PF2α and PGE2 Their Effects on Cell Respiration and Steroidogensis in the Bovine Corpus Luteum

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Mitochondria are essential to the process of cell respiration and steroidogenesis. Both of these processes impact the viability of the corpus luteum, which is required to maintain early pregnancy. In cattle and other species, progesterone supports gestation, regulates embryogenesis and works in concert with estrogen to maintain the reproductive tract. Many investigators have sought to understand the processes that control regression of the corpus luteum. Two prostaglandins, PGF2α and PGE2, have been implicated in the functioning of this transient gland. It is accepted by most researchers that the primary luteolysin responsible for functional and structural demise of the corpus luteum is PGF2α. However, there is ambiguity between reported effects of PGF2α and PGE2 on whole cells and on animals in vivo. Therefore, the specific effects of these prostaglandins on oxygen consumption and progesterone secretion in mitochondria isolated from bovine corpora lutea are the focus of this dissertation. A decrease in oxygen consumption and a concomitant decrease in ATP may contribute to luteolysis. These in vitro studies using isolated mitochondria may help to resolve these questions. The first objective was to determine the influence of PGF2α and PGE2 on the oxygen consumption of mitochondria isolated from bovine corpora lutea. The second objective was to determine the effect of both of these prostaglandins on mitochondrial progesterone production. This is the first time that the direct effects of PGF2α on mitochondrial cellular respiration and steroidogenesis have been studied in the corpora lutea of any species. These results will expand information in the area of ovarian physiology and may clarify the roles of PGF2α and PGE2 in corpus luteum function.
PGF2α and PGE2
Their Effects on Cell Respiration and Steroidogenesis in the Bovine Corpus Luteum

Charlotte Bidwell Bacon

B.S., Denison University, 1957
M.A., Bowling Green State University, 1960

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Connecticut 2013
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Charlotte Bidwell Bacon

2013
Doctor of Philosophy Dissertation

PGF2α and PGE2
Their Effects on Cell Respiration and Steroidogenesis in the Bovine Corpus Luteum

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2013
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To be always learning something new is the greatest of pleasures.

-Aristotle

The members of my committee have guided me through what has been a long, challenging process. I have cherished the hope of obtaining my Ph.D. for years. As a single mother raising three children alone and helping to care for an elderly parent, I postponed this aspiration until late in life.

So much has changed since my earliest schooling: ball-point pens have replaced stick pens, video games are the choice of amusement rather than marbles and jump rope. Children now-a-days spend their time on Facebook rather than listening to Jack Benny on the radio. I have had a lot of catching up to do and new techniques to master.

Tom Hoagland has painstakingly reviewed my many drafts and made helpful suggestions. John McCracken provided funds for the purchase of corpora lutea and kept me amused with fish stories. Also, he and I share a love of Scotch. Dr. Richard Mancini made his laboratory and equipment available for all of our respiration and steroidogenesis trials. John Riesen’s Reproductive Physiology course and Bob Milvae’s Essential Reproduction course provided relevant information about physiology, which filled a gap in my knowledge, since my background has been in human anatomy. Jake Harney and I have known each other for years in the Biology Department at the University of Hartford and I am grateful to have his perspective and input in reviewing this dissertation.
Thanks are due to many others. Oklahoma State University professor, Dr. Robert Wettemann and his graduate assistant, Brit Boehmer conducted the radioimmunoassays of our progesterone data.

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I dedicate this dissertation to Ranjith Ramanathan, “A gentleman and a scholar”, who has been absolutely essential to its culmination! Before the time of the poets Burns and Wordsworth, there was the belief that to be both a gentleman and a scholar were the worthiest of ideals. Ranjith is both. Everyone should be lucky enough to have Ranjith as a mentor.
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Chapter I. Introduction

Most of our knowledge of reproductive physiology has transpired during the past 85 years. Advances such as artificial insemination, estrous synchronization, embryo transfer, in-vitro fertilization, and contraception, to mention just a few, have all emerged in the past few decades (Alexander et al., 2010; Abeydeera, 2002). Now that we have achieved some control over reproduction, it has become conceivable to consider optimum population size, whether the reference point is the number of cattle or the number of humans (Sande et al., 2008; Myrsky et al., 2009). A balance needs to be achieved to avoid degradation of the quality of life and the erosion of resources.

In 1855 a rapid increase in knowledge led to the beginning of what is called modern reproductive physiology. It was recognized that the gonads produce steroid hormones that affect reproductive tissues and that the anterior pituitary controls the function of the gonads. In the 1940s and 50s the understanding of the physiology of sperm led to successful artificial insemination of many species. In the 1960s it became apparent that PGF2 alpha regulates the length of the estrous cycle in most mammals (Mellin and Busch, 1976; McCracken et al., 1999). Now the cycle could be manipulated to control the timing of ovulation. In 1960, the development of radioimmunoassay made possible the precise measurement of hormones (Yalow and Berson, 1960; see also Yalow, 1978; 1995). The discovery that ovarian stem cells can replenish oocytes in postnatal mammalian females is a revolutionary breakthrough. It overturns the belief that oogenesis does not occur in postnatal life. Stem cells in the ovary can supply functional follicles in post-natal ovaries. The evidence for
neo-oogenesis under normal conditions remains controversial, however. Manipulation of stem cells can have tremendous applications in animal production (Mooyotto S, 2011). Despite the multitude of modern advances, much remains to be elucidated. The result of our investigations into the effect of prostaglandins on respiration and steroidogenesis will complement and extend previously published data on ovarian physiology.

**Primary Reproductive Hormones in the Female**

Quiescent neurons located in the hypothalamus become active at puberty (Plant and Barker-Gibb, 2004). At the initiation of puberty, gonadotropin releasing hormone (GnRH) is produced by neurons in the surge center and tonic center of the hypothalamus. Communication between the hypothalamus and the anterior pituitary occurs via a portal system that consists of a capillary bed. This allows minute quantities of releasing hormone to act directly on the cells of the anterior pituitary before dilution by the systemic circulation (Charlton, 2008). The primary hormones secreted by the anterior pituitary are follicle stimulating hormone (FSH) and luteinizing hormone (LH). Luteinizing hormone targets receptors in the theca and follicle stimulating hormone targets receptors in the granulosa cells. After an increase in the secretion of estradiol by the granulosa cells, luteinizing hormone peaks, culminating in ovulation and stimulation of the corpus luteum to produce progesterone. Progesterone and estrogen have important roles in regulating various physiological functions related to growth, development, and reproduction in domestic animals. Recent research suggests that these two hormones can alter target cell responses nongenomically via a
membrane receptor (Blesson et al., 2012). Estrogen can suppress LH and progesterone, also acting via a membrane receptor and has been shown to inhibit binding of oxytocin to its receptors in vitro (Stormshak and Bishop, 2008).

As the name implies, follicle stimulating hormone causes the growth of follicles in the ovary. The neurohormone, oxytocin, is produced in the hypothalamus and released from the posterior pituitary, exerting its effects on the ovary, uterus, and mammary gland. Along with estrogen, it is involved in inducing the endometrial synthesis of PGF2α, causing regression of the corpus luteum (Flint et al., 1986; McCracken et al., 1999).

**Dissertation Chapters**

Chapter 1, Introduction, lists the primary topics discussed in succeeding chapters and includes a review of the literature. The morphology of mitochondria, their involvement in corpus luteum function, their role in apoptosis, steroidogenesis and respiration is discussed in Chapter 2. In Chapter 3 the biosynthesis of prostaglandins is examined, as well as their interaction with nitric oxide, endothelin-1 (ET-1), and oxytocin in contributing to luteolysis. In addition, in Chapter 3 the effect of PGF2α and PGE2 is emphasized, since our investigation is concerned with the influence of these two prostaglandins on progesterone synthesis and their effect on luteal cell respiration. Cholesterol and its conversion to steroid hormones is discussed in Chapter 4, which includes various enzymes involved in steroidogenesis and the importance of steroidogenic acute regulatory protein (StAR) and translocator protein (TSPO) in the entrance of cholesterol into the mitochondria. Chapter 5, focuses on respiration and the
locus where known inhibitors of the process act. Chapter 6 contains statements of our hypothesis and objectives. Materials, methods, and statistical analysis are the subject of Chapter 7. Chapter 8 summarizes our results and conclusions, including the effects of PGF2α and PGE2 on luteolysis.

References Cited


Aristotle, 350 BC. On the generation of animals. Translated by Arthur Platt. eBOOKS@Adelaide.


Part 1
Review of the Literature
Chapter II. Mitochondria

Historical Review

The earliest records of structures which probably represent mitochondria are found in the 1840s (Henle, 1841; Retzius, 1890). This is only a few years after the cell nucleus was discovered. Altmann, in 1890 was the first to realize that these “bioblasts” could be organisms living inside cells that are capable of performing important functions. The name “mitochondrion” was first used in 1898 by Benda and originates from the Greek word for thread, “mitos” and granule, “chondros”.

In 1925, Keilin discovered the cytochromes, leading to the conclusion that the respiratory chain was a sequence of enzymes with the dehydrogenases on one end and an iron-containing respiratory enzyme on the other end (Keilin, 1925). Several experiments in the late 1930s uncovered the reaction pathways of aerobic respiration. In 1937 Sir Hans Krebs (Krebs and Johnson, 1937) described the biochemistry of the citric acid cycle. In 1939 Belitser and Tsybakova found that two molecules of ATP are formed per atom of oxygen consumed. These experiments led to the speculation that phosphorylation probably occurs as part of the respiratory chain. In the late 1940s, Friedkin and Lehninger verified the supposition that the respiratory chain and phosphorylation are linked. It is significant that all these important developments occurred before it was known that these intracellular “particles” were mitochondria (James and Arney, 1939). Detailed information on mitochondrial ultrastructure awaited the development of thin-sectioning techniques in the 1950s. In 1952, Palade
discovered that the mitochondrion is surrounded by a membrane which is folded into “cristae”. The micrographs of Sjostrand (Sjostrand and Rhodin, 1953) revealed a double membrane surrounding the mitochondrion.

Research of the past thirty years has strengthened the hypothesis that mitochondria are symbionts of a primitive eukaryote (Margulis, 1981). A more recent theory called the **serial endosymbiotic theory** suggests that a protoeukaryotic cell lacking mitochondria evolved first and was able to incorporate a proteobacterium by endocytosis. Transfer of genes from the bacterium to the nucleus of the protoeukaryotic cell resulted in the distribution of genes, which are currently seen in the two genomes (Gray, 1993; Gray et al., 1999).

Many mitochondrial DNAs (mtDNA) from different organisms have now been completely sequenced (Scheffler, 2000). Most genomes encode proteins of **Complexes I, III, IV**, and **Complex V** of the electron transport chain. Evolutionary studies are now concentrating on the nucleotide sequences of specific genes with the intention of further elucidating the evolutionary origins of the mitochondria (Scheffler, 2000). Absolute proof to determine the monophyletic versus multiphyletic origins of the mitochondria or to ascertain their relationship to present day bacteria may not be possible, because we lack a representative of their common ancestral form (Scheffler, 2000).

**Morphology**

The basic morphology of mitochondria as a matrix enclosed by an inner and outer membrane separated by an intermembrane space was revealed when
the earliest electron microscope was developed. Transmission electron microscopy permitted more detailed views, showing that the inner membrane is folded into what are now called “cristae” (Karbowski and Melino, 2003).

Decades after this early view was accepted, a revisionist view of the mitochondrial membrane is currently being explored (Mannella, 2000; Perkins and Frey, 2000). Electron microscopic tomography, which is analogous to “computerized axial tomography” (CAT scan), has permitted the reconstruction of Leydig cell mitochondria in three dimensions (Prince and Buttle, 2004). The unusual morphology of mitochondria in Leydig cells may be typical of mitochondria in other steroidal tissue (Prince, 1999). In human Leydig cells a form of the cristae termed the lamellar association (LA) is apparent. The cristae are arranged in apposed layers within the inner mitochondrial membrane, each layer separated by a 4 nm gap. The LA appears to be unique to steroid-producing cells. It is surmised that the LA are present in a region of the cristae which is not involved in ATP production, since the dimensions do not allow for complexes on the matrix side of the cristae. One of the subunits of ATP synthetase is too large to be accommodated in the membrane (Prince, 2000).

In human Leydig cells, the surface area of the inner membrane is divided into two domains. One domain adheres closely to the outer membrane, making contacts with the outer membrane in several locations. This domain has been termed the “inner boundary domain”. The second domain of the inner membrane forms the cristae, which are now interpreted to form lamellar structures, which connect to the first domain of the inner membrane by tubular structures named
“crista junctions” (Perkins et al., 1997). In many respects, the structure of the cristae is similar to previous studies of nonsteroid-producing cells. Unique to steroid-producing mitochondria, however, is a type of crista in which the lamellae are closely applied to each other. These interconnections form an extensive internal membrane system. This system has an extensive surface area with few openings to the inner boundary membrane. The significance of this form of the cristae is unknown (Prince and Buttle, 2004).

These investigations into the morphology of the cristae give rise to several questions. Are proteins limited in their distribution between the two domains? Does the membrane potential vary from one region of the inner membrane to another? What maintains these two domains of the inner membrane (Scheffler, 2000)? A final answer remains elusive (Ernster and Schatz, 1981).

Variations can be found in the number, size, and shape of mitochondria (Racker, 1970; Tandler and Hoppel, 1972; Novikoff, 1961). Examples of variation can be observed in insect flight muscles, where mitochondria are arranged in a slab-like orientation (Edwards and Ruska, 1955). In the proximal kidney tubule cells and in the flagella of sperm, there is a single, large mitochondrion at the base of the cell. Intracellular distribution of mitochondria depends upon several circumstances, including cell shape, organization of the cytoskeleton, and energy demands of the cell (Sjostrand and Rhodin, 1953). The morphology of mitochondria in human oocytes varies from oogenesis through embryonic stages. The mitochondria are initially rounded, becoming fewer in number and elongate after implantation (Nicholls and Budd, 2000).
Understanding alterations in mitochondria morphology can explain many disease conditions. Abnormal mitochondria cause neuronal synaptic loss and cell death in Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease (Seo et al., 2010).

Movement Capability

Mitochondria are capable of movement. This is especially apparent during mitosis. However, the significance of these movements is unknown (Frederic and Chevremont, 1952). In 2006, cultured mammalian cells were tagged with green fluorescent dye to investigate mitochondrial movement. Because of the diverse roles of mitochondria, their distribution within the cell is of great importance. Lysophosphatic acid, acting via GTPase RhoA, was found to inhibit fast movement. Investigators concluded that mitochondrial movement is controlled by GTPase RhoA and this control is mediated by formins (Minin et al., 2006).

