Glucocorticoid Regulation of Intrauterine Prostaglandin Production and Uterine Contractility at the Onset of Parturition in the Sheep and the Human

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Institute of Medical Science, University of Toronto.

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Abstract

The purpose of this thesis was to examine the relationship between fetal glucocorticoid synthesis, intrauterine prostaglandin production and uterine activity during late gestation and the onset of labor. We hypothesized that sustained activation of the fetal hypothalamic-pituitary-adrenal axis leads to elevated fetal adrenal glucocorticoid production that in turn increases intrauterine prostaglandin production and uterine contractility. In the sheep, we have demonstrated that increased intrauterine glucocorticoid concentration both directly increases fetal trophoblast derived prostaglandin production and indirectly increases maternal intrauterine tissue prostaglandin production through the stimulation of placental estradiol synthesis. Comparing the events of ovine parturition with those of human parturition, we found that glucocorticoids also directly regulate prostaglandin production within human trophoblast-derived amnion epithelial and mesenchymal cells. We also conclude that a similar indirect effect of glucocorticoid on prostaglandin production exists within this tissue but is mediated by glucocorticoid-stimulated intrauterine corticotropin releasing hormone synthesis. Furthermore, we determined that glucocorticoid regulated prostaglandin production within the epithelial cells is influenced by 11βhydroxysteroid dehydrogenase activity. We conclude that paracrine interactions between the amnion epithelial and mesenchymal cells regulate the net production of prostaglandin by this tissue. Glucocorticoid-induced prostaglandin production in turn stimulates the onset of labor events including cervical ripening, membrane rupture and uterine contractility. Thus, parturition follows a tissue specific progression of events from a fetal signal to a maternal labor response.
To laugh often and love much;
To win the respect of intelligent
persons and the affection of children:
To earn the approbation of honest
citizens and endure the betrayal of false friends:
To appreciate beauty:
To find the best in others:
To give of one's self;
To leave the world a bit better,
whether by a healthy child, a garden patch
Or a redeemed social condition:
To have played and laughed with enthusiasm
and sung with exultation:
To know even one life has
breathed easier because you have lived-

This is to have succeeded.

_Ralph Waldo Emerson_
To my family...
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To members of the Program Advisory Committee- Dr. S.J. Lye, Dr. J. Kingdom, Dr. W. Gibb and Dr J.W.K. Ritchie- thank you for your guidance and support in the development of the thesis and my development as a clinician scientist.

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To my family- I am proud to be your daughter and sister. To my Dad, thank you for showing me the thrill of learning and giving me the skills-confidence, discipline, enthusiasm, honesty-to be successful! To my Mom, thank you for your kindness, your encouragement and your friendship. To my Brother, thank you for your friendship and teaching me about courage and strength.

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PUBLICATIONS


ABSTRACTS


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  b. Society For Obstetricians and Gynecologists of Canada Annual Meeting; Ottawa, Canada; 1999. (Oral Presentation)

Whittle W.L., Gibb W., Challis J.R.G.; Characterization of Human Amnion Cells and the Cellular Localization of Prostaglandin H Synthase Type II
  a. SGI-Society of Gynecological Investigation 1999 Annual Meeting; Atlanta, USA; 1999. (Oral Presentation)
  b. Society For Obstetricians and Gynecologists of Canada 1999 Annual Meeting; Ottawa, Ontario; 1999. (Oral Presentation)
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<td>11β hydroxysteroid Dehydrogenase</td>
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<tr>
<td>11βHSD-I</td>
<td>11β hydroxysteroid Dehydrogenase Type I</td>
</tr>
<tr>
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<tr>
<td>4OHA</td>
<td>4 hydroxyandrostendione</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
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<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
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<td>ACTH-R</td>
<td>adrenocorticotropic hormone receptor</td>
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<td>AVP</td>
<td>arginine vasopressin</td>
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<td>CAM</td>
<td>calmodulin</td>
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<td>cyclic adenosine monophosphate</td>
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<td>contraction associated protein</td>
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<td>corticosteroid binding globulin</td>
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<td>CD68</td>
<td>cluster determinant 68</td>
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<td>cDNA</td>
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<td>cyclic guanosine monophosphate</td>
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<td>corticotrophin-like intermediate lobe peptide</td>
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<td>cPLA</td>
<td>cytosolic phospholipase A</td>
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<tr>
<td>cpm</td>
<td>counts per minute</td>
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<td>CRE</td>
<td>cAMP response element</td>
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<tr>
<td>CRH</td>
<td>corticotropin releasing hormone</td>
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<td>corticotropin releasing hormone binding protein</td>
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<td>cAMP response sequence</td>
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<td>connexin 43</td>
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<tr>
<td>DCC</td>
<td>dextran coated charcoal</td>
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<td>DEX</td>
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<td>DHEA</td>
<td>dehydroepiandrosterone</td>
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<td>dehydroepiandrosterone sulphate</td>
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<td>DMEM</td>
<td>Dubelcco's Modified Essential Media</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>E₂:P₄</td>
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<td>EGF</td>
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<td>EMG</td>
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<td>FAK</td>
<td>focal adhesion kinase</td>
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<td>gram</td>
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<td>GC</td>
<td>glucocorticoid</td>
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<tr>
<td>G-CSF</td>
<td>gram colony stimulating factor</td>
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GR  glucocorticoid receptor
GRE  glucocorticoid response element
h  hour
HPA  hypothalamic-pituitary-adrenal
hsp  heat shock protein
IL  interleukin
Ir-PGHS  immunoreactive PGHS
LDL-R  low density lipoprotein receptor
ml  milliliter
MLCK  myosin light chain kinase
mm  millimeter
MMP  mettallomatrix proteinase
MR  mineralocorticoid receptor
mRNA  messenger RNA
NADPH  nicotinamide adenine dinucleotide phosphate
NPY  neuropeptide Y
°C  degree Celcius
OT  oxytocin
OTR  oxytocin receptor
P450arom  P450aromatase
P450c17  P450 17 hydroxylase/C17 lyase
P450sc  P450 side chain cleavage
PBS  phosphate buffered saline
PBSG  phosphate buffered saline and gelatin
PBS-T  phosphate buffered saline and gelatin
PC1  proconvertase 1
PC2  proconvertase 2
PD  putdown
PG  prostaglandin
PGDH  prostaglandin Dehydrogenase
PGE2  prostaglandin E2
PGFM  13,14 dihydro-15 keto prostaglandin F2
PGG2  prostaglandin G2
PGH2  prostaglandin H2
PGHS  prostaglandin H synthase
PGHS-I  prostaglandin H synthase Type I
PGHS-II  prostaglandin H synthase Type II
PKA  protein kinase A
PLA  phospholipase A
PLC  phospholipase C
PLD  phospholipase D
POMC  proopiomelanocortin
PR  progesterone receptor
PTL  preterm labor
rpm  revolutions per minute
SEM  standard error of the mean
sPLA  secretory phospholipase A
TGFβ  transforming growth factor β
TLC  thin layer chromatography
TNFα  tumor necrosis factor α
αMSH  α melanocyte stimulating hormone
Chapter One

General Introduction

A portion of this chapter has been accepted for publication in the journal Biology of Reproduction (2001: in press) and appears here with the permission of the journal (refer to attached authorization).
1.1. Introduction and Hypothesis

Parturition is defined as the development of regular, coordinated uterine contractions that start at the fundus and spread down through the body of the uterus with increasing frequency and strength (Cunningham et al., 1993). These contractions are associated with progressive thinning and dilatation of the uterine cervix and lead to the descent of the fetus into the birth canal (Cunningham et al., 1993). The mechanisms governing the spontaneous onset, progression and regulation of parturition are not well understood. In most mammalian species towards the end of gestation and the onset of labor, there is an increase in fetal plasma glucocorticoid concentration due to maturation and sustained activation of the fetal hypothalamic-pituitary-adrenal (HPA) axis (Fowden et al., 1998). Under normal conditions, the fetal tissues are exposed to increasing levels of bioactive glucocorticoids in the final 10-15 days prior to delivery. These glucocorticoids induce maturational changes in the fetal organs in preparation for a successful transition from intra to extra-uterine life (Fowden et al., 1998). In addition, this surge in glucocorticoid production appears to be integral to the cascade of events leading to the onset of parturition (Challis et al., 1997). Concurrent with the rise in fetal glucocorticoid levels, there is a progressive increase in plasma, amniotic fluid and intrauterine tissue concentrations of prostaglandin E\(_2\) (PGE\(_2\)) followed by an increase in prostaglandin F\(_{2\alpha}\) (PGF\(_{2\alpha}\)) (Gyomory et al., 2000; Challis et al., 1997). These PGs have been identified as key mediators of the events of labor, including uterine contractility, cervical thinning and dilatation, membrane rupture and the maintenance of utero-placental blood flow; as well these PGs act as mediators of the adaptations that the fetus undertakes in response to the process of labor (Challis et al., 1997). Recent evidence has suggested that the
rise in fetal glucocorticoid production directs the increase in intrauterine prostaglandin production and leads to that cascade of events defined as labor (Challis et al, 1997).

The purpose of this thesis is to examine the relationship between fetal glucocorticoid synthesis, intrauterine prostaglandin production and uterine activity during late gestation and the onset of labor. We will suggest that sustained activation of the fetal HPA axis leads to elevated fetal adrenal glucocorticoid production that increases intrauterine prostaglandin production. In addition, we will suggest that glucocorticoids both directly increase fetal placental prostaglandin production and indirectly increase maternal intrauterine tissue prostaglandin production through the stimulation of placental estradiol synthesis. We further hypothesize that the increase in cortisol will induce the expression of specific contraction associated proteins required for uterine contractility. We will compare the events of ovine parturition with those of human parturition and hypothesize that glucocorticoids directly regulate prostaglandin production by the human fetal membranes, in particular within the amnion. We proposed that a similar indirect effect of glucocorticoids on prostaglandin production exists in humans but is mediated by glucocorticoid-stimulated intrauterine corticotropin releasing hormone (CRH) synthesis. Studies have found that glucocorticoid bio-availability within human fetal membranes may be regulated by the autocrine/paracrine activity of the enzyme 11β hydroxysteroid dehydrogenase type I (11βHSD-I). We therefore hypothesize that the direct and indirect effects of cortisol on amnion PG output are determined by the activity of 11βHSD-I. We will examine these hypotheses in parallel experiments using an in vivo ovine preparation and an in vitro human amnion cell culture preparation. An outline of these hypotheses is presented in Figure 1-1.
Figure 1-1: General Thesis Hypothesis

We hypothesize that glucocorticoids produced by the fetal adrenal due to sustained activation of the fetal hypothalamic-pituitary-adrenal axis leads to increased intrauterine prostaglandin production. In the sheep we suggest that glucocorticoids both directly increase fetal placental prostaglandin production and indirectly increase maternal intrauterine tissue prostaglandin production through the stimulation of placental estradiol synthesis. We further hypothesize that the increase in cortisol will induce the expression of specific contraction associated proteins required for uterine contractility. We will compare the events of ovine parturition with those of human parturition and hypothesize that glucocorticoids directly regulate prostaglandin production by the human fetal membranes, in particular within the amnion. We proposed that a similar indirect effect of glucocorticoids on prostaglandin production exists in humans but is mediated by glucocorticoid-stimulated intrauterine corticotropin releasing hormone synthesis. We therefore hypothesize that the direct and indirect effects of cortisol on amnion PG output are determined by the activity of 11β hydroxysteroid dehydrogenase type I.
1.2. Clinical Relevance for the Study of Labor at Term

Despite the advances in perinatal medicine over the past thirty years there has been very little improvement in the incidence and outcome of premature labor. The preterm birth rate (defined as the number of deliveries prior to the completion of 37 weeks of gestation) among singleton gestations in Canada has modestly increased from 5.61% in 1981-83 to 5.86% in 1992-94 (Joseph et al. 1998). However, in the same time period there has been a dramatic, 25% increase in the preterm birth rate among multiple gestations; currently the Canadian preterm birth rate is 51.5% and 95.9% for twin and triplet gestations respectively (Joseph et al. 1998). These preterm deliveries account for greater than 75% of the perinatal mortality and morbidity rates not associated with lethal anomalies (Creasy, 1991). In North America, the cost of maintaining premature neonates in the intensive care nursery for the first months of life has been estimated at $5-6 billion annually; this does not include the cost of chronic care and special education needs of those infants left with residual major motor and/or mental handicaps (Mountquin et al. 1996). Furthermore, these figures and statistics do not account for the extraordinary financial and emotional stress to the family of a premature infant.

Preterm labor (PTL) is a symptom of a multifactorial disease process where labor is the final step in a biochemical cascade of events. The current methods of diagnosis of PTL are unsatisfactory: only one third of women admitted to hospital with the diagnosis of PTL will deliver within 24-48h of admission (Creasy, 1991). This statistic indicates that the current clinical practices for the determination of PTL are inadequate and thereby the precise diagnosis of true PTL difficult and often overestimated. The causes of PTL and subsequent delivery fall into three main categories: (1) iatrogenic- where there is a clear, demonstrable
maternal and/or fetal complication that demands delivery; (2) premature rupture of the fetal membranes with or without an associated intrauterine infection and; (3) idiopathic, where there is no immediate cause identifiable (Cunningham et al, 1993). Numerous predisposing factors for idiopathic PTL have been characterized including, subclinical intrauterine infection, fetal hypoxia, and endocrine/paracrine changes within the placenta and fetal membranes (Creasy, 1991). Many of these situations may in fact be detrimental to the fetus, and the onset of PTL could be considered a mechanism for the fetus to escape a hostile intrauterine environment (Creasy, 1991). Thus, each clinical situation of PTL must be critically evaluated to determine the probability of delivery within a defined period of time and, whether the etiology of the increased uterine activity warrants an attempt to delay delivery. Therefore, PTL presents two critical clinical challenges: (1) early, accurate diagnosis; and (2) an understanding of the underlying cause and subsequent appropriate management.

Unfortunately, once given the diagnosis of true PTL and the opportunity to safely delay delivery, there is no therapeutic intervention currently available that meets the criteria for optimal tocolysis, defined as (1) an attenuation of labor progress for greater than 24-48 hours, (2) an improvement of neonatal outcome and (3) limited deleterious fetal and maternal side-effects (Hannah, 2000). In 1995, a consensus statement on tocolytic use by the Society of Obstetricians and Gynecologists of Canada stated that offering patients presenting with PTL the option of no tocolytic treatment is reasonable and appropriate care. Currently the only therapeutic intervention shown to improve neonatal survival and outcome is the administration of glucocorticoid to the mother to induce fetal lung surfactant production and prevent neonatal respiratory disease (Katz and Farmer, 1999). The optimal therapeutic effect
of single course of steroid administration is greatest if the steroid is administered between 24 and 34 weeks of gestation and administered more than 24h and less than 7d before delivery (Katz and Farmer, 1999). The benefit of this treatment has led to the use of repeated courses of steroid in the event of continued or renewed threat of premature delivery. However, the efficacy and safety of repeated antenatal steroid administration has not been established. Recent evidence has suggested that antenatal exposure to glucocorticoids may have adverse effects on the fetus including decreased birth weight, impaired neuronal development and function and increased potential for adult onset diseases (Sloboda et al, 2000; Barker, 1995). A recent Canadian study reported that only 25% of patients considered to be in PTL received optimal antenatal steroid therapy and that 61% of patients were not in true PTL and received multiple doses of steroid (Skoll et al, 2000). These data highlight two important points: (1) the difficulty of predicting which patients are in true PTL and thus would benefit from steroid administration and; (2) a significant number of fetuses are exposed to the potentially detrimental effects of steroids (both single and multiple doses) with no apparent clinical justification. In an effort to both limit antenatal glucocorticoid exposure and prevent premature delivery, studies have determined thirteen risk factors for PTL which include low socioeconomic class, previous preterm delivery, uterine anomalies, and multiple gestation (Mountquin et al, 1996). Unfortunately, the majority of these risk factors are non-modifiable; increased antenatal surveillance and intervention (including home uterine monitoring, bed rest, work termination) in cases of increased risk has had little effect on the incidence and outcome of PTL (Mountquin et al, 1996). To complicate matters further, 30-60% of all PTL cases present without identifiable risk factor(s) (Mountquin et al, 1996). By defining the biochemical cascade involved in the spontaneous onset and progression of normal term labor,
a better understanding of the risk factors, the triggers and the mechanism(s) of PTL can be established. This information can then be used to direct the development of appropriate diagnostic, therapeutic and preventative strategies for PTL and delivery. Thus, PTL provides the clinical rationale for the study of term labor.

1.3. General Characteristics of Pregnancy and Parturition

Throughout gestation the uterus is a relatively quiescent organ due to both the inhibition of myometrial contractility, and the stimulation of myometrial relaxation (Lye, 1994). Key mediators of uterine quiescence are progesterone, prostacyclin, parathyroid-like-hormone and nitric oxide (Lye, 1994). Towards term there is an evolution of uterine activity from quiescence to contractility with associated cervical ripening; this evolution occurs in two distinct yet integrated phases: uterine activation and uterine stimulation. Uterine activation involves a series of changes within the myometrial muscle that allow for the muscle to function as a single, coordinated contractile unit with polarized waves of contraction, to possess spontaneous contractile activity and to have increased responsiveness to uterotonins (Cunningham et al, 1993). These changes are mediated by a group of proteins collectively referred to as “contraction associated proteins” (CAPs) (Lye, 1994). These CAPs include the gap junction component connexin 43 (Cx43), prostaglandin receptors (FP and EP1-4), ion channel components and the oxytocin receptor (Lye, 1994). The coordinated expression of the CAPs permits a wave of contraction to be initiated at the fundus of the uterus and rapidly spread through the uterine body towards the cervix (Cunningham et al, 1993). In contrast to the contraction of the fundus, there is a relaxation of the lower segment of the uterus and dilatation of the cervix to create the birth canal (Cunningham et al, 1993).
Progressive shortening of the fundal muscle fibers and the repeated waves of contraction direct the descent of the fetus into this birth canal (Cunningham et al., 1993). Uterine stimulation refers to the local and systemic production of uterotonins (factors which stimulate uterine contractility) and their subsequent induction of uterine contractions. Two important uterotonins are oxytocin (OT) and prostaglandins (PGs) (Lye, 1994). Both OT and PGs are produced within the intrauterine tissues and are under complex regulation by fetal, placental and maternal factors (Challis et al., 2000).

Two key initiating factors involved in the evolution of uterine activity have been determined: (1) increased uterine wall tension and (2) alterations in the feto-placental endocrine profile (Challis et al., 2000). During the first half of pregnancy, uterine myocytes undergo rapid growth due to both hypertrophy and hyperplasia. Through the second half of gestation the uterus continues to increase in size due mainly to myocyte hypertrophy. This hypertrophy is induced by a stretch due to the growing fetus, the placenta and increasing amniotic fluid volume. The Law of Laplace would dictate that this stretch force should generate an increase in uterine wall tension. However, a constant process of remodeling of the myocyte attachments to the extracellular matrix mediated by a focal adhesion kinase (FAK) allows the myocytes to detach from the matrix, stretch to accommodate the increasing uterine contents and reform focal extracellular matrix adhesions for stability (Macphee et al., 2000). Through this process the uterus increases in size without generating tension within the muscle wall. Evidence from studies using rodents indicate that FAK activity increases through gestation and is maintained by placental progesterone; at the end of gestation, as the placental endocrine profile changes FAK activity decreases and the remodeling process becomes inhibited (Macphee et al., 2000). Thus, in the final stages of gestation increasing
fetal growth generates uterine stretch and this force becomes translated into an increase in muscle wall tension. This wall tension has been implicated in the induction of CAPs and the evolution of uterine contractility (Ou et al, 1998).

Preceding the onset of uterine contractility the fetus undergoes a sustained activation of its hypothalamic-pituitary-adrenal axis (HPA) leading to an increase in adrenal cortisol and C19 estrogen precursor production (see section 1.4) (Pepe and Albrecht, 1995). Cortisol has been suggested to induce an alteration in placental steroidogenesis leading to a decrease in progesterone production and surge in placental estrogen synthesis (Anderson et al, 1975; Flint et al, 1975; Steele et al, 1976; France et al, 1988; Mason et al, 1989). The rise in cortisol and estrogen and concomitant the fall or attenuation of progesterone production have been both linked to the regulation of CAP expression and the production of PGs, OT and other uterotonic agents (Challis et al, 2000). Therefore, the fetus, through increased growth and HPA activity, provides both mechanical and endocrine signals capable of overcoming the maternal and placental factors acting to maintain uterine quiescence which provides a trigger for the cascade of events defined as parturition (Challis et al, 2000). The remainder of this introduction will focus on the endocrine events associated with the onset of parturition, and in particular will examine the role of the fetal hypothalamic-pituitary-adrenal axis in the regulation of uterine activation and stimulation.

1.4. Steroid Hormone Events Associated with Pregnancy and Parturition

Throughout gestation both the placenta and fetal adrenal gland produce large quantities of steroids necessary for pregnancy maintenance, fetal development and the onset of parturition; these hormones are progesterone, estrogen and glucocorticoids (Pepe and
To better understand the potential role of cortisol, estrogen and progesterone in the regulation of PG production and CAP expression at the onset of labor, the synthesis of these steroids through gestation and parturition will be reviewed.

1.4.1. Intrauterine Progesterone Synthesis Through Gestation and the Withdrawal of Progesterone Action at Parturition

Progesterone is a key steroid involved in the maintenance of uterine quiescence throughout gestation. Progesterone uncouples the myometrial excitation-contraction apparatus by inhibiting both CAP expression and uterotonin production (Lye, 1994). As well, progesterone stimulates the production of factors that promote uterine quiescence and inhibit uterine contractility (Challis et al, 2000; Lye, 1994). In humans, progesterone is initially produced by the corpus luteum, then at 5-6 weeks of gestation a “luteal-placental shift” occurs as the placenta becomes the main site of progesterone synthesis (Cunningham et al, 1993). However, there are some species where the corpus luteum continues as the major site of progesterone synthesis for the remainder of gestation. The trophoblast tissue expresses the enzymes necessary for the synthesis of progesterone and this production continues to increase through gestation. Cholesterol, acquired from the maternal circulation and the fetal liver bound with its carrier lipoprotein (LDL) binds to the low-density lipoprotein receptor (LDL-R) in the placenta and is incorporated into the trophoblast cell. Placental LDL-R expression is increased by estrogen and thus cholesterol incorporation into the trophoblast tissue is increased as a function of gestational age (Pepe and Albrecht, 1995). Estrogen further contributes to progesterone production by stimulating cholesterol synthesis within the fetal liver. The cholesterol side chain cleavage enzyme P450scc located in the mitochondria mediates the first rate-limiting step of progesterone synthesis. This enzyme catalyzes three
sequential reactions at a single active site: a 20α-hydroxylation, a 22-hydroxylation and a scission of the 20-22 carbon bond to yield pregnenolone and isocaproic acid. Placental P450scc expression and activity is increased by estrogen, and therefore also increases as a function of gestational age. Pregnenolone subsequently undergoes a 3β-hydroxysteroid dehydrogenation and an isomerization of the double bond from its B ring ($\Delta^3$) to its A ring ($\Delta^4$) to form progesterone. This reaction is catalyzed by the enzyme 3β-hydroxysteroid dehydrogenase II (3βHSD-II); its expression and activity are not affected by estrogen and remain constant through gestation. The placental steroidogenic pathway through gestation, prior to the onset of labor events, is outlined in red in Figure 1-2a.

