phospholipase A₂, may initiate structural aspects of regression. Cytokine and prostaglandin secretion within the corpus luteum is viewed as a form of paracrine, and possibly autocrine, communication between immune cells and luteal cells which facilitates regulation of the luteolytic process. Future research will hopefully clarify the role(s) of immune cells and their secretory products in ovarian function, including regression of the corpus luteum. At the same time, these studies may provide a basis for improving the control of fertility in animals.
REFERENCES


62. Milvae RA, Alila HW, Hansel W. Involvement of
lipoxigenase products of arachidonic acid metabolism in

63. Hansel W, Dowd JP. New concepts of the control of

64. Zelinski-Wooten MB, Sargent EL, Molskness TA, Stouffer
RL. Disparate effects of the prostaglandin synthesis
inhibitors, meclofenamate, and flurbiprofen on monkey
luteal tissue in vitro. Endocrinology 1990;
126:1380-1387.

65. Hu Y, Sanders JD, Kurz SG, Ottobre JS, Day ML. In vitro
prostaglandin production by bovine corpora lutea
destined to be normal or short-lived. Biol Reprod 1990;
42:801-807.

66. Houmard BS, Ottobre JS. Progesterone and prostaglandin
production by primate luteal cells collected at various
stages of the luteal phase: Modulation by calcium
ionophore. Biol Reprod 1989; 41:401-408.

67. Sargent EL, Baughman WL, Novy MJ, Stouffer RL.
Intraluteal infusion of a prostaglandin synthesis
inhibitor, sodium meclofenamate, causes premature
luteolysis in rhesus monkeys. Endocrinology 1988;
129:2261-2269.

68. Milvae RA, Hansel W. Prostaglandin F2α,
and progesterone production by bovine luteal cells
during the estrous cycle. Biol Reprod 1983;
29:1063-1068.

69. Milvae RA, Hansel W. The effects of prostacyclin (PGI2)
and 6-keto-PGF1α on bovine plasma progesterone and LH

70. Roby KF, Weed J, Lyles R, Terranova PF. Immunological
evidence for a human ovarian tumor necrosis factor-α. J

71. Benyo DF, Pate JL. Tumor necrosis factor-alpha alters
bovine luteal cell synthetic capacity and viability.
Endocrinology 1992; 130:854-860.

72. Fairchild DL, Pate JL. Modulation of bovine luteal cell
synthetic capacity by interferon-gamma. Biol Reprod


92. Hutchison JS, Zeleznik AJ. The rhesus monkey corpus luteum is dependent on pituitary gonadotropin secretion throughout the luteal phase of the menstrual cycle. Endocrinology 1984; 115:1780-1786.


116. Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA, Serhan CH. Leukotrienes and lipoxins: structures,


127. Kieborz KR, Silvia WJ, Edgerton LA. Changes in uterine secretion of prostaglandin F₂α and luteal secretion of


137. Chegini N, Lei ZM, Rao CV. Nuclear volume and chromatin conformation of small and large bovine luteal cells: effect of gonadotropins and prostaglandins and


139. Jacobs AL, Homanics GS, Silvia WJ. Activity of phospholipase C in ovine luteal tissue in response to PGF$_{2\alpha}$, PGE$_{2}$ and luteinizing hormone. Prostaglandins 1991; 41:495-500.


141. Davis JS, Weakland LL, Weiland DA, Farese RV, West LA. Prostaglandin F$_{2\alpha}$ stimulates phosphatidylinositol 4,5-bisphosphate hydrolysis and mobilizes intracellular free calcium in bovine luteal cells. Proc Natl Acad Sci 1987; 84:3728-3732.


147. Mitchell DE, Lei ZM, Rao Ch V. The enzymes in cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism in human corpora lutea: Dependence on


228. Wong WYL, Richards JS. Evidence for two antigenically distinct molecular weight variants of prostaglandin H