Interactions of proteins on the outer membrane and components of the cytoskeleton, including actin filaments, microtubules, and intermediate filaments are involved in movement of the mitochondria (Scheffler, 1999). An obvious problem ensues regarding the equal distribution of mitochondria during cell division. Outer membrane structures identified in yeast suggest that there is interaction between cytoskeletal structures and mitochondria in diverse cytoskeletal structures in different organisms (Yaffe, 1999).
Replication – Fission and Fusion

The observation has been made that mitochondria undergo both fission and fusion (Bereiter-Hahn and Voth, 1994). Images of mitochondria that appear to be dividing show a septum that can be seen by electron microscopy. Components of the outer membrane in yeast lend credence to the idea that mitochondria can interact with cytoskeletal structures in different organisms (Yaffe, 1999). Evidence from yeast and fungi support the idea that mitochondria form a reticulum or network that is constantly undergoing fissions and fusions. It is known that proteins are continuously mixed and exchanged between mitochondria or within the reticulum (Suelmann and Fischer, 2000). Other evidence supports the hypothesis that mitochondria in mammalian cells may form an interconnected network that may be involved in the delivery of energy or channel calcium between areas of the cell (Frey and Mannella, 2000).

Mitochondria provide a buffering system, which controls the amount of calcium in the cytosol versus the amount required within the mitochondria for the action of dehydrogenases in Krebs cycle (Hansford, 1994), a system which may be dependent upon a network of microtubules.

In eukaryotes, mitochondria may replicate their DNA and undergo fission in response to energy needs of the cell. When energy needs are low, mitochondria are destroyed, but if ATP is needed, mitochondria increase in number by fission. A spiral of dynamin forms a helical tube around the organelle, constricts when GTP is added, pinching the mitochondrion into two halves (Mears et al., 2011). Two mitochondria can also merge into one, a process called
fusion. There appears to be a balance between fission and fusion, but how the balance is maintained is unknown.

Mitochondria are apparently randomly distributed to daughter cells during cytokinesis. A daughter cell that fails to receive mitochondria will not survive, since mitochondria cannot be synthesized \textit{de novo}. Their number may be increased by fission, but since their genome is uniparental, they will lack variation. Even if recombination occurs, it will not result in greater genetic diversity, since an individual's mitochondrial genes are inherited from the female parent. Although mitochondria in the sperm enter the egg, they are destroyed by ubiquitin in the embryo (Sutovsky et al., 1999). The study of maternal inheritance has been useful for divulging the evolutionary history of populations (Lunt et al., 1997).

\textbf{Mitochondria in the Corpus Luteum}

Mitochondria are essential for luteal cell function in three ways. They generate most of the ATP required by the cell for energy. Secondly, they are the site of the initial stage of steroidogenesis during which pregnenolone is synthesized from cholesterol and subsequently metabolized to produce progesterone. Thirdly, they release pro-apoptotic proteins, e.g. cytochrome c, that activates the caspase cascade which triggers apoptosis and programmed cell death (Niswender, 2002).

All three of these mitochondrial roles interact during the life and demise of the corpus luteum. After ovulation the theca interna and granulosa cells of the follicle become transformed into luteal cells. This structural and functional
remodeling is partially dependent upon the apoptotic capability of the mitochondria. The cells of the corpus luteum synthesize steroids, require ATP, and regress to allow the initiation of the next estrous cycle. Mitochondria within the corpus luteum initiate steroidogenesis via the production of pregnenolone, a portion of which is subsequently metabolized to progesterone, the primary hormone produced by the corpus luteum and which is essential for the maintenance of pregnancy (Stocco and Sodeman, 1991). All these cellular transformative events require energy. The energy is supplied by oxidative phosphorylation within the mitochondria (Niswender and Nett, 1994). It is logical to study the mitochondria and the corpus luteum in concert, since their roles are intertwined.

**Involvement of Mitochondria in Cell Respiration**

Cells store and use the energy in the bonds of ATP, which is synthesized primarily in the mitochondria. The first stage of cell respiration, called glycolysis, is anaerobic and occurs in the cytosol. The product of this first stage, pyruvate, which enters the mitochondrial matrix, is oxidized to acetyl-CoA, which enters the second stage, the “citric acid cycle” or “Krebs cycle”. The two products synthesized in the second stage, (NADH and FADH$_2$) donate electrons and hydrogen atoms which provide energy for the generation of ATP which occurs in the third stage (Nelson and Cox, 2005).

The third and last stage of aerobic respiration, oxidative phosphorylation (electron transport chain), is pertinent to our investigation since it culminates in the production of the energy-rich compound, ATP. It occurs on the inner
membrane of the mitochondria, where enzymes are lined up in an efficient sequence. The first protein in the transport chain to receive the electrons from NADH (which was synthesized in the Krebs cycle) is an enzyme embedded in the membrane, NADH dehydrogenase located in Complex I. A mobile carrier called ubiquinone passes the electrons from Complex I to Complex II. Succinate enters the transport chain at Complex II and also donates electrons to ubiquinone. Complex III, also called the Cytochrome bc1 Complex, passes the electrons to cytochrome c. From cytochrome c, the electrons are passed to Complex IV (cytochrome c oxidase). Each passage of electrons is accompanied by the expulsion of a proton into the space between the inner and outer membrane of the mitochondrion. Hydrogen protons are combined with oxygen at Complex IV, producing water (Butterworth PJ, 2005).

Protons that have accumulated in the intermembrane space enter the ATP synthase complex, sometimes designated Complex V. The parts of this complex are arranged in such a way that it resembles a rotary engine. As hydrogen protons enter this structure from an area of high concentration to an area of low concentration, the ATP rotator turns, producing the energy for the synthesis of ATP from ADP and phosphate (Nelson and Cox, 2005). ATP is available to provide energy for all the processes required to maintain life. Thus growth and function of the corpus luteum are dependent upon the respiratory role of the mitochondria.

State IV oxygen consumption rate (OCR) of the mitochondria is defined as in vitro oxygen consumption by isolated mitochondria in a substrate
(frequently succinate) in the absence of ADP or any inhibitors. Determination of the OCR provided verification that our mitochondria were alive and able to utilize succinate as a substrate. In addition, determination of the OCR allows us to ascertain what effect the addition of PGF2α and PGE2 would have on oxygen consumption prior to ATP production.

**Involvement of Mitochondria in Steroidogenesis**

Steroid hormones are made from cholesterol. The conversion of cholesterol to pregnenolone occurs inside the mitochondria where the cholesterol side-chain-cleavage enzyme, (P450scc), resides on the matrix side of the inner mitochondrial membrane (Bose et al., 2002; Stocco, 2001). Thus, P450scc and enzymes of the electron transport chain both occupy the inner mitochondrial membrane. It has been shown that P450scc can function only in the mitochondria (Black et al., 1994). Once thought to be the rate-limiting step in biosynthesis of steroids, P450scc enzymatic activity is now known to rely upon the steroidogenic acute regulatory protein (StAR), which has been localized to the outer mitochondrial membrane. It is StAR which makes cholesterol available to the P450scc enzyme (Stocco, 2001; Strauss et al., 1999). A partner in the transport of cholesterol is translocator protein (TSPO), which binds to StAR and assists in the movement of cholesterol through the outer mitochondrial membrane.

The first two enzymes in the steroidogenic pathway are P450scc and 3 β hydroxysteroid dehydrogenase (3 β HSD). The latter enzyme converts pregnenolone to progesterone and is found primarily in the cytosol. However,
recently it has been located within the mitochondria, inferring that some progesterone may be synthesized within the mitochondria as well (Stocco, 2000; Chapman et al., 2005). Earlier evidence that HSD is located within mitochondrial fractions of ovarian tissue was reported by Jackanicz and Armstrong, 1968; Sulimovici and Boyd, 1969; and Flint and Armstrong, 1971). Data from these investigations confirm that conversion of cholesterol to progesterone can occur within the mitochondria of rat and bovine corpora lutea and perhaps within mitochondria of other species as well. Further metabolism of progesterone occurs in the cytosol of tissue that produces steroids further down the steroidogenic pathway, such as glucocorticoids, estrogen and testosterone (Li et al., 1997).

Several comparisons can be made between respiration and steroidogenesis. First of all, both processes occur on the inner mitochondrial membrane. The manner in which both processes are accommodated on the inner membrane is a matter of current speculation.

The path of electron flow in steroidogenesis involves NADPH and FADH$_2$ (nicotinamide nucleotides or flavin nucleotides). A series of oxidation/reduction reactions is initiated. The path of electron flow in steroidogenesis involves a compound similar to NADH, NADPH and the same steroidogenic electron acceptor, FADH$_2$. The respiratory electron acceptors carry electrons from catabolic reactions to the electron transport chain and the steroidogenic electron acceptors carry electrons to anabolic reactions. Cells maintain separate groups of NADH (respiratory electron acceptor) and NADPH (steroidogenic electron acceptor).
acceptor) and these two types of acceptors differ in their capability to perform reduction and oxidation reactions. NADH is found in mitochondria and associated with catabolic reactions, e.g. oxygen consumption; whereas NADPH is an enzyme complex found in the plasma membrane and associated with anabolic reactions. Both NADH and NADPH decrease the permeability of the outer mitochondrial membrane to ADP. Neither can cross the inner mitochondrial membrane unassisted. However, the outer mitochondrial membrane is easily crossed, since its pores are permeable to molecules smaller than 10 kDa. Both of these electron acceptors are required for the processes of respiration and steroidogenesis (Labedzka, 2006).

**Involvement of Mitochondria in Apoptosis**

All cells have a certain lifespan and eventually become candidates for what is termed “programmed cell death” or apoptosis. Apoptotic factors affecting the inner membrane necessarily will compromise cellular respiration and steroidogenesis. Mitochondria play an important role in the process, which is distinct from necrosis, allowing for the removal of old, damaged cells without the destruction of neighboring tissues and permitting the conservation and reuse of cell components such as amino acids and nucleotides. Apoptosis was described by Kerr as long ago as 1972 (Kerr et al., 1972). Morphologic changes during embryonic development, DNA damage, viral infections, accumulation of oxidative stress (ROS), all require cell death and replacement. The process of functional luteolysis requires uterine PGF2α, whereas structural luteolysis requires luteal
PGF2α synthesis (apoptosis). A marker of apoptosis is activation of endonucleases, which cleave DNA. The appearance of endonuclease activity in the CL indicates that apoptosis occurs during luteal regression in cattle (Juengel et al., 1993). There are two apoptotic pathways, but only the intrinsic pathway involves the mitochondria, whereas the extrinsic pathway is initiated by transmembrane death receptors such as Fas, located on the cell membrane. The intrinsic pathway prevails if DNA damage or heat shock occurs. Ultimately, cytochrome c is released by mitochondria and an amplification of the caspase cascade is generated (Raff, 1998).

One early response to stress is an increase in permeability of the outer mitochondrial membrane, allowing the release of cytochrome c into the cytosol (Labedzka et al., 2006). In the cytosol, cytochrome c interacts with inositol triphosphate (IP3) on the ER, resulting in activation of calcium channels on the membrane of the ER. The sustained increase in calcium creates a positive feedback mechanism whereby additional cytochrome c is released from the outer mitochondrial membrane (Breckenridge et al., 2003).

Pro-apoptotic factors located on the outer membrane, are responsible for the change in permeability, which permits the release of both cytochrome c and the proapoptotic protein “second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein” (SMAC/DIABLO). If not subject to inhibition, SMAC/DIABLO activates caspases (Chai et al., 2000).

Caspases are cysteine-aspartic acid proteases discovered in the 1990s that are active in necrosis, apoptosis, and inflammation. Essentially, they cleave
cell proteins, resulting in death of the cell. Caspases are active in both the extrinsic and intrinsic pathways of apoptosis. Once released, cytochrome c binds to the “apoptotic protease-activating factor-1”, (APAF-1), causing the formation of an apoptosome composed of seven Apaf-1 and seven cytochrome c molecules. It is the union of APAF-1 and cytochrome c that forms this large multi-protein structure (Cereghetti et al., 2006). Within the apoptosome, pro-caspase 9 activates an initiator caspase, turning on a caspase cascade. Caspase 9 subsequently activates downstream effector caspases: 3, 6, and 7, thus destroying the cells of the corpus luteum from within. Steroidogenetic activity of the mitochondria is therefore compromised. As stated in the chapter on respiration, cytochrome c carries electrons between Complex II and Complex IV. There should be an awareness that cytochrome c has a completely separate role as an instigator of apoptosis. Since it is a small, water-soluble enzyme, it is mobile. When released into the cytoplasm from either the inner mitochondrial membrane or the endoplasmic reticulum in response to pro-apoptotic stimuli, it is involved in the initiation of apoptosis by binding to an apoptotic protease factor (Skulachev, 1998). Thus, the processes of respiration and apoptosis both rely upon cytochrome c. Cytochrome c may be involved in the apoptotic effects of PGF2α as well. Peterson (1988) found that PGE1, PGE2, and PGI2 all decreased the synthesis of cytochrome c. However, PGF2α failed to decrease the apoptotic effects of cytochrome c. Peterson’s study may therefore support the luteotropic influence of PGE2 and the luteolytic influence of PGF2α, which
has been well documented in numerous studies (Pratt et al., 1977; Fitz et al., 1993).

References Cited


Chapter III. Prostaglandins

Historical Review

In 1934, prostaglandin was discovered by von Euler of Stockholm during a search for adrenaline in animal tissue. The term “prostaglandin” arises from its discovery in seminal vesicles from the prostate (Euler 1934, 1936). Understanding the characteristics of prostaglandins was hindered by the lack of methods to purify lipid-soluble substances. In 1959, two prostaglandins, PGE1 and PGF1α, were isolated by Bergstrom and Samuelsson, an achievement for which Bergstrom, Samuelsson, and Vane received the Nobel Prize in 1982 (Bergstrom and Samuelsson, 1965).

The accepted nomenclature for the prostaglandins assigns letters to denote functional groups in the cyclopentane ring. The subscript numeral indicates the number of double bonds, thus the degree of unsaturation. If the hydroxyl group at position 9 in the cyclopentane ring is oriented below the plane of the ring, the subscript α is used; a hydroxyl group above the plane of the ring is denoted by β (Zreik and Behrman, 2008; Craig, 1975).