In the majority of mammalian species, with the exception of humans and non-human primates, there is a decrease in the placental (or ovarian) production of progesterone at term. This withdrawal has been considered to be the common triggering event directing the onset of parturition (Liggins et al., 1983). The administration of exogenous progesterone can prolong gestation and administration of the progesterone receptor antagonist RU486 induces premature labor even in the primate (Haluska et al., 1994). In species where the corpus luteum remains the main site of progesterone synthesis, ovariectomy will induce myometrial contractility and labor (Sugimoto et al., 1997). In the sheep, the prepartum decrease in the maternal peripheral plasma progesterone does not coincide with an increase in the progesterone metabolic clearance rate but does reflect a pronounced decrease in progesterone concentration in the utero-ovarian circulation (Anderson et al., 1975). These data suggest that an attenuation of intrauterine progesterone production rate must be responsible for the decline in the plasma progesterone level.
Both pregnenolone and progesterone can be metabolized by the enzyme P450C17 hydroxylase (P450C17) to form 17-hydroxylated pregnenolone and progesterone respectively; subsequent scission of the C17-20 bond yields dehydroepiandrosteredione (DHEA) and androstenedione respectively. Both of these reactions are mediated by the enzyme P450C17 at two distinct catalytic sites (Miller et al. 1998). Several studies have reported that steroid 17α-hydroxylase activity within the ovine placenta increases immediately prepartum; this induction has been attributed the increase in fetal adrenal glucocorticoid production due to activation of the fetal hypothalamic-pituitary-adrenal axis (Mason et al. 1989; France et al. 1988; Steele et al. 1976; Anderson et al. 1975; Flint et al. 1975). Mason et al (1988) demonstrated that intraplacental cortisol administration elicited a ~600-fold increase in placent al P450C17 activity through either an increase in the P450C17 gene transcription or mRNA stability. Concomitant with the increase in placental P450C17 activity, fetal and maternal estrogen plasma concentrations rise suggesting that increased placental P450C17 activity leads to elevated placental estrogen output by increasing the intrauterine production of aromatizable C19 steroids (DHEA and androstenedione) the substrates for estrogen synthesis (Mason et al. 1989; France et al. 1988; Steele et al. 1976; Anderson et al. 1975; Flint et al. 1975). Mason et al (1989) established that the ovine placenta inefficiently metabolizes 17αOH-progesterone to androstenedione but that pregnenolone is readily converted to DHEA. These data suggest that the decline in placenta progesterone production with the onset of ovine parturition is caused by the increased expression of P450C17 which diverts pregnenolone from the production of progesterone to preferentially produce DHEA which provides substrate for increased placental estrogen formation. An outline of this shift in placental steroidogenesis is presented in blue in Figure 1-2b.
1-a. Ovine Placental Progesterone Synthesis Through Gestation

cholesterol
1 20α-hydroxylase
2 22-hydroxylase
3 20,22-desmolase
→
P450ccc
→
P450c17 17-OH
→
P45017,20 lyase
→
pregnenolone
→
P450c17 17-OH
→
P45017,20 lyase
→
pregnenolone
→
P45017,20 lyase
→
androstenedione
→
testosterone
→
P450arom
estrone
→
P450arom
estradiol

1-b. Shift in Ovine Placental Steroidogenesis at Term

cholesterol
1 20α-hydroxylase
2 22-hydroxylase
3 20,22-desmolase
→
P450ccc
→
P450c17 17-OH
→
P45017,20 lyase
→
pregnenolone
→
P450c17 17-OH
→
P45017,20 lyase
→
pregnenolone
→
P45017,20 lyase
→
androstenedione
→
testosterone
→
P450arom
estrone
→
P450arom
estradiol

Figure 1-2: Ovine Placental Steroidogenesis Through Gestation and the Onset of Parturition

P450c17: P450 c17 hydroxylase
P450arom: P450 aromatase

17 keto reductase

3βHSD-II: 3β hydroxysteroid dehydrogenase type II
However, in primates a decline in progesterone production at term has not been documented. The primate placenta does not express P450c17 at any time in gestation, and the intrauterine expression of 3βHSD does not decrease at the end of gestation (Riley et al., 1993). Moreover, the levels of the 5α and 5β reduced progesterone metabolites that have been shown to bind to the GABA-A and oxytocin receptors causing inhibition of smooth muscle contractility have not been found to decrease with labor (Challis et al., 2000). However, a withdrawal of progesterone activity could be mediated by a decrease in the intrauterine expression of the progesterone receptor. Three isoforms of the progesterone receptor have been identified: PR-B is a full length, active receptor and PR-A and PR-C are truncated receptor isoforms that modulate PR-B and other steroid receptors (Peter, 2000). Although a decrease in overall PR immunoreactivity within the myometrium has been suggested, the isoform involved has not been reported (How et al., 1995). These data imply that an alternative mechanism of progesterone withdrawal may occur that is not reflected as a change in plasma concentration. However, progesterone may play a role in the mechanism of primate labor. Recent evidence has suggested that a ‘functional’ withdrawal of progesterone activity could occur through the production of endogenous PR antagonists; the inflammatory cytokine TGFβ has been considered as a candidate inhibitor (Casey et al., 1996). In addition, although each steroid hormone has its respective receptor, considerable cross-reactivity between steroids and receptors has been reported. The ratio of cortisol to progesterone concentration in the plasma, amniotic fluid and intrauterine tissues increases at term suggesting that cortisol could interfere with progesterone action by acting at the progesterone receptor or by competing with progesterone at the glucocorticoid receptor. For example, in human chorion trophoblast cells endogenous progesterone tonically stimulates the activity of
prostaglandin dehydrogenase (PGDH) (Patel et al. 2000). Increasing cortisol levels inhibit this basal level of PGDH activity; the inhibition appears to be mediated through the displacement of progesterone activity by cortisol at the glucocorticoid receptor (Patel and Challis; unpublished results). Thus, an increase in the cortisol:progesterone ratio can cause a functional withdrawal of progesterone activity.

Recently it has been suggested that a complete withdrawal of progesterone activity may in fact impede primate parturition. With labor, the primate uterus undergoes a unique regionalization of contractile activity: the fundus becomes contractile and the lower segment relaxes to create the birth canal. Increased expression of connexin 26, a progesterone responsive contraction associated protein that promotes uterine relaxation, within lower segment human myometrium at term suggests that progesterone may be required to mediate the relaxation of the lower segment (Ciray et al. 2000). Progesterone has been found to bind directly to the oxytocin receptor to inhibit signaling suggesting a second mechanism by which progesterone could mediate uterine relaxation (Zingg et al, 1998). Within human lower segment myometrium the expression of the PR chaperone heat shock proteins HSP-90 and 56 and the expression of the steroid receptor co-activators SRC-1 and TIF-2 are increased at term suggesting an increase in PR transcriptional activity in this area (Erb and Lye. unpublished results). These data imply that progesterone activity may be important at term to mediate the regionalization of uterine activity.

In summary, a decline in placental (and ovarian) progesterone synthesis at the end of gestation is not a common event across all species. Although the withdrawal of progesterone has been suggested to be a key trigger to the events of parturition, the maintenance of progesterone production and activity may be important for primate parturition. On the basis
of this evidence, a prepartum fall in progesterone should not be considered as the common signal for the onset of labor across the species.

1.4.2. Estrogen Synthesis by the Feto-Placental Unit

Estrogen is regarded as an uterotrophin with a central and integrative role in the mechanisms of pregnancy maintenance, fetal maturation and the onset of labor (Pepe and Albrecht, 1995). Estrogen facilitates implantation and continued pregnancy by inducing local intrauterine immunosuppression, promoting growth and development of the uterus and regulating utero-placental blood flow (Pepe and Albrecht, 1995). Estrogen protects the fetus from the precocious maturational effects of maternal glucocorticoids through the upregulation of the placental enzyme 11βhydroxysteroid dehydrogenase type II (11βHSD-II) (Baggia et al. 1990). 11βHSD-II converts cortisol to its inactive metabolite cortisone thereby limiting the transplacental transfer of maternal cortisol into the fetal circulation (Stewart and Krozowski, 1999; Pepe and Albrecht, 1995; Baggio et al, 1990b). In addition, estrogen indirectly maintains uterine quiescence by stimulating the enzymes responsible for progesterone biosynthesis (see section 1.3.2). Paradoxically, at term as placental estrogen production peaks, estrogen induces the expression of the CAPs and the production of uterotonins to contribute to the induction of uterine activation and stimulation (Lye, 1994).

The enzyme P450arom (P450arom) catalyzes the biosynthesis of estrogen; this enzyme binds a C19 androgen precursors and catalyzes a series of reactions leading to the formation of the phenolic A ring characteristic of estrogens. France et al (1987) established that during the last four weeks of ovine gestation, placental aromatase activity remains relatively constant. However, a modest increase in aromatase expression and activity occurs immediately prepartum with natural and glucocorticoid induced labor (Mason et al, 1989;
France et al., 1987). The significance of this increase in \( P450_{\text{arom}} \) expression and activity remains unclear as the expression of this enzyme within the placenta does not appear to be a rate-limiting step in the production of estrogen. In fact, across the species basal levels of \( P450_{\text{arom}} \) appear to be adequate to accommodate estrogen production throughout gestation and immediately prepartum. It is the availability of the aromatizable C19 substrates that is a rate limiting factor for placental estrogen production (Mason et al., 1989; France et al., 1987; Rosenfeld et al., 1980).

In primates, placental estrogen production depends upon the provision of C19 steroid precursors from exogenous sources. Approximately 50% of the estrone and estradiol present in the maternal circulation is produced from dehydroepiandrosterone sulphate (DHEAS) supplied by the fetal zone of the fetal adrenal. This zone expresses \( P450_{\text{c17}} \) but not \( 3\beta\text{HSD-II} \) and therefore predominately produces the \( \Delta^5 \) steroid DHEA (secreted as its sulphonyl conjugate DHEAS) which is subsequently aromatized by the placenta. The remaining plasma estrone and estradiol are derived from C19 precursors supplied by the maternal adrenal (Pepe and Albrecht, 1995). In the human, estrone and estradiol are detectable in maternal serum by 8-10 weeks of gestation indicating that the fetal zone of the adrenal gland is functional by this time (Pepe and Albrecht, 1995). A 100-fold increase in fetal plasma DHEA concentration occurs by week 12 of gestation concurrent with increasing fetal pituitary ACTH secretion; by term the fetal adrenal is producing ~200mg of DHEA per day (Messiano et al., 1997). Maternal plasma estriol is derived almost exclusively from fetal adrenal DHEAS that has undergone a 16-hydroxylation within the fetal liver, and subsequent aromatization within the placenta. Throughout gestation, as the fetal HPA axis activity increases, the supply of C19 precursors also increases and this is reflected as a continued rise in the maternal plasma estrogen.
concentrations (Peter et al. 1994; Natanielsz et al. 1982). At term, the levels of estriol, estrone and estradiol increase exponentially reflecting a sustained activation of the fetal HPA axis.

In other species, such as the sheep, the fetal adrenal cortex also directs placental estrogen production throughout gestation. As in primates, the sheep placenta does not express P450c17 for majority of gestation, and produces estrogens (mainly sulfoconjugates) from Δ5 and Δ4 C19 precursors supplied by the developing zona fasiculata and zona reticularis of the fetal adrenal cortex (Challis and Brooks, 1989). However, late in gestation increased glucocorticoid production by the fetal HPA axis induces the placental expression of P450c17 (see section 1.4.1). Consequently, in the final days of ovine gestation there is a surge in placental estrogen biosynthesis due to both increased fetal adrenal and local placental production of aromatizable C19 steroids. The key differences between ovine and primate placental estrogen synthesis are the source of estrogen precursors at the end of gestation and measurable decline in progesterone production by the ovine placenta. However, both species are dependent upon the activity of the fetal HPA axis for the provision of estrogen precursors. Therefore, the maturation and sustained activation of the fetal HPA axis could be considered the common trigger for the events of parturition across the species.

1.4.3. Maturation of the Fetal Hypothalamic-Pituitary-Adrenal Axis (HPA)

The critical role of the fetal HPA in the events of parturition was first established more than thirty years ago, based on studies by Liggins and colleagues who recognized that pregnant ewes fed V. californicum at a particular time in pregnancy had prolonged gestation length and their fetuses had markedly hypoplastic pituitary and adrenal glands (Liggins et al., 1973). Further in vivo studies found that fetal hypophysectomy or adrenalectomy, disruption
of the fetal hypothalamic-pituitary stalk and/or lesions of the paraventricular nucleus of the fetal hypothalamus similarly caused a prolongation of gestation length (McDonald et al., 1992; Gluckman et al., 1991; McDonald et al., 1991). In addition, intrafetal administration of adrenocorticotropin hormone (ACTH), cortisol or the synthetic glucocorticoid dexamethasone was found to prematurely induce delivery of the ovine fetus (McLaren et al., 1996; Thorburn et al., 1991). These in vivo studies highlighted the critical role of the fetal HPA axis in the determination of gestation length and the timing of labor.

Recent studies of the ovine fetus have led to a greater understanding of the relationship between fetal HPA function and the onset of labor. Corticotropin releasing hormone (CRH) is produced by the parvocellular neurons of the hypothalamus; its secretion into the hypophyseal portal system drives the pituitary to synthesize proopiomelanocortin (POMC), the precursor of adrenocorticotropin hormone (ACTH) and to subsequently secrete ACTH. Fetal hypothalamic CRH expression is present by d60 of gestation, increases gradually until d120 and then increases markedly over the final 20d of gestation (Matthews and Challis, 1996). CRH-R1 receptor expression and CRH binding are present in the fetal ovine pituitary by d100 of gestation, increase by d135 and then decrease towards term (Green et al., 2000; Lu et al., 1991). In vivo, fetal pituitary responsiveness to intrafetal CRH infusion increases from d110 until d125 then decreases until term; these findings are consistent with decreased pituitary CRH-R1 expression and CRH binding in late gestation (Green et al., 2000; Norman et al., 1985). These data suggest that hypothalamic CRH has its greatest effect on pituitary function from d120-135 of gestation and that the marked increase in CRH expression in late gestation may reflect a decreased pituitary sensitivity to CRH at that time. In addition, CRH and cortisol down-regulate CRH receptor expression and CRH binding; in
late gestation CRH and cortisol levels are high and may contribute to the decreased sensitivity of the fetal pituitary to CRH (Lu and Challis, 1991). Thus, it appears that although hypothalamic CRH may initiate HPA activation, CRH action may be blunted at term and may not be the key to sustained function of the axis at this time.

Arginine vasopressin (AVP) is produced by the supraoptic and paraventricular nuclei (magnocellular and parvocellular neurons) of the hypothalamus; AVP production by the parvocellular neurons has been linked to the regulation of pituitary ACTH synthesis and secretion (Matthews et al, 1995). AVP expression is present in the parvocellular neurons of the fetal sheep pituitary gland by d60 of gestation, however, this expression does not alter with gestational age (Matthews et al, 1995). AVP acts via V1b and V3 receptors: the expression of these receptors in the fetal sheep pituitary throughout gestation has not been studied. In vitro, AVP increased POMC mRNA expression and ACTH output in a dose dependent manner from cultured fetal sheep pituitary cells (Matthews et al, 1995; Durand et al. 1986). AVP had a greater stimulatory effect compared with CRH in cells at d120-138 of gestation but at term the potency of CRH and AVP was similar. In vitro CRH and AVP together had an additive effect on fetal pituitary cell ACTH output (Durand et al, 1986). Through the end of gestation, intrafetal administration of either AVP or CRH increased pituitary ACTH production: although responsiveness to CRH decreased and responsiveness to AVP increased as a function of gestational age (Carr et al, 1995; Norman et al, 1985). At d120 of gestation, AVP and CRH had a synergistic effect on ACTH production while at all other ages the effect was simply additive (Carr et al, 1995). Therefore, the sustained increase in fetal pituitary ACTH synthesis and secretion through late gestation may be differentially regulated by both AVP and CRH. AVP and CRH may be involved in the early rise in ACTH.
synthesis and secretion. AVP may stimulate the rise in ACTH through the last 20d of gestation. AVP and CRH effects, mediated by separate post receptor mechanisms, may be additive and act to maintain the high levels of ACTH at term and with the onset of labor.

ACTH is produced by the corticotroph cells within the pars distalis and pars intermedia of the anterior pituitary. After cleavage of POMC by the action of prohormone convertase 1 (PC1), ACTH can then be cleaved by prohormone convertase 2 (PC2) to form smaller molecular weight products including corticotrophin-like intermediate lobe peptide (CLIP) and α-melanocyte stimulating hormone (αMSH). Within the ovine fetal pituitary, POMC mRNA is expressed by d60 of gestation; POMC expression in the pars intermedia increases progressively from d60 until d100 and then remains relatively constant through the end of gestation (Matthews et al. 1996; Matthews et al. 1994). POMC expression increases from d60 until d120 within the superior and inferior aspects of the pars distalis. After d120 of gestation, POMC expression is localized mainly to the inferior aspect of the pars distalis and this expression is increased markedly by term but no further increases are observed with the onset of labor (Holloway et al., 2000; Matthews et al. 1994). These increases in POMC expression in the pars distalis are reflected in increased immunoreactive ACTH content within the corticotroph cells and increased circulating fetal plasma levels of ACTH_{(1-39)} (Norman et al., 1985; Hennessy et al. 1982). The progressive rise in pituitary ACTH synthesis may be mediated by a change in the expression of prohormone convertase enzyme PC1. The level of PC1 expression within the fetal pituitary increases towards the end of gestation suggesting that increased ACTH output could be attributable in part to increased cleavage of its precursor POMC (Holloway et al., 2000). In addition, the level of fetal pituitary PC2 does not change through gestation, suggesting that the ACTH produced is not increasingly cleaved.
to form αMSH and CLIP (Holloway et al. 2000). Thus, despite decreasing CRH responsiveness and maintained AVP action, POMC expression and ACTH secretion from the fetal pituitary continues to rise through the end of gestation and the onset of labor. The potential mechanism(s) of this sustained production and secretion will be discussed later.

In fetal sheep, plasma levels of ACTH(1,39) increase gradually from d110 of gestation and a further surge in output is observed with the progression of labor (Holloway et al., 2000; Norman et al., 1985). The rise in plasma ACTH concentration is followed by sustained increases in fetal plasma cortisol levels approximately 10d later (Magyar et al., 1980). ACTH induces expression of the key adrenal steroidogenic enzymes involved in cortisol synthesis, P450ox and P450c17. Therefore it has been suggested that increasing ACTH drives cortisol synthesis by adrenal cortical cells in late gestation (Conley et al., 1997; Durand et al., 1982). However, under conditions of low, constant plasma ACTH levels, adrenal cortisol production continued to increase through the end of gestation suggesting that developmental changes occurring within the fetal adrenal also contribute to the rising cortisol levels in late gestation (Jacobs et al., 1994). Expression of adrenal ACTH receptor mRNA increases modestly after d130 of gestation (Fraser et al., 2000). ACTH also increases its own receptor signal transduction by enhancing receptor coupling to adenylate cyclase thereby facilitating increased adrenal sensitivity to ACTH through the end of gestation (Durand et al., 1981).

Several studies have examined the trigger(s) of fetal HPA activation and the mechanism(s) of sustaining this activation. Leptin, the adipocyte hormone, has been shown to reduce expression of neuropeptide Y (NPY) mRNA in the arcuate nucleus. NPY stimulates HPA axis function by increasing CRH production. Thus, a decrease in leptin at the end of gestation would release the tonic inhibition of NPY and lead to HPA axis activation.
Support for this possibility comes from a recent study in which intraventricular infusion of leptin to the late gestation ovine fetus caused a decrease in fetal plasma ACTH and cortisol concentrations. Presumably reduced output of endogenous leptin would allow HPA activity to increase (Howe et al, 2000). OT is expressed in the parvocellular and magnocellular neurons of the fetal hypothalamus and OT expression in the hypothalamus increases with gestational age. OT increased ACTH output by cultured fetal pituitary cells and the administration of CRH and OT together caused a greater ACTH output compared to either secretagogue alone. Thus OT could play a role in HPA activation and exert synergistic effects with CRH in late gestation (Matthews, 1999).

There are several mechanisms by which HPA activity could be sustained. Firstly, there appears to be an attenuation of the normal negative feedback regulation of HPA function (Challis and Brooks, 1989). Fetal plasma and pituitary levels of the high affinity cortisol binding protein, corticosteroid binding globulin (CBG) increase through gestation. CBG may act to bind cortisol and thereby limit the local bio-availability of free cortisol within the pituitary to exert negative feedback (Challis et al, 1995; Berdusco et al, 1993; Challis et al. 1985; Ballard et al, 1982). The enzyme 11βhydroxysteroid dehydrogenase (11βHSD) which inter-converts cortisol and its inactive metabolite cortisone, is expressed within the fetal pituitary and preferentially metabolizes cortisol to cortisone, thereby reducing the effects of cortisol on pituitary ACTH production (Yang et al, 1995). At present, however, the nature of fetal pituitary isoform(s) of 11βHSD is unclear. Cortisol acts through GR: fetal pituitary GR mRNA and cortisol binding can be detected from d60 of gestation and increases from d135 until term. Levels of GR mRNA in the anterior pituitary then decrease with the progression of labor (Holloway et al, 2000; Yang et al, 1992). Fetal hypothalamic GR also
decline in late pregnancy (Andrews and Matthews, 2000; Yang et al, 1992). Collectively these data suggest that pituitary ACTH output may be incompletely inhibited by the increasing cortisol levels due to reduced hypothalamic and/or pituitary GR expression.

Estrogen may also influence activity of the fetal HPA axis. Intrafetal estrogen infusion increased hepatic CBG and 11βHSD-I expression in late gestation (Wang et al, 1998). Fetal plasma concentrations of estrone sulfate increase progressively during ovine pregnancy and in some studies physiologic increases in fetal plasma estrogen concentrations increase fetal plasma ACTH concentrations. It is suggested that this effect is mediated by estrogen increasing hypothalamic CRH (Wood et al, 1997). The fetal hypothalamus, hippocampus and brainstem have been found to express high levels of estrogen sulfatase activity (Purniton et al 1999) suggesting that within the fetal brain, local estrogen production may increase and potentially influence HPA function. Furthermore, estrogen has been shown to decrease hypothalamic GR expression, suggesting that the changes in pituitary and/or hypothalamic GR expression in late pregnancy may reflect increased local or systemic estrogen production [Holloway, Wang and Challis, unpublished results].

PGs, specifically PGE₂ may also exert a positive feed-forward effect on the fetal HPA axis that is not subject to negative feedback regulation. Plasma cortisol and PGE₂ concentrations increase with similar time-course in fetal sheep during late pregnancy (Fowden et al, 1987; Challis et al 1976). Intrafetal PGE₂ infusion stimulated fetal plasma ACTH and cortisol production (Thorburn and Challis, 1979; Louis et al, 1976); inhibition of PGE₂ production at both term labor and RU486 induced preterm labor decreased fetal plasma ACTH and cortisol levels (McKeown et al, 2000; Unno et al, 1998; Young et al, 1996). Young et al (1996) showed that disconnection of the hypothalamic-pituitary stalk blocked the
effects of fetal PGE$_2$ on the fetal HPA axis suggesting that PGE$_2$ exerted its effects at or above the level of the pituitary. In addition, PGE$_2$ has been shown to increase adrenal cortical cell P450c17 expression and activity suggesting that PGE$_2$ could directly increase adrenal cortisol synthesis (Rainey et al. 1991). Clearly, the mechanisms regulating the sustained activation of fetal HPA activity through the end of gestation are complex and multi-factorial reflecting a complex interplay between the developmental and endocrine changes occurring at this time. A summary of the key events of ovine fetal hypothalamic-pituitary-adrenal activation is depicted in **Figure 1-3**.
Figure 1-3: Summary of the Events Regulating the Sustained Activation of the Fetal Hypothalamic-Pituitary-Adrenal Axis at the End of Gestation and the Onset of Parturition.

Neuropeptide Y, NPY; Corticotropin releasing hormone, CRH: arginine vasopressin, AVP; prostaglandin E\(_2\), PGE\(_2\); proopiomelanocortin, POMC; adrenocorticotropic. ACTH: prohormone convertase-1, PC-1; prohormone convertase-2, PC-2; corticotrophin like intermediate lobe peptide, CLIP: a melanocyte stimulating hormone, a MSH; glucocorticoid receptor, GR; 11\(\beta\)-hydroxysteroid dehydrogenase Type 1, 11\(\beta\)-HSD-1; corticosteroid binding globulin, CBG.
It has been more difficult to assess whether sustained activation of the fetal HPA axis occurs during late gestation in the human and the non-human primate. Both prematurity and post-maturity are complications associated with anencephalic human fetuses and provide conflicting evidence for the role of fetal HPA maturation in the onset of primate parturition (Ryan et al. 1974; Novy, 1977; Tulchinsky et al. 1972). In the rhesus monkey, fetal plasma levels of DHEAS (presumably from the fetal zone of the adrenal gland) increase with a similar time course to that of cortisol in the fetal sheep suggesting a comparable pattern of ontogenic development of fetal adrenal steroidogenesis (Novy, 1977). The administration of exogenous ACTH has been shown to induce labor at term and preterm in the rhesus monkey (Mecenas et al. 1996; Novy, 1977). In monkeys, placental retention time following fetectomy was increased compared to controls; fetectomy in baboon pregnancy did not alter gestation length but did decrease plasma estrogen and progesterone concentrations (Albrecht et al. 1989). These data imply that the primate fetus contributes signals for the initiation of labor similar to that of the ovine fetus. In the human, maternal plasma levels of estriol increase through the end of gestation and a surge in estriol concentration is observed with labor at any time in gestation (Tulchinsky et al. 1972). Because the fetal adrenal and liver are the major source of C19 steroid precursor for estriol synthesis, these data provide indirect evidence of increased human fetal adrenal activity at term and with preterm labor. Since the adrenal is under the direction of the hypothalamus and pituitary, these data also provides indirect evidence of coordinated HPA activity at labor suggestive of HPA maturation. Fetal plasma and amniotic fluid concentrations of cortisol also increase at term in both the human and non-human primate which further supports the hypothesis of increased fetal HPA activity at this time (Pearson-Murphy et al, 1975). However, the fetal membranes can synthesize cortisol
from cortisone (see section 1.6.2) and may contribute to the amniotic pool of cortisol. In the early 1970's maternal and intra-amniotic administration of cortisol was used to induce labor in post-mature human pregnancies (Katz et al, 1979; Pearson-Murphy et al, 1975). The current use of antenatal corticosteroid for the purpose of fetal lung maturation has been linked with transient increases uterine contractility (Yeshaya et al, 1996; Elliot and Radin, 1995). These studies provide evidence in support of a role for cortisol in the onset of uterine activity. In summary, substantial direct and indirect evidence supports the hypothesis that sustained activation of the primate fetal HPA axis at the end of gestation determines the timing of labor onset similar to that observed in the ovine and other species.