Biosynthesis

Prostaglandins are all derived from arachidonic acid, a 20 carbon polyunsaturated fatty acid containing a cyclopentane ring between C8 and C12 and two side chains (Sun et al., 1977). The corpus luteum contains abundant arachidonic acid (Smith and Dewitt, 1996). Prostaglandins affect every organ in the body. They regulate physiological processes such as pregnancy, ovulation, luteolysis, and uterine contractions. Their gastro-intestinal effects include the
production of pain, inhibition of acid secretion and stimulation of mucus secretion (Ophardt, 2003).

**Cyclooxygenase Pathway**

Arachidonic acid may be metabolized by either of two pathways. One pathway oxidizes the substrate via lipoxygenase, resulting in the formation of leukotrienes. In contrast, the second pathway begins with the metabolism of arachidonic acid by cyclooxygenase, resulting in the production of prostaglandins and thromboxanes (Lands, 1979). Cyclooxygenase-1 maintains normal gastric mucosa and influences kidney function, whereas cyclooxygenase-2 promotes inflammation. Both of these enzymes are able to convert arachidonic acid to prostaglandins, although investigators have reported that cyclooxygenase-1 (COX-1) requires a higher concentration of arachidonic acid than does cyclooxygenase-2 (COX-2) (Arosh et al., 2004) and COX-1 causes platelet aggregation, whereas COX-2 produces prostacyclin, which prevents platelets from adhering to vessel walls.

Several enzymes control the production of individual prostaglandins, such as cytosolic phospholipase A2 (cPLA2), cyclooxygenases, and prostaglandin synthase (Smith and Dewitt, 1996). Phospholipase A2 initiates the release of arachidonic acid from the phospholipids in the cell membrane, exposing it to the action of COX-1 and COX-2. The action of these enzymes is dependent upon the availability of calcium, which activates phospholipase A2. The COXs convert arachidonic acid into prostaglandin H2 (PGH2), (Gijon and Leslie, 1999). Various synthases catalyze PGH2, resulting in the formation of several
prostaglandins, including PGF2α and PGE2. Both of these prostaglandins use G protein-coupled receptors to stimulate target tissue. These receptors are coupled to phospholipase C, giving rise to two second messengers, inositol triphosphate (IP3) and diacyl glycerol (DAG). Inositol triphosphate diffuses into the cytosol and liberates intracellular Ca++ (Narumiya and Ushikubi, 1999) and DAG remains within the plasma membrane where it activates protein kinase C. Results of a study by Wiltbank in 1992 suggest that PGF2α acts via the protein kinase C second messenger system to exert antisteroidogenic effects upon large luteal cells.

**Various Effects**

The difficulty in investigating the effect of prostaglandins on physiological processes is compounded by their transient nature (Lands, 1979). Degrading enzymes in many tissues result in a loss of activity within one circulation of the blood (Hansen, 1976; Newcombe, 1975).

The eicosanoids, a term encompassing both the prostaglandins and thromboxanes, readily break down into inactive products. Thromboxane (TXA2) is a vasoconstrictor and platelet aggregator, therefore it is antagonistic to prostacyclin (PGI2), which is a vasodilator and inhibitor of platelet aggregation (Gryglewski et al., 1976). The arterial wall, corpus luteum, follicle, and uterus produce copious quantities of PGI2 (Bunting et al., 1977). *In vitro* PGI2 production in cardiac tissue is inhibited by nicotine. It is unclear whether this effect is the result of nicotine per se or the action of prostacyclin synthase (Wennmole, 1978). Two other prostaglandins interact antagonistically, but are
also capable of similar effects. PGE2 relaxes smooth muscle of the oviduct, whereas PGF2α contracts the muscle of the oviduct (Lindblom and Wikland, 1982). However, both of these prostaglandins are capable of causing uterine contractions, although the threshold concentration of PGF2α required for contraction is less than that of PGE2 (Villar et al., 1986).

Milvae, in Reviews of Reproduction (2000) enumerates the following effects of PGF2α: a decrease in luteal blood flow and decrease in number of small luteal cells, altered activity of steroidogenic enzymes, decreased gene expression for StAR, increased gene expression of prostaglandin G/H synthase, inhibition of steroidogenesis, changes in membrane fluidity, and release of oxytocin by the CL. Conclusions to be drawn from these data are clouded by the fact that PGF2α effects vary according to species, culture conditions, and cell types. For example, if PGF2α is added to dispersed bovine luteal cells, it actually stimulates progesterone production (Hansel et al., 1991). Possibly, the action of PGF2α is influenced by the presence of endothelial cells (Auletta and Flint, 1988) or via the presence of oxytocin (Rodgers, 1990).

Although PGs are lipids, they cannot easily cross cell membranes due to the presence of charged anions (Schuster VL, 2002). Cellular transporters aid their passage through membranes. These transporters have 12 transmembrane domains and are members of the anion transport class of transmembrane proteins (Tsai and Wiltbank, 1997). Prostaglandin transporter protein (PGT) aids the relocation of PGF2α and PGE2 from the uterus to the ovary via the utero-ovarian plexus. Impaired transport of PGF2α could affect luteolysis and fertility in
ruminants (Schuster, 1998; 2002; Lee et al., 2010). Transporter components are primarily expressed in the large luteal cells (Arosh et al., 2004).

PGE2 expresses its effects primarily via the G protein coupled receptors designated EP1, EP2, EP3, and EP4. Recently, it has been determined that EP2 and not EP4 is the main cAMP- producing PGE2 receptor. The receptor which binds PGF2α is designated the FP receptor (Arosh et al., 2004).

A viable CL and secretion of progesterone is required for maintenance of pregnancy in many species. PGE2 stimulates progesterone as efficiently as luteinizing hormone (LH) in both the bovine and ovine according to several sources (Weems et al., 2002 and 1997; Kim et al., 2001). PGE2 increases progesterone by activation of the cAMP-PKA pathway (Marsh and LeMaire, 1974; Boiti et al., 2001). The signaling pathway which leads to luteal regression is complicated and differs among species. PGF2α receptor (FP) causes an increase in Ca++ and activation of PKC. Intracellular calcium causes cytosolic phospholipase A2 to be activated and targeted to the nuclear membrane, where it causes phospholipids in the membrane to release arachidonic acid, which is converted to PGF2α. Activation of PKC also inhibits progesterone production, probably by inhibiting StAR protein (Diaz et al., 2002; Anderson et al., 2001; Tsai et al., 1998).

**Intraluteal Production**

A current concept asserts that after the secretion of extraluteal and endometrial prostaglandin, luteal sources of prostaglandin regulate the CL (Davis and Rueda, 2002). Investigations of Arosh et al., (2004) suggest that locally
produced luteal PGs complement endometrial and extraluteal availability of PG, thus influencing the function of the CL.

In the large luteal cells (LLC), arachidonic acid is metabolized into PGH2 by COX-1 and COX-2. PGH2 is then converted into either PGE2 or PGF2α by PGE synthase or PGF synthase. Prostaglandin transport (PGT) is responsible for both the egress and entrance of PGE2 and PGF2α through their receptors. In addition PGT is involved in the transport of PGE2 and PGF2α from the uterus to the ovary. There are ten known PG receptors, but those of importance in luteal cells are EP2 and EP3 and FP.

Both PGE2 and PGF2α have specific roles in the maintenance and regression of the CL. In the bovine, COX-1, PGF synthase, and PG 15-dehydrogenase are present at constant levels. However, COX-2, PGE synthase, PG transporter, EP2, EP3, and FP levels vary depending upon the phases of the corpus luteum. For example, PGE2 biosynthesis is greater in growing CLs and lesser in regressing CLs. Biosynthesis of PGF2 increases during regression of the CL. Thus, PGE2 is active during the maintenance phase of the CL and PGF2α is active primarily during luteal regression. Biosynthesis of both PGs occurs primarily in the large luteal cells (Arosh et al., 2004).

There is abundant evidence to support the contention that luteal cells secrete prostaglandins (Arosh et al., 2004). During early development of the CL and during luteolysis, secretion of PGs is elevated. Several factors are involved in this regulation. In several species an “autoamplification” pathway has been identified which enables PGF2α to induce luteal cells to secret additional PGF2α.
In various species differing expression of Cyclooxygenase-2 (Cox-2) and phospholipase A2 may control the process. Decreased progesterone, increased endothelin-1, as well as increased cytokines, all ensue as a result of PGF2α secreted by the CL (Arosh et al., 2004).

In the absence of pregnancy, PGF2α from the endometrium initiates luteolysis between day 15 and 17 of the 21-day cycle in the bovine (McCracken et al., 1999; Niswender et al., 2000). It has been confirmed by other investigations that in ruminants, more than 80% of the total progesterone is produced in the large luteal cells independent of any stimulus from LH (Diaz et al., 2002; Niswender, 2002). It remains to be determined why the large luteal cells produce the largest amount of progesterone (Niswender, 2002). In ruminants the mechanism responsible for refractoriness of the early CL to PGF2 is unknown. It is speculated, however, that limited availability of PG transport in combination with increased secretion of PGE2 may explain this refractoriness of the early CL to luteolysis.

**Influence of Various Drugs**

The modus operandi of common drugs has been clarified by knowledge of the biological activities of the eicosanoids. Aspirin, the oldest and most common drug, inhibits prostaglandin synthesis (Vane, 1971). Indomethacin blocks the cyclooxygenase pathway when administered twice daily on day 4-6 of the estrous cycle in heifers (Milvae and Hansel, 1985). It is speculated that prostacyclin may exert a luteotropic effect early in the luteal phase. Phospholipase, which acts upon the phospholipids in the cell membrane, making
arachidonic acid available, depends upon calcium for eicosanoid synthesis. Therefore, agents that inhibit calcium availability, such as local anesthetics, reduce prostaglandin production, probably by adversely affecting the phospholipase activity (Kunzl et al., 1974). Acting in a similar manner, glucocorticoids inhibit phospholipase A2, thereby decreasing the availability of arachidonic acid. This action is probably the reason why glucocorticoids exert an anti-inflammatory effect (Kantrowitz et al., 1974).

**Influence of Endothelin-1 (ET-1)**

Luteal endothelial cells produce endothelin 1, a potent vasoconstrictor that interacts with PGF2\(\alpha\) to cause luteolysis (Fukada, 1988 and Levin, 1996). There is abundant evidence that endothelin-1 (ET-1) also inhibits steroidogenesis and reduces the biosynthesis of both progesterone and prostacyclin (Milvae, 2000). Support for this theory is afforded by the discovery of receptors for ET-1 on both large and small bovine luteal cells. Endothelin 1 reaches its greatest concentration when PGF2\(\alpha\) levels are also increased, at the time of luteolysis, lending credence to theory that both ET-1 and PGF2\(\alpha\) have a role in luteolysis. When ET-1 was administered to ewes, jugular venous progesterone was reduced, while the luteal lifespan was not affected. If ET-1 and PGF2\(\alpha\) were combined, however, complete luteolysis and a reduction in the lifespan of the CL were achieved (Milvae, 2000). However, ET-1, in the absence of PGF2\(\alpha\) does not cause luteolysis in cows (Choudhary et al., 2004a) or sheep (Hinckley and Milvae, 2001).

**Influence of Nitric Oxide (NO)**
Nitric oxide is thought to mediate the luteolytic effect of PGF$_2\alpha$ via unknown mechanisms. An NO donor (SNAP) when injected directly into the CL caused an increase in blood flow (Miyamoto and Shirasuna, 2009). Studies in the cow using the NOS inhibitor, L-NAME, suggest that L-NAME acts by blocking PGF$_2\alpha$-induced luteolysis (Jaroszewski and Hansel, 2000). In 2003, evidence supported the contention that L-NAME caused prolongation of the lifespan of the CL in heifers and increased progesterone (Jaroszewski, et al., 2003).

The earliest physiological sign of the luteolytic cascade in the bovine is apparent at day 8-12 of the estrous cycle. At this phase of the cycle, PGF$_2\alpha$ reduces progesterone concentrations and decreases the volume of the CL. PGF$_2\alpha$ does not induce luteolysis during the early luteal phase up to day 5 of the estrous cycle (Hendricks et al., 1974). If PGF2 is available endogenously or exogenously at day 10, when the CL is mature, there is an increase in blood flow at the periphery of the CL, (Miyamoto and Shirasuna, 2009), which is followed by a gradual decrease in blood flow (Acosta et al., 2002). The increased blood flow in the periphery of the CL on day 17-18 was accompanied by peak levels of plasma 13, 14 dihydro 15 keto PGF$_2\alpha$ (PGFM), a metabolite of PGF$_2\alpha$. Immediately subsequent to the peak levels of PGFM, progesterone levels declined (Miyamoto et al., 2005; Shirasuna et al.; 2008c). Blood flow in the CL increases with each PGFM pulse. This increased blood flow induced by NO is one of the earliest events during luteolysis in the cow (Miyamoto and Shirasuna, 2009).
The effect of PGF2α and eNOS on the peripheral and central regions of the early CL, which is resistant to both PGF2α and luteolysis, was compared to the mid CL, which is sensitive to PGF2α. Although it appears that PGF2α does not affect blood flow in the early CL, it is known that PGF2α stimulates the expression of eNOS mRNA in the periphery, but not in the center of the mid CL (Shirasuna et al., 2008b). It is hypothesized that PGF2α action depends upon the phase of the cycle (early versus midcycle) and also on the region of the CL (periphery versus center of the CL). In conclusion, PGF2α stimulates the synthesis of NO which results in increased blood flow in the periphery of the mature CL (Miyamoto and Shirasuna, 2009). Corroborating the conclusions of these investigations is an article published in March 2012. The regulation of angiogenesis genes by prostaglandin in luteal endothelial cells (ECs) varied depending upon the stage of luteolysis. An increase in prostaglandin on day 11 of the CL adversely affected blood vessels and reduced hormonal output of the CL (Zalman et al., 2012).

*Influence of Oxytocin (OT)*

In 1906, Sir Henry Dale found that extracts from the human posterior pituitary contracted the uterus in a pregnant cat. He named the extract “oxytocin”. Forty-seven years after Dale’s discovery, oxytocin was sequenced and synthesized by Vincent du Vigneaud (Magon and Kaira, 2011). Although oxytocin exerts multiple effects on various tissues, it is primarily associated with hastening labor contractions after distension of the cervix and in causing milk to
be released from mammary glands and enter the ducts of the breast after stimulation of the nipples (du Vigneaud et al., 1953).

Oxytocin is a nonapeptide (nine amino acids) synthesized in the corpus luteum (McCracken, 1999) and in the supraoptic and paraventricular nuclei of the hypothalamus (Ross et al., 2009). It is released into the circulation from the posterior pituitary. In addition to its role in the labor process, it promotes generalized feelings of contentment and aids bonding of the infant and mother (Meyer, 2007; Marazziti et al., 2006).

In the bovine CL, the expression of oxytocin mRNA is elevated during the early luteal phase (Ivell et al., 1985; Furuya et al., 1990; Wathes and Denning-Kendal, 1992). In sheep, oxytocin mRNA is low during the mid to late estrous cycle (Ivell et al., 1990). PGF2α stimulates OT secretion from the CL (Flint and Sheldrick, 1982). Oxytocin stimulates uterine secretion of PGF2α (Roberts and McCracken, 1976). Therefore, there exists a positive feedback mechanism between these two hormones.

**Summary of the Effects of ET-1, NO, and OT**

A consideration of current research has led to the proposal of a possible sequence of events promoting luteolysis in the bovine. PGF2α is transported from the ovarian artery to luteal microvessels. PGF2α activates eNOS and the peptide apelin in the outer, peripheral area of the CL. Apelin promotes the formation of new blood vessels and the activation of apelin receptors induces the release of nitric oxide. This results in an acute increase in blood flow in the peripheral area as a result of vasodilation induced by NO. The increase in blood
flow is one of the earliest physiological signs of luteolysis in the bovine. After the
increase in blood flow, PGF2α stimulates the production of endothelin 1 (ET-1)
and angiotensin II as well as additional PGF2α from luteal cells (Milvae, 2000).
These vasoactive molecules (NO, ET-1, angiotensin II, and OT) regulate blood
flow in the CL, thereby expediting luteolysis (Miyamoto and Shirasuna, 2009).
Apoptotic factors, and perhaps PGF2α as we hypothesize in this dissertation,
cause the release of cytochrome c from the inner mitochondrial membrane and
the caspase cascade is generated (Raff, 1998). The direct relationship between
PGF2α and release of cytochrome c remains tenuous, but PGF2α had no
inhibitory effect on the promotion of apoptosis by cytochrome c (Peterson, 1988).
After the onset of the luteal cascade, there exists a positive feedback between
PGF2α and OT. Oxytocin released in a pulsatile manner on days 13-14 in sheep
induces luteolytic pulses of PGF2α (McCracken et al., 2012). The strong
vasoconstriction which ensues from ET-1, angiotensin II, and OT is accompanied
by a decrease in progesterone and an increase in apoptosis in both luteal cells
and endothelial cells.

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sheep, cows, non-human primates, and women especially in relation to the time

in the corpus luteum: recent information on the gene, messenger ribonucleic


Chapter IV. Steroidogenesis

Cholesterol

Steroid hormones are made from cholesterol, a 27 carbon molecule which is an important constituent of the cell membrane and a precursor molecule for the synthesis of steroidal hormones. Steroid compounds contain three cyclohexane rings and one cyclopentane ring. Functional groups which attach to this structure confer a variety of functions (Heffner and Schust, 2010).

In 1769, Francois Poulletier de la Salle first identified cholesterol while observing gallstones (Magee, 1998). The name “cholesterol” originates from the Greek word for bile and the word “stereos” for solid, reflecting the fact that cholesterol becomes concentrated in the bile and may settle out, forming gallstones.

The major site of cholesterol biosynthesis is the liver. Twenty to twenty-five percent of daily cholesterol production occurs in the liver. Other sites of its synthesis include the intestines, adrenal glands, and reproductive organs. Its synthesis is governed by homeostatic mechanisms that depend upon dietary intake and liver production. The greater the dietary consumption of cholesterol from animal fat sources, the less the liver will synthesize endogenously (Ness and Chambers, 2000). The primary regulatory mechanism for the synthesis of
cholesterol is sterol regulatory element-binding protein 1 and 2 (SREBP) (Grosshans et al., 2006). This sterol is able to sense the amount of intracellular cholesterol in the endoplasmic reticulum of eukaryotes (Espenshade and Hughes, 2007). When SREBP is cleaved, it migrates to the nucleus and functions as a transcription factor for intracellular cholesterol in the endoplasmic reticulum. The SREBP pathway regulates the expression of genes that control all lipid formation (Eberle et al., 2004).

Because cholesterol is unique to animals, it is essential for herbivores, such as ruminants, to have a cholesterol synthesis pathway. The first compound in the pathway is acetate, from which all of cholesterol’s carbon atoms are derived. Synthesis of cholesterol is initiated by acetyl-CoA, an organic compound in which an acetyl group is attached to a coenzyme. Acetyl-CoA is converted to hydroxymethylglutaryl-CoA (HMG-CoA) in the cytosol, whereas all subsequent steps occur in the smooth ER. When enzymatically reduced, HMG-CoA synthesizes mevalonate. From this step on, substrates are committed to become steroids. It is for this reason that attempts to regulate the production of cholesterol are concentrated on manipulating the enzyme HMG-CoA reductase. Mevalonate, via phosphorylation, produces squalene. Squalene, in turn, synthesizes lanosterol, the first sterol in the pathway. Lanosterol is the immediate sterol precursor of cholesterol (Dayspring, 2008).

After formation in the liver, cholesterol is concentrated during storage in the gall bladder, and then released into the small intestine in response to the presence of lipids in the tract. Its chief function in digestion is the emulsification of
lipids. Fifty percent is reabsorbed from the intestine and returned to the blood. In addition to excretion as bile, cholesterol may leave the liver as a lipo-protein complex called “very low density lipoproteins” (VLDL). As VLDL circulates in the blood, some of the protein moieties may be removed, forming low density lipoproteins (LDL). Lipoproteins have cell-targeting signals that guide the transport of cholesterol to its target tissues. The peripheral tissues obtain most of their cholesterol from LDL by endocytosis. Liver and peripheral tissues therefore have two means of obtaining cholesterol: synthesis from acetyl-CoA by the de novo pathway discussed in the preceding paragraph, or they may obtain it from the blood via endocytosis. Dietary cholesterol is converted within the intestine to lipid-protein complexes called chylomicrons. After removal of triglycerides from the chylomicrons at the peripheral tissues, remnants of the chylomicrons may be taken up by receptors in the liver in a manner similar to the uptake of LDL by the liver (Payne and Hales, 2004).

Cholesterol actually moves back and forth between the liver and peripheral tissues. While LDL removes cholesterol from the liver, high-density lipoproteins (HDL) return cholesterol to the liver where excess is excreted as bile salts into the intestine. In this manner, accumulation of detrimental amounts of cholesterol may be avoided.

In addition to its role in digestion, cholesterol is an essential component of the cell membrane. In contrast to plasma membranes, in which cholesterol constitutes approximately 20% of the mass of the membrane, in mitochondria it makes up about 3% of the mass of the membrane. Within the cell membrane,
the hydroxyl group on cholesterol interacts with the polar head of the phospholipids of the membrane. The hydrocarbon chain of the cholesterol molecule is embedded in the membrane in association with nonpolar portions of other lipids. Cholesterol confers fluidity to the membrane and also reduces the permeability of the membrane to hydrogen and sodium ions (Rone et al., 2009). It accomplishes this feat by separating the phospholipids so that the fatty acid chains can’t come together and crystallize. Because of the way the cholesterol molecule is shaped, part of the steroid ring is attracted to the fatty acid tail on the nearest phospholipid. This helps to stabilize the outer surface of the membrane and make it less soluble to small hydrophilic molecules. To function properly, the membrane must be neither too fluid nor too firm. Greater firmness is conferred by the formation of lipid rafts that associate with larger proteins. A lipid raft contains concentrated cholesterol and sphingolipids, a type of phospholipid with longer and more saturated fatty acid tails. These rafts aggregate, making the membrane thicker to accommodate proteins which must be in close association with other proteins in order to function properly. Which proteins exist in lipid rafts and which do not has not been determined (Alberts, 2002).

**Conversion of Cholesterol to Steroid Hormones**

The conversion of cholesterol to pregnenolone occurs inside the mitochondria where the cholesterol side-chain-cleavage enzyme (P450scc) resides on the matrix side of the inner mitochondrial membrane (Bose et al., 2002; Stocco, 2001). Thus, P450scc and enzymes of the respiratory electron transport chain both occupy the inner mitochondrial membrane. It has been
demonstrated that P450scc can function only in the mitochondria (Black et al., 1994). Once thought to be the rate-limiting step in biosynthesis of steroids, P450scc enzymatic activity is now known to partially rely upon the steroidogenic acute regulatory protein (StAR), which has been localized to the outer mitochondrial membrane (Stocco, 2001; Strauss et al., 1999).

The first two enzymes in the steroidogenic pathway are P450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD). The latter enzyme converts pregnenolone to progesterone and is found primarily in the cytosol. However, recently 3β-HSD has been located within the mitochondria, inferring that some progesterone may be synthesized within the mitochondria as well (Stocco, 2000; Chapman et al., 2005). As early as the 1960s, it was determined that 3β-HSD was located within mitochondrial fractions of ovarian tissue (Jackanicz and Armstrong, 1968; Sulimovici and Boyd, 1969; and Flint and Armstrong, 1971). Their data confirmed that conversion of cholesterol to progesterone can occur within the mitochondria of rat and bovine corpora lutea and perhaps within mitochondria of other species as well.

**P450 Enzymes**

With the advent of the 21st century, our understanding of the identification and characterization of the enzymes involved in steroid hormone biosynthesis has advanced substantially. Multiple isoforms of 3β-hydroxysteroid dehydrogenase (HSD), which is essential for the synthesis of all steroid hormones, and 17β - HSD, which is necessary for the final step in biosynthesis have been identified and cloned (Schuster et al., 2008). By utilization of
alternative promoters, it has been possible to determine the mode of action of tissue-specific P450 aromatase, a member of the P450 superfamily, which converts androgen to estrogen. Although steroidogenic enzymes are associated primarily with the adrenals and gonads, these enzymes have recently been identified in the cardiovascular and nervous systems, where they may act in an autocrine or paracrine fashion (Payne and Hales, 2004).

Although our emphasis in this dissertation is upon those enzymes which are important in the biosynthesis of steroids in the gonads, there has been evidence in recent years of de novo synthesis in the nervous system and in cardiac tissue. These neurosteroids function as paracrine factors within the nervous system in contrast to gonadal and adrenal steroids, which are released into the circulatory system to be transported to their target sites. As new techniques become available, it is likely that more peripheral sites of enzyme expression will be discovered (Mellon and Griffin, 2002).

All the enzymes involved in the biosynthesis of the adrenal steroids: corticosterone, cortisol, adosterone as well as those involved in biosynthesis of gonadal steroids: progesterone, estradiol, and testosterone, fall into two major classes of proteins. These classes are: the cytochrome P450 heme-containing proteins and the hydroxysteroid dehydrogenases. Isoforms of 3β - HSD are homologous in their amino acid composition, unlike the 17β - HSDs that show very little amino acid homology (Curnow et al, 1991). Adrenal and gonadal trophic hormones differ in their pathways. Gonadal hormones act via cAMP
through a protein kinase A pathway, whereas adrenal glands use a protein kinase C pathway (Tremblay and Viger, 2003; Kuri, 2009).

The P450 enzymes (re-named the Cytochrome P450 or CYP) are membrane-bound proteins in contact with either the mitochondrial membranes: CYP11A, CYP11B1, and CYP11B2 or in contact with the endoplasmic reticulum, i.e. microsomal: CYP17, CYP19, and CYP21. These heme-containing proteins are found in bacteria, fungi, plants, and animals (Nelson et al., 1996). When combined in vitro with carbon monoxide, they absorb light maximally at 450 nm. The P450s accept two oxygen atoms from nicotinamide adenine dinucleotide phosphate (NADPH). One oxygen atom binds to the substrate as a hydroxyl group and the other oxygen atom is reduced to water. Two distinct electron transfer systems are involved in this transfer. Initially, the mitochondrial transfer system passes an electron to adrenodoxin reductase, then to adrenodoxin, then to P450 and finally to the substrate. The microsomal transfer system, which takes place in the endoplasmic reticulum, involves cytochrome oxidoreductase, a protein which contains two flavins. In this case electrons are transferred from NADPH to a flavinadenine dinucleotide, then to flavinmononucleotide, next to P450 and finally to the substrate (Payne and Hales, 2004).

**CYP11A (P450scc)**

CYP11A catalyzes the first step in the biosynthesis of all steroid hormones. Three molecules of oxygen, three molecules of NADPH, and the mitochondrial electron transfer system are required. The side-chain on cholesterol is cleaved between carbons 20 and 22, resulting in the C21 steroid
pregnenolone (Bose et al., 2002). CYP11A has been detected in all three zones of the adrenals, the theca interna and granulosa cells of ovulatory follicles, testes, placenta, central and peripheral nervous systems, and in the human and rodent heart (Kaves-Wandover and White, 2000; Young et al., 2001).

**CYP17 (P450c17)**

CYP17 utilizes the microsomal electron transfer system of the ER to catalyze the hydroxylation of the C21 steroids (pregnenolone and progesterone), followed by cleavage of the C17-20 bond, to produce the C19 steroids: androstenedione, androgens, and testosterone. Each reaction requires one molecule of NADPH and one molecule of molecular oxygen (Payne and Hales, 2006). CYP17 is expressed in Leydig cells and thecal cells, but not in granulosa cells (Sasano et al., 1989).

**CYP19 (P450arom)**

CYP19 converts the C19 androgens, androstenedione, and testosterone to the C18 estrogens, estrone, and estradiol. Three molecules of oxygen and three molecules of NADPH are required for this reaction, which uses the microsomal electron transfer system of the ER (Payne and Hales, 2004).

**Regulation and Expression of P450 Steroidogenic Enzymes**

The nuclear binding protein, steroidogenic factor -1 (SF-1) (Lala et al., 1992), also referred to as Ad4BP, (Morohashi et al., 1992), is essential for cell-specific gonadal and adrenal expression of P450 enzymes. ACTH stimulates the release of the adrenal hormones, whereas LH in the theca, corpus luteum, and Leydig cells and FSH in the granulosa cells activate adenylate cyclase, thereby
increasing cAMP and stimulating the synthesis of P450 enzymes. This stimulation is affected via G protein-coupled receptors (Waterman, 1994; Waterman and Keeney, 1996). With the exception of CYP17, whose secretion is wholly dependent upon cAMP, other factors aid in maintaining maximal expression of the other P450s (Anakwe and Payne, 1987).