The regulation of primate fetal HPA maturation is not as well understood as that of the ovine fetus. Betamethasone administered in late gestation to baboons suppressed fetal pituitary POMC expression, lowered fetal adrenal ACTH-R and steroidogenic enzyme levels and decreased overall fetal adrenal mass (Aberdeen et al, 1998). These data imply that primate fetal adrenal maturation requires fetal pituitary ACTH stimulation. Pepe and Albrecht (1995) have hypothesized that a complex interplay between placental steroidogenesis and maternal plasma cortisol concentration governs fetal primate HPA maturation. They suggest that intrauterine cortisol metabolism regulated by placental estrogen synthesis is responsible for the suppression of fetal HPA activity through gestation. 11β-hydroxysteroid dehydrogenase (11βHSD), expressed in the fetal membranes and placenta, is the enzyme responsible for intrauterine cortisol metabolism; two isoforms of this enzyme exist within the intrauterine tissues. 11βHSD type I (11βHSD-I) is capable of the bi-directional inter-conversion of cortisone and cortisol and the type II isoform of this enzyme (11βHSD-II) preferentially catalyzes the oxidation of cortisol to cortisone. Based on activity
measurements. *in situ* hybridization and immunochemical localization studies in the baboon and the human it has been established that an 11βHSD-II oxidative system is present within the placenta (Pepe et al., 2000; Pepe et al., 1995). 11βHSD-II is extensively expressed throughout the syncytiotrophoblast including the basal membrane in contact with the fetal blood but has a lower level of expression at the microvillus membrane (Pepe et al., 2000). Given that the fetal primate adrenal does not develop the enzyme capacity to synthesize cortisol *de novo* until the end of gestation, the cortisol present in the fetal circulation throughout gestation must be exclusively maternal in origin regulated by the activity of 11βHSD-II within the placenta. Pepe and Albrecht (1995) propose that this maternally derived cortisol suppresses fetal primate HPA activity, in particular, the fetal pituitary production of ACTH to limit the growth and development of the fetal adrenal definitive zone and the expression of 3βHSD-I required for cortisol synthesis. However, as gestation progresses increasing placental estrogen production causes increased 11βHSD-II oxidative activity leading to a marked increase in the placental conversion of maternal cortisol to cortisone and a decrease in the proportion of maternal cortisol entering the fetal circulation. As a consequence the tonic inhibition of the fetal HPA axis by maternally derived cortisol is released leading to an increase in fetal pituitary ACTH production and the maturation of the fetal adrenal gland (Pepe and Albrecht, 1995). In support of this proposal, pituitary ACTH expression, adrenal 3βHSD-I and P450C17 expression and the ontogenesis of *de novo* cortisol production was found to increase with gestational age and after the premature induction of placental 11βHSD-II by estradiol (Pepe and Albrecht, 1995). An outline of this proposal is presented in *Figure 1-4*. 

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Figure 1-4: Proposed Mechanism of Primate Fetal Hypothalamic-Pituitary-Adrenal Axis Activity Through Gestation and the Onset of Parturition

11βHSD-II: 11β hydroxysteroid dehydrogenase Type II
In summary, there is compelling evidence across the species in support of the hypothesis that sustained activation of the fetal hypothalamic-pituitary-adrenal axis occurs at the end of gestation and provides the trigger for the onset of uterine activation and stimulation. The consequent rise in fetal adrenal activity provides increased C19 steroid substrate for placental estrogen synthesis as well as increased fetal plasma cortisol concentration. In some species this rise in cortisol triggers the induction of placental P450C17 expression diverting pregnenolone away from the production of progesterone towards the intrauterine production of additional C19 estrogen precursors. The increase in fetal adrenal glucocorticoid production occurs concurrently with increased concentrations of prostaglandins in the plasma, amniotic fluid and intrauterine tissues. The purpose of this thesis will be to examine the glucocorticoid regulation of prostaglandin production and uterine activity during late gestation and the onset of labor in the sheep and the human.

1.5. Prostaglandins: Key Mediators of the Events of Parturition

Across the species intrauterine prostaglandin production increases towards the end of gestation and further increases are observed with the onset of labor. This introduction will review the structure, synthesis, metabolism and mechanism of action of prostaglandins and in particular examine the regulation of prostaglandin production and action within the intrauterine tissues through the end of gestation and onset of labor.

2.5.1. Prostaglandin Structure

Prostaglandins are classified as “eicosanoids” referring to a family of oxygenated fatty acids composed of the prostanoids [prostaglandins (PG), prostacyclin and thromboxanes], the leukotrienes and the epoxides (Smith et al, 1996). Eicosanoids are
synthesized from naturally-occurring C20 polyunsaturated fatty acids that contain three to five double bonds in the cis position; in the majority of mammalian species the most prevalent eicosanoid precursor is arachidonic acid, although other precursors include 8,11,14-eicosatrienoic acid, 5,8,11,14,17-eicosapentenoic acid and diacyl glycerol (Smith et al. 1996). PGs have a characteristic hairpin structure with a cyclopentane ring and 2 sidechains. α and ω, extending from the ring; modifications extending from the ring determine the subclass and activity of the PG (Smith et al. 1996). PGA, B and C are synthetic products not existing in nature; PGG and PGH are transient intermediates of the prostanoid biosynthetic pathway (Smith et al, 1996). The F type PGs are the most polar class of PGs with two hydroxyl groups extending from the ring and the E type PGs have intermediate solubility with one ketone and one hydroxyl group extending from the ring (Smith et al, 1996). The series of the PG is determined by the number of double bonds in the structure: PG2 series have 13 trans and 5 cis double bonds (Smith et al, 1996). Two main PGs are critical to the labor process, PGE2 and PGF2α. Figure 1-5 presents an overview of the eicosanoid biosynthetic pathway.
Phospholipids

- Phospholipase A (PLA)
- Phospholipase C (PLC)
- Phospholipase D (PLD)

Arachidonic acid

- Prostaglandin H synthase-I (PGHS-I)
- Prostaglandin H synthase-II (PGHSII)

PGG₂ / PGH₂

PG or Thromboxane synthases

- PGE₂
- PGF₂α
- PGI₂
- Thromboxane A₂

15-keto PG metabolites

15 hydroxy Prostaglandin Dehydrogenase (PGDH)

*Figure 1-5: The Prostaglandin Biosynthetic Pathway.*
1.5.2. Prostaglandin Synthesis: Substrate Availability and Lipase Activity

Prostanoids are synthesized in response to a specific proteolytic or hormonal stimulus (Vane et al. 1998; Smith et al. 1996; Goppelet-Streube, 1995; Mitchell et al. 1995; Marshall et al. 1987). Under normal conditions, cells synthesize basal level of prostanoid; increases in prostanoid production are correlated temporally with an increase in arachidonic acid release, suggesting that substrate availability is an important control point in the regulation of prostanoid biosynthesis (Vane et al., 1998; Smith et al., 1996; Goppelet-Streube, 1995; Mitchell et al. 1995; Marshall et al. 1987). Arachidonate is stored within cellular membranes as glycerophospholipid and released through the action of lipase enzymes. The arachidonate release is rapid and accompanied by a turnover of inositol containing phospholipids within the cell: the major sources of arachidonate are phosphatidylcholine and phosphatidylethanolamine (Vane et al., 1998; Smith et al., 1996; Goppelet-Streube, 1995; Mitchell et al. 1995; Marshall et al. 1987). Several isoforms of the lipase enzyme exist including phospholipase A1 (PLA1), A2 (PLA2), phospholipase C (PLC), and phospholipase D (PLD) (Vane et al., 1998; Smith et al., 1996; Goppelet-Streube, 1995; Mitchell et al. 1995; Marshall et al. 1987).

PLA2, the most prevalent lipase enzyme, hydrolyzes the acyl bond in the sn-2 position of the glycerol backbone of phospholipids resulting in the C20 free fatty acid prostanoid precursor and a lysophosphatide which also can be used as precursor for inflammatory mediators (Dennis, 2000; Bingham et al. 1999; Rice, 1998). Certain PLA2 isoforms not only have lipase catalytic function but also serve as ligands for specific membrane bound receptors (Dennis, 2000; Bingham et al. 1999; Rice, 1998). PLA2 I (B), II (A and C), V and X contain a "secretory" signal peptide, are stored in secretory vesi

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are referred to as the secretory or sPLA₂ enzymes (Dennis, 2000; Bingham et al. 1999; Rice, 1998). Both a membrane bound and a soluble form of the mammalian sPLA₂ receptor have been isolated (Dennis, 2000; Bingham et al. 1999; Rice, 1998). Once bound, the extracellular receptor-sPLA₂ complex becomes internalized and may serve to traffic secreted PLA₂ back inside the cell (Dennis, 2000; Bingham et al, 1999; Rice, 1998). Alternatively, an intracellular form of the receptor may act as an inhibitor of soluble sPLA₂ or function as part of a signal transduction cascade (Dennis, 2000; Bingham et al. 1999; Rice, 1998). Group VI PLA₂, a cytosolic lipase, is rapidly translocated to a cell membrane, in close proximity to its substrate and catalyzing the release of arachidonate after stimulation by Ca²⁺. Recently, it has been suggested that because cPLA₂ activity can be rapidly initiated, this lipase may be an acute phase enzyme responsible for the immediate mobilization and release of arachidonate for constitutive or basal eicosanoid synthesis (Dennis, 2000; Bingham et al. 1999; Rice, 1998). In contrast, because of the time lag between stimulation and assembly, sPLA₂ has been labeled as the late phase enzyme responsible for induced eicosanoid production and/or as an autocrine/paracrine amplifier of eicosanoid generation (Dennis, 2000; Bingham et al, 1999; Rice, 1998). Within the intrauterine tissues, PLA₂ (secretory and cytosolic forms) has been identified as the key lipase enzyme through gestation as will be discussed in section 1.6 (Dennis, 2000; Bingham et al, 1999; Rice, 1998).

1.5.3. Prostaglandin Synthesis: Prostaglandin H Synthase Expression and Activity

Once arachidonate is liberated to the cytoplasm, two synthetic reactions catalyzed by a single enzyme complex occurs within an intracellular membrane to culminate in the cytoplasmic release of the obligate precursor for all 2-series prostanoids, prostaglandin H₂ (PGH₂) (Vane et al, 1998; Smith et al, 1996; Goppelet-Streube, 1995; Mitchell et al, 1995;
Marshall et al. 1987). Prostaglandin H synthase (PGHS) is an heme containing, glycosylated protein composed of two identical subunits each with two separate catalytic sites (Vane et al., 1998; Smith et al. 1996; Goppelet-Streube, 1995; Mitchell et al. 1995; Marshall et al. 1987). PGHS is a membrane associated protein anchored by four short amphipathic helices that interdigitate with a leaflet of the lipid bilayer; PGHS has been localized to the luminal membrane of the endoplasmic reticulum and the inner or the outer nuclear envelope (Smith et al. 1996).

PGHS forms PGH₂ through sequential cyclooxygenase and peroxidase reactions catalyzed at separate active sites. The cyclooxygenase activity has an absolute requirement for a hydroperoxide activator to oxidize a heme prosthetic group interposed between the cyclooxygenase and peroxidase sites (Vane et al. 1998; Smith et al. 1996; Goppelet-Streube, 1995; Mitchell et al. 1995; Marshall et al. 1987). Once oxidized this heme group will oxidize a neighboring tyrosine₁₃₈₅ residue located within the cyclooxygenase site; the resulting tyrosyl radical removes a hydrogen from arachidonate to form an arachidonate radical (Vane et al. 1998; Smith et al. 1996; Goppelet-Streube, 1995; Mitchell et al. 1995; Marshall et al. 1987). This two step process is referred to as 'cyclooxygenase activation'. Following the formation of the arachidonate radical, sequential additions of oxygen to C₁₁ and C₁₅ yields prostaglandin G₂ (PGG₂). PGG₂ is translocated to the peroxidase active site where it undergoes a two-electron reduction of its 15-hydroxyperoxyl group to form PGH₂ (Vane et al. 1998; Smith et al. 1996; Goppelet-Streube, 1995; Mitchell et al. 1995; Marshall et al. 1987). This PGH₂ intermediate is the obligate precursor of all other prostanoids.

PGHS activity is a rate limiting step in the formation of prostanoids and is regulated in a cell specific manner (Smith et al. 1996). Generally, the PGHS activity of a cell is
determined by the cellular PGHS protein concentration and an increase in prostanoid production requires the de novo synthesis of the enzyme (Smith et al, 1996). However, recently post-translational modification (phosphorylation and/or glycosylation), interference with dimerization or repression of either of the two catalytic sites has been suggested to alter enzyme activity and may be a mechanism of maintaining an intracellular pool of inactive enzyme (Vane et al. 1998). Several constraints act to limit the daily amount of prostanoids produced by a given cell. Substrate availability restricts the rate of PGH₂ synthesis; although there are high concentrations of free arachidonate within the tissues it is protein bound and therefore not available for prostanoid production (Vane et al, 1998; Smith et al, 1996; Goppelet-Streube. 1995; Mitchell et al, 1995; Marshall et al, 1987). Thus, lipase expression and activity is an important regulator of PG biosynthesis. Another factor regulating prostanoid production is the availability of hydroperoxide. The cyclooxygenase activity of PGHS requires nanomolar quantities of hydroperoxide as an activator and the main mechanism restricting the level of intracellular hydroperoxide is endogenous peroxidase activity (Vane et al. 1998; Smith et al, 1996; Goppelet-Streube, 1995; Mitchell et al, 1995; Marshall et al. 1987). In addition, cellular prostanoid biosynthesis is limited by the self-catalyzed inactivation of the PGHS enzyme; the half-life of the PGHS enzyme is less than ten minutes (Vane et al. 1998; Smith et al, 1996; Goppelet-Streube, 1995; Mitchell et al. 1995; Marshall et al, 1987). Each PGHS dimer consumes approximately 400 arachidonate molecules before becoming inactivated through a process of substrate incorporation followed by intra-molecular cross-linking (Vane et al, 1998; Smith et al, 1996; Goppelet-Streube, 1995; Mitchell et al, 1995; Marshall et al, 1987). The precise mechanism governing inactivation has not yet been defined; however, the accumulation of enzyme product has been
clearly determined not to be responsible for enzyme inhibition (Vane et al. 1998; Smith et al. 1996; Goppelet-Streube. 1995; Mitchell et al, 1995; Marshall et al, 1987). Thus, a combination of suicide inactivation, peroxide suppression and substrate availability places an upper limit on the amount of prostanoid produced by a cell under normal physiologic conditions.

The prostaglandin H synthase enzyme exists as two distinct isoforms: PGHS type I (PGHS-I) and PGHS type II (PGHS-II). PGHS-I is expressed within most mammalian tissues but not necessarily in all cells of that tissue (Smith et al., 1996; Inoue et al., 1995; Xu et al., 1995). The level of PGHS-I expression varies across cell types although its expression within a given cell remains relatively constant throughout the cell cycle, simply increasing or decreasing within a narrow 2-4 fold range (Smith et al., 1996; Inoue et al., 1995; Xu et al., 1995). PGHS-I gene expression can be stimulated by serum, cytokines and growth factors (Xu et al., 1997). The human PGHS-I gene, mapped to chromosome 9, is a single copy gene, 22 kilobases long containing 11 exons (Smith et al., 1996; Inoue et al., 1995; Xu et al., 1995). A PGHS-I cDNA probe detects a 2.7 kb mRNA transcript as well as a 5.5 kb transcript representing an immature form (Smith et al., 1996; Inoue et al., 1995; Xu et al., 1995). The GGC rich 5' region is characteristic of a housekeeping gene, containing multiple transcription start sites and lacking canonical CAAT and TATA sequences (Smith et al., 1996; Inoue et al., 1995; Xu et al., 1995). Xu et al (1997) demonstrated that two SP-1 elements are essential for basal PGHS-I transcription and contribute 70-75% of the promoter activity. However, it is unclear how these two regions drive PGHS-I expression under physiological and pathophysiological conditions. Several other 5' putative transcription factor binding sites have been characterized including AP-1, AP-2, PEA (viral oncogene site), a negative
glucocorticoid response element and several shear stress response elements although the functional role of these sites has not yet been determined (Smith et al., 1996; Inoue et al., 1995; Xu et al., 1995). A relatively short 3' region contains a single polyadenylation site and contributes to the relative stability of the PGHS-I transcript (Smith et al., 1996; Inoue et al., 1995; Xu et al., 1995). The translation product is a ~600 amino acid, 72 kD protein which has four glycosylation consensus sequences and a hydrophobic, 24 amino acid N-terminal signal peptide (Smith et al., 1996; Inoue et al., 1995; Xu et al., 1995). In addition, PGHS-I possesses signal peptide at its C-terminus that directs the localization of the enzyme to the membrane. PGHS-I activity appears to be involved in the production of prostanoids that act extracellularly as local paracrine/autocrine factors to mediate acute “housekeeping” responses to circulating hormones (i.e., in the regulation of normal renal and stomach function and vascular homeostasis) (Smith et al., 1996; Inoue et al., 1995; Xu et al., 1995). Thus, the PGHS-I isoform is often referred to as the constitutive or housekeeping PGHS enzyme.

Conversely, PGHS-II expression is undetectable in most mammalian cells but can be rapidly and transiently increased 20-30 fold in response to growth factors, mitogens, hormones, cytokines and bacterial endotoxin. Thus PGHS-II is referred to as the “inducible” isozyme (Smith et al. 1996; Inoue et al., 1995; Xu et al., 1995). However, in some tissues including the brain, the testes, the epithelium of the trachea and the macula densa cells of the kidney. PGHS-II is constitutively expressed (Smith et al., 1996; Inoue et al., 1995; Xu et al., 1995). PGHS-II expression is cell specific as well as cell cycle specific in cells where PGs are required for replication (Smith et al., 1996; Inoue et al., 1995; Xu et al., 1995). The human PGHS-II gene is mapped to chromosome 2 and is a single copy gene 8 kb in length with 10 exons (Smith et al., 1996; Inoue et al., 1995; Xu et al., 1995). PGHS-II cDNA probes detect a
4-4.5 kb mRNA transcript; a second smaller 2-2.3 kb transcript has been reported and attributed to different polyadenylation sites in the PGHS-II gene (Smith et al. 1996; Inoue et al. 1995; Xu et al. 1995). The 5' promoter contains a TATA box and a single start site suggestive of strict control of gene expression (Smith et al, 1996; Inoue et al, 1995; Xu et al, 1995). Multiple transcription factor binding sites have been characterized including a cAMP response element (CRE), CEBPβ (NF-IL6), AP-2, SP-1, PEA-3 and a glucocorticoid response element (GRE) (Smith et al. 1996; Inoue et al, 1995; Xu et al, 1995). Deletion studies suggest that CRE activity may be obligatory for gene expression, in particular during cell differentiation (Smith et al, 1996; Inoue et al, 1995; Xu et al, 1995). The 3' untranslated region spans more than 2 kb, expresses multiple polyadenylation sites, seventeen Shaw Kamen sequences (ATTTA) and other non-sequenced regions within the cDNA open reading frame which confer instability (Smith et al, 1996; Inoue et al, 1995; Xu et al, 1995). The PGHS-II gene possesses the characteristics of an early response gene: (1) rapid induction of mRNA, protein and activity followed by rapid decline in protein level (2) inhibition by cyclohexamide indicating that increased activity is due to increased gene expression and (3) parallel induction with other acute response elements including c-fos and c-jun (Smith et al, 1996; Inoue et al, 1995; Xu et al, 1995). The translation product of this gene is a ~600 amino acid protein with 5 glycosylation sites (3 sites conserved from PGHS-I). Glycosylation at amino acid 509 may cause two molecular weights, 72 and 74 kD (Smith et al, 1996; Inoue et al. 1995; Xu et al, 1995). As mentioned, many factors regulate PGHS-II gene expression and its post-transcriptional/post-translational modification to affect enzyme activity.

Despite certain similarities, PGHS-I and II have distinct differences in their structure, biochemical properties, expression and function. PGHS-I and II gene expression differs
through the cell cycle: PGHS-I is constitutively expressed whereas PGHS-II expression rapidly increases and decreases throughout the cycle (Smith et al, 1996; Inoue et al, 1995; Xu et al. 1995). This difference in gene expression is attributable to the larger promoter region of the PGHS-II and its multiple response elements representing complex regulatory control. Both forms of the enzyme have superimposable crystalline structures however at the amino acid level there is only ~60% homology between the isoforms. There is a unique 18 amino acid sequence at the C-terminus of PGHS-II that allows for the development of specific PGHS-II antibodies. There is conservation of the functionally relevant sites including: the heme ligand, the residues that form the substrate binding channel and the catalytic sites (same Km and Vmax). However, the cyclooxygenase active site between the two isoforms is differentially regulated. PGHS-I exhibits co-operative changes in cyclooxygenase activity in response to concentrations of arachidonate where as PGHS-II displays saturable kinetics. Swinney et al (1997) reported that PGHS-II was more active at low concentrations of arachidonate (<0.5μM) as compared with PGHS-I which was more active at arachidonate concentrations greater than 2.5 μM. Chen et al (1999) reported a similar intrinsic difference between the two isoforms regarding the ‘cyclooxygenase activation’ efficiency; PGHS-II is initiated at much lower hydroperoxide concentrations than PGHS-I. The biological significance of this effect maybe to enhance the selectivity of PGHS-II activity over PGHS-I activity in tissues where the two forms are co-expressed and substrate availability is limited. Alternatively the co-operative activity could serve two roles: firstly inactive PGHS-I would not consume intracellular arachidonate required for other cellular processes and secondly it could prevent suicide inactivation allowing PGHS-I to remain in a quiescent intracellular pool capable of rapidly responding to increased cellular demand for PGs (Chen et al, 1999;
Swinney et al. 1997). The PGHS-II isoform also has a larger size cycloxygenase active site as compared to PGHS-I. Immediately adjacent to the catalytic site, two isoleucine residues, (433, 523) are substituted by valine in PGHS-II; this substitution opens the hydrophobic channel and creates a larger cycloxygenase active site (Vane et al, 1998; Smith et al, 1996; Inoue et al. 1995; Xu et al, 1995). As a consequence PGHS-II has a wider range of substrate tolerability. Both isozymes have a serine residue within the cycloxygenase catalytic site (530 and 516 respectively) that becomes acetylated by aspirin or other non-steroidal anti-inflammatory drugs. This acetylation sterically hinders substrate accessibility and inhibits enzyme activity (Smith et al, 1996; Inoue et al, 1995; Xu et al, 1995). Aspirin has a greater inhibitory effect on PGHS-I than PGHS-II because the larger active site on PGHS-II requires more than an acetyl group to block access to the site (Smith et al. 1996; Inoue et al, 1995; Xu et al, 1995). Thus, the large active site has directed the development of specific PGHS-I and II inhibitors. In summary, PGHS-I and II catalyzed the same reaction but are under differential regulation with regards to gene expression, specific function, substrate affinity and enzyme inhibition. Both PGHS isoforms are expressed within the intrauterine tissues through gestation. The role and regulation of these enzymes during parturition will be discussed in the upcoming section 1.6.