**Hydroxysteroid Dehydrogenase**

One of the chief differences between the P450 enzymes and the hydroxysteroid dehydrogenases is the fact that all P450 enzymes are coded by a single nuclear gene. The several isoforms of the 3β-HSDs and the several isozymes of 17β-HSDs are each coded by separate genes. Both the 3β-HSDs and the 17β-HSDs are involved in the reduction and oxidation of steroid hormones that require NAD+/NADP+. The 17β-HSDs catalyze the final reaction in the biosynthesis of estradiol and testosterone. The isomerases are found in both mitochondrial and microsomal membranes depending upon the type of cell in which they are expressed. Since they are numbered in the order of their discovery, it is not possible to ascribe the same characteristics to similarly numbered isoforms found in different species (Simard et al., 1996; Payne et al., 1997; Ahhaszade et al., 1997). The 3β-HSDs are required for the production of all steroid hormones except pregnenolone. The localization of 3β-HSDs within the mitochondria has been determined in bovine adrenal cells and rat adrenal cells (Cherradi et al., 1997). The significance of finding 3β-HSD within the mitochondria leads to the supposition that some pregnenolone is converted to
progesterone before exiting the mitochondria. The HSDs have been studied much more extensively in the human, mouse, and rat than in other species.

**Intracellular Pathways of Cholesterol Transport**

The 37 kDa precursor of steroidogenic acute regulatory protein (StAR) is synthesized in the cytoplasm, accompanied by chaperone proteins which prevent its folding. In the unfolded form, the precursor is able to gain entrance through the outer mitochondrial membrane. Upon entrance, cleavage of the precursor by proteases in the mitochondrion convert the immature 37 kDa form to the mature 30 kDa form of StAR.

The presence of a “sterol-binding pocket” on StAR can accommodate one molecule of cholesterol when it is in contact with the outer membrane (Miller and Auchus, 2011). When StAR ceases to be in contact with the outer membrane, cholesterol transport ceases (Stocco, 2000). StAR is dependent upon transporter protein (TSPO) for its import into the inner membrane (Rone, 2009).

Translocator Protein (TSPO) is an 18 kDa protein located on the outer mitochondrial membrane. Initially determined to be a secondary binding site for diazepam, it was called Peripheral Benzodiazepine Receptor (PBR), a term which is still in use. However, subsequent research has expanded greatly what is known regarding its many functions. It is expressed throughout the body and in small amounts in the brain. TSPO is a biomarker of gliosis and inflammation associated with several neuropathologic disorders. Recent investigations support the idea that TSPO ligands may act to facilitate healing of brain injury. In
addition, TSPO acts to reduce anxiety and panic attacks without causing sedation or withdrawal problems (Chen and Guilarte, 2008). Upregulation of TSPO has been confirmed in multiple sclerosis, Parkinson’s disease, Huntington’s disease and Alzheimer’s disease, thus it has been possible to use TSPO as a marker to diagnose and follow the progression of many diseases (Rone et al., 2009).

In 1986, TSPO was localized to the outer mitochondrial membrane (Anholt et al., 1986). In addition to its association with steroid synthesis in adrenal, placental, testicular, ovarian and glial cells (Brown and Papadopoulos, 2001; Papadopoulos et al., 2001; Lacapere and Papadopoulos, 2003; Giatzakis and Papadopoulos, 2004), it has a role in such disparate functions as induction of apoptosis in colorectal cancer cells, (Batarseh and Papadopoulos, 2010), modification of the immune system, regulation of cell proliferation, transport of porphyrin. In addition, it assists the entrance of cholesterol through the outer mitochondrial membrane (Casellas et al., 2002). A ligand of TSPO, Ro5-4864, reduces reactive oxygen species (ROS) cell damage. Therefore, a beneficial effect of this TSPO ligand may prevent aging-associated peripheral nerve damage (Leonelli et al., 2005).

Of special interest to us is the ability of TSPO to bond with strong affinity to cholesterol and to aid in transport of cholesterol across the outer mitochondrial membrane (Papadopoulos et al., 1997). It is an essential partner with StAR in the early steps in steroidogenesis. There are several steps whereby StAR is transferred through the outer membrane and into the inner membrane of the
mitochondrion, while TSPO regulates the translocation of cholesterol into the inner mitochondrial membrane. It is unclear how cholesterol binds to P450scc after it moves to the membrane (Rone, 2009).

Research since the early 2000s has clarified the role of both StAR and TSPO. StAR cannot accomplish the transport of cholesterol without the assistance of TSPO (Jefcoate and Artemenko 2004). The outer mitochondrial membrane of steroidogenic cells contains abundant receptors for TSPO. These receptors have been localized in the mitochondrial permeability transition pore of the outer mitochondrial membrane (Marselli et al., 2004), where the receptors associate with the porin family of channel proteins. When porins are activated by agonists or acetyl CoA binding protein, their permeability is altered. Thus, mitochondria are able to alter permeability of these channel proteins (Jefcoate and Artemenko, 2004). Entrance of cholesterol into the mitochondria may be affected by the activity of these porins, since receptors for TSPO are located in their vicinity,

It may be assumed that transport of cholesterol across the aqueous intermembrane space occurs with difficulty. This is just one of the many questions which require further investigation. The study of the process in yeast has yielded some insights, which may be applied to other organisms (Cyr and Neupert, 1996; Koehler et al., 2000). After translation in yeast, immature StAR p37 is phosphorylated, causing the enzyme to unfold from its prior conformation. In its unfolded state it is able to pass through channels in the outer mitochondrial membrane and inner mitochondrial membrane and refold in the mitochondrial
matrix, forming the mature StAR p30. It is hypothesized that mature StAR p30 aids the relocation of cholesterol from inner mitochondrial membrane sites which are in close proximity to the outer membrane to tubular sites on the inner membrane where P450scc is located. Tubular protrusions and buds can be seen on the inner mitochondrial membrane with scanning electron microscopy. These buds increase the surface area, creating more space for steroidogenic enzymes and possibly allowing for separation from respiratory domains (Jecoate and Artemenko 2004; Riva et al., 2003). It is further conjectured that stimulatory effects of free fatty acids on the metabolism of cholesterol by P450scc may assist translocation of StAR to the inner membrane (Dhariwal et al., 1989). Thus, fatty acids may accumulate in proximity to StAR.

Many questions remain to be answered by further investigation. The complex process of cholesterol transport is regulated at many points by several signaling pathways. A balance must be maintained between cytoplasmic transfer of cholesterol and its transfer into the mitochondria to avoid the accumulation of excess cholesterol within the mitochondria. Reference has been made to the protein-protein complex involved in cholesterol transport. A water-soluble form of cholesterol, 22-R-hydroxycholesterol, induced pregnenolone synthesis 20-fold (Lui, et al., 2006. Questions arise regarding the origin of the cholesterol used in steroid synthesis. It has long been assumed that cholesterol is made available via lipid droplets and the plasma membrane. DiBartolomeis and Jefcoate (1984) proposed the presence of pools of cholesterol in the mitochondria that are able to support steroidogenesis for the first few minutes. It is currently thought that
these pools can sustain steroidogenesis longer than a few minutes (Lui, 2006). Recent technical advances must be brought to bear on other questions. Characteristics of the two primary forms of cholesterol, LDL (low density lipoprotein) and HDL (high density lipoprotein), vary considerably. The main function of LDL is to transport cholesterol to tissues and arteries, whereas HDL transports cholesterol from tissues and arteries to the liver and other organs for storage. LDL is a larger compound than HDL and therefore may enter mitochondria via endocytosis. Since Cholesterol in the form of HDL is a smaller compound, it may pass into the mitochondria directly bound to esters, compounds produced by a reaction between an acid and an alcohol (Maxfield and Wustner, 2002).

Experiments in yeast suggest that the second messenger, cAMP binds to protein kinase A and subunits are released, causing phosphorylation of StAR (Cyr and Neupert, 1996; Koehler et al., 2000). Hormonal signals are thereby amplified. The mechanism whereby amplification occurs is unknown. A binding protein located mainly in the Golgi apparatus (PAP 7) binds to both PKA and TSPO. Li et al., (2001) hypothesizes that PAP7 may be involved in p37 StAR phosphorylation. Cholesterol likewise binds to TSPO and enters the inner mitochondrial membrane via voltage dependent anion channels (VDAC). There are contact sites between the outer and inner membrane at the location of TSPO binding. Located in the inner membrane is the side-chain cleavage enzyme, P450, which cleaves the bond between carbon 20 and 22, removing these from the cholesterol molecule and yielding pregnenolone (P). Failure at any point in
this chain of events would prevent the synthesis of pregnenolone and the subsequent synthesis of progesterone. Extrapolation of these data to organisms exclusive of yeast should be considered with caution.

Several comparisons can be made between the processes of steroidogenesis and respiration. First of all, both occur on the inner mitochondrial membrane. The path of electron flow in steroidogenesis involves NADPH and FADH2 (nicotinamide nucleotides or flavin nucleotides) and a series of oxidation/reduction reactions. The path of electron flow in respiration involves a compound similar to NADPH, NADH. The respiratory electron acceptors carry electrons from catabolic reactions to the electron transport chain and the steroidogenic electron acceptors carry electrons to anabolic reactions. Cells maintain separate groups of NADH (respiratory electron acceptor) and NADPH (steroidogenic electron acceptor). Neither acceptor can cross the inner mitochondrial membrane. Presumably, they are located in disparate positions on the membrane. The exact location remains to be determined. The inner membrane is thus essential for these two processes that have an effect upon the corpus luteum: respiration and steroidogenesis.

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Chapter V. Respiration

Overview

Cellular respiration consists of a series of metabolic reactions that take place primarily in the mitochondria for the purpose of converting biochemical energy from nutrients into adenosine triphosphate (ATP), releasing carbon dioxide and water as waste products. Cells store and use the energy in the bonds of ATP, which is synthesized primarily in the mitochondria. Adenosine triphosphate (ATP) supplies the energy required to maintain all body processes. The first stage of cell respiration, glycolysis, occurs in the cytosol. Since oxygen is not involved, this initial stage is anaerobic. Although a total of 4 ATP molecules are generated, 2 ATP molecules are expended in the formation of two molecules of nicotinamide adenine dinucleotide (NADH) as well as the synthesis of one molecule of pyruvate. Pyruvate enters the mitochondrial matrix where it is oxidized to acetyl-CoA. It is within the mitochondrial matrix that the second stage, the “citric acid cycle” or “Krebs cycle” takes place. One glucose molecule undergoes a series of oxidation reactions that generate 2 ATP, 6 NADH and 2 FADH$_2$ upon the completion of the cycle. The first carrier is nicotinamide adenine dinucleotide (NAD), which becomes reduced to 6 molecules of NADH. The second carrier is flavin adenine dinucleotide (FAD), which becomes reduced to two molecules of FADH$_2$ (Verkhovsky et al., 1996). These last two products of the Krebs cycle (NADH and FADH$_2$) are released from the mitochondrial matrix and participate in the last phase of cell respiration, the electron transport chain, donating electrons and hydrogen atoms which provide energy for the generation
of almost all the ATP required for cell processes by the process of chemiosmosis (Babcock and Wikstrom, 1992). This process creates a proton gradient that enables the synthesis of ATP from ADP and phosphate. Since ATP molecules cannot be stored for long periods of time, cellular respiration must constantly regenerate ATP. Each ATP molecule releases 7.3 kilocalories of energy per mole (Crofts A, 1996).

In 1948, Eugene Kennedy and Albert Lehninger discovered that mitochondria are the site of oxidation phosphorylation in eukaryotes. Thus began the modern study of biological energy transductions. Mitochondria have two membranes. The outer mitochondrial membrane (OMM) is easily permeable to small molecules and ions, which can move freely through transmembrane channels formed by "porins", integral membrane proteins. The inner mitochondrial membrane (IMM) is relatively impermeable. Specific transporters are required to traverse the IMM. Protons cross the membrane as part of antiport transport mechanisms, for example Ca++, Fe++, K+, and Na+ (Scheffler, 2000).

The mitochondrial respiratory chain contains sequentially acting electron carriers, most of which are integral proteins that accept one or two electrons. In addition to nicotinamide adenine dinucleotide (NAD) or NADP, other electron carriers function in the chain: hydrophobic ubiquinone, which is lipid-soluble and cytochromes. Ubiquinone can accept one or two electrons. It can freely diffuse within the lipid bilayer of the IMM. Because it can carry both electrons and protons, it can couple electron flow to proton flow. Mitochondria contain three
classes of cytochromes: a, b, c, named for differences in their light-absorption spectra.

In the electron transport chain, electrons move from NADH, succinate, ubiquinone, cytochromes and eventually to oxygen. Each carrier down the chain is more electronegative than the preceding carrier.

The constituents within the inner mitochondrial membrane that perform oxidation/reduction reactions are referred to as “complexes”. The first protein to receive two electrons from NADH is NADH dehydrogenase located in Complex I. This electron transfer drives the expulsion from the matrix of four protons. A mobile carrier called ubiquinone passes the electrons to Complex II, but unlike subsequent carriers, ubiquinone does not expel any protons into the space between the inner and outer membranes. Succinate (which was synthesized in the Krebs cycle) is the only component of the transport chain that takes part in both the Krebs cycle and the electron transport chain. Succinate enters the transport chain at Complex II and donates electrons to FAD. Each passage of electrons is accompanied by the expulsion of a proton into the space between the membranes. The electrons are carried from Complex II to Complex III by another carrier, cytochrome c. Cytochrome c is a small, heme-containing soluble protein, which freely diffuses within the membrane, carrying one electron from Complex II to Complex III. However, unlike the other carriers, cytochrome c transports only one electron at a time (Voet and Voet, 2004).

Some of the hydrogen protons are combined with oxygen at Complex IV, producing water. Oxidation of NADH and FADH$_2$ and the phosphorylation of
ADP are coupled by a proton gradient, which has developed across the inner membrane. When electrons are transferred from donor to acceptor through this series of protein complexes, the energy released as they flow through the transport chain is used to transport protons across the inner membrane, where they accumulate in the space between the membranes in a process called “chemiosmosis” (Mitchell, 1961). Chemiosmosis generates potential energy in the form of a pH and electrical gradient formed by ions, which pass through the large, mushroom-shaped ATP synthase complex sometimes designated Complex V. This enzyme completely penetrates the membrane. The “stem” portion of ATP synthase is capable of rotating and with each rotation ADP is phosphorylated to ATP (Campbell et al., 2006). The current model of ATP synthase (alternating catalytic model) uses the force of movement of protons across the inner membrane to drive the synthesis of ATP. ATP synthase undergoes a conformational change, which results from the rotation of one of its subunits. As hydrogen protons enter this structure from an area of high concentration to an area of low concentration, the ATP rotator turns, producing the energy for the synthesis of ATP from ADP and phosphate. Thus, energy stored in the proton gradient drives the synthesis of ATP from ADP and phosphate (Nakamoto et al., 2008). Estimates of the number of protons required to synthesize one ATP molecule range from three to four (Voet and Voet, 2004). As a result of these oxidation-reduction reactions approximately 36-38 molecules of ATP are synthesized.
The original model for how complexes are arranged has recently been questioned. The complexes may form supercomplexes or “respirasomes” (Heinemeyer et al., 2007). According to this model, the complexes form organized sets of interacting enzymes. Possibly, this might permit more efficient channeling of substrates between complexes. However, the disagreement over these respirasomes is on-going, since some data do not conform to this model (Schagger and Pfeiffer, 2000).

**Inhibitors**

Several common drugs and toxins are known inhibitors of oxidative phosphorylation. An explanation of how some of the more commonly used inhibitors affect the respiratory process will aid understanding of some research paradigms.

Cyanide binds to the iron groups in cytochrome oxidase, thereby inhibiting the electron change by halting all oxidation/reduction reactions. Proton pumps cannot function (Illingworth, 2012). Azide exerts an effect which is similar to cyanide. It binds more strongly than oxygen to cytochrome c oxidase, thereby preventing the reduction of oxygen (Tsubaki and Yoshikawa, 1993).

Rotenone impairs *Complex I* by preventing the transfer of electrons from *Complex I* to ubiquinone by blocking its binding site (Lambert and Brand, 2004).

The affinity of the electron chain for carbon monoxide is lower than that for oxygen. However, it still exerts a detrimental effect by inhibiting cytochrome oxidase (Illingworth, 2012).
Antimycin inhibits the flow of electrons from cytochrome b to cytochrome c₁ at Complex 3, thus preventing the reduction of cytochrome c and the oxidation of both NADH and succinate (Illingworth, 2012).

Another disruptor of cell respiration is 2,4-dinitrophenol, which interferes with the proton gradient by carrying protons across the inner mitochondrial membrane, thereby uncoupling proton pumping from the synthesis of ATP (Heytler, 1979).

Oligomycin is an antibiotic which binds to ATP synthase, the last enzyme in the electron transport chain, thus preventing State III respiration. State III is defined as ADP-stimulated respiration. As energy in the electrical gradient is used up, ADP binds to ATP synthase. In the presence of inorganic phosphate, which also binds ATP synthase, a channel is opened in the ATP synthase complex that allows protons into the matrix from the inner mitochondrial membrane. As energy in the gradient is removed, the electron transport chain speeds up, producing more ATP. Oligomycin blocks the proton channel in the ATP synthase, thereby completely preventing STATE III respiration, which requires ATP (Joshi and Huang, 1991). The respiratory effects are not immediate, but become apparent after several minutes (Joshi and Huang, 1991).

Malonate and oxaloacetate are competitive inhibitors of Complex II. Malonate inhibits succinate dehydrogenase by binding to its active site without reacting and therefore competes with succinate. Oxaloacetate is also an inhibitor of Complex II (Heytler, 1979).
Comparison of Electron Carriers

There are both similarities and dissimilarities between electron carriers involved in the respiratory chain and electron carriers in the steroid P450 system. Although some enzymes are embedded in the inner mitochondrial membrane, in both the respiratory and steroidogenic systems there are small peripheral enzymes that act as electron carriers. It is postulated that this may afford faster diffusion of surface proteins in contrast to those within the membrane. The membrane has a high protein content, which may impede electron transport. For example, the diffusion rate of cytochrome c is ten times faster than complexes embedded in the membrane (Gupte, et al., 1984).

Mitochondrial P450 systems bear similarities to the respiratory oxidative phosphorylation system in several respects. The respiratory system is composed of four complexes (I-IV), which are embedded in the inner mitochondrial membrane. Cytochrome c is a small protein, which is not embedded, but is located on the side of the intermembrane space. This is also true of another small electron transfer protein, ubiquinone, of the respiratory transfer system. All three proteins of the mitochondrial P450 systems are integral membrane proteins, whereas adrenodoxin and adrenodoxin reductase are mobile and soluble in the matrix (Hanukoglu et al., 1981a, 1981b). In this regard, therefore, they may be compared to cytochrome c and ubiquinone of the respiratory transfer chain.

Electron transfers between Complex III and Complex IV are mediated by cytochrome c. Transfers between Complex I, II, and III are mediated by
ubiquinone, another small protein. Since cytochrome c binds both to its reductase on Complex III and oxidase on Complex IV it must be capable of moving between the two complexes. In this regard, cytochrome c in the respiratory chain behaves in a similar manner to adrenodoxin in the steroidogenic system. Both are capable of movement and are not, therefore, integral components of the membrane (Capaldi, 1982 and Gupte et al., 1984).

Since oxidative phosphorylation enzymes and steroidogenic enzyme levels increase in concert, it seems likely that their regulation is correlated. This is understandable, since increased steroid synthesis necessitates an increase in the requirement for energy.

**Reactive Oxygen Species (ROS)**

Reactive oxygen species are generated during cellular respiration and steroidogenesis.

Electron transfer may be "coupled" (all electrons from NADPH used to hydroxylize the substrate) or "leaky" (some electrons transferred to oxygen). In the latter instance, oxygen radicals are generated as a result of the amount of NADPH oxidized exceeding the amount of substrate hydroxylated. The reactivity of these oxygen radicals is due to their unpaired valance electrons. Experiments have shown that P450 proteins can oxidize NADPH even when steroid substrate is not present (Rapoport et al., 1995). Since NADPH is required for many reactions within the mitochondria in addition to its role in the respiratory transport chain, a basal level is necessary for normal cell function. It appears, therefore, that the creation of oxygen radicals is unavoidable. Oxygen is the acceptor of...
leaky, uncoupled electrons, producing a superoxide radical (Hanukoglu et al., 1993). Two superoxide radicals are able to react with two protons, producing hydrogen peroxide in a reaction termed a “dismutation”. Hydrogen peroxide may be converted to benign substances such as oxygen and water by catalase and superoxide dismutase. However, it is likely that some peroxides will persist in the cell. The leakage of electrons from mitochondria during the respiratory electron transfer chain is the primarily source of these reactive oxygen radicals (ROS), (Genova et al., 2003).

Cells defend themselves against ROS damage with enzymes such as superoxide dismutases, catalases, lactoperoxidases, and small molecule antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E), uric acid and glutathione. These substances either neutralize or scavenge free radicals (Hanukoglu, 2006).

The deleterious effects of ROS include damage to proteins, lipids and DNA. ROS is generated when cells are actively proliferating and progesterone is being synthesized. The generation of free radicals contributes to apoptotic cell death, which occurs during luteolysis (Behrmann et al., 2001). Evidence from several sources indicates a decrease in antioxidant levels as the corpus luteum regresses, although the results are not consistent (Hanukoglu, 2006). In addition to ROS, there are many other factors involved in luteolysis (Amsterdam et al., 2003; McCracken et al, 1999).
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Part 2

Rationale and Methodology
Chapter VI. Hypothesis and Objectives

Rationale for Investigating Effects of PGE2 and PGF2α

Our investigations have focused on two prostaglandins: PGE2 and PGF2α and their effects upon cell respiration and progesterone synthesis. This choice was partially dictated by a literature search, which revealed the complex and conflicting influence of these two prostaglandins upon cell respiration and steroidogenesis. It is apparent that there is ample ambiguity to justify further investigation. The present study has been conducted to clarify the mode of action of these two prostaglandins in bovine corpora lutea. It is noteworthy that we have found no mention in the literature of the effect of PGE2 or PGF2α upon oxygen consumption of mitochondria isolated from bovine corpora lutea. In view of the fact that respiratory distress could contribute to the process of luteolysis, it was decided to pursue this avenue in our research. We believe that the current study constitutes new, important data on the influence of PGE2 and PGF2α on cellular respiration.

Hypothesis

The addition of PGE2 or PGF2α to mitochondria obtained from bovine corpora lutea will affect oxygen consumption and the secretion of progesterone.
Objectives

The overall objectives of this study were:

1. To determine the effect of PGF2α or PGE2 on oxygen consumption by mitochondria isolated from bovine corpora lutea.

2. To determine the effect of PGF2α or PGE2 on the synthesis of progesterone by mitochondria isolated from bovine corpora lutea.
Chapter VII. Materials and Methods

Mitochondrial Isolation

Mitochondria were isolated from firm, well-developed bovine corpora lutea obtained from Applied Reproductive Technology, Madison, WI. All procedures were carried out at 4°C. Corpora lutea were removed from ovarian tissue, minced, and washed with isolation buffer (250 mM sucrose, 1mM EGTA, 10 mM Tris, 0.1% BSA, and the pH adjusted to 7.4). Following washing, 15 g of minced tissue was suspended in 150 ml of isolation buffer and subjected to two homogenization processes. The first of these was accomplished in a Kontes Duall grinder (Vineland, New Jersey) with several passes, whereas the second homogenization was performed using a Wheaton Potter-Elvehjem grinder (Millville, New Jersey) with several passes. Pestles for these grinders were driven by a heavy-duty drill at 1400 rpm. After homogenization, contents were centrifuged for 10 minutes at 3000 rpm (452.5 g) to remove cellular debris. The pellet was discarded and the supernatant centrifuged for 10 minutes at 7500 rpm (2830 g). After the second centrifugation, the pellet was saved, and washed with suspension buffer. The pellet was transferred to an eppendorf tube with 1000 µl of suspension buffer (250 mM sucrose, 10 mM HEPES, pH 7.4). Protein content was measured using a Bicinchoninic Acid Protein Assay Kit B9643 (Sigma Chemical Co, St. Louis MO). The Bicinchoninic Assay consists of two reagents: Reagent A (Sigma B9643) and Reagent B (Sigma C2284). Reagent A contains 1 g sodium bicinchoninate, 2 g sodium carbonate, 0.16 g sodium tartrate, 0.4 g NaOH, and 0.95 g sodium bicarbonate. These components were added to 100
ml distilled water and adjusted to pH 11.25. Reagent B contains 0.4 g cupric sulfate in 10 ml distilled water. Reagent A, 100 volumes, is mixed with 2 volumes of reagent B. Samples are read at 562 nm. This test is based upon the principle that complexes between copper ions and peptide bonds will produce a purple end product.

**Characterizing Mitochondrial Function**

In the current investigation, mitochondrial function was characterized using oxygen consumption studies and also via staining to visualize outer membrane integrity.

**Oxygen Consumption Measurement**

Mitochondrial oxygen uptake was determined using a Clark electrode. The Clark electrode operates on the principle that reduction of oxygen will generate a current. The current decreases if oxygen consumption increases. Therefore, the current is directly proportional to the amount of oxygen in a solution. Oxygen consumption was expressed as nanomoles of oxygen consumed per minute per mg of mitochondria. The resulting decrease in voltage (mV) was graphed and analyzed to determine oxygen consumption.

The Clark electrode was attached to a water bath maintained at 37 °C and connected to a computer and data logger. Incubation buffer provided oxygen to the incubation chamber, which was stirred with a 10 mm magnetic bar at 600 rpm. Oxygen consumption of mitochondria was recorded at pH 7.4 in incubation buffer (250 mM sucrose, 5mM KH$_2$PO$_4$, 5mM MgCl$_2$, 0.1 mM EDTA, 0.1% BSA, 20 mM HEPES).
**Mitochondrial Staining**

Further verification of the health of the mitochondria and the integrity of the outer membrane was obtained by use of a hemocytometer to view mitochondria at 440 magnification. The mitochondria were stained with Janus Green B (Ward’s #9458601; 1% aqueous solution), a vital stain, which is the preferred stain for mitochondria, since it changes color according to the amount of oxygen present. Mitochondria stain blue in its presence, indicating the presence of oxygen. Our intention was to verify the presence of healthy mitochondria, since the mitochondria stain pink if the membrane is compromised.

Our diluted solution contained 10 µl mitochondria (14,425 mitochondria), 10 µl Janus Green B dye, and 80 µl suspension buffer. The suspension buffer consists of 21.4 g sucrose, 0.691 g HEPES at pH 7.4. Mitochondria were observed via the use of a hemocytometer. The area of each small square = 0.04 mm$^3$, depth = 0.1 mm. Area x depth = volume per square. Volume per square multiplied by the number of squares counted = the number of mitochondria per mm$^3$. Lastly, this number is multiplied by the dilution factor. When oxygen is present in the medium, the dye takes on a blue color (Lazarow and Cooperstein, 1953).

It is extremely difficult to view mitochondria, whose average size ranges from 0.5 to 10 µm and whose numbers are estimated to average 1000 – 2000 per liver cell (Marinos and Billet, 1981). Liver cells contain the greatest number of mitochondria, although the number varies greatly depending upon type of cell and species. Our estimate was approximately 1,442.50 mitochondria/µl of
suspension buffer and dye (~ 100 µl). The mitochondria were stained a dark blue, which facilitated observation. It was doubly difficult to observe pink mitochondria, but it can be stated that there were very few. The chief value of this staining exercise was to verify that the outer membranes of the mitochondria were intact.

**Determining the Oxygen Consumption Rate**

Oxygen consumption rate (OCR) was calculated. OCR respiration is defined as *in vitro* oxygen consumption by isolated mitochondria in a substrate (frequently succinate) in the absence of ADP or any inhibitors. The rationale for determining the OCR is to permit measurement of the rate of electron transport when dissolved oxygen is reduced to water. The significance of oxygen reduction is that it removes electrons, allowing the carriers, NADH, which donates electrons to *Complex I*, and FADH2, which donates electrons to *Complex II*, to be re-oxidized. Thus, the electron chain is essentially an energy converter that harnesses the flow of electrons to the pumping of hydrogen ions across the inner membrane and into the intermembrane space. This separation of electrons on one side of the inner membrane and hydrogen ions on the other side of the membrane results in an electro-chemical gradient. Damaged mitochondria cannot maintain the gradient. Every *in vitro* preparation will include a small proportion of damaged mitochondria. Such mitochondria are referred to as “uncoupled”, since the gradient is no longer coupled to the synthesis of ATP. Although damaged, such mitochondria are capable of consuming oxygen but not capable of synthesizing ATP. OCR respiration does not exist *in vivo*, because
undamaged mitochondria will not halt activity at Complex IV, but continue the process to Complex V and the synthesis of ATP.