1.5.4. Prostaglandin Synthesis: Prostaglandin Synthase and Isomerases

Prostaglandin H₂ (PGH₂) is the precursor of all biologically active and physiologically important prostanoids; this cyclic endoperoxide intermediate is acted upon by various isomerases or synthases to produce the different prostanoid subclasses (Vane et al, 1998; Smith et al, 1996; Goppelet-Streube, 1995; Mitchell et al, 1995; Marshall et al, 1987). The prostanoids are generally synthesized in a cell specific manner such that any given PG
forming cell tends to produce only one type of prostanoid as its major product (Smith et al. 1996). Relatively little is known about the enzymes that mediate PGH₂ metabolism. PGE₂ synthesis is catalyzed by an enzyme referred to as PGE synthase; this enzyme has been recently recognized as a member of the protein super-family of membrane associated proteins involved in eicosanoid and glutathione metabolism, the MAPEG family (Jakobsson et al. 1999; Meyer et al. 1997). In addition, members of the glutathione-S-transferase family have been found to also express PGE isomerase activity (Jakobsson et al. 1999; Meyer et al. 1997). A microsomal 180 kD PGE synthase has been immunoprecipitated and found to contain both PGHS synthase and PGE synthase suggesting that these two enzymes can be associated within the same membrane system (Jakobsson et al. 1999; Meyer et al. 1997). PGF₃₄ can be synthesized from PGE₂, PGD₂ or PGH₂ by 9-ketoreductase. PGD-11-ketoreductase and PGH-9,11-endoperoxide reductase respectively. Both PGD-11-ketoreductase and PGH-9,11-endoperoxide reductase activities are expressed in the same protein and comprise the enzyme PGF synthase (aka PGF reductase) (Suzuki et al. 1999). This enzyme is a member of the aldo-keto reductase family and requires NADPH for activity (Suzuki et al. 1999). Two forms of PGF synthase have been identified: a lung type and a liver type but there is scant information available regarding the distribution and regulation of these enzymes. Once formed, prostanoids are not stored within the cell but are immediately transported out of the cell through carrier mediated transport (Suzuki et al. 1999). Little information is available regarding the expression of the PG isomerases and transporters within the intrauterine tissues.
1.5.6. Prostaglandin Metabolism

PG metabolism is an important determinant in the regulation of the biological activity of prostanoids in the circulation and at the tissue level. PGs are metabolized by three different enzymes: a type I NAD$^+$-dependent-15-hydroxy-prostaglandin dehydrogenase (PGDH), a type II NADP$^+$-dependent carbonyl reductase and a cytochrome P450-dependent ω/20-hydroxylase (Okita and Okita, 1996; Ensor et al., 1995). However, PGDH is the key enzyme regulating PG catabolism. It has been purified from the lung, kidney, hematopoietic tissue and placenta (Okita and Okita, 1996; Ensor et al., 1995). This cytoplasmic protein is a short chain nonmetalloenzyme member of the alcohol dehydrogenase family which catalyzes the reversible oxidation of the 15-hydroxyl group to form a 15-keto group to reduce the biological activity of PGs (Okita and Okita, 1996; Ensor et al., 1995). The human PGDH gene is located on chromosome 4 but has not been cloned except for a portion of its promoter and a 3' portion containing 3 exons. The gene sequence of these exons suggests that several truncated PGDH mRNA transcripts may exist due to alternate splicing sites (Greenland et al., 2000; Delage-Mourroux et al., 1997; Okita and Okita, 1996; Ensor et al., 1995; Picaud et al., 1995). In support of these observations the human PGDH cDNA hybridizes to two species of polyA+ mRNA (2 kb and 3.4 kb) isolated from human placental tissue (Greenland et al., 2000; Delage-Mourroux et al., 1997; Okita and Okita, 1996; Ensor et al., 1995; Picaud et al., 1995). The 2.0 kb mRNA is the type I PGDH. The 3.4 kb transcript corresponds to a second type I PGDH isoform- PGDHr- that was recently characterized and sequenced from the promyelocytic HL-60 cell line but also found in the liver and placenta (Greenland et al., 2000; Delage-Mourroux et al., 1997; Okita and Okita, 1996; Ensor et al., 1995; Picaud et al., 1995). PGDHr codes for a C-terminal truncated form of PGDH. When expressed in E. coli, PGDHr
was unable to catalyze the 15-dehydrogenation of PGE$_2$ suggesting that PGDHr may be an inactive isoform or an active isoform with a greater affinity for an alternative substrate (Greenland et al. 2000; Xun et al. 1991). This information also suggests that the C terminus is critical for enzyme activity. Recently, portions of the human PGDH promoter have been cloned and compared with the previously cloned mouse PGDH gene promoter. Striking homology was observed including conservation of the transcription start site, an upstream TATA box and potential binding sites for myoD/AP-1 and SP-1/AP-2 (Greenland et al. 2000). These similarities suggest a functional relevance of these sites in the regulation of PGDH gene expression. In addition, the mouse promoter also contained several regulatory elements including CRE, GRE, AP1, AP2, NF-IL6 and ERE and the human promoter was found to have 5 AP-1, 2 Ets and 2 CREB sites (Greenland et al. 2000; Okita and Okita, 1996). These multiple transcription factor binding sites suggest that PGDH gene expression is tightly regulated.

Peptide analysis of human placental PGDH revealed a 266 amino acid protein; however depending on the source the purified PGDH protein can have a molecular weight ranging between 20 and 70 kD suggesting that PGDH may exist in multiple forms (Okita and Okita, 1996; Ensor et al. 1995). Human placental PGDH has a molecular weight of 28-29 kD as well as a 56 kD protein which may reflect a tight dimerization (Okita and Okita, 1996; Ensor et al. 1995). However, studies examining the regulation of PGDH expression have only documented changes in the 28-29kD protein suggesting that this is the active form of the enzyme (Ensor et al. 1995). In addition to the C-terminus, a tyrosine residue$^{151}$ appears to be essential for PGDH activity because a base substitution at this site abolishes enzyme activity (Okita and Okita, 1996; Ensor et al. 1995). Not all PGs are substrate for PGDH;
PGD₂, PGB₂, thromboxane A₂ are poor substrates but PGDH does readily metabolize the E and F series PGs and ω-6 hydroxy fatty acids (ie. hydroxy-heptadecatrienoic acid) (Okita and Okita, 1996; Ensor et al, 1995). PGDH has a greater affinity and catabolic activity for E series PGs with respect to PGF series (Kₘ 30 μM (PGE₁); 59 μM (PGE₂) and 133μM (PGF₂α) but has the greatest affinity for the ω-6 hydroxy fatty acids (Kₘ range 12-16 μM) (Okita and Okita, 1996; Ensor et al, 1995). Substitutions at the ω end of the prostanoid decreases the rate of PGDH metabolism indicating a key zone involved in enzyme catalysis and potentially directing the development of PG analogs with increased half-life. Pulse chase experiments in HL-60 cells found that PGDH had a rapid turnover rate (t½ ~47 seconds) indicating that its activity depends on continued protein synthesis (Xun et al, 1991). PGDH has been characterized within the intrauterine tissues through gestation and its role in the regulation of parturition will be discussed in the upcoming section 1.6.

1.5.7. Mechanism of Prostaglandin Action: Prostaglandin Receptors

Once synthesized prostanoids are immediately released to either the cytoplasm or the extracellular space to act in an autocrine/paracrine manner and in some instances PGs can be carried in the bloodstream to a target tissue to mediate an endocrine effect (Narumiya et al, 1999; Smith et al, 1996; Coleman et al, 1994). The PG receptor is coupled to a seven trans-membrane domain G protein. Each PG type binds to a specific receptor but there is considerable cross-reactivity between ligands and receptors (Narumiya et al, 1999; Smith et al. 1996; Coleman et al, 1994). E series PGs bind with the greatest affinity to EP receptors for which there are 4 isoforms: EP₁-4; there are 8 identified splice variants of the EP₃ receptor (Narumiya et al, 1999; Smith et al, 1996; Coleman et al, 1994). The F series PGs bind to the FP receptor for which two isoforms have been identified: FPₐ and FPₐ (Narumiya
et al. 1999; Smith et al. 1996; Coleman et al. 1994). Each receptor is ~300-500 amino acids in length with a molecular weight ~40-57 kD; several consensus sequences directing the N-glycosylation of asparagine residues within the extracellular portion of the amino terminus account for the range of molecular weights (Narumiya et al., 1999; Smith et al., 1996; Coleman et al., 1994). In addition to traditional ionic ligand binding, hydrogen bonds between the ligand and PG receptor can form. The hydrogen bond can be strong enough to secure ligand binding but may not result in signal transduction, depending on the type of G protein coupled to the receptor (Narumiya et al., 1999; Smith et al., 1996; Coleman et al., 1994). This may explain the different responses of a given receptor to various ligands. However, despite many conserved sequences, there is limited homology, ~20-30%, among the receptors; in fact the homology of a given receptor type across the species is greater than across receptor types within a species (Narumiya et al., 1999; Smith et al., 1996; Coleman et al., 1994). Translation initiation sites vary between species affecting the length of the amino terminal extracellular domain and creating differences in receptor structure. This may dictate why PGs have different actions across species (Narumiya et al., 1999; Smith et al., 1996; Coleman et al., 1994). Through various G proteins, PG receptors affect intracellular Ca^{2+} or cAMP levels. The FP receptor is coupled to a G_{i} protein linked to phospholipase C activation (Narumiya et al., 1999; Smith et al., 1996; Coleman et al., 1994). In certain cells the FP receptor has also been found to inhibit cAMP (Narumiya et al., 1999; Smith et al., 1996; Coleman et al., 1994). The G protein coupled with the EP1 receptor has not been determined but ligand binding to this receptor does cause an increase in intracellular Ca^{2+} mobilized from an extracellular source presumably through a ligand gated Ca^{2+} channel (Narumiya et al., 1999; Smith et al., 1996; Coleman et al., 1994). EP2 and EP4 are coupled to a G_{s} protein and ligand binding
results in an increase in intracellular camp. In general EP3 is linked to a $G_i$ protein with ligand binding leading to a decrease in cAMP level (Narumiya et al. 1999; Smith et al. 1996; Coleman et al. 1994). The various splice variants of EP3 can be linked to different G proteins and one particular isoform negatively regulates G protein activity (Narumiya et al. 1999; Smith et al. 1996; Coleman et al. 1994). Thus, the same PG can have multiple effects depending on the type of receptor isoform present. Minimal information is available regarding the regulation of PG receptor expression and regulation. PG receptors have been identified within the intrauterine tissues, in particular, the EP and FP receptors are expressed in the myometrium and considered to be CAPs. The role of the PG receptor during parturition will be discussed in the upcoming section 1.7.

1.6. Prostaglandins and Parturition

A significant role for PGs in the mechanisms of parturition across species has been well established. PGs mediate cervical ripening, uterine contractility, maintenance of utero-placental blood flow and fetal adaptations to labor. PG synthesis inhibitors have been shown to block uterine activity and prolong gestation length in a variety of species. In contrast, the administration of natural or synthetic prostaglandins at any time through gestation leads to delivery of the fetus (Song et al. 2000). Perhaps the most striking evidence in support of the integral role of PGs comes from the fact that amniotic fluid, serum and urine levels of PGs and their metabolites increase prior to the onset of uterine activity, indicating that PGs are causal to and not the consequence of the labor process (Gomez et al. 1997). However, it has recently been determined using transgenic mouse models with null mutations for the PG synthesizing enzymes and/or the FP receptor that labor can occur in the absence of PG
production or activity. These data suggest that PGs may not be obligatory to the labor process (Sugimoto et al. 1997). Alternatively, PGs may have species specific roles in the mechanism of labor and the evidence from transgenic rodent models may not be applicable to all mammals. Regardless of the obligatory or redundant nature of PG activity at parturition, there is substantial evidence that PGs do participate in the labor events.

1.6.1. *Ovine Parturition and Prostaglandins*

The current hypothesis of ovine parturition suggests that cortisol produced by the activation of the fetal HPA axis acts to induce the expression P450c17 within fetal placental trophoblast tissue diverting pregnenolone away from the production of progesterone and towards the production of C19 steroids required for increased placental estradiol synthesis (Mason et al. 1989; Mason et al., 1988, Flint et al, 1976; Anderson et al., 1975). This increased expression and activity of P450c17 leads to additional local production of C19 precursors triggering a surge in placental estradiol production and an associated decline in progesterone secretion (Mason et al., 1989; Mason et al., 1988, Flint et al, 1976; Anderson et al., 1975). In turn, it has been suggested that estradiol increases the expression and activity of PGHS within the intrauterine tissues to produce PGE2 and PGF2a (Challis et al., 1997). Increased estradiol output also induces the expression of the contraction associated proteins (CAPs) in particular connexin 43, the oxytocin receptor, and the PG receptors (Lye, 1994). However, closer examination of these endocrine changes during late pregnancy suggests that this may not be the correct sequence of events that leads to the onset of labor and delivery of the ovine fetus.

In sheep, placental PGHS content and activity increases through the final third of gestation with a rapid increases at term and the onset of labor (Gyomorey et al., 2000; Wu et
Prostanoid production by dispersed ovine trophoblast cells is minimal prior to d110 of gestation however increases dramatically through the end of gestation (Risbridger et al, 1985). Rice et al (1988) demonstrated that prostanoid production by ovine placental microsomes increased 25 fold between early gestation and term and Fowden et al (1987) reported increased placental PGE₂ secretion into maternal and fetal circulation late in gestation. These data suggest that induction of PGHS activity in the ovine placenta leads to the increased production of prostaglandin in late gestation.

Fetal plasma PGE₂ concentration gradually rises from d120 of gestation with a time course similar to the increase in fetal plasma cortisol level. The temporal pattern of trophoblast PGHS-II induction parallels the activation of the fetal HPA axis and precedes the induction of placental P450C₁₇ expression and the surge in placental estrogen synthesis (Challis et al. 1976; Magyar et al, 1980; Gyomorey et al, 2000). Furthermore, intrafetal infusion of estradiol did not increase placental PGHS-II expression implying that estrogen does not regulate trophoblast PGHS expression/activity (Challis et al, 1997). However, the intrafetal administration of either cortisol or betamethasone increases placental PGHS-II expression and fetal plasma PGE₂ concentration (McLaren et al, 2000; Challis et al, 1997; McLaren et al, 1996). In particular, 14h after fetal glucocorticoid injection a 3-fold increase in PGHS-II protein expression was detected. By the time of active labor, placental PGHS-II expression was increased by 12 fold (McLaren et al, 2000). The glucocorticoid receptor (GR) has been localized within the placental mononuclear trophoblast cells and this expression increased with the onset of term labor (Gupta et al, 2000). These observations suggest activation of the fetal HPA axis and increased fetal adrenal glucocorticoid elicits a receptor-
mediated induction of ovine trophoblast PGHS-II and leads to the rise in fetal plasma PGE₂ level observed in the final days of gestation.

Several investigators have reported that intrafetal glucocorticoid administration increased placental P₄₅₀ᵢ₇ mRNA and protein expression co-incident with a dramatic increase in 17-hydroxylase activity; this cortisol effect mimicked the prepartum increase in P₄₅₀ᵢ₇ expression and activity suggesting that fetal HPA activity directs placental P₄₅₀ᵢ₇ expression at term (Anderson et al, 1975; Flint et al, 1975; Steele et al, 1976; France et al, 1988; Mason et al, 1989). However, these in vivo studies were observational and did not examine the mechanism by which glucocorticoids increase P₄₅₀ᵢ₇ expression. Thus, it is possible that fetal cortisol may not directly increase P₄₅₀ᵢ₇ gene expression, but could influence P₄₅₀ᵢ₇ indirectly through a glucocorticoid-stimulated intermediate. The increase in trophoblast PGHS-II expression clearly precedes the induction of P₄₅₀ᵢ₇ (Gyomorey et al, 2000; Anderson et al, 1975; Flint et al, 1975; Steele et al, 1976; France et al, 1988; Mason et al, 1989) suggesting that PGE₂ may be the intermediary compound. Two studies have shown that PGs can increase the expression and activity of this enzyme; PGF₂α induced P₄₅₀ᵢ₇ in cultured bovine preovulatory cells and PGE₂ induced P₄₅₀ᵢ₇ in cultured bovine adrenal cells (Wijayagunawarde et al, 1991; Rainey et al, 1991). These data suggest that PGE₂ produced by the fetal trophoblast tissue in response to glucocorticoid stimulation could act in an autocrine/paracrine manner to increase placental P₄₅₀ᵢ₇ expression/activity. The infusion of nimesulide, a potent PGHS-II inhibitor, following the spontaneous onset of term labor elicited a significant decrease in fetal plasma PGE₂ concentration as well as a significant decrease in placental P₄₅₀ᵢ₇ expression (Ma et al, 1999). Although a decrease in fetal plasma cortisol also occurred, this observational study could not exclude the possibility
that placental PGE₂ may act as a glucocorticoid stimulated intermediate capable of the autocrine/paracrine induction of P450c₁₇.

A pronounced increase in the maternal plasma concentration of 13,14-dihydro-15keto-prostaglandin F₂α (PGFM) occurs at the onset of labor well after the progressive rise in PGE₂ concentration in the fetal plasma. These data imply that PGE₂ and PGF₂α synthesis is regulated by separate mechanisms and that these PGs may be produced by different intrauterine tissues (Gyomorey et al, 2000; Mitchell et al, 1979). The rise in PGFM correlates with elevated maternal plasma estradiol levels and increased endometrial and myometrial PGHS-II expression (Gyomorey et al, 2000a; Wu et al, 1999). Temporally, the increase in endometrial PGHS-II expression occurs in association with the increase in placental P450c₁₇ expression and precedes the increase in myometrial PGHS-II expression (Gyomorey et al, 2000; Wu et al, 1999). Estradiol has been shown to increase PGHS-II expression significantly in non-pregnant ovine myometrium and in non-pregnant ovine endometrium after progesterone priming (Wu et al, 1998). The estrogen receptor (ER) is exclusively localized to the luminal endometrial epithelium and the myometrium in late gestation (Riley et al. 2000; Leung et al, 1999; Wu et al, 1997). The expression of ER was found to increase with term labor and with glucocorticoid induced preterm labor (Leung et al, 1999; Wu et al, 1997). In addition, PGF synthase expression has been reported in the endometrium and myometrium but not the fetal placenta, precluding the production of PGF₂α by the fetal placenta (Wu et al, 2000). These data suggest that the maternal intrauterine tissue expression of PGHS-II and the production of PGF₂α are induced by the rise in placental estrogen synthesis through a direct, receptor-mediated mechanism. In addition, endometrial ER mRNA expression was found to decrease following the administration of the PGHS-II
inhibitor during spontaneous term labor implying a positive, feed-forward interaction between PG production and estrogen receptor activity (Wu et al, 1999).

Although PGHS-II expression within the intrauterine tissues is an important regulator of PGE\(_2\) and PGF\(_{2\alpha}\) synthesis in late gestation, the expression and activity of this enzyme may not be the sole factor regulating PGE\(_2\) and PGF\(_{2\alpha}\) production at the end of gestation. In the sheep immunoreactive PGHS-I is more strongly expressed in the fetal membranes and cervix with respect to the placenta, endometrium and myometrium suggesting that PGHS-I may be responsible for the majority of fetal membrane prostanoid production (McLaren et al, 2000). Intrafetal betamethasone injection elicited a modest 3.5 fold increase in PGHS-I expression in the ovine placenta but did not affect PGHS-I expression in the other intrauterine tissues (McLaren et al, 2000). These data suggest that PGHS-I may contribute to the intrauterine pool of PGs through gestation but likely does not represent a major contribution to the increase in PG output at parturition (McLaren et al, 2000). PGDH immunoreactivity has been detected in the uninucleate trophoblast cells of the placenta, the luminal epithelium of the endometrium and the smooth muscle of the myometrium. In the placenta, PGDH activity did not change from d70-d135 of gestation but paradoxically was increased significantly with active labor (Riley et al, 2000). PGDH activity appeared to be independent of changes in fetal plasma estradiol concentration (Riley et al, 2000). However, these studies did not chronicle the pattern of PGDH expression or activity through the final days of gestation or comment on the expression and regulation of PGDH expression within the endometrium or myometrium. In the human, in vitro studies have suggested progesterone tonically maintains trophoblast PGDH expression through gestation; at term an increase in cortisol concentration elicits a decrease in PGDH expression and
activity by inhibiting the effects of progesterone at the glucocorticoid receptor (Patel and Challis; unpublished results). A similar increase in cortisol and withdrawal of progesterone occurs within the ovine intrauterine tissues suggesting that PGDH expression may decrease towards the end of gestation and contribute to the increase in intrauterine PG output at term. There is little information is available regarding the regulation of lipase activity within the intrauterine tissues: cPLA₂ expression in the endometrium was found to increase with advancing gestational age and with glucocorticoid induced PTL suggesting that increased substrate availability may contribute to the increased intrauterine PG production at the onset of parturition (Zhang et al, 1996).

1.6.2. Primate Parturition and Prostaglandins

The current hypothesis of primate parturition suggests that prostaglandins produced by the intrauterine tissues, particularly the fetal membranes, act in an autocrine/paracrine manner to mediate extracellular matrix, myometrial contractility and cervical ripening (Challis et al. 2000). PGs indirectly mediate extracellular matrix remodeling through metalloproteinases (MMPs). MMPs digest the major components of basement membranes and the extracellular matrix including type IV collagen, laminin and fibronectin. Degradation of the fetal membranes leads to spontaneous rupture of the membranes and digestion of the placental-maternal tissue interface allows for placental separation following delivery (Riley et al. 1999: Tsatas et al, 1999). The intrauterine tissue specific localization of MMPs has recently been characterized. MMP-9 is present within the placenta and connective tissue of the fetal membranes; MMP-2 is present within fetal membranes (Tsatas et al. 1999: Xu, Alfaidy and Challis; unpublished results). Increased MMP-9 activity in both rat and human fetal membranes at the time of labor has been reported (Vadillo-Ortega et al,
1990: Lei et al. 1995, Riley et al. 1996). In particular, human fetal membranes and placenta demonstrated increased immunoreactive MMP-9 expression and activity following the onset of term labor (Tsatas et al., 1999). In pregnant rats, in vivo PGE$_2$ infusion at term and preterm caused marked elevations in plasma and intrauterine tissue concentrations of MMP-2 (Lyons et al., 2000). In addition, PGE$_2$ infusion at preterm elevated plasma levels of MMP-9 and PGE$_2$ infusion at term increased intrauterine tissue levels of MMP-9 (Lyons et al., 2000). In vitro, PGE$_2$ mediated an increase in the secretion of proMMP-9 by cultured placental trophoblast cells (Xu, Alfaidy and Challis, unpublished results). These data suggest that PGs produced by the fetal membranes and placenta act in an autocrine/paracrine manner to increase the expression, secretion and activity of membrane and placental MMPs leading to extracellular matrix remodelling.

PGs have been found to be potent mediators of both cervical ripening and myocyte contractility. In the human, administration of synthetic PGs at any stage in gestation will induce myometrial contractions (Song, 2000); however, depending on the type of PG receptor present, PGs can exert either a contractile or a relaxant effect. EP1, EP3 and FP receptors mediate uterine contractility through an increase in intracellular Ca$^{2+}$ signaling, whereas EP2 and EP4 cause an increase in intracellular cAMP that mediates myometrial relaxation (Narumiya et al., 1999). EP2, EP3, EP4 and FP receptors have been characterized within primate myometrium. Lower segment baboon myometrium has a high level of EP2 receptor expression and a low level of EP3 receptor expression as compared to the fundal region (Smith et al., 1998). FP and EP4 isoforms are expressed at both sites however the level of expression of these receptors does not differ between the fundus and the lower segment (Smith et al., 1998). Strips of upper segment baboon myometrium treated with PGE$_2$ and
PGF$_{2\alpha}$ elicited a potent contractile response: strips of lower segment myometrium contracted in response to PGF$_{2\alpha}$ but not PGE$_2$ (Smith et al., 1998). Within the human myometrium, lower segment EP2 expression decreased with gestational age. FP expression also decreased with gestational age but was paradoxically increased with labor (Brodt-Eppley and Myatt, 1999). This characteristic distribution of PG receptors suggests that PGE$_2$ contributes to the relaxation of the lower segment, and both PGF$_{2\alpha}$ and PGE$_2$ mediate the fundal contractility observed with labor. Therefore, PGs act in an autocrine/paracrine manner to effect uterine contractility and extracellular matrix remodeling.

Integral to this hypothesis is the assumption that PGs are transported through the fetal membranes and decidua. Carrier mediated PG transport has been postulated to occur in many tissues however specific PG transport systems(s) with the fetal membranes have not be characterized (Schuster, 1998). Given that both PGE$_2$ and PGF$_{2\alpha}$ are present in the amniotic fluid it is assumed that these PGs can pass through the membranes in a bi-directional manner presumably by passive diffusion (Nakla et al., 1986; Bennett et al., 1990). The total prostanoid transfer rate in either direction has been determined to be ~2-3% per hour with ~40-50% PG transferred intact, i.e. not metabolized; the remaining metabolized portion was attributed to membrane PGDH activity (Nakla et al., 1986; Bennett et al., 1990). Critics suggest that the paracrine action of PGs would be limited by this relatively low transfer rate; however, these studies were conducted in vitro and thus may not accurately depict in vivo events. Further, these studies did not measure the level of PG retained at either of the decidual or amnion surfaces and regional differences in the transfer rate were not considered. In particular, the area overlying the internal os of the cervix reportedly has increased membrane permeability (Bryant-Greenwood, 1998). This area is under mechanic stress from the forbag of amniotic
fluid and is exposed to a variety of inflammatory mediators from cervico-vaginal fluids: these factors contribute to degenerative membrane changes leading to increased intercellular space and permeability. In addition, the ability of the membranes to metabolize PGs will affect the PG transfer rate. PGDH is expressed within the fetal membranes and this expression is lower within the membranes overlying the cervix contributing to a diminished metabolic barrier to PG transfer at this site (van Meir et al, 1997). The metabolic barrier to membrane transfer of PGs is dramatically decreased with the onset of PTL with associated chorioamnionitis, due to both a decrease in PGDH expression and an inflammatory degradation of the membranes (van Meir et al, 1997b). Thus, an increase in membrane-derived PGs output appears to play a multifactorial autocrine/paracrine role critical to the successful cascade of events defined as labor; an outline of this hypothesis is presented in Figure 1-6.
Prostaglandin synthase type II (PGHS-II) expression/activity in the amnion and chorion increases as prostaglandin dehydrogenase (PGDH) expression/activity in the chorion decreases leading to a net increase in prostaglandin (PGE₂ and PGF₂α) produced by the fetal membranes. These prostaglandins are suggested to act in a local paracrine manner to mediate membrane rupture, cervical ripening and uterine contractility. It is speculated that idiopathic preterm labor is associated with a diminution in chorionic PGDH thereby allowing the transmembrane transfer of prostaglandin from amnio-chorion to the underlying decidua and myometrium.

Figure 1-6: Diagrammatic Representation of the Sites of Prostaglandin Synthesis and Metabolism within Human Intrauterine Tissues at Preterm and Term Labor.
1.6.2.1. Prostaglandin Production by the Fetal Membranes

The human fetal membranes are composed of the amnion- the innermost layer, which is a single layer of epithelial cells overlying a thick layer of connective tissue embedded in which are fibroblast (mesenchymal cells)- and the chorion trophoblast layer which lies adjacent to the maternal decidua (Bryant-Greenwood et al, 1998). The fetal membranes express both PGHS-I and PGHS-II. However, at both term and preterm labor, only the expression of PGHS-II expression within amnion epithelium, amnion mesenchyme and the chorion trophoblast layer is increased (Feuntes et al, 1996; Gibb et al, 1996; Hirst et al, 1995; Teixera et al, 1994). In contrast, PGDH is localized to the chorion trophoblast layer and its expression decreases with term and preterm labor (Patel et al, 2000; Sangha et al, 1994; Cheung et al, 1992). Thus, fetal membrane PG output at term and preterm labor is attributed to both an increase in PG synthesis and a decrease in PG metabolism. In association with these changes, a 6-10 fold increase in the amniotic fluid arachidonic acid concentration and increased phospholipase activity within the intrauterine tissues has also been documented suggesting that increased substrate availability affects PG output (MacDonald et al, 1974). Both sPLA2 and cPLA2 are present within the amnion epithelium, chorion and placental vasculature (Aitken et al, 1996; Skannal et al, 1997). sPLA2 is also localized to the mesenchymal tissue of the amnion, chorion and placenta; its receptor has been identified within these sites and the concentration of this receptor in the plasma increases with preterm labor (Aitken et al, 1996; Skannal et al, 1997). However, the mRNA and protein levels of sPLA2 were not found to change with labor (Aitken et al, 1996; Skannal et al, 1997). cPLA2 activity was decreased after labor at term and preterm, suggesting that a depletion of arachidonic and free fatty acid stores occurs with the process of labor (Aitken et al, 1996;
Skannal et al. 1997). PLC has also been found within the fetal membranes and the upregulation of this enzyme has been linked to increased PGE$_2$ levels (Aitken et al. 1996; Skannd et al. 1997). On the basis of this evidence it has been postulated that elevated PGHS-II expression correlated with increased intrauterine substrate availability and decreased PGDH expression contributes to the increased output of PGE$_2$ and PGF$_{2\alpha}$ by the fetal membranes. This introduction will focus on the regulation of prostaglandin synthesis within the amnion epithelium and mesenchymal tissue.

2.6.2.2. Regulation of Amnion Prostaglandin Synthesis

Primary culture preparations and the established amnion epithelial-derived cell line (the WISH cell) have been used to study the regulation of amnion PG synthesis. Both types of amnion cells produce PGE$_2$ in culture and this production decreases as a function of time in culture (Gibb et al. 1988). Cytokines, peptide hormones, cAMP, bacterial endotoxin and steroids have been identified as positive regulators of amnion PG output. The peptide hormone corticotropin releasing hormone (CRH) and ACTH increase amnion PGHS-II expression and PGE$_2$ output; CRH effects will be further discussed in section 2.6.2.3. Cytokines play a complex and integrative role in the regulation of amnion PG production. In particular IL-1$\alpha$, IL-1$\beta$ and TNF-$\alpha$ have been found to increase PGE$_2$ output and PGHS-II expression in both primary amnion cells and WISH cells (Hansen et al. 1998; Xue et al. 1995). At term, amniotic fluid contains IL-6, IL-8 and G-CSF; the concentration of these cytokines increases with the onset of labor suggesting that amnion PG production associated with labor may reflect a pro-inflammatory process mediated by cytokines. Both term primary amnion and WISH cells secrete IL-6 and IL-8 in culture; IL-6 output remains constant while IL-8 production decreases with time in culture with a profile similar to that of PGE$_2$. 

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suggesting that cytokine and PGE₂ production may be related (Keelan et al. 1997). In WISH cells, IL-6 and IL-8 output was increased by IL-1β and TNF-α suggesting that cytokine induced PGE₂ secretion may not be simple, direct effect but may reflect a positive feed-forward cascade of cytokine production. Keelan et al (1999) reported that IL-4 and IL-13 inhibited IL-1β and TNF-α stimulated PGE₂ production as well as basal and stimulated IL-6 and IL-8 production. In addition, Keelan et al (2000) found that thromboxane A₂ but not the other prostanoids could increase WISH cell cytokine secretion and had a synergistic effect with IL-1β. These data suggest that amnion PG production represents a complex balance between pro- and anti-inflammatory cytokine activity. An increase in pro-inflammatory cytokines can elicit an autocrine/paracrine positive feed-forward cascade that leads to increases in both cytokine and prostanoid output.

Growth factors also increase amnion PG production. Epidermal growth factor (EGF) in the presence or absence of exogenous arachidonic acid increased WISH cell PGE₂ output; a similar effect of EGF in the presence of arachidonic acid was observed in primary amnion cells (Perkins et al. 1997). Perkins et al (1997) demonstrated in WISH cells that EGF rapidly elicited a transient, dose dependent, de novo production of PGHS-II mRNA and protein. EGF has also been found to cause a modest increase in amnion IL-6 and IL-8 (Keelan et al., 1997). A novel pathway of amnion PG production has been suggested by Johnson et al (1999) who reported that PAP-1, a lipid intracellular mediator, increased PGHS-II mRNA expression and initiated a cascade of events leading to the induction of group IV phospholipase A₂. Similarly, TNF-α and IL-1β have been found to increase amnion cell cytosolic phospholipase A₂ expression and activity (Hansen et al 1999; Xue et al, 1996). These data suggest that amnion PG production may be regulated by a complex feed-forward interplay between cytokines,
growth factors and lipid mediators that act to increase not only synthase expression and activity but also substrate availability.

Steroid hormones, in particular glucocorticoids, affect amnion PG production. Gibb et al (1993) reported that glucocorticoids decreased PGE₂ production by freshly dispersed amnion cells but caused a significant increase in PGE₂ output by cultured cells. This stimulatory effect was determined to be receptor-dependent, linked to the activity of tyrosine kinase and involved the de novo production of PGHS-II mRNA and protein (Zakar et al, 1998; Zakar et al, 1997). This effect is in contrast to studies in WISH cells that reported a decrease in basal PG output by dexamethasone (Deval et al, 1998). Dexamethasone has also been found to suppress cytokine and EGF induced PGE₂ production in both primary amnion cells and WISH cells (Perkins et al, 1997). As well, dexamethasone treatment decreased IL-6 and IL-8 output by freshly dispersed and cultured amnion cells (Keelan et al. 1997). These data suggest that glucocorticoids can exert both pro and anti-inflammatory effects on amnion PG output depending upon the length of time in culture; this biphasic response may reflect the level of PGHS-II expression at the time of glucocorticoid exposure and an interaction with endogenous cytokine production.

Primary amnion cell cultures contain both epithelial and mesenchymal cells in varying proportions. Interestingly, these two cell types respond differently to steroid and cytokine treatment. Using immunocytochemistry and in situ hybridization techniques, both dexamethasone and IL-1β were found to increase PGHS-II mRNA and protein expression exclusively in the mesenchymal cells of a mixed amnion cell preparation (Gibb et al. 1998; Economopoulos et al, 1996). These data suggest that the amnion mesenchymal cells may be responsible for a significant portion of the whole amnion PG output. Keelan et al (1997)
reported that enriched mesenchymal cell and enriched epithelial cell cultures had different characteristics such that basal PGE₂ production by mesenchymal cells was less than that of the epithelial cells. Mesenchymal cell PGE₂ output did not respond to cytokine treatment but epithelial cell PGE₂ output was increased by IL-1, TNFα and EGF. However, mesenchymal cell PGE₂ output was increased to a level exceeding that of the epithelial cells by dexamethasone. Blumstein et al (2000) also isolated epithelial and mesenchymal cells. Although the epithelial cells produced seven fold greater levels of PGE₂ with respect to the mesenchymal cells, these epithelial cells did not respond to dexamethasone whereas dexamethasone did cause an increase in the mesenchymal cell PGHS-II expression and PGE₂ production. However, the mesenchymal cells had undergone two passages and the effects of dexamethasone were lost with a passage of the cells suggesting that the dexamethasone response may reflect the culture technique and impaired cellular function and not accurately represent the activity of these cells in vivo. These studies highlight the unique characteristics of amnion epithelial and amnion mesenchymal cells as well as the effect of time and culture technique on these characteristics.

1.6.2.3. Amnion Epithelial and Mesenchymal Cell Paracrine Interactions

The juxtaposition of the amnion epithelial and mesenchymal cells within the fetal membranes may allow paracrine interactions between the cell types and in turn, influence net amnion PG production. However, direct evidence of such paracrine interactions has not yet been demonstrated. One candidate paracrine mediator is CRH. Intrauterine CRH is homologous to hypothalamic CRH and is produced by the fetal membranes and placental syncytiotrophoblast tissue (Riley et al, 1991). CRH expression can be detected by 10 weeks of gestation and increases progressively through normal pregnancy (Riley et al, 1991).
Maternal plasma levels of CRH follow the pattern of increased placental CRH mRNA expression; however, CRH does not appear to affect the maternal HPA axis because it is bound in the maternal circulation to a specific CRH binding protein (CRH-BP). The expression of CRH-BP also increases through normal gestation (Maclean and Smith, 1999) but then decreases in the 3-4 weeks prior to the onset of labor at term or preterm. Total CRH and free (active) CRH increases exponentially during the 3-4 weeks prior to parturition consistent with the hypothesis that placenta CRH production is stimulated by increased fetal adrenal cortisol production and suggesting that CRH could act as a trigger for the events of parturition (MacLean and Smith, 1999).

CRH acts through specific membrane-bound, G protein coupled receptors that exist as two subtypes, CRH-R1 and CRH-R2: in the fetal membranes these receptors are coupled with the IP3 and DAG second messenger system (Karteris et al, 2000). These receptors arise from separate genes and have multiple splice variants. CRH-R1α, R1c and R2β expression has been localized to the amnion epithelium but not the amnion mesenchyme (Grammatopoulos et al. 1999). In mixed amnion cell cultures CRH stimulated PGE2 output however, the cell type involved has not yet been identified (Jones et al, 1989). These data suggest that CRH produced within the amnion mesenchymal cells could act as a paracrine mediator to increase amnion epithelial cell PGHS-II expression and PGE2 output. In addition, cortisol increases CRH mRNA and CRH output in vitro by cultured amnion, chorion and placental cells (Jones et al, 1989). In vivo, the antenatal administration of maternal glucocorticoid increased maternal plasma concentration and intrauterine tissue expression of CRH (Marioni et al. 1998; Korebrits et al, 1998). These data suggest that cortisol could
stimulate amnion CRH production which in turn could act as an autocrine/paracrine mediator of amnion epithelial PG production.

1.6.2.4. Role of Amniotic 11βHydroxysteroid dehydrogenase Type I Expression

The evidence presented suggests that cortisol plays a central role in the regulation of amnion PG production at term. This cortisol may be derived from sustained secretion by the fetal HPA axis, the maternal adrenal, or it could be generated locally within the fetal membranes through the activity of the enzyme 11βHSD. There are two major isoforms of the 11βHSD enzyme, 11βHSD-I that can act bi-directionally, but generally operates as a reductase converting cortisone to cortisol, and 11βHSD-II that acts predominantly as a dehydrogenase (Stewart and Krozowski, 1999). 11βHSD-I mRNA and protein have been localized to the human fetal membranes, in particular to the chorion trophoblast cells, amnion epithelium and scattered amnion mesenchymal cells with the highest level of expression within the chorion (Sun et al., 1997). The enzyme was determined to function almost exclusively in the reductase direction to produce cortisol from cortisone (Sun et al., 1997). Addition of cortisone to cultured chorion trophoblast cells decreased PGDH activity and PGDH mRNA expression; this effect was blocked in the presence of carbenoxolone, an inhibitor of 11βHSD-I activity (Patel et al., 1999). In addition, 11βHSD-I activity in the chorion cells was increased by both PGE₂ and PGF₂α (Alfayed et al., 2000). Thus, chorion trophoblast cells appear to utilize “inactive” cortisone to produce cortisol that may then act in an autocrine/paracrine fashion to decrease PGDH catabolism of PGs within the chorion. This creates a positive feedback loop between chorion PG production and cortisol bio-availability. The 11βHSD-I enzyme expression is also present within the amnion epithelial and
mesenchymal cells suggesting that a similar inter-relationship between 11βHSD-I and PGHS-II could occur in the amnion. Here cortisol produced locally from cortisone could act in an autocrine/paracrine fashion to increase amnion PGHS-II expression/activity and PGE₂ synthesis. In turn, PGE₂ could act in an autocrine/paracrine manner to increase 11βHSD-I activity in the amnion as well as the chorion to create a positive feed-forward loop between cortisol and PG production within the entire fetal membrane.

1.7. Contraction Associated Proteins and Parturition

At the onset of labor, uterine activity evolves from long duration, low amplitude, low frequency and poorly coordinated “contractures” to high frequency, high amplitude and short duration contractions associated with increased intrauterine pressure (Lye, 1994). This myometrial contractility is dependent upon conformational changes in the actin and myosin molecules that allow these filaments to slide over each other leading to shortening of the myocyte (Larsson et al., 1999). The process is initiated when an intracellular influx of Ca²⁺ binds to the protein calmodulin (Ca-CAM) which in turn binds to the enzyme myosin light chain kinase (MLCK) (Larsson et al., 1999). Ca-CAM induces a conformational change in the MLCK enabling the enzyme to phosphorylate the 20 kD light chains of myosin and induce the conformational changes required for the actin/myosin interaction (Larsson et al., 1999). Thus, MLCK is central to the initiation of the myocyte contraction, and regulation of this enzyme controls uterine contractility. MLCK is subject to upregulation by Ca²⁺ (through Ca-CAM activation) and downregulation through phosphorylation by PKA (Larsson et al., 1999). In general uterotonic agents act to increase intracellular Ca²⁺ through the increased influx of Ca²⁺ via receptor gated channels or by the release of Ca²⁺ from intracellular stores.
Antagonists of uterine activity generally act by increasing the intracellular levels of cyclic nucleotides (cAMP or cGMP) which either inhibit the release of Ca^{2+} or reduce the activity of MLCK (Lye, 1994).

The evolution from myometrial quiescence to contractility has been attributed to the increased expression of a specific cassette of proteins referred to contraction associated proteins (CAPs) (Lye, 1994). A primary role of the CAPs is the facilitation of the influx of Ca^{2+} and the inhibition of intracellular levels of cyclic nucleotides. This effect is achieved through the insertion of specific uterotonic receptors in the myometrial cell membrane, in particular the oxytocin receptor and the PG receptor (Lye, 1994). Recent evidence has suggested that PGHS-II expressed within the myometrium should be considered a CAP as it provides a local source of uteronin, namely PGs (Gyomorey et al., 2000). Ion channel components are also members of the CAP family; the insertion of these channels into the cell membrane decreases the resting potential of the myocyte towards its threshold such that the myocyte becomes spontaneously active and increasingly responsive to uteronic stimulation (Lye, 1994). Another function of CAP expression is the facilitation of cell-cell communication and coordination of the contractile signal. The insertion of gap junctions between adjacent myocytes permits myocyte-myocyte intercommunication that allows signals to spread easily and rapidly between the cells such that the uterus can contract as a single polarized unit (Garfield et al., 1988). Thus, the myometrial expression of CAPs plays a critical role in regulation of MLCK activity and the coordination of the uterine contraction. This present thesis will examine the glucocorticoid regulation of a specific CAP: connexin 43, the component of the gap junction.
1.7.1. **Connexin 43: The Gap Junction Protein**

Structurally gap junctions are formed when a hexameric connexin unit (connexon) inserted in one cell membrane becomes associated with a connexon in an adjacent cell membrane to create an intercellular pore that non-specific, small molecular weight molecules and ions can pass through (Garfield et al., 1988). Within the smooth muscle of the uterus, the gap junction provides a low resistance path for the passage of ions and therefore increases the electrical conductivity between the cells. X-ray diffraction and confocal microscopy studies suggest that a gap junction plaque is composed of hundreds of connexon channels (Garfield et al., 1988). In the sheep, gap junctions are generally absent from the myometrium through gestation but are increased in both size and number at the end of gestation with further increase during term or preterm labor (Garfield et al., 1979). The major protein component of these gap junctions is connexin 43 (Cx43) (Garfield et al., 1979). McNutt et al (1994) demonstrated that Cx43 mRNA and proteins increase two fold at the onset of ACTH induced preterm labor corresponding to a switch in the pattern of uterine activity from contractures to contractions. It was suggested that this change in Cx43 expression could be attributed to an increase in the maternal plasma estrogen:progesterone (E$_2$P$_4$) ratio. This hypothesis was consistent with the observation by Garfield et al (1979) that gap junction number and density were correlated with an increase in the E$_2$:P$_4$ ratio. Similarly, in the human Cx43 is expressed in both the upper and lower segment of the uterus (Sparey et al, 1999). Based on these observational studies, the endocrine regulation of Cx43 expression has been studied using the rodent as the experimental model. Rat myometrium Cx43 expression increases nine fold in association with an increase in the E$_2$:P$_4$ ratio at the onset of labor (Ou et al., 1997). Ovariectomy causing a fall in luteal progesterone production or the administration of the
progesterone antagonist Ru486 induces a premature rise in rat myometrial Cx43 expression and triggers preterm labor (Petrocelli et al, 1993). Similarly, exogenous progesterone administration either at term or during ovarieectomy-induced preterm labor blocked the increase in Cx43 mRNA expression (Petrocelli et al, 1993). Estradiol triggered an increase in Cx43 mRNA levels in the ovarrectomized rat; this effect was attenuated by the administration of progesterone (Petrocelli et al, 1993). These data suggest that progesterone tonically inhibits Cx43 expression and that either an increase in the E2:P4 ratio or simply a decline in progesterone can induce Cx43 expression. However, the Cx43 gene does not have an estrogen response element within its 5’ promoter region implying that estradiol may not directly regulate Cx43 gene expression. In fact, the stimulatory effect of estradiol was inhibited by cyclohexamide suggesting that new protein synthesis may be required to mediate the effect of estradiol (Piersanti et al, 1995). Expression of the acute phase reactant protein c-fos has been correlated with the changes in Cx43 expression at term and the increase in c-fos expression also followed the endocrine changes used to manipulate Cx43 expressin (Piersanti et al, 1995). c-fos acts at AP-1 transcription factor sites with the 5’ promoter region; Cx43 expresses this site and mutation at the AP-1 site decreases the basal activity of the Cx43 promoter (Piersanti et al. 1995). These data suggest that an increase in the E2:P4 ratio increases the expression of c-fos which in turn increases the expression of Cx43. Similar effects of progesterone and estradiol have recently been observed in human myocyte cell cultures suggesting that the changes in the feto-placental endocrine profile at the end of gestation regulate the expression of Cx43 across the species (Kilarski et al, 2000). Although these studies have examined the effects of estradiol and progesterone, the role of cortisol in the regulation of Cx43 expression has not yet been considered.
1.8. Summary

In most mammalian species towards the end of gestation and the onset of labor, the fetus experiences a maturation and sustained activation of its hypothalamic-pituitary-adrenal axis leading to an increase in fetal adrenal glucocorticoid production (Fowden et al, 1998). This activation appears to be a common triggering event leading to the onset of parturition (Challis et al, 1997). Substantial evidence across the species has linked fetal adrenal glucocorticoid output with increases in plasma, amniotic fluid and intrauterine tissue prostaglandin concentration. These prostaglandins have been identified as key mediators of events integral to the labor process including myometrial contractility, cervical ripening, membrane rupture and fetal adaptation to labor (Challis et al, 1997). The general purpose of this thesis is to examine the relationship between fetal adrenal glucocorticoid production, intrauterine prostaglandin output and uterine activity during late gestation and the onset of labor.
Chapter Two

Rationale
The maturation and sustained activation of the fetal HPA axis appears to be the common fetal endocrine signal triggering the onset of parturition across the species. This thesis will explore the hypothesis that fetal adrenal GC output directs the increased intrauterine PG production and uterine contractility observed at the end of gestation and onset of labor. We will examine this hypothesis in parallel experiments using an in vivo ovine preparation and an in vitro human amnion cell culture preparation.

In the sheep, the current hypothesis of parturition suggests that cortisol induces the placental expression and activity of P450c17 leading to a shift in placental steroidogenesis towards increased placental estrogen production (Mason et al, 1989; France et al. 1988; Steele et al. 1976; Anderson et al, 1975; Flint et al, 1975). In turn, estrogen increases the expression and activity of PGHS-II within the intrauterine tissues to produce PGE2 and PGF2α (Challis et al, 1997). Estrogen also induces the expression of contraction associated proteins within the myometrium to promote uterine activity (Lye, 1994). However, recent evidence has led us to re-evaluate this hypothesis.

The temporal pattern of fetal placental trophoblast PGHS-II induction and the rise in fetal plasma PGE2 parallels the activation of the fetal HPA axis and fetal adrenal GC production (Gyomorey et al. 2000). The increase in both PGHS-II expression and plasma PGE2 level clearly precedes the induction of placental P450c17 expression and the terminal surge in placental estradiol synthesis (Gyomorey et al. 2000). Furthermore, the intrafetal administration of estradiol did not induce the placental expression of PGHS-II, but the intrafetal administration of cortisol did increase trophoblast PGHS-II expression and fetal plasma PGE2 concentration (McLaren et al, 2000; Challis et al, 1997; McLaren et al. 1996). These observations suggest that fetal placental PGHS-II expression and PGE2 synthesis
increase in response to increased fetal adrenal cortisol production independent of increases in placental estradiol synthesis.

The increase in the maternal plasma 13,14-dihydro-15keto PGF$_{2\alpha}$ (PGFM) concentration follows the rise in fetal plasma PGE$_2$ concentration suggesting that PGE$_2$ and PGF$_{2\alpha}$ are regulated by separate mechanisms and may be produced by different intrauterine tissues. Maternal plasma PGFM concentration correlates with increasing maternal estradiol concentration, and the increased expression of PGHS-II expression within the maternal intrauterine tissues- the endometrial epithelium and the myometrium (Gyomorey et al, 2000). Furthermore, estradiol has been found to induce the expression of PGHS-II in the non-pregnant ovine endometrium and myometrium (Wu et al, 1997). These observations suggest that the increase in maternal intrauterine tissue expression of PGHS-II and subsequent PGF$_{2\alpha}$ production are dependent upon cortisol-induced increases in placental estradiol synthesis.

On the basis of these observations we hypothesize that there may be two separate pathways of intrauterine PG synthesis: a cortisol dependent/estradiol independent path within the fetal placental trophoblast tissue and an estradiol dependent path within the maternal intrauterine tissues. To test this hypothesis we have established the following specific objectives.

1. To determine the fetal plasma profile of PGE$_2$ and the maternal plasma profile of PGFM, the 13,14-dihydro-15 keto metabolite of PGF$_{2\alpha}$ in response to increased fetal plasma cortisol concentration in the presence and absence of cortisol-induced placental estradiol synthesis.

2. To determine the fetal trophoblast and maternal intrauterine mRNA and protein expression of the PG synthesizing enzymes PGHS-I and II in response to increased fetal plasma cortisol concentration in the presence and absence of cortisol-induced placental estradiol synthesis.
3. To determine the pattern of uterine contractility in response to increased fetal plasma cortisol concentration in the presence and absence of cortisol-induced placental estradiol synthesis.

These objectives will be addressed in *Chapter Three* of the thesis. The results of these studies have been published in *Endocrinology* (41:3783-3791, 2000) and appear in this thesis with the permission of the journal (refer to the attached authorization form).

Although intrauterine PG production has been associated with increased PG synthesis, a decrease in intrauterine PG metabolism could also contribute to the rise in plasma PGE₂ and PGF₂α concentrations. PGDH is expressed within the fetal trophoblast and maternal intrauterine tissues; however, the expression, activity and regulation of this enzyme within these tissues at the onset of labor has not yet been characterized (Riley et al., 2000; Kierse et al., 1978). In humans, intrauterine PGDH expression and activity is tonically maintained by progesterone (Patel et al., 1999). At term, increasing cortisol levels inhibit this basal level of PGDH activity; the inhibition appears to be mediated through the displacement of progesterone activity by cortisol at the GC receptor (*Patel and Challis, unpublished results*). These data raise the possibility that increased fetal adrenal cortisol production may similarly decrease the expression and activity of PGDH within the ovine intrauterine tissues contributing to the rise in PGE₂ and PGF₂α observed at term and with the onset of labor. Recently, the GC receptor has been localized to the uninucleate fetal trophoblast tissue, the endometrium and the myometrium whereas the estradiol receptor has been localized to the maternal intrauterine tissues including the endometrium and the myometrium (Gupta et al., 2000; Leung et al., 1999; Wu et al., 1997). The expression of these receptors increases with
the onset of labor at term. These data suggest that the steroid induced alterations in the intrauterine expression of the PG synthesizing and metabolizing enzymes may be mediated in a receptor dependent manner. Although fetal cortisol has been suggested to increase the expression of P450C17, a direct interaction between cortisol and P450C17 gene expression or activity has not been documented. Thus, the possibility of a GC stimulated intermediate leading to P450C17 induction can not be excluded. The temporal pattern of trophoblast PGHS-II and P450C17 induction suggests that increased PGE2, produced as a result of GC stimulation, could induce P450C17 leading to increased placental estradiol synthesis.

In addition to elevated plasma PG concentrations, the rise in maternal plasma estradiol concentration is also correlated with an increase in uterine activity consistent with active labor (Gyomorey et al. 2000). Myometrial gap junction size and number as well as the expression of connexin 43 is increased with labor in a number of species including the sheep. These changes have been attributed to an increase in the plasma estradiol:progesterone ratio (Lye. 1994). McNutt et al (1994) demonstrated that Cx43 expression in the ovine myometrium was increased at the onset of ACTH induced preterm labor. This increase was correlated to an increase in the estradiol:progesterone ratio however, plasma cortisol concentrations were also increased. However, the possibility that fetal adrenal GC production may have increased myometrial connexin 43 expression and uterine activity independent of alterations in placental estrogen and progesterone production can not be excluded.

On the basis of these observations we hypothesize that increased fetal adrenal GC production decreases the intrauterine tissue expression and activity of PGDH contributing to the increase in the intrauterine PG production observed at term and with the onset of labor. Furthermore, we hypothesize that these cortisol-dependent and estrogen-dependent
alterations in intrauterine PG production are mediated by an increase in the intrauterine expression of the GC and estradiol receptors respectively. We suggest that cortisol induced PGE₂ production leads to the increased expression of placental trophoblast P450C₁₇ expression. In addition to increased PG production, we hypothesize that fetal adrenal cortisol output contributes to increased uterine activity through the induction uterine connexin 43 expression independent of changes in the plasma estradiol:progesterone ratio. To study these hypotheses we have established the following specific objectives.

1. To determine the intrauterine tissue expression of PGDH in response to increased fetal plasma cortisol concentration in the presence and absence of cortisol-induced placental estradiol synthesis.

2. To determine the intrauterine localization and expression of the GC and estradiol receptors in response to increased fetal plasma cortisol concentration in the presence and absence of cortisol-induced placental estradiol synthesis.

3. To examine the regulation of placental trophoblast P450C₁₇ expression in response to increased fetal plasma cortisol concentration in the presence and absence of cortisol-induced placental estradiol synthesis.

4. To determine the uterine expression of connexin 43 in response to increased fetal plasma cortisol concentration in the presence and absence of cortisol-induced placental estradiol synthesis.

These objectives will be addressed in Chapter Four of the thesis.

In the human, the current hypothesis of parturition suggests that an increase in PG synthesis, and a decrease in PG metabolism within the fetal membranes leads to the increase
in intrauterine PG concentration (Challis et al. 1997). Within the trophoblast-derived fetal membranes, the amnion, is the main site of PG synthesis at the onset of labor (Challis et al., 1997). Both amnion epithelial and mesenchymal cells express PGHS-II and this expression increases with the onset of labor at term and preterm (Feuntes et al., 1996; Gibb et al., 1996; Hirst et al., 1995; Teixiera et al., 1994). Using primary culture preparations GCs have been found to stimulate amnion cell PGHS-II expression and PGE₂ production in a receptor-dependent manner (Blumsyein et al. 2000; Economopoulos et al., 1996; Potestio et al., 1988; Smeijla et al., 1993; Zakar et al., 1993). Mixed amnion cell culture preparations suggest that this stimulatory effect of GCs may occur only within the amnion mesenchymal cells (Economopoulos et al., 1996). However, the effect of GCs on isolated amnion epithelial and mesenchymal cell PGHS-II expression and PGHS activity has not yet been addressed. Therefore, we hypothesize that GCs increase amnion epithelial and mesenchymal cell PG production. To test this hypothesis we have developed the following specific aims:

1. To determine the basal PGE₂ output by the two amnion cell types in the presence and absence of exogenous substrate.

2. To determine the protein expression of PGHS-II and the GC receptor within separately cultured amnion epithelial and mesenchymal cells.

3. To determine the pattern of GC regulation of PGE₂ production by separately cultured amnion epithelial and mesenchymal cells.

These objectives will be addressed in Chapter Five of the thesis. The results of these studies have been published in Placenta (21:394-401,2000) and appear in this thesis with the permission of the journal (refer to the attached authorization form).
The intimate juxtaposition of these two cell types in vivo suggests that paracrine interactions between the epithelial and mesenchymal cells may occur. One possible paracrine mediator is corticotropin releasing hormone (CRH). CRH expression has been localized to both the amnion epithelium and mesenchyme and chorion (Riley et al, 1991). This expression increases with the onset of labor at term and preterm and is increased both in vivo and in vitro following GC exposure (Marioni et al, 1998; Korebriks et al, 1998; Riley et al, 1991; Jones et al, 1989). In vitro, CRH caused a dose dependent increase in mixed amnion cell PGE₂ output; however, the specific amnion cell type responding to CRH stimulation has not been identified (Jones et al, 1989a; Jones et al, 1989b). CRH acts through specific membrane-bound receptors; the CRH-R₁α, R₁c and R₁d isoforms have been exclusively identified with the amnion epithelium. These data suggest that CRH produced by the amnion epithelial and mesenchymal cells in response to GC stimulation may act as an autocrine/paracrine mediator to increase amnion epithelial cell PGE₂ output.

At term, the activity of the fetal HPA axis, the concentration of cortisol in maternal plasma and the activity of 11βHSD within the intrauterine tissues regulates the intrauterine concentration of cortisol. Within the fetal membranes, 11βHSD-I expression is present within the chorion trophoblast cells; in vitro chorionic 11βHSD-I acts as a reductase to produce cortisol that in turn acts in an autocrine/paracrine manner to decrease PGDH expression and activity (Patel et al, 1999; Sun et al, 1997). 11βHSD-I is also expressed within both amnion epithelial and mesenchymal cells. These observations suggest that 11βHSD-I expression within the amnion epithelial and mesenchymal cells may influence amnion cell PG synthesis.
On the basis of these observations we hypothesize that the effects of GCs on amnion cell PG production are regulated by the expression of 11βHSD-I within the epithelium and mesenchyme. We further hypothesize that paracrine interactions between the amnion epithelial and mesenchymal would regulate amnion PG output and in particular, CRH may be a paracrine mediator affecting amnion epithelial cell PG production. To test these hypotheses we have developed the following specific aims:

1. To determine if 11βHSD-I protein is expressed in separately cultured amnion epithelial and mesenchymal cells.

2. To determine if the activity of 11βHSD-I in separately cultured amnion epithelial and mesenchymal cells affects amnion epithelial and mesenchymal cell PGE2 output.

3. To determine whether separately cultured amnion epithelial and mesenchymal cells produce PGE2 in response to CRH treatment.

4. To determine the effect of amnion epithelial cell conditioned media on amnion mesenchymal cell PGE2 output and vice versa.

These objectives will be addressed in Chapter Six of the thesis.
Chapter Three

Prostaglandin Production at the Onset of Ovine Parturition is Regulated by Both Estrogen-Independent and Estrogen Dependent Pathways

The contents of this chapter have been published (Endocrinology; 41:3783-3791, 2000) and appear here with the permission of the journal (refer to attached authorization).

The experiments were designed, implemented and interpreted solely by Whittle WL. The co-author Holloway AC assisted with the surgical procedure and animal euthanasia. The co-authors Lye SJ, Gibb W and Challis JRG were the principal investigators who supervised the experimental design, implementation and interpretation.
3.1. INTRODUCTION

The current model of ovine parturition suggests that the surge in fetal adrenal cortisol production towards the end of gestation alters placental steroidogenesis causing a decline in progesterone output and an increase in estradiol production through the induction of the placental P450c17 enzyme (Mason et al., 1989; France et al., 1988; Steele et al., 1976; Anderson et al., 1975; Flint et al., 1975). Estrogen, in turn, stimulates intrauterine PG production, in particular PGE2 and PGF2α, as well as triggers the expression of a specific cassette of CAPs within the myometrium (Challis et al., 1997; Lye, 1994). Consequently, myometrial contractility is stimulated and labor and delivery of the fetus ensues. However, recent evidence has led us to question this concept.

Studies examining the ontogeny of intrauterine PG production during late gestation and the onset of labor demonstrated that the rise in fetal plasma PGE2 concentration occurred with a similar time course to the rise in fetal plasma cortisol and preceded the rise in both fetal and maternal plasma estradiol concentrations (Gyomorey et al., 2000; Wu et al., 1999, Mitchell et al., 1979). The expression of P450c17, a rate-limiting enzyme of estradiol synthesis from C21 precursors in the ovine placenta through gestation, did not increase until the onset of early labor, well after the rise in fetal plasma PGE2 concentration and the increase in placental PGHS-II expression. In addition, intrafetal estradiol infusion failed to increase expression of PGHS-II mRNA in the sheep placenta although intrafetal administration of cortisol did increase placental PGHS-II expression and plasma PG levels (Challis et al., 1997; McLaren et al., 1996). These observations suggested that cortisol but not estradiol might increase placental trophoblast PGHS-II expression and activity to produce PGE2.
In related studies we also found that changes in maternal plasma concentrations of PGFM were correlated with increased endometrial PGHS-II expression, maternal plasma estradiol levels and uterine activity (Gyomerey et al, 2000a; Gyomerey et al, 2000b). Recently, estradiol has been shown to increase PGHS-II expression significantly in non-pregnant ovine myometrium and in non-pregnant ovine endometrium after progesterone priming (Wu et al, 1997). These observations suggested that placental estradiol may stimulate non-trophoblast intrauterine tissue PGHS-II expression/activity to produce PGF2α that in turn may contribute to uterine activity.

Therefore, we hypothesized that there might be two separate pathways of intrauterine PG production: a cortisol dependent/estradiol independent pathway within fetal placental trophoblast tissue and an estradiol dependent pathway within maternal intrauterine tissues. To test this hypothesis, we infused late gestation sheep fetuses with cortisol in the presence and absence of the aromatase inhibitor 4-hydroxyandrostendione (4-OHA) and determined changes in placental and uterine PGHS-II expression and PG output.

3.2. MATERIALS AND METHODS

3.2.1. Animal Care

Pregnant singleton ewes of mixed breeds and known gestational age were purchased from Boxwood Farms (London, Canada). Gestational age was calculated from the date of insemination and number of fetuses was confirmed by ultrasonography. The animal care/experimental protocol was approved by the Animal Care Committee at the University of Toronto and was in accordance with the guidelines of the Canadian Council on Animal Care.
3.2.2. **Surgery and Post-Operative Care**

Following transport to the research facility, the animals underwent a recovery period for 5d. Ewes were then fasted for a period of 24h and water was withheld for 12h prior to the surgery. In the surgical preparation room the ewe received an injectable induction anesthetic, 40-50 mL 2.5% sodium pentothal (1.00-1.25 g; Abott Laboratories Ltd.; Montreal, Canada) administered via external jugular venipuncture. Following induction, the animal was intubated with a cuffed, size 9mmID endotracheal tube (Mallinckrodt Medical Inc.; St Louis, USA) and a maintenance inhalational anesthetic, 1-3% halothane (Wyeth-Aerst; Montreal, Canada) was administered with 5-7 L oxygen. The abdominal and bilateral groin regions of the ewe were sheared and scrubbed three times with iodine soap (ProviDine™, Rougier Inc.; Chably, Canada). The animal was transferred to the surgical suite and secured to the operating table with leg restraints. The surgical field was rinsed with iodine soap and sprayed with 100% ethanol.

The surgical procedure was performed under aseptic conditions. The animal was draped with two layers of sterile cloth exposing the midline abdominal area. A vertical midline incision through the skin and underlying fascia was made and extended ~10 cm above and below the umbilicus. From within the abdominal cavity, a 3-4 cm diameter incision was made through the right abdominal wall using a 3 mm trochar and shield. The gas sterilized catheter/electromyographic electrode package was advanced through the trochar shield and pulled through the abdominal cavity to the midline incision. The uterus was identified and exposed through the midline incision; the number of fetuses was confirmed by palpation. The head of the fetal lamb was identified through the uterine wall and a 5-8 cm uterine incision was made over this area, avoiding uterine vessels and placental
The fetal head was delivered through the incision. Atraumatic Babcock clamps were used to secure the edges of the uterine incision (including the myometrial and fetal membrane edges) to the fetal neck to minimize the loss of amniotic fluid and maintain the integrity of the incision edge. A 4-5 cm horizontal skin incision was made over the area of the external jugular vein on the fetal neck. Blunt dissection was used to isolate the carotid artery and external jugular vein. Each vessel was isolated individually and cleared from adjacent connective tissue using blunt dissection. An ~1mm incision was made though the anterior wall of each vessel and a polyvinyl catheter [1.2 mmOD/0.6 mmID (Scientific Commodities Inc.; Lake Havasua City, USA)] flushed with heparinized saline [1x10^6 IU heparin sodium (Organon Teknika Inc.; Toronto, Canada)/250 mL sterile 0.9% sodium chloride (Baxter Inc.; Toronto, Canada)] was advanced ~6 cm along each vessel and secured to the vessel with 2-0 silk (Ethicon™, Johnson and Johnson Medical Products; Peterborough, Canada) ligatures above and below the incision. Each catheter was attached to a three-way stopcock and catheter patency was confirmed. The skin incision was closed with a running, non-locking stitch using 2-0 silk. The catheters were individually anchored to the fetal skin with 2-0 silk ties. The fetus was returned to the amniotic cavity; the uterine incision was closed with a running, locking stitch using 2-0 silk; the fetal membranes were included in each bite to ensure continuity of the amniotic sac. A second imbricating stitch using 2-0 silk was used to bury the running stitch and ensured strength and hemostasis of the incision.

Three uterine electromyographic (EMG) leads (Cooner Wire Co.; Chatsworth, USA) were sewn in a triangular pattern to the outer myometrial layer. The fascial incision was closed with a running stitch using 2-vicryl (Ethicon™, Johnson and Johnson Medical Products, Peterborough, Canada) reinforced with intermittent figure eight ties of the same suture. The
skin was closed with simple running stitch using 0-vicryl. An ~10-15 cm horizontal incision was made in the right maternal groin region. The femoral artery and vein were identified by blunt dissection. The vessels were individually catheterized as described above using polyvinyl catheters [2.3mmOD/1.5 mmID (Scientific Commodities Inc.; Lake Havasca City, USA)] flushed with heparinized saline and advanced ~20 cm along the vessel. The catheters were attached to one-way stopcocks and catheter patency was confirmed. The groin skin was closed with simple running stitch using 0-vicryl. The catheters were anchored individually at two sites along the inside flank. The incision in the right abdominal wall was closed with a purse-string stitch around the catheters using 0 vicryl. Maternal and fetal catheters and the EMG wires were placed in a plastic bag and secured to the ewe's back. The inhalational anesthetic was discontinued and the ewe was extubated when she was able to chew and maintain airway patency. Preoperatively (0.15 mg) and prior to extubation (0.3 mg) Temgesic™ (buprenorphine hydrochloride; Reckett and Coleman Inc.; North Humberside, UK) respectively was administered subcutaneously for postoperative analgesia.

The ewes recovered for a 5d period prior to the infusion protocol. Ewes received 3 ml Trivetrim™ (trimethoprine/sulfadioxide (1:5); Serring Plough Animal Health Ltd.; Philadelphia, USA) intraoperatively via the venous catheter and for 3 consecutive postoperative days. Penicillin G sodium [1 000 000 IU (Novopharm Ltd.; Toronto, Canada)] was administered via the fetal jugular catheter intraoperatively and for 3 consecutive postoperative days. All catheters were flushed with sterile heparinized saline daily during the recovery period. Animals were housed in pens for the initial 24h postoperative period after which they were transferred to metabolic cages where they remained until the termination of the protocol. The cage permitted forward and backward movement, standing and sitting
leaving the catheters intact. The animals were housed in a 14h light cycle (0600-2000h) with a 10h dark period. Ewes were fed Ewe Chow™ 3297s (14% protein, 2% fat, 12% fibre; 0.5kg daily; Agribands Purina Canada Inc.; Woodstock, Canada) and were supplemented with hay and water ad libitum.

3.2.3. Infusion Protocol

Beginning on d125-128 of gestation, fetuses received a continuous intravenous infusion of saline (3ml/h; n=10) or cortisol (1.35mg/h; n=10) (Steraloids; Wilton, USA). Following 24h of infusion five animals in each group received an additional intrafetal infusion of Lentaron™ (1.44 mg/h) 4-OHA: 4-hydroxyandrostendione, a competitive suicide inhibitor of the \( \text{P}450_{\text{aromatase}} \) enzyme; CIBA-Geigy; Basel, Switzerland] (Nathanielsz et al. 1998; Brodie et al. 1991). From preliminary studies (data not shown) it was determined that an intrafetal cortisol infusion (1.35mg/h in 3 ml saline) for 80h was sufficient to induce an evolution in the uterine activity to a pattern consistent with labor (contraction frequency: \( \geq 50 \) contractions/2h period) for a minimum of 1h. When the infusion period was extended for greater than 80h fetuses and intrauterine tissue were lost due to delivery. On the basis of these preliminary timecourse experiments the animals in each of the treatment and control groups were euthanized following an infusion period of 80h.

3.2.4. Blood Sampling and Uterine Monitoring

Fetal and maternal arterial blood samples were collected at 12h intervals beginning 24h prior to the start of the infusion protocol and continued through the infusion period. Blood to be used for the determination of estradiol and progesterone was collected into syringes rinsed previously with heparinized saline; blood to be used for the determination of PGE\(_2\) and PGFM was collected in heparinized syringes and then transferred to vials.
containing indomethacin [200 μl of 1 mg/ml INDOCID™ (Merck, Sharp and Doeme: Kirkland, Canada)]. Plasma was separated from blood cells by centrifugation at 1500 g for 10 min at 4°C. Plasma samples were frozen at −20°C for subsequent assay.

Uterine muscle EMG activity was processed by a Grass wide-band AC preamplifier, Model 7P511J and recorded using a Grass 78D EEG and polygraph data recording system (Grass Instruments; Quincy, USA). Uterine activity monitoring began 24h prior to the start of the infusion protocol and continued for the infusion period. Uterine contractures were defined as a low amplitude uterine activity pattern (duration 5-8 min, frequency ~2-3 per h); uterine contractions were defined as a high amplitude activity pattern (duration <1 minute, frequency ≥ 30 per h) (Gyomory et al, 2000; Nathanielsz et al, 1976).

3.2.5. Euthanasia and Tissue Collection

Following completion of the infusion period, the ewes were euthanized with an overdose of Euthanyl™ (24% Sodium Pentobarbital, MTC Pharmaceuticals; Cambridge, Canada) and intrauterine tissues were collected. Placental cotyledons were dissected from the uterine muscle and fetal membranes; intercotyledonary endometrium was bluntly peeled from the myometrium. A cross-sectional piece of uterine tissue including myometrium, endometrium, chorion and amnion was cut, rolled and collected. Tissues were snap frozen in liquid nitrogen and stored in PolyQ scintillation vials (Beckman Instruments Inc.; Toronto, Canada) at −80°C or slow frozen over dry ice, wrapped with two layers Parafilm™ (American National Can: Menasha, USA) and stored at −80°C.
3.2.6. Plasma Hormone and Prostaglandin Assays

3.2.6.1. Fetal Plasma Total Cortisol Concentration

Fetal plasma cortisol was quantified as previously described by Challis et al. (1981). Plasma samples (100μL) were pipetted into glass test-tubes (16x125 mm) containing diethyl ether [5 mL (BDH Inc.; Toronto, Canada)] mixed with a vortex (~20 sec) and then incubated under agitation for ~1h. The aqueous phase was separated from the organic phase by freezing the samples in dry ice cooled acetone. The organic phase was decanted into 12 x 75 mm glass test-tubes, air dried at 37°C and then reconstituted in 1 mL phosphate buffered saline with gelatin [PBSG: 0.4 M NaH₂PO₄·2H₂O; 0.6 M Na₂HPO₄·7H₂O; 0.15 M NaCl; 0.1% w/v gelatin; pH 7.1 (Sigma; St Louis, USA)]. Extraction efficiency was calculated by extracting 100 μL of [1,2,6,7-³H] cortisol (~10 000 cpm; NEN Dupont Canada Inc.; Mississauga, Canada) as described above and comparing initial radioactivity with recovered radioactivity [(cpm recovered)/ (cpm initial)- (cpm blank)] x 100%. The extraction efficiency for the assay was 85%.

Aliquots (100μL and 400 μL) of the reconstituted samples were transferred to assay glass test-tubes (12x75 mm) and incubated with rabbit anti-cortisol antiserum (100μL; final dilution 1:30 000; titrated to give a B/Bo of ~35-40%) and [1,2,6,7-³H] cortisol (100μL; ~10 000 cpm; purified as described below) at 4°C for ~18h. The antiserum bound cortisol was separated from the unbound cortisol using dextran-coated charcoal [DCC: 0.625% w/v dextran: (Pharmacia KLB; Baie d’Urfe, Canada), 6.25% w/v charcoal (Fisherbrand, Fisher Scientific; Nepean, Canada) in PBSG; pH7.1] and centrifugation at 15 000 g at 4°C for 10 min. The supernatant containing the bound cortisol was decanted to PolyQ scintillation vials (Beckman Instruments Inc.; Toronto, Canada) containing 4 mL CytoScint™ (ICN
Biomedicals Inc.; Costa Mesa, USA) and counted on a beta counter (Tri-Carb 21000TR Liquid Scintillation Analyzer; Camberra Packard Instrument Co.; Meriden, USA). Cortisol concentration in the extracted aliquot was determined and the fetal plasma total (free and bound) cortisol concentration was calculated accounting for the PBSG dilution factor, the extraction efficiency and the initial volume of plasma extracted. The antiserum used for the assay was generated by this laboratory and has been previously characterized (Challis et al. 1981). The cross-reactivities reported are as follows: 4-pregnen-11β,17,21-triol-3,20-dione sulphate 76%; 4-pregnen-17,21-diol-3,20-dione 35%; 4-pregnen-17-ol-3,11,20-trione 33%; 3-pregnen-17,21-diol-3,11,20-trione 6%; 4-pregnen-11β,21-diol-3,20-dione 0.84%; 4-pregnen-3,20-dione 0.31% and 5-pregnen-3β-ol-20-one, 5-pregnen-3β,17-diol-20-one, 4-pregnen-11α-ol-3,20-dione were less than 0.1%. All samples were measured in the same assay and the intra-assay coefficient of variation was 7.5%; the mean assay sensitivity was 4 ng/ml.

Thin layer chromatography (TLC) was used to purify the cortisol tracer: specifically [1,2,6,7-3H] cortisol (specific activity 80-100 Ci/mmol) was separated from the breakdown products of the labeled steroid on a 20x20 cm TLC plate (Fisherbrand, Fisher Scientific; Nepean, Canada) placed in an incubation chamber with 200 ml chloroform:ethanol (95:5: BDH Inc.; Toronto, Canada). The solvent front advanced to the top of the plate. The TLC plate was then air-dried. [1,2,6,7-3H] cortisol (40 μL) and non-labeled cortisol (20 μg) were spotted at the same level, ~5 cm from the bottom, on opposite sides of the plate. The TLC plate was returned to the incubation chamber and the solvent front was allowed to rise to the top of the plate. The TLC plate was then air-dried and the unlabeled steroid was visualized using UV light. The area corresponding to the radio-labeled cortisol on the opposite side of
the plate was scrapped off and mixed with 4 mL ethyl acetate (BDH Inc.; Toronto, Canada). The mixture was vortexed (~20 sec), centrifuged (10 min, 4°C, 1500g) and the supernatant collected. The pellet was re-suspended in an additional volume of ethyl acetate (4 mL), re-vortexed and centrifuged; the supernatant was collected and added to the first volume. A 1μL aliquot of the pooled supernatant was added to a scintillation vial containing 4 mL CytoScintTM and counted on a beta counter to determine the concentration of radioactivity in the stock cortisol tracer.

3.2.6.2. Fetal and Maternal Plasma Estradiol Concentration

The radio-immunoassay for fetal and maternal plasma estradiol concentrations was performed using a commercially available [125I] RIA kit (ImmuChemTM Double Antibody 17βEstradiol 125I RIA Kit; ICN Biomedicals Inc.; Costa Mesa, USA). All samples were measured in the same assay; the intra-assay coefficient of variation was 3% and the mean assay sensitivity was 15 pg/ml. The specificity was provided by the manufacturer; the main cross-reacting steroids were estrone, estriol, and estradiol-17α with 20, 1.51, and 0.68 % cross-reactivity respectively.

3.2.6.3. Fetal Plasma Androstenedione Concentration

Fetal plasma androstenedione concentrations were measured using a commercially available [125I] RIA kit (ImmuChemTM Double Coated Tube Androstenedione 125I RIA Kit; ICN Biomedicals Inc.; Costa Mesa, USA). All samples were measured in the same assay; the intra-assay coefficient of variation was 12% and the mean sensitivity of the assay was 120 pg/ml. The specificity was provided by the manufacturer; the main cross-reacting steroids
were DHEA, androsterone, testosterone and estrone with 2.08, 1.96, 0.83, and 0.2 % cross-reactivity respectively.

3.2.6.4. Maternal Plasma Progesterone Concentration

Maternal plasma progesterone concentration was determined as previously described by Olson et al (1985). Maternal plasma was extracted in an identical manner as described for fetal plasma (section 3.2.6.1.) Extraction efficiency was calculated using [1,2,6,7-^3H] progesterone (NEN Dupont Canada Inc.; Mississauga, Canada) and determined to be 80%. Aliquots (250μL) of the reconstituted samples were transferred to glass test-tubes (12x75 mm) and incubated with rabbit anti-progesterone antiserum (100μL; final dilution 1:8000; titrated to give a B/Bo of ~35-40%) and [1,2,6,7-^3H] progesterone (100μL; ~10 000 cpm) at 4°C for ~18h. The antiserum bound progesterone was separated from the unbound progesterone using DCC and centrifugation at 2500 rpm at 4°C for 10 min. The supernatant containing the bound progesterone was decanted to scintillation vials containing 4 mL CytoScint™ and counted on a beta counter. Progesterone concentration in the extracted aliquot was determined and the maternal plasma total (free and bound) progesterone concentration was calculated accounting for the PBSG dilution factor (4x), the extraction efficiency and the initial volume of plasma extracted. The antiserum used for the assay was obtained from Endocrine Sciences Products (Calabasa Hills; USA); the specificity was provided by the manufacturer. The main cross-reacting steroids were: 4-pregnen-21-ol-3,20-dione, 4-pregnen-20βol-3-one, 4-pregnen-20αol-3-one, 4-pregnen-11β,21diol-3,20-dione, 4-pregnen-17-ol-3,20-dione and 4-pregnen17,21diol-3,20-dione with 3.3, 1.3, 0.8, 0.6, 0.6, 0.4% cross-reactivity respectively. All samples were measured in the same assay and the intra-assay coefficient of variation was 10%; the mean assay sensitivity was 1ng/ml.
3.2.6.4. **Fetal Plasma Prostaglandin E\textsubscript{2} Concentration**

Fetal plasma prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) was quantified as previously described by Olson et al. (1985). Plasma samples (300\mu L) were pipetted into glass test-tubes (16x125 mm) with diethyl ether (5 mL) and HCl [6.0 N, 10 \mu L (ACP Chemicals Inc.; Montreal, Canada)] pH~3.0, mixed with a vortex (~20 sec) and incubated under agitation for ~1h. The aqueous phase was separated from the organic phase by freezing the samples in dry ice cooled acetone. The organic phase was decanted to glass test-tubes (12 x 75 mm), air dried at 37°C and then reconstituted with 1 mL PBSG. Extraction efficiency was calculated using [5.6,8,11,12,14,15(n)-\textsuperscript{3}H] PGE\textsubscript{2} (~10 000 cpm) as previously described (section 3.2.6.1.) and determined to be 64%.

Aliquots (300\mu L) of the reconstituted samples were transferred to glass test-tubes (12x75 mm) and incubated with rabbit anti-PGE\textsubscript{2} antiserum (100\mu L; final dilution 1:4000; titrated to give a B/Bo of ~35-40%) and [5,6,8,11,12,14,15(n)-\textsuperscript{3}H] PGE\textsubscript{2} (100\mu L; ~10 000 cpm) at 4°C for ~18h. The antiserum bound PGE\textsubscript{2} was separated from the unbound PGE\textsubscript{2} using alkaline DCC and centrifugation at 2500 rpm at 4°C for 10 min. The supernatant containing the bound PGE\textsubscript{2} was decanted to scintillation vials containing 4 mL CytoScint\textsuperscript{TM} and counted on a beta counter. PGE\textsubscript{2} concentration in the extracted aliquot was determined and the fetal plasma PGE\textsubscript{2} concentration was calculated accounting for the PBSG dilution factor (3.3x), the extraction efficiency and the initial volume of plasma extracted. The antiserum used for the assay was generated by the laboratory of Dr T. Kennedy (University of Western Ontario; London, Canada). The antibody cross-reacts with PGF\textsubscript{2\alpha} by 0.18%. All samples were measured in the same assay and the intra-assay coefficient of variation was 8%.
3.2.6.5. ***Maternal Plasma 13,14-dihydro15-keto-prostaglandin F_{2\alpha} Concentration***

Maternal plasma 13,14-dihydro15-keto-prostaglandin F_{2\alpha} (PGFM) was quantified as previously described by Olson et al. (1985). Plasma samples (250μL) were extracted as described in section 3.2.6.4. The organic phase was decanted to glass test-tubes (12 x 75 mm), air dried at 37°C and then reconstituted with 250μL PBSG. Extraction efficiency was calculated using [5,6,8,9,11,12,14,15,(n)-^{3}H] PGFM (~10 000 cpm) as described (section 3.2.6.1.) and determined to be 63%. Aliquots (100μL) of the reconstituted samples were transferred to glass test-tubes (12 x 75 mm) and incubated with rabbit anti-PGFM antiserum (100μL; final dilution 1:500; titrated to give a B/Bo of ~35-40%) and [5,6,8,9,11,12,14,15,(n)-^{3}H] PGFM (100μL; ~10 000 cpm) at 4°C for ~18h. The antiserum bound PGFM was separated from the unbound PGFM using alkaline DCC and centrifugation at 1000 g at 4°C for 15 min. The supernatant containing the bound PGFM was decanted to scintillation vials containing 4 mL CytoScint™ and counted on a beta counter. PGFM concentration in the extracted aliquot was determined and the maternal plasma PGFM concentration was calculated accounting for the extraction efficiency and the initial volume of plasma extracted. The antiserum used for the assay was obtained from PerSeptive Biosystems (Framingham, USA). The specificity was provided by the manufacturer; the main cross-reacting eicosanoids were prostaglandin F_{2\alpha} and 13,14-dihydro15-keto-prostaglandin E_{2} with 1.7 and 0.14% cross-reactivity respectively. All samples were measured in the same assay and the intra-assay coefficient of variation was 4%.
3.2.7. *In situ Hybridization Analysis of PGHS-II mRNA Expression*

Sense and antisense probes based on the structure of the human PGHS-II gene were synthesized by the University of Ottawa Molecular Biology Department (Ottawa, Canada) using an Oligo1000 DNA synthesizer (Beckman Instrument Inc.; Mississauga, Canada). The oligonucleotide sequence for the PGHS-II probe was GGG ACA GCC CTT CAC GTT ATT GCA GAT GAG AGA CTG AAT TGA GGC AGT GT corresponding to nucleotides 1734-1783 of the human PGHS-II. Northern analysis was used to confirm that the probe recognized the 4.5 kb transcript of the PGHS-II. The probe was labeled with terminal deoxynucleotidyl transferase (Gibco BRL; Burlington, Canada) and 33P-labeled deoxyadenosine 5'p(α-thio) triphosphate (1300 Ci:mmol; NEN, Dupont Canada Inc.; Mississauga, Canada). The probe was used at a concentration of ~600 cpm/μl.

The method used for the *in situ* hybridization has been described previously by Matthews et al. 1991. Tissue sections (10μm) were mounted on Fisher Superfrost glass slides (Fisher Scientific; Nepean, Canada), fixed with 4% paraformaldehyde, dehydrated through graded ethanol and stored in 95% ethanol at 4°C. Slides were removed from ethanol and allowed to air dry at room temperature. Tissues were incubated overnight in a moist incubation chamber at 42°C with the radiolabeled oligonucleotide PGHS-II probe diluted in hybridization buffer. Hybridization buffer was composed of 4xSSC [single strength 1xSSC: 150 mM sodium chloride, 15mM sodium citrate (Sigma Chemical Co.; St. Louis, USA)], 50% deionized formamide (Gibco BRL; Burlington, Canada), 0.02% BSA (Boehringer Mannheim; Dorval, Canada), 10% dextran sulfate (Pharmacia KLB; Baie d'Urfe, Canada), 200 μg hydrolyzed salmon sperm DNA/ml, 0.02% polyvinylpyrrolidone, 40mM dithiothreitol, 50 mM sodium phosphate (pH 7.0) (Sigma Chemical Co.; St. Louis, USA).
Following incubation, slides were washed with 1xSSC at room temperature for 30 min and then 1xSSC at 45°C for 45 min. Slides were washed with decreasing strength SSC, dehydrated in ethanol, air dried and exposed to X-ray film (Kodak Eastman BioMax MR; Rochester, USA). Placental tissue was exposed for 38h and myometrium/fetal membranes were exposed for 270h. The autoradiographic films were developed using standard procedure. Linearity of the mRNA signals was established by simultaneous exposure of the samples with ^14C standards in the appropriate range (RPA504 Amersham LifeScience; Buckinghamshire, England). The optical density of PGHS-II mRNA expression was determined relative to a curve established by these ^14C standards. Nonspecific binding was established using a 45-mer nonsensical sequence oligonucleotide probe and the signal subtracted from the antisense PGHS-II mRNA signal; the specificity of the antisense PGHS-II probe was established by incubation with two positive tissue controls—term ovine placenta and cultured human amnion cells, both previously shown to express PGHS-II mRNA (Economopoulos et al, 1996). The autoradiograms were then analyzed using computerized image analysis software (Image Research Inc; St Catherines, Canada; Laser Scanner by Molecular Dynamics; software by ImageQuant). The relative optical density of placental and endometrial PGHS-II expression was assessed using 9 tissue sections per animal.

3.2.8. Western Blot Analysis of PGHS Protein Expression

Frozen tissue samples were homogenized on ice for 1 min in lysis buffer [50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% (w/v) sodium deoxycholate, 0.1% sodium lauryl sulfate (SDS), 100μM sodium orthovanadate (Sigma; St Louis, USA), 1%(v/v) Triton X-100 (Fisher Chemicals; Fairlawn, USA) and Complete™ MiniEDTA-free Protease Inhibitors
Homogenates were centrifuged at 4°C, 15,000g for 15 minutes; supernatants were collected. Protein concentrations were determined by the Bradford Assay (Bradford, 1976) using bovine serum albumin (Bio-Rad; Richmond, USA) as the standard and protein absorbance at 595 nm.

Protein samples (25-100 µg) were separated by polyacrylamide gel (4%-10% gradient) electrophoresis as described by Laemmli et al, 1973. Proteins were electrophoretically transferred to a 0.45μm pure nitrocellulose membrane (Bio-Rad; Richmond, USA); equivalency of protein loading and transfer were confirmed by protein visualization with Ponceau S solution and densitometric quantification (Sigma; St Louis, USA). Blots were washed with PBS-T [150 mM NaCl, 10 mM Na2HPO4, 1.5 mM NaH2PO4, 0.1% Tween-20; pH 7.5 (Sigma; St Louis, USA)] and incubated overnight with blocking solution (5% skim milk powder in PBS-T). Blots were incubated with primary antibody for PGHS-II [1:500 dilution in blocking solution (PG27, Oxford Biomedical Research Inc; Oxford, USA)] or PGHS-I [1:4000 dilution in blocking solution (PG19, Oxford Biomedical Research Inc; Oxford, USA)]. Blots were then rinsed 5x5min with PBS-T and incubated with secondary antiserum conjugated with horseradish peroxidase for 1h [1:1000-1:3000 dilution in blocking serum (Amersham Life Science; Buckinghamshire, England)]. Blots were washed 6x5min and the antibody-antigen complex was detected using the Amersham ECL detection system (Amersham Life Science; Buckinghamshire, England). Blots were exposed to X-ray film (Eastman Kodak X-Omat Blue; Rochester, USA) for serial exposure times to determine the appropriate concentration of protein loading and exposure time to ensure that the protein signal was within the linear response range. The intensity of the protein signal
was quantified using computerized image analysis software (Image Research Inc; St Catherines, Canada; Laser Scanner by Molecular Dynamics; software by ImageQuant).

3.2.9. Statistical Analysis

All data were analyzed using parametric statistical tests after the data was submitted to both normality and equal variance testing. Uterine contractility data were analyzed using two-way analysis of variance (repeated measures) followed by a post hoc, all pairwise multiple comparison Tukey’s test to determine significant interactions. The contraction frequency at putdown was also compared between the cortisol and cortisol +4-OHA treated animals using a Student’s t-test. Plasma data were analyzed by two-way analyses of variance (repeated measures) followed by a post hoc, all pairwise multiple comparison Tukey’s test to determine significant interactions. mRNA and protein data were analyzed by one way analyses of variance followed by a post hoc, all pairwise multiple comparison Tukey’s test to determine significant interactions. Significance was set at p<0.05. Data are presented as the mean +/- standard error of the mean (SEM) for n=4 or 5 per group.

3.3. RESULTS

3.3.1. Maternal and Fetal Plasma Steroid Levels

Mean fetal plasma cortisol concentration was increased significantly during 80h of cortisol infusion in presence or absence of 4-OHA (Figure 3-1). Mean fetal and maternal plasma estradiol concentrations were increased significantly during 80h of cortisol infusion. Fetal plasma estradiol concentration increased from 21 +/- 8 pg/ml to 259 +/- 33 pg/ml at the time of sacrifice (Figure 3-2); maternal plasma estradiol concentration was increased from 15 +/- 2 pg/ml to 217 +/- 59 pg/ml (Figure 3-3). The increases in both fetal and maternal plasma estradiol concentrations were attenuated significantly by co-infusion with 4-OHA
(Figure 3-2, 3-3). At the time of euthanasia, fetal plasma estradiol concentration was 64% less and maternal plasma estradiol concentration was 54% less than that of the cortisol infusion group. Fetal plasma estradiol (Figure 3-2) and maternal plasma estradiol were not altered significantly at any time during the infusion period in either control group. Androstenedione concentration in the fetal plasma was 342 +/- 41 pg/ml prior to the start of the infusion. The concentration of androstendione rose to 5340 +/- 1427 pg/ml in the animals treated with cortisol+4-OHA but did not change significantly in any of the other animal groups (Figure 3-4). Mean maternal progesterone concentration was decreased significantly from 33 +/- 3 ng/ml to 9 +/- 3 ng/ml at the time of sacrifice in the cortisol treated animals. Maternal plasma progesterone also decreased in the cortisol + 4-OHA treated animals (Figure 3-3). The ratio of estradiol:progesterone (E2:P4) in maternal plasma was increased from 0.46 to 23.4 x 10^-3 pg/ng by cortisol infusion. The E2:P4 ratio change in the animals co-infused with 4-OHA was attenuated and rose from 0.32 to only 2.3. There were no significant changes in maternal plasma P4 before and at the end of the infusion period in either control group of animals.

3.3.2. Maternal and Fetal Plasma Prostaglandin Levels

Mean concentrations of PGE2 in fetal plasma were increased significantly during both cortisol and cortisol+4-OHA infusion (Figure 3-5). In the cortisol treated animals, basal PGE2 levels (757 +/- 49 pg/ml) rose to 1618 +/- 471 pg/ml at time of sacrifice. In the cortisol+4-OHA treated animals, fetal plasma PGE2 increased from 564 +/- 37 pg/ml to 1241 +/- 91 pg/ml at the time of euthanasia. The rise in PGE2 concentrations in fetal plasma was not statistically different between the two groups of animals. Mean maternal plasma PGFM concentration increased significantly from 83 +/- 40 pg/ml to 423 +/- 85 pg/ml at the time of
sacrifice in the cortisol infused animals. However, in the cortisol+4-OHA treated animals, basal PGFM concentration was not increased significantly at the end of the infusion period (basal PGFM concentration: 149 +/- 22 pg/ml versus terminal PGFM concentration: 157 +/- 49 pg/ml) (Figure 3-6). The plasma PGE_2 and the PGFM concentrations in the control animals did not change through the infusion period (Figure 3-5, Figure 3-6).

3.3.3 Intrauterine PGHS mRNA and Protein Expression

Placental trophoblast PGHS-II mRNA expression was increased significantly by both cortisol and cortisol+4OHA infusion (Figure 3-7); there was a corresponding increase in placental PGHS-II immunoreactive protein expression in both of these groups of animals (Figure 3-8). PGHS-II mRNA expression in the intercotyledonary endometrium was increased significantly after cortisol infusion but not after cortisol+4OHA infusion (Figure 3-7). Similarly, endometrial PGHS-II immunoreactive protein expression was increased after cortisol but not after cortisol+4-OHA infusion (Figure 3-8). Placental and endometrial immunoreactive PGHS-II protein migrated an equivalent distance under electrophoresis (data not shown). Levels of PGHS-I immunoreactive protein expression in placenta and endometrium were not significantly different amongst the four groups of animals (Figure 3-9).

3.3.4 Uterine Contractility

In pregnant sheep whose fetuses were infused with cortisol alone, labor-type uterine contractions were present by 68h (12h prior to putdown, PD) and had increased significantly by the time of euthanasia (80h; PD) (Figure 3-10). Labor-type uterine contractions were present following 68h of cortisol+4OHA infusion and had increased significantly by the time of euthanasia (80h; PD) (Figure 3-10). However, during the last 2h prior to euthanasia, the
cortisol treated animals had a significantly greater contraction frequency (56.6 +/- 6.7 contractions/2h) compared with the animals treated with cortisol +4-OHA (26.1 +/- 9.0 contractions/2h). The uterine contracture pattern was not altered at any time during the infusion in either control group (Figure 3-10).
**Figure 3-1: Fetal Plasma Cortisol Concentration**

Values are presented as mean +/- SEM over the time period for n=5 animals in each group. Statistical analysis was performed using a two way analyses of variance (repeated measures) followed by a post hoc Tukey's test; significance was set at p ≤ 0.05. Fetal plasma cortisol concentration in the cortisol and cortisol+4-OHA treated animals at the end of the infusion period (80h; PD) was increased significantly compared to basal (0h) and increased significantly as compared to both basal (0h) and PD in the control groups (b). Fetal plasma cortisol concentrations at PD in the cortisol and cortisol+4-OHA treated animals were not statistically different. Fetal plasma cortisol concentrations in the control groups did not change over the infusion period. PD= putdown (80h); 4-OHA= 4-hydroxyandrostendione.
Figure 3-2: Fetal Plasma Estradiol Concentration

Values are presented as mean +/- SEM over the time period for n=5 animals in each group. Statistical analysis was performed using a two way analysis of variance (repeated measures) followed by a post hoc Tukey's test, significance was set at p≤0.05. Fetal plasma estradiol concentration in the cortisol treated animals at the end of the infusion period (80h; PD) was increased significantly compared to basal (0h) (*) and to the final (80h) fetal plasma estradiol concentration of the cortisol+4OHA and control treated animals (a). Fetal plasma estradiol concentrations in the cortisol+4OHA and control groups did not change significantly over the infusion period. PD= putdown (80h); 4-OHA= 4-hydroxyandrostenedione.
Figure 3-3: Maternal Plasma Estradiol and Progesterone Concentrations
Values are presented as mean +/- SEM over the time period for n=5 animals in each group. Statistical analysis was performed using a two way analysis of variance (repeated measures) followed by a post hoc Tukey’s test; significance was set at p≤0.05. Mean maternal plasma estradiol concentration in the cortisol treated animals was increased significantly at the end of the infusion period (PD; 80h) as compared to basal (0h) (b) and compared to the fetal plasma estradiol concentration of the cortisol+4OHA treated animals (basal and PD); plasma estradiol was not increased during the infusion period in the cortisol +4-OHA treated animals. Mean maternal plasma progesterone was significantly decreased as compared with basal (0h) at the end of the infusion period (PD; 80h) in the cortisol and cortisol+4OHA treated animals.
**Figure 3-4: Fetal Plasma Androstenedione Concentration**

Values are presented as mean +/- SEM over the time period for n=5 animals in each group. Statistical analysis was performed using a two way analysis of variance (repeated measures) followed by a post hoc Tukey's test; significance was set at p<0.05. Fetal plasma androstenedione concentration in the cortisol+4-OHA treated animals at the end of the infusion period (80h; PD) was increased significantly compared to basal (0h) (*) and compared to the final (80h) fetal plasma androstenedione concentration of the cortisol and control treated animals (b). Fetal plasma androstenedione concentrations in the cortisol and control groups did not change over the infusion period. PD= putdown (80h); 4-OHA= 4-hydroxyandrostenedione.
**Figure 3-5: Fetal Plasma Prostaglandin E\(_2\) (PGE\(_2\)) Concentration**

Values are presented as mean +/- SEM over the time period for n=5 animals in each group. Statistical analysis was performed using a two way analysis of variance (repeated measures) followed by a post hoc Tukey's test; significance was set at p<0.05.

Fetal plasma PGE\(_2\) concentration was increased significantly at the end of the infusion period (PD; 80h) (*) in both the cortisol and cortisol+4OHA treated animals. Fetal plasma PGE\(_2\) concentration at PD in cortisol and cortisol+4OHA treated animals was increased significantly as compared with the control treated animals (b). Plasma PGE\(_2\) concentration at PD in the cortisol and cortisol+4-OHA treated animals was not statistically different. There was no change in plasma PGE\(_2\) concentration in the two control groups. PD= putdown (80h); 4-OHA= 4-hydroxyandrostenedione.
**Figure 3-6: Maternal Plasma 13,14-dihydro-15-keto Prostaglandin F2α (PGFM) Concentration**

Values are presented as mean +/- SEM over the time period for n=5 animals in each group. Statistical analysis was performed using a two way analysis of variance (repeated measures) followed by a post hoc Tukey’s test; significance was set at p<0.05. Maternal plasma PGFM was increased significantly at end of the infusion period (PD; 80h) in the cortisol treated animals (*). Maternal plasma PGFM in the cortisol+4OHA treated animals did not change over the infusion period and was significantly less at the end of the infusion period as compared with the cortisol treated animals (b). There was no change in plasma PGFM concentrations in the two control groups.

PD= putdown (80h); 4-OHA= 4-hydroxyandrostenedione.
**Figure 3-7: Placental and Intercotyledonary Endometrial PGHS-II mRNA Expression**

Values are presented as mean +/- SEM for n=4 animals in each group. Statistical analysis was performed using a one way analysis of variance followed by a post hoc Tukey's test; significance was set at p<0.05. Placental tissue had a 38h exposure time; endometrial tissue had a 270h exposure time. Placental PGHS-II mRNA expression was increased significantly by both cortisol and cortisol + 4-OHA infusion (a) as compared to the control groups. Endometrial PGHS-II mRNA expression was increased significantly by cortisol infusion (a) but not by cortisol + 4-OHA infusion.

4-OHA = 4-hydroxyandrostenedione
Figure 3-8: Placental and Intercotyledonary Endometrial Immunoreactive PGHS-II Expression

Values are presented as mean +/- SEM for n=4 animals in each group. Statistical analysis was performed using a one way analysis of variance followed by a post hoc Tukey's test; significance was set at p<0.05. Placental immunoreactive PGHS-II expression was increased significantly by cortisol and by cortisol+4-OHA infusion (a). Endometrial immunoreactive PGHS-II was increased significantly by cortisol infusion (a) but not by cortisol+4-OHA infusion. 4-OHA= 4-hydroxyandrostenedione
Figure 3-9: Placental and Intercotyledonary Endometrial PGHS-1 Expression

Values are presented as mean +/- SEM n=4 animals in each group. Statistical analysis was performed using a one way analysis of variance; significance was set at p<0.05. Placental and intercotyledonary endometrial immunoreactive PGHS-1 expression remained unchanged and was not significantly different amongst the four groups of animals. 4-OHA= 4-hydroxyandrostenedione
Figure 3-10: Uterine Activity Pattern

Uterine activity measured as number of contractures or contractions per 2h interval. Values are presented as mean +/- SEM; n=5 per group. Statistical analysis of the contraction frequency was performed using two way analysis of variance followed by a post hoc Tukey's test and Student's t-test; significance was set at p<0.05. Uterine contractions were present in both the cortisol and cortisol+4-OH1A treated animals following 68h of infusion (12h prior to putdown); this contraction frequency was increased significantly in the final 8h of the infusion period (ANOVA; a). However, in the cortisol treated animals, contraction frequency during the final 2h prior to putdown was significantly greater than cortisol +4-OHA treated animals (Student's t-test; *). There was no change in the uterine contracture pattern in either control group.

4-OHA= 4-hydroxyandrostenedione.
3.4. DISCUSSION

In this study, we have shown that intrafetal cortisol infusion increased fetal trophoblast PGHS-II mRNA and protein expression, fetal plasma PGE$_2$ concentration, endometrial PGHS-II mRNA and protein expression and maternal plasma PGFM concentration. In the absence of increased placental estradiol production, intrafetal cortisol infusion did not lead to an increase of maternal endometrial PGHS-II expression or maternal plasma PGFM concentration. Uterine activity was attenuated when the prepartum increase in placental estradiol synthesis was prevented. Therefore, we suggest that within the ovine intratuterine environment, the increased placental PGHS-II expression and PGE$_2$ production associated with elevated fetal cortisol concentrations are independent of increased placental estradiol synthesis but endometrial PGHS-II expression and PGF$_{2\alpha}$ production are dependent upon increased placental estradiol synthesis. Estrogen synthesis is also required for the development of a full labor-like pattern of uterine contractility.

We have used Lentaron$^\text{TM}$, 4-OHA, to inhibit placental aromatase activity. 4-OHA is a competitive inhibitor that irreversibly binds to the active site of the aromatase enzyme (Brodie, 1991). Nathanielsz et al (1998) showed that maternal intravenous 4-OHA infusion inhibited androgen induced estradiol synthesis in pregnant rhesus monkeys consistent with a 4-OHA mediated inhibition of placental aromatase activity. France et al (1987) showed that 4-OHA could inhibit ovine placental aromatase activity \textit{in vitro} with a K$_i$ of 0.05 µM; we presume that this is the mechanism of the 4-OHA effect in the present study. There was no effect on basal steroid or PG concentrations in the animals receiving 4-OHA but not cortisol. Androstenedione concentration was increased in the plasma of fetuses treated with cortisol+4-OHA. This increase was not observed in the other treatment groups, consistent
with precursor build up after cortisol infusion in the presence of 4-OHA and effective inhibition of placental aromatase activity. Using this protocol we were able to block the cortisol induced increase in placental estradiol production and thereby we were able to determine the effects of intrafetal cortisol on intrauterine PGHS-II expression and PG output in the presence and absence of increased placental estradiol production. However, we can not exclude the effects of basal placental estradiol production nor can we exclude the possibility that the increase in androstenedione production and/or the 4-OHA may have influenced endometrial PGHS-II expression and PGF$_{2\alpha}$ output in the animals treated with cortisol+4-OHA.

GC stimulation has been reported to enhance the metabolism of arachidonic acid and increase PG output in fetal rat lungs, rat gastric mucosa, murine fibroblasts, bone marrow derived myeloid leukemia cells and rat reno-medullary cells (Avunduk et al. 1992; Tsai et al. 1983; Honma et al. 1980; Russo-Marie et al. 1980; Chandabrose et al. 1978). In particular, GCs have also been found to upregulate PGHS-II expression and activity in some trophoblast derived cells (Blumstein et al. 2000; Economopoulos et al. 1996; Zakar et al. 1994; Potestio et al. 1988): although inhibition of PGHS-II in amnion WISH cells treated with GC has also been reported (Deval et al., 1998). The effect of cortisol on ovine placental PGHS-II expression and PGE$_2$ output occurred in the absence of a cortisol-induced increase in the fetal or maternal plasma estradiol concentration. Ovine placental PGHS-II mRNA and protein expression has been localized previously to the mononuclear trophoblast cells (Gibb et al. 1996), which also express the GC receptor (Gupta et al. 2000). The post receptor mechanism by which GCs could regulate PGHS-II is not well understood; a specific GC response element within the 5' promoter region of the PGHS-II gene has been reported by two groups...
of investigators (Xu et al. 1995; Tazawa et al. 1994). The PGHS-II promoter also contains other transcription factor binding sites including two NFκB sites, 2 SP-1 sites, a 2 C/EBP (NFIL-6) sites, 1 AP-2 site and a cAMP response element (Inoue et al. 1995; Xu et al. 1995; Tazawa et al. 1994). Recently, evidence has been presented in support of synergistic interactions between transcription factors; in particular GR could interact with a member of the AP-1, NFκB or C/EBP families at either of their respective transcription factor DNA binding sites or at a unique, composite response element (McKay et al. 1999; Gottlicher et al. 1998; Bamberger et al. 1996). Alternatively, GCs could increase PGHS-II expression through interference with repressor transcription factors, stimulation of a unique trophoblast transcription factors or by effecting an increase in the stability of PGHS-II mRNA (McKay et al. 1999; Economopoulos et al. 1996; Smeija et al. 1993; Zakar et al. 1994; Potestio et al. 1988). Our present findings mimic the effects of GCs observed in some studies of human amnion cells. Mixed cultures of human amnion cells increased PGE₂ output in response to cortisol and dexamethasone stimulation (Economopoulos et al. 1996; Smeija et al. 1993; Zakar et al. 1994; Potestio et al. 1988). This stimulatory effect was found to be to be receptor dependent and involved an increase in PGHS-II expression. Thus, we suggest that the prepartum increase of fetal adrenal cortisol output increases expression of PGHS-II within the fetal ovine placental trophoblast cells leading to PGE₂ production; this PG synthesis occurs independently of an increase in placental estradiol output.

In addition, we found that endometrial PGHS-II expression and intrauterine PGF₂α output, reflected in maternal PGFM concentrations, were dependent upon increased placental estradiol synthesis. Previously, we were unable to demonstrate an increase in sheep placental PGHS-II expression with intrafetal estradiol infusion (Challis et al. 1997). In addition,
PGHS-II expression and activity could not be stimulated by estradiol in cultured human trophoblast derived cells (Gibb et al., 1988). However, PGHS-II expression can be stimulated by estradiol in other tissues including human monocytes, bovine oviduct, nonpregnant ovine endometrium and myometrium (Wu et al., 1997; Wijayagunawardane et al., 1991; Fu et al., 1990). These data support a role for estradiol in the regulation of PG synthesis by intrauterine, non-trophoblast tissue while arguing against a role for estradiol in the regulation of trophoblast PGHS-II. Recent studies have reported the presence of the estrogen receptor (ER) within non-trophoblast intrauterine tissues including the maternal placental villi, endometrium and myometrium of the sheep in late gestation. The ER was absent from placental trophoblast cells (Leung et al., 1999). These data argue against an estradiol effect on PGHS-II within the fetal trophoblast tissue but are consistent with ER-mediated, estradiol regulation of PGHS-II within the endometrium of pregnant sheep. The PGHS-II promoter does not contain an estrogen response element but does contain an AP-1 site (Inoue et al., 1995; Xu et al., 1995; Tazawa et al., 1994). Estradiol has been shown to interact with the AP-1 site to induce gene expression (McKay et al., 1999). Thus, we suggest that placental estradiol upregulates maternal endometrial PGHS-II expression and PGF\textsubscript{2\alpha} output at the onset of ovine parturition; this effect may be direct, mediated by the ER. Early studies using non-pregnant sheep showed that progesterone treatment increased intrauterine prostaglandin synthetic activity and was a prerequisite to the additional effects of estradiol (Louis et al., 1977). Therefore, we should not exclude the role progesterone plays in the regulation of prostaglandin production.

In addition to the observed changes in PGHS-II expression within the fetal trophoblast and maternal endometrial tissue we cannot exclude possible changes in the
expression and activity of other key enzymes in the PG biosynthetic pathway including PGE isomerase, PGF synthase and prostaglandin dehydrogenase (PGDH). To date little information is available regarding the expression and activity of these enzymes within the ovine intrauterine tissues. PGF synthase mRNA has recently been identified in the ovine maternal placenta, endometrium and myometrium (Wu et al, 2000). The expression of this enzyme decreased within the endometrium during betamethasone induced preterm labor and remained unchanged in all three tissues with spontaneous term labor (Wu et al, 2000). These data suggest PGF synthase expression does not regulate PGF$_{2\alpha}$ production and also precludes the production of PGF$_{2\alpha}$ from the fetal placental. PGDH expression has been localized to the uninucleate trophoblast tissue of the placenta, the endometrium and myometrium. Based on the inhibitory effect of cortisol on PGDH expression/activity in human chorionic and placental trophoblast cells we would expect that intrafetal cortisol infusion would decrease PGDH expression within the ovine intrauterine tissues. The regulation of this key enzyme requires further investigation.

Placental PGE$_2$ production might play an important role in mediating fetal HPA axis activation and placental steroidogenesis at the onset of labor. Fetal plasma cortisol and PGE$_2$ concentrations increase with a similar time course over the last twenty days of gestation and are associated in a positive feedback manner (Challis et al, 1993). Intrafetal PGE$_2$ infusion increased fetal plasma cortisol and adrenocorticotropic hormone (ACTH) concentrations in late gestation (Ratter et al 1979; Louis et al 1976). Recently, we have shown that specific inhibition of PGHS-II blocked the increase in fetal plasma cortisol and ACTH concentrations induced by RU486 administration in late gestation sheep (McKeown et al, 2000). These data
suggest that placental PGE_2 may be important for sustaining activation of the HPA axis at the end of gestation and onset of labor.

Through gestation, the ovine placenta does not express the enzyme P450C17 and cannot convert C21 steroids to the C19 substrate required for estrogen synthesis; the placenta depends upon the provision of C19 steroid precursors by the fetal adrenal (Mason et al., 1989; Anderson et al., 1975). A prepartum induction of placental P450C17 expression leads to the local production of C19 steroids and a surge in placental estrogen synthesis (Mason et al., 1989; Anderson et al., 1975). The current hypothesis of ovine parturition suggests that the increase in fetal adrenal GC output directs the induction of placental P450C17; however, the mechanism responsible for this effect has not been documented. The expression of placental P450C17 occurs well after the increase in placental PGHS-II expression and PGE_2 production (Gyomory et al., 2000a; Gyomory et al., 2000b; Wu et al., 1999). Two studies have shown that PGs can increase the expression/activity of this enzyme: PGF_2α induced P450C17 in cultured bovine preovulatory cells and PGE_2 induced P450C17 in cultured bovine adrenal cells (Wijayagunawarde et al., 1991; Rainey et al., 1991). These data suggest that PGE_2 could act as a GC-stimulated intermediate capable of mediating an autocrine/paracrine induction of P450C17. This hypothesis remains to be examined.

The frequency of uterine contractions was attenuated in the absence of increased placental estradiol production, although, we did not find a delay in the activation of uterine contractility from contractures to contractions. Myometrial contractility is associated with induction of a specific cassette of contraction associated proteins (CAPs) including connexin 43, oxytocin receptor, ion channels and prostaglandin receptors (Lye, 1994). These proteins are responsible for the evolution of uterine activity from quiescence to contractility. We
suggest that endometrial PGHS-II may also be considered a 'contraction associated protein' contributing to this evolution of uterine activity. Once CAP expression has been initiated, the uterus can be stimulated to contract by a variety of uterotonins, in particular oxytocin and prostaglandin (Challis et al, 1984; Lye, 1994; Challis et al, 1997). CAP expression appears to be regulated by an increase in the ratio of estrogen to progesterone (E₂/P₄) in late gestation (Lye, 1994). In the present study, the E₂/P₄ ratio in the cortisol treated animals increased 24 fold at the end of the infusion period while the E₂/P₄ ratio of the cortisol+4OHA treated animals increased only 2 fold. Thus, the lack of change in the E₂/P₄ ratio observed in the cortisol +4-OHA treated animals may have failed to induce CAP expression thereby attenuating uterine activity. Furthermore, the inhibition of intrauterine PG production during spontaneous term labor decreased myometrial expression of the oxytocin receptor suggesting that PGs act in a positive feed-forward manner to increase CAP expression (Wu et al, 1997).

The lack of increase in PGF₂α may have limited CAP expression and contributed to the attenuation of uterine contractility in the cortisol+4-OHA treated animals. Alternatively, CAPs may have been induced but uterine contractility not initiated because the production of PGF₂α did not increase. These possibilities remain to be evaluated.

Based on the observations of the present study we propose a new model for the onset of parturition in sheep (Figure 3-11). We suggest that towards the end of gestation there is a gradual and sustained increase in the placental trophoblast expression of PGHS-II expression and PGE₂ production under the regulation of fetal cortisol produced from the maturation of the fetal HPA axis. Placental PGE₂ in turn mediates an autocrine/paracrine increase in placental P450c₁₇ expression/activity to promote placental estrogen production. PGE₂ also acts to sustain fetal HPA axis activation. Estrogen up-regulates the expression of maternal
endometrial PGHS-II and PGF$_{2\alpha}$ output as well as induces the expression of CAPs. Consequently, myometrial contractility is stimulated and labor ensues. This hypothesis follows a tissue specific progression of parturition events from a fetal signal to a maternal labor response.
Figure 3-11: Proposed Hypothesis of Ovine Parturition
Towards the end of gestation, fetal cortisol increases placental trophoblast expression of prostaglandin synthase type II (PGHS-II) expression and activity leading to PGE₂ production. Placental PGE₂ in turn mediates an autocrine/paracrine increase in placental P450C₁₇ expression/activity resulting in a surge in placental estrogen production that is superimposed on a gestation-dependent increase in estrogen output. Placental PGE₂ is also secreted into the fetal compartment where it acts to sustain fetal hypothalamic-pituitary-adrenal axis activation. Estrogen up-regulates the expression of maternal endometrial PGHS-II, leading to increased PGF₂α output. Estrogen also induces the expression of contraction associated proteins (CAPs). Consequently, myometrial contractility is stimulated and labor and delivery of the fetus ensues.
Chapter Four

Regulatory Mechanisms Involved in the Separate Estradiol Independent and Estradiol Dependent Pathways of Intrauterine Prostaglandin Production and Uterine Activity
4.1. INTRODUCTION

We have demonstrated that intrafetal cortisol infusion leads to increased fetal trophoblast expression of PGHS-II and PGE₂ production independent of increases in placental estradiol synthesis. In contrast, we found that the maternal endometrial expression of PGHS-II, PGF₂α production and uterine contractility were dependent upon increased placental estradiol production. The differential effects of cortisol and estradiol could be due to the different distribution of the GC and estradiol receptors (GR and ER) within the intrauterine tissues. It has been demonstrated previously that placental PGHS-II expression and activity precedes the rise in plasma estradiol concentration and the induction of placental P450c17 expression, the critical enzyme responsible for the conversion of C21 steroids to C19 steroids providing an intrauterine source of estradiol precursor (Gyomorey et al. 2000). The model we have established will allow us to further examine the role of cortisol in the regulation of P450c17 expression. Intrauterine PG production is determined by both PG synthesis and PG metabolism. 15-dihydro prostaglandin dehydrogenase (PGDH) is the main enzyme responsible for PG metabolism; its expression and activity in other tissues is regulated by steroids, in particular cortisol and progesterone (Okita and Okita, 1996). We will determine the role of cortisol and estradiol in the regulation of ovine intrauterine PGDH expression. And finally, both increased placental estrogen synthesis and decreased intrauterine progesterone production regulate connexin 43, a critical CAP gene. We will examine the effect of cortisol in the presence and absence of increased placental estradiol synthesis on connexin 43 expression to elucidate the essential nature of estradiol in the regulation of uterine connexin 43 expression.
Recently the intrauterine expression of the GR and the ER at the end of ovine gestation and the onset of labor has been characterized (Gupta et al, 2000; Riley et al, 2000; Leung et al, 1999). GR was found to be co-expressed with PGHS-II in the uninucleate trophoblast cells of the ovine placentome (Gupta et al, 2000). The ER was localized to the endometrial epithelium and the uterine myocytes; PGHS-II expression was similarly localized to these maternal tissues (Gyomorey et al, 2000; Riley et al, 2000). The intrauterine expression of GR and ER as well as PGHS-II was found to increase with the onset of labor at term (Gyomorey et al, 2000; Gupta et al, 2000; Wu et al, 1999; Leung et al, 1999). These data suggest that the differential effects of cortisol and estradiol on intrauterine PGHS-II expression and PG production we documented in Chapter 3 may be due to the tissue specific expression of the GR and ER. We will examine the intrauterine tissue localization and level of expression of these receptors; we will correlate these findings with the intrauterine expression of PGHS-II to determine the potential relationship of intrauterine GR and ER expression in the cortisol-dependent and estradiol-dependent changes in intrauterine PG production.

The current hypothesis of ovine parturition suggests that fetal adrenal cortisol increases expression and activity of P450C17 within the placental trophoblast tissue (Mason et al, 1989. Mason et al, 1988; Anderson et al, 1975). However, a direct interaction between cortisol and P450C17 gene expression or activity has not been documented. Thus, the possibility of a GC stimulated intermediate leading to P450C17 induction can not be excluded. Studies examining the progression of parturition determined that the increase in trophoblast PGHS-II expression and PGE2 production preceded the induction of P450C17 expression and the terminal surge in estradiol production (Gyomorey et al, 2000). Furthermore, we
demonstrated that cortisol increased PGE\(_2\) production independent of increases in placental estradiol synthesis (*Chapter 3*). These findings suggest that PGE\(_2\) could act as a GC stimulated intermediate to elicit the increase in P450\(_{17}\) in an autocrine/paracrine manner. We will examine the expression of P450\(_{17}\) in the animals infused with cortisol +/- 4-OHA to further define the role of cortisol and estradiol in the regulation of P450\(_{17}\) expression.

Accelerated PG synthesis may not be the only mechanism responsible for the increase in PG production at the end of gestation. PGDH catalyzes the initial rate-limiting step in the conversion of active PGs to their inactive 15-keto metabolites (Okita and Okita, 1996). In the pregnant sheep, PGDH activity is present in the placenta, the endometrium and the myometrium between days 116 and 132 of gestation and at term (Riley et al. 2000; Kierse et al. 1978). Within these tissues immunoreactive PGDH was detected in the uninucleate placental trophoblast cells, the luminal endometrial epithelium and the smooth muscle of the myometrium (Riley et al. 2000). A decrease in the expression or activity of PGDH at the end of gestation would contribute to a net increase in intrauterine PG production. In the human, decreased chorionic, placental and myometrial expression of PGDH occurs with labor at term and preterm (*Giannoulias and Challis; unpublished results*: Patel et al. 1999). Trophoblast PGDH expression/activity is chronically maintained by progesterone (Patel et al, 1999). With labor, the increased intrauterine concentration of cortisol is thought to compete with progesterone at the GR leading to a down-regulation of PGDH and a decrease in the rate of PG metabolism (*Patel and Challis; unpublished results*). In the sheep at the end of gestation intrauterine progesterone production decreases and cortisol concentrations increase suggesting that intrauterine PGDH expression may be decreased by these steroid changes similar to the decrease in PGDH expression observed in
the human. We will examine the intrauterine tissue expression of PGDH in the animals treated with cortisol in the presence or absence of 4-OHA to determine the role of cortisol and estradiol in the regulation of ovine intrauterine tissue PG metabolism.

At term, in response to uterotonin stimulation, the uterus functions as a contractile syncytium attributed to the increased size and density of myometrial gap junctions. Various studies in a number of species have indicated that gap junctions and the expression of the gap junction protein connexin 43 may be modulated by an increase in placental estradiol synthesis and a decrease in intrauterine progesterone production. In particular, the mRNA and protein levels of connexin 43 in ovine myometrium were increased significantly with the onset of ACTH induced preterm labor: this increase was attributed to an increase in the plasma estradiol:progesterone ratio. However, this increase also occurred with a similar time course to the rise in fetal adrenal cortisol production suggesting that cortisol may play a role in the regulation of gap junction expression and the onset of uterine contractility. We will examine the effect of intrafetal cortisol infusion in the presence and absence of increased placental estradiol synthesis to determine the essential nature of estradiol and the potential role of cortisol in the regulation of gap junction expression and uterine contractility.

In summary, the objectives of the present study were fourfold: 1) to determine the role of intrauterine steroid receptor expression in the estrogen independent and dependent regulation of PG production; 2) to further define the role of cortisol in the regulation of P450c17 expression; 3) to determine the effects of cortisol in the presence or absence of increased placental estradiol synthesis on the regulation of intrauterine PGDH expression and 4) to determine the role of estradiol and cortisol in the regulation of connexin 43 expression and uterine activity.
4.2. METHODS

4.2.1. Animals, Experimental Protocol and Tissue Collection

The animals used, treatment protocol (intrafetal infusion of cortisol +/- 4-OHA, saline +/- 4-OHA for 80h; n=5 per group) and intrauterine tissue collection were described in section 3.2. Placental, endometrial and myometrial tissue to be used for Northern and Western Blot analysis were snap frozen in liquid nitrogen and stored at -80°C. Placentomes and cross sectional pieces of myometrium, endometrium and fetal membranes to be used for immunohistochemical studies were fixed in 4% paraformaldehyde/0.2% glutaraldehyde.

4.2.2. Western Blot Analysis of Protein Expression

Western blots were prepared using protein extracted from frozen tissues as described in section 3.2.8. Blots were incubated with primary antibodies (GR\text{total} [5 \mu g/ml dilution in blocking solution; GR\text{a} [5 \mu g/ml dilution in blocking solution (Affinity Bioreagents; Neshanic Station, USA)]; PGDH [1:200-1:500 dilution in blocking solution (gift from Dr H-H. Tai)]; P450\text{C17} [1:500 in blocking solution (gift from Dr F. Labrie)]; ER\text{a} [1:100 dilution in blocking solution (Novacastra Laboratories Ltd.; Newcastle upon Tyne, UK); ER\text{b} [1:200 dilution in blocking solution (Santa Cruz Biochemical; SanFrancisco, USA)] for 1h at room temperature. Blots were then rinsed 5x5min with PBS-T and incubated with secondary antiserum conjugated with horseradish peroxidase for 1h [1:1000-1:4000 dilution in blocking serum (Amersham Life Science; Buckinghamshire, England)]. Blots were washed 6x5min and the antibody-antigen complex was detected using the Amersham ECL detection system (Amersham Life Science; Buckinghamshire, England). Blots were exposed to X-ray film (Eastman Kodak X-Omat Blue; Rochester, USA) for serial exposure times to determine the appropriate concentration of protein loading and exposure time to ensure that the protein
signal was within the linear response range. The intensity of the protein signal was quantified using computerized image analysis (ScanJet 6200C Hewlett Packard: software by Scion Image, National Institute of Health; USA). Positive tissue controls were used for each protein analyzed: rat liver was used for GR, rat ovary was used for ER, human placenta was used for PGDH, fetal sheep adrenal was used for P450c17.

4.2.3. Immunohistochemical Protein Localization

Tissue samples were paraffin embedded [Department of Pathology, University of Toronto Health Network, General Division (Toronto; Canada)]. The tissues were cut in 5 μm sections, mounted on Fisher Superfrost slides (Fisher Scientific; Nepean, Canada), deparaffinized with three serial washes of xylene (BDH Inc.; Toronto, Canada) and rehydrated with serial increasing dilutions of ethanol (100-50%) ending with two washes of PBS. Endogenous peroxidase activity was quenched by pretreatment with 0.3% hydrogen peroxide (ACP Chemicals Inc.; Montreal, Canada) in PBS. Tissues were then washed 2x5min in PBS and incubated with immune serum that served as a blocking agent for nonspecific binding. Antibodies (as described in section 4.2.1. and section 3.2.8.) were diluted in PBS with 0.2% bovine serum albumin (GRα 1:100; PGHS-II 1:500; ERβ 1:400).

A second ERα antibody (Santa Cruz Biochemical; San Francisco, USA) was used at a dilution of 1:100. Primary antibody was applied and the tissues were incubated at 4°C for 18-24h. Primary antibody binding was visualized as per the Vectastain ABC Kit (Vector Laboratories; Burlingame, USA). Tissues were washed with 2x5min PBS, incubated with biotinylated secondary antibody for 120 min, washed again and incubated with the ABC (avidin biotin-peroxidase complex) for 120 min. Following a final 2x5min PBS wash, the immunoreactive protein was visualized after the addition of the peroxidase substrate
3.3'diaminobenzidine (Sigma Chemical Co., St. Louis, MO) for 2-5 min. Tissues were counterstained with hematoxylin, dehydrated in graded ethanol, cleared and cover slips applied. Control tissues were treated identically with the omission of the primary antibody.

4.2.4. *Northern Blot Analysis of Messenger RNA Expression*

Frozen tissue samples (~160 mg) were homogenized for 1-2 min in 2mL Trizol\textsuperscript{TM} (Gibco BRL: Burlington, Canada); tissue homogenate aliquots (~1 ml) were pipetted into flip-cap vials, layered with 200 \mu L chloroform (Sigma Chemical Co.; St. Louis, USA), mixed with a vortex and centrifuged at 4\degree C, 12 000g for 15 min. The supernatants were collected, rinsed with 500 \mu l isopropyl alcohol (BDH Inc.; Toronto, Canada) and centrifuged at 4\degree C, 12 000g for 15 min. The supernatants were poured off, the total RNA pellet re-suspended in 75% ethanol and the samples were centrifuged a third time at 7 500g for 5 min. The RNA pellet was suspended in 100 \mu l DEPC treated water [0.01% v/v diethyl-pyrocarbonate in deionized, distilled water, autoclaved (Sigma Chemical Co., St. Louis, MO