The OCR is determined by measuring points on the graph generated by the Clark electrode, indicating the decline of millivolts. See Figure 1 for a pictorial representation of the graph and the equation used to determine the OCR.

**Progesterone Determination**

Preparation of mitochondria for the steroidogenesis trials was identical to the mitochondrial preparation for the respiration trials, as stated in Chapter VII.

A hydroxylated derivative of cholesterol, 22R-Hydroxycholesterol (Sigma HP384) was chosen in preference to cholesterol for our substrate, since it had been used successfully in investigations similar to ours (Fitz et al., 1993; Arikan and Yigit, 2009). Hydroxycholesterol is slightly soluble in water, but readily soluble in ethanol. Therefore, 6-7 µl ethanol was added to our protocol.

Progesterone formation was determined according to Vizcarra et al. 1997. Progesterone was quantified using a solid phase RIA (Coat-A-Count progesterone kit, Diagnostic Products Corp., Los Angeles, CA). When different concentrations of plasma were assayed, concentrations were parallel to the standard curve.

**Standardizing the Substrates and Prostaglandin Concentration for Oxygen Consumption and Progesterone Determination**

Incubation buffer (1000 µl 1 ml) was added to the Clark electrode as our control (see page 85 for chemical constituents). Isolated mitochondria (30 µl; 1.5 mg/ml 45 µg; 1.5mgml) were added to the incubation buffer and allowed to
stabilize for one minute. In the incubation medium either PGF2α or PGE2 (0.75 or 1.0 µM, (final concentration ~ 3500 pg/ml) was added and the contents stabilized for 3 minutes. Succinate (20 µl; 20 mM, final concentration), (Fisher S413) was dissolved in distilled water and added after 5 seconds to the Clark electrode. Ten µl of a known respiratory inhibitor, antimycin, (.02 mM, final concentration in ethanol) (Sigma A-864), was added after 5 seconds in one or two of our trials to ascertain activity of our mitochondria.

The concentration of antimycin and succinate was based upon that used by other investigators. We were guided by the results of an experiment by Bowolaksono (2008), in our choice of 1 µM concentrations for both PGFα and PGE2. Beta Nicotinamide adenine dinucleotide 2’ phosphate, NADPH (25 µl; 1mM, final concentration), (Sigma N6505) was added to our progesterone trials.

We determined the appropriate molar concentration of our reagents by performing an initial experiment (Figure 3). The number of µl of mitochondria used in each trial was dependent upon the results of our Bicinchonic Acid (BCA) analysis, which determined the protein content of the mitochondria.

**Statistical Analysis**

The experimental design was a completely randomized design and the data were analyzed using Proc Mixed procedure of SAS using type 3 tests of fixed effects. Results were considered significant at p < 0.05.

Trials were performed in duplicate and repeated three times in respiration experiments. Trials were performed in duplicate and repeated four times in progesterone experiments.
References Cited


Part 3

Results and Discussion
Chapter VIII. Results and Discussion

Prostaglandin Influence on Oxygen Consumption

Figure 1 aided our analysis of data in the determination of oxygen consumption rate. Two points on the curve just after succinate addition were selected and the oxygen consumption rate was calculated using the equation shown. This calculation was performed on 115 graphs from each individual experiment and used to support our conclusions regarding the effect of succinate, antimycin, and prostaglandins on mitochondrial oxygen consumption.

Additional details pertaining to oxygen consumption are illustrated in Figure 2. This experiment was repeated a minimum of ten times. Incubation buffer was added to the Clark electrode and allowed to stabilize for 60 seconds prior to the addition of mitochondria. After the addition of mitochondria, a slight decrease in voltage (indicative of increased oxygen consumption) was noted. The addition of succinate caused an increase in OCR ($p < .0001$) in the average oxygen consumption. Mean oxygen consumption prior to the addition of succinate as measured by mV, was $100.0 \pm 0.5$ mV compared to $74.5 \pm 0.5$ mV after succinate addition. Antimycin, a known inhibitor of oxygen consumption, was added after 120 seconds. As time continued, it was clear that oxygen consumption was compromised with the addition of antimycin ($p < .0001$) even when a known stimulator (succinate) was added, averaging $96$ mV $\pm$ 0.5. The drop in voltage as oxygen consumption increased was attributed to the addition of succinate, indicating that our mitochondria are viable, as succinate is a known stimulator of mitochondrial function. Antimycin reduced the drop in mV caused
by succinate as oxygen consumption decreased, again confirming that our mitochondria are functional. All further data were converted to nanomoles of oxygen consumption instead of mV, as in Figure 2.

The influence PGF2α on OCR from mitochondria isolated from bovine corpora lutea is illustrated in Figure 3. Nanomoles of oxygen consumed per minute per mg of mitochondria were significantly increased by the addition of succinate, averaging 2 ± 3.5 versus 84 ± 3.5 nm respectively prior to and after succinate addition. PGF2α was added to achieve 0.01 μM PGF2α, which failed to inhibit succinate stimulated OCR, averaging 79.0 ± 3.5 nm. However, when PGF2α was added to achieve 1 μM or 10 μM PGF2α, OCR was reduced (p < .0001) by approximately 50%. Oxygen consumption was 38.0 ± 3.5 and 36.0 ± 3.5 nm respectively for 1 μM and 10 μM PGF2α (p < .0001). The addition of antimycin to this preparation of mitochondria reduced OCR to control levels. Since both prostaglandins and antimycin were dissolved in ethanol prior to to addition to the media, influence of ethanol on succinate stimulated OCR was determined and it was minor.

Clearly, the addition of PGF2α to these mitochondria was inhibitory and reduced OCR, therefore reducing the production of ATP and perhaps the ability to produce progesterone. Since both 1.0 μM and 10 μM PGF2α had similar inhibitory effects on OCR, further experiments used 0.75 μM and 1.0 μM.

The influence of 0.75 μM and 1.0 μM PGF2α on OCR from mitochondria isolated from bovine corpora lutea is depicted in Figure 4. Succinate stimulated OCR was evident in both control treatments, averaging 82.9 ±11.5 nm. When
PGF2α was added at 0.75 µM (p < 0.038) or 1.0 µM (p < 0.047) in the presence of succinate, OCR was reduced more than 50%, averaging 46.0 ± 4.0 and 52.0 ± 8.9 nm respectively for 0.75 µM and 1.0 µM PGF2α. All molarities of PGF2α, 0.75 µM, 1.0 µM, and 10 µM (Figures 3 and 4) reduced OCR, indicating that luteal cells exposed to PGF2α will not function properly. Luteolysis caused by PGF2α may in part be associated with a direct effect of PGF2α on the luteal cell mitochondria.

The influence of the addition of PGE2 to isolated mitochondria is depicted in Figure 5. Succinate stimulated OCR was evident in both control treatments, averaging 82.9 ± 11.8 nm of oxygen consumed per minute per mg of mitochondria. The addition of 30 µl of PGE2 (0.75 µM) in the presence of succinate to a similar dispersion of mitochondria significantly (p < 0.021) inhibited OCR levels, averaging 38.2 ± 10.2 nm. The addition of 30 µl of PGE2 (1.0 µM) also significantly (p < 0.022) inhibited OCR levels, averaging 38.5 nm ±11.8 nm. This was surprising, since PGE2 has been implicated as a stimulus of progesterone production, which arises from mitochondrial activity. The positive influence of PGE2 on luteal function cannot be explained by its negative effect on the oxygen consumption of isolated mitochondria.

**Prostaglandin Influence on Progesterone Secretion**

The influence of PGF2α or PGE2 on progesterone production from mitochondria isolated from bovine corpora lutea is shown in Figure 6. 22R-Hydroxycholesterol, at a final concentration of 144 µM, was added as a precursor for progesterone production. The amount of progesterone produced was low and
similar across treatments, averaging 94.6 ± 18.7 ng. The addition of 22R-hydroxycholesterol had a minimal effect on progesterone production, averaging 93 ± 18.7 ng versus 86.2 ± 18.7 ng respectively for 0 and 144 µM 22R-hydroxycholesterol. In this unstimulated model of mitochondrial progesterone production, the addition of PGF2α or PGE2 had only minor influences.

The influence of succinate, which stimulated OCR and electron movement in the other experiments, is depicted in Figure 7. The addition of cholesterol and succinate did not improve the amounts of progesterone produced, averaging 65.9 ± 10.6 ng/ml across all treatments, versus mitochondria alone which averaged 84.5 ± 10.6 ng/ml. However, the addition of 30 µl of PGF2α (1 µM) decreased progesterone synthesis compared to the control with succinate and cholesterol, averaging 46.5 ± 10.6 ng/ml versus 58.1 ± 10.6 ng/ml respectively for the 1 µM PGF2α versus 0 µM. Although succinate stimulated electron movement in the respiratory electron transport chain, it was ineffective in stimulating the synthesis of progesterone. The portion of the mitochondrial inner membrane where steroidogenesis occurs was not stimulated by the addition of succinate. The inhibition of progesterone production by PGF2α might be associated with its inhibition of oxygen consumption and adverse effect upon ATP synthesis. If mitochondria are deprived of sufficient ATP, there exists the possibility of a resulting decrease in progesterone production.

The low basal progesterone production obtained in the previous experiments was unexpected, so the addition of NADPH, an electron donor associated with cholesterol metabolism was added to stimulate progesterone
production. The influence of the addition NADPH on progesterone synthesis is depicted in Figure 8. When NADPH was added (1.0 mM) to the protocol, greater amounts (p < .0001) of progesterone were produced compared to controls, averaging 3100 ± 109.9 for 1.0 mM NADPH versus and 73.3 ± 109.9 for 0.0 mM NADPH groups.

The influence of 30 µl of 0.75 µM or 1.0 µM PGE2 and PGF2α on NADPH stimulated progesterone production is depicted in Figures 9, 1f0, 11, and 12. NADPH stimulated large amounts of progesterone as seen in Figure 9, averaging 3448.0 ± 399.0 ng/ml of progesterone in the presence of 1.0 mM NADPH compared to 236.4 ± 399.0 ng/ml of progesterone in the absence of NADPH. The addition of 0.75 or 1.0 µM PGE2 (Figures 9 and 10) had minimal influence (p < 0.7 or 0.9) respectively on progesterone production. The influence of 0.75 and 1.0 µM of PGF2α is illustrated in Figure 11. Clearly, as before, the addition of NADPH stimulated (p < 0.0001) large amounts of progesterone. The addition of 1.0 µM PGF2α had minimal influence, whereas 0.75 µM reduced (p < 0.04) progesterone production by 18%.

The standard error of the mean associated with NADPH stimulated progesterone production was 399.0, which is 10% of the average progesterone produced in the positive control treatment. Variances this large mask treatment effects such as the 18% (600.0 ng/ml) drop in progesterone production in the 0.75 µM PGF2α treatment. To reduce the influence of this variance, progesterone values were expressed as a fraction of the average control progesterone production and are depicted in Figures 10 and 12. Nanograms of
progesterone produced per ml for each treatment were divided by the average of the positive controls and expressed as a fraction prior to statistical analysis.

In summary, the fraction of progesterone produced was reduced (p < 0.04) by the addition of 0.75 \( \mu \)M PGF2\( \alpha \) (Figure 12). The addition of PGF2\( \alpha \) reduced the fraction of progesterone produced by 28%. The addition of 1.0 \( \mu \)M PGF2\( \alpha \) or 0.75 and 1.0 \( \mu \)M PGE2 did not significantly influence progesterone production (Figures 10 and 12).

**OCR Data**

Crucial to an understanding of luteolysis is the study of those processes which impinge upon the viability of the corpus luteum: cell respiration and steroidogenesis. Our in vitro studies on the effect of PGF2\( \alpha \) and PGE2 on respiration and steroidogenesis in bovine mitochondria have produced some results which are similar and other results which differ from conclusions reached in *in vivo* studies.

The decrease in oxygen consumption by PGF2\( \alpha \) would be expected to cause a subsequent decrease in ATP production. If the energy needs of the CL are compromised, a loss of viability of luteal cells will ensue. What was unanticipated about these results was the inhibition of respiration by PGE2 (Figure 5).

Since PGE2 is luteotropic, our results indicating inhibition of oxygen consumption were surprising. However, there is support for inhibition of oxygen consumption by PGE2 from other investigations. Lear et al., 1990, found that exogenous PGE2 at a concentration of 30 \( \mu \)M inhibited transport-dependent
oxygen consumption in the rabbit kidney by 70% and caused an increase in cAMP by approximately 100%. Thus PGE2 may be interfering with the phosphorylation of AMP to ATP. Rainsford (1980) found that PGE2 in a dose of 0.125 – 1.25 µg/ml inhibited oxygen consumption in rat gastric mucosal slices in vitro, thereby reducing the capacity of the mucosa to produce ATP.

Both PGE2 (10⁻⁶ g/kg/min) and PGF2α (10⁻⁷ g/kg/min) decreased oxygen consumption when infused into the carotids of anesthetized baboons, thus providing evidence for the inhibitory effects on oxygen consumption of these two prostaglandins in primates (Pickard et al., 1977).

Additional support for inhibition of oxygen consumption by PGE2 is evident in a study of rat myocardial tissue (Noguchi et al., 2001). Oxygen consumption was reduced in cardiac muscle by the protein kinase C (PKC) pathway. Both PKC and PKA pathways are involved in the metabolism of arachidonic acid. Arachidonic acid has been shown to have a generalized inhibitory effect on the sodium/potassium pump. The pump, also called the Na⁺ - K⁺ - ATPase, maintains the gradients of these ions across the plasma membrane via active transport against their concentration gradients. Therefore, interference with the function of the pump results in a diminution of ATP. Investigations indicate that inhibition of the pump is primarily due to PGE (Therien and Blostein, 2000). It is known that hypoxic conditions adversely affect the generation of ATP. Although the exact mechanism by which PGE exerts its inhibitory effect remains unknown, the possibility exists that a decrease in oxygen consumption
caused by PGE may explain the multiple inhibitory effects of PGE2 through its inhibition of electron transport and reduced ATP production.

At a molarity of 0.75 µM, inhibition of oxygen consumption by PGF2α is significant at p < .0377 (Figure 4). This is approximately a 50% reduction. It is evident that this inhibition reduces the amount of ATP available for luteal cell activity and could contribute to the luteolytic effects of PGF2α. A thorough search of the literature has failed to disclose any prior investigations on the effect of PGF2α on oxygen consumption. However, it is commonly accepted that PGF2α is the primary luteolysin. Since this study has indicated a significant reduction in oxygen consumption by PGF2α, thus impacting ATP synthesis, our results support a possible link between depletion of ATP and luteolysis.

**Progesterone Data**

Cholesterol alone is incapable of stimulating progesterone production in this model system (Figure 6.) The addition of succinate (Figure 7) was also ineffective in stimulating steroidogenesis. Succinate, although increasing oxygen consumption, did not increase progesterone production. This may be the result of a clear separation between electron movement in the ATP transport chain and electron movement associated with progesterone synthesis. The cristae of the mitochondrial inner membrane are not identical in arrangement in steroid-producing tissue and non-steroidal tissue. The steroidal substructure of the lamellar cristae contains a gap between membranes of approximately 4 nanometers. This is significant because the size of this gap is not sufficiently
large to accommodate one of the subunits of ATP synthase (Prince and Scheffler, 2002), thus precluding oxidative phosphorylation.

The addition of NADPH (Figure 8) provides the essential component for successful steroidogenesis. The increase in progesterone production is highly significant (p < .0001). NADPH is required to stimulate the chain of events which culminate in the synthesis of progesterone. Stein and Tesone, 1992, confirmed that the specific site of the action of NADPH is at the conversion of cholesterol to pregnenolone within the mitochondria. The entrance of cholesterol is also dependent upon the availability of oxygen and electrons, both of which are contributed by NADPH (Sulimovici and Boyd, 1968). Thus NADPH is essential for the process of steroidogenesis.

Many investigations have confirmed that PGF2α inhibits progesterone secretion (Conley and Ford, 1989; Pate and Condon, 1989; (Gregoraszczuk and Michas, 1999; Juengel et al., 2000; Fitz et al., 1993). There is a common consensus that PGF2α decreases progesterone in intact animals (Fitz et al., 1993; Girsh et al., 1996a; McCracken et al., 1972; Niswender and Nett, 1994). PGF2α decreases progesterone from purified preparations of ovine and bovine large luteal cells (Wiltbank et al., 1991). In vivo, very low levels of PGF2α (~250 pg/ml) are required to depress progesterone secretion in sheep (McCracken, 1999). The evidence that PGF2α has an inhibitory effect is substantial. When PGF2α was added to the mitochondria, progesterone production was inhibited even in the presence of NADPH. PGF2α inhibited both baseline (Figure 7) and NADPH-stimulated progesterone production (Figure 11). PGF2α clearly inhibited
the movement of electrons in the ATP transport chain (Figure 4), which might have contributed to the inhibition of baseline progesterone production. In the presence of NADPH, which stimulated a 10-fold increase in progesterone production, PGF2α reduced progesterone production by 28%, indicating that PGF2α was influencing the movement of electrons along the steroidogenic transport chain.

PGF2α decreases the synthesis of progesterone via multiple mechanisms. It is speculated that progesterone could be decreased by several intracellular events, including down-regulation of receptors, decreased uptake of cholesterol, decreased transport of cholesterol across mitochondrial membranes, and decreased activity of the enzymes required for the synthesis of progesterone. These anti-steroidogenic effects of PGF2α appear to be mediated by the protein kinase C secondary messenger pathway (Niswender et al., 2000). When PGF2α binds to receptors on the large luteal cells, a chain of events is set in motion which ultimately results in an increase in calcium (Juengel et al., 1996; Abramovitz et al., 1994; Graves et al., 1995). Calcium promotes the synthesis of additional PKC and inhibits synthesis of cholesterol (Niswender et al., 2000; McGuire et al., 1994; Boujrad and Hudson, 1993). The decrease in cholesterol may be another of the mechanisms whereby PGF2α inhibits the synthesis of progesterone. Confusion results from recent reports that both PGE2 and PGF2α at concentrations of 1µM stimulate progesterone synthesis in bovine luteal cells in vitro when incubated for 24 hours (Bowolaksono et al., 2008). This result is in contrast with our results in Figure 7 and Figure 10. Also surprising was the
conclusion of Bowolaksono et al., that PGE2 and PGF2α exert luteoprotective roles by suppressing apoptosis in the large luteal cells.

*In vitro*, PGF2α prevents cholesterol from interacting with the side-chain enzyme (P450scc), resulting in an initial drop in progesterone in incubated whole luteal cells (Grusenmeyer and Pate, 1992). After this initial drop in progesterone, StAR mRNA declines, compounding the loss of progesterone (Pescador et al., 1996). The decrease in StAR suppresses the availability of cholesterol, since StAR is required for the transfer of cholesterol to the tubular sites of the mitochondria where much of the P450scc is located (Jefcoate and Artemenko, 2004). The decrease in StAR mRNA occurs after the initial drop in progesterone, and therefore, fails to explain the initial drop in progesterone synthesis. Attempts to locate the precise site within the luteal cell where PGFα exerts its effects continue to be illusive (Pate, 2012). However, in the present study, PGF2α directly influenced the mitochondria in some manner such that less progesterone was produced.

**Membrane Permeability and Prostaglandins**

How do prostaglandins diffuse into a luteal cell and influence mitochondria? Because of their lipid nature, prostaglandins were originally thought to traverse cell membranes by passive diffusion. Although PGE2 and PGF2α are able to pass through membranes by diffusion, diffusion is too slow to be suitable for cellular needs. Since prostaglandins are organic anions, they traverse membranes inefficiently. This is the result of the nature of both the plasma membrane and the mitochondrial membrane, which both consist of a
bilayer of phospholipids. The hydrophilic heads of the phospholipids are exposed to the aqueous milieu outside the mitochondrion and the hydrophobic tails inside the membrane, apposed to the inner membrane, where the phospholipids are similarly arranged. The externally oriented phosphate heads are negatively charged, as are the prostaglandins. The positioning of these two negative charges impedes entrance of negatively charged substances such as prostaglandins.

Prostaglandins typically enter membranes with the aid of receptors and transporters. The mitochondria in our investigation are intact, and therefore the mitochondrial membrane would possess receptors. Carrier-mediated transport enables prostaglandins to cross cell membranes faster than diffusion (Schuster, 2002). Bovine prostaglandin transporter (PGT) assists the entrance and exit of both PGE2 and PGF2α with equal affinity. PGT is involved in transport within the bovine uterus and from the uterus to the uterine-ovarian plexus, where the prostaglandins primarily affect the large luteal cells (Arosh 2004). When transported to the luteal cell by PGT, both PGE2 and PGF2α enter the cell via G protein-coupled receptors. In the bovine these are primarily PGE2 receptors EP2 and EP4 and PGF2α receptor FP, which are located on the luteal cell membrane. Unfortunately, there is a lack of information regarding the structure of the EP2 and EP4 receptors (Regan, 2003).

Prostaglandins may either bind to luteal cells to exert their effects or be synthesized within luteal cells. However, their influence upon mitochondrial function is dependent upon second messengers, since PGE2 and PGF2α are
excluded from the inner mitochondrial membrane. The outer mitochondrial membrane (OMM) contains integral proteins called “porins”. Porins form channels 2-3 nm in diameter, which permit molecules of 5000 Daltons or less to freely diffuse across the membrane (Alberts et al. 1994). Since the inner mitochondrial membrane is devoid of porins, ions and molecules require transporters (Alberts et al., 1994). These transporter proteins allow the membrane to be selectively permeable to small molecules such as ATP.

**Mitochondria and Luteolysis**

Crucial to an understanding of luteolysis is the study of those processes which impinge upon the viability of the corpus luteum: cell respiration and steroidogenesis.

Mitochondria control the corpus luteum in three ways. They generate most of the ATP required by the cell for energy. They are the site of the initial stage of steroidogenesis during which pregnenolone is metabolized to progesterone. They release pro-apoptotic proteins that activate a cascade which culminates in regression of the CL.

The thousands of mitochondria in the average cell generate most of the ATP required by the cell for energy. Deprived of oxygen, cellular respiration will cease and the amount of ATP available to the cell will diminish, since oxygen functions as the terminal electron acceptor in the process of oxidative phosphorylation. Low oxygen affects not only the energy needs of the cell, but adversely affects cultured bovine luteal cells. The processes of cellular respiration and steroidogenesis are allied and therefore, these processes are not
distinct from each other. Whatever affects oxygen consumption will affect steroidogenesis. In support of this contention is the finding that mitochondria in Leydig cells must be actively respiring to support steroidogenesis (Allen et al., 2006). Pregnenolone, the precursor of progesterone, is inhibited in low oxygen conditions, resulting in functional luteolysis (Nishimura et al., 2006). Both of these processes occur on the mitochondrial inner membrane, although in separate regions.

Structural luteolysis of the CL occurs when the mitochondria release apoptotic proteins, such as cytochrome c. If apoptosis occurs at the appropriate time in the ovarian cycle, it is beneficial, causing regression of the CL and the initiation of a new cycle. Luteolysis in ruminants is initiated by the binding of PGF2α to its FP receptor on the luteal membrane. As a result, free calcium is released and protein kinase C is activated (PKC). Subsequent events await further investigation, but could include PGF2α transport to the mitochondria, a decrease in steroidogenic enzymes and a loss of structural integrity of the CL (Diaz et al., 2000).

The collective results from our experiments verify the luteolytic effects of PGF2α. For the first time, this study has documented the detrimental effects on cell respiration of PGF2α, which doubtless contributes to the inhibition of steroidogenesis. Our determination of the effect of PGF2α on cellular respiration in luteal mitochondria represents new data. To our knowledge, this study is the first to investigate the effect of PGF2α on mitochondrial oxygen consumption in ruminants.
The mechanism of the role of PGF2α within the corpus luteum remains a conundrum (Pate, 2012). However, the pieces of the puzzle are beginning to come together. The dual processes of cellular respiration and steroidogenesis both occur on the inner mitochondrial membrane. Future studies need to be designed to elucidate how both electron transport mechanisms are positioned on the membrane and how both PGE2 and PGF2α influence them.

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Figure 1. Equation for determination of oxygen consumption rate.

The Clark electrode communicates with a computer to produce a trace similar to the one pictured above. Analysis of the graphs generated in our respiration trials permitted determination of the amount of oxygen consumed. Measurements between the points indicated on the graph were factored into the equation shown.
Figure 2. Effect of succinate (20 mM) and antimycin (0.02 mM) on oxygen consumption rate in mitochondria (1.5 mg/ml) isolated from bovine luteal cells.
**Figure 3.** Effect of various concentrations of PGF2α (0, 0.01, 1.0, or 10 µM) on oxygen consumption in mitochondria (1.5 mg/ml) isolated from bovine luteal cells with succinate (20 mM), ethanol (0 µl added to 0 PGF2α, 4 µl added to 0.01 µM PGF2α, 40 µl added to 1 µM PGF2α, 400 µl added to 10 µM PGF2α), antimycin (0.02 mM) was added as indicated.
Figure 4. Effect of PGF2α (0.75 and 1.0 μM) on oxygen consumption in mitochondria (1.5 mg/ml) isolated from bovine luteal cells with ethanol (30 – 40 μl), succinate (20 mM) at 37° C. Control 1 = 30 μl ethanol, mitochondria, succinate (20 mM). Control 2 = 40 μl ethanol, mitochondria, succinate (20 mM).
Figure 5. Effect of PGE2 (0.75 and 1 μM) on oxygen consumption in mitochondria (1.5 mg/ml) isolated from bovine luteal cells with ethanol (30-40 μl) and with succinate (20 mM). Control 1 = 30 μl ethanol, mitochondria, succinate (20 mM). Control 2 = 40 μl ethanol, mitochondria, succinate (20 mM).
Figure 6. Effect of PGE2 and PGF2α (0.75 and 1 µM) on synthesis of progesterone from mitochondria (1.5 mg/ml) isolated from bovine luteal cells with or without cholesterol (144 µM), and ethanol (6-7 µl).
Figure 7. Effect of PGE2 AND PGF2α (0.75 and 1 µM) on synthesis of progesterone from mitochondria (1.5 mg/ml) isolated from bovine luteal cells with or without cholesterol (144 µM), succinate (20 mM), and ethanol (6-7 µl).
Figure 8. Effect of the addition of NADPH (1 mM) on synthesis of progesterone in mitochondria (1.5 mg/ml) isolated from bovine luteal cells with cholesterol (144 μM), succinate (20 mM) and NADPH (1 mM).
Figure 9. Effect of PGE2 (0.75 and 1 µM), antimycin (0.02 mM), cholesterol (144 µM), ethanol (6-7 µl), and NADPH (1 mM) on progesterone synthesis in mitochondria isolated from bovine luteal cells at 37°C. Negative control = mitochondria, and ethanol; positive control = mitochondria, cholesterol, and NADPH expressed as ng/mL.
Figure 10. Effect of PGE2 (0.75 and 1 µM), antimycin (0.02 mM), cholesterol (144 µM), ethanol (6-7 µl), and NADPH (1 mM) on progesterone synthesis in mitochondria isolated from bovine luteal cells at 37°C. Negative control = mitochondria, and ethanol; positive control = mitochondria, cholesterol, NADPH expressed as relative value.
Figure 11. Effect of PGF2α (0.75 and 1 µM) on progesterone synthesis in mitochondria (1.5 mg/ml) isolated from bovine luteal cells with ethanol (6-7 µl), cholesterol (144 µM), NADPH (1 mM), antimycin (0.02 mM) at 37°C. Negative control = mitochondria, and ethanol; positive control = mitochondria, cholesterol, and NADPH expressed as ng/mL.
Figure 12. Effect of PGF2α (0.75 and 1 µM) on progesterone synthesis in mitochondria (1.5 mg/ml) isolated from bovine luteal cells with ethanol (6-7 µl), cholesterol (144 µM), NADPH (1 mM), antimycin (0.02 mM) at 37°C. Negative control = mitochondria, and ethanol; positive control = mitochondria, cholesterol, NADPH expressed as relative value.
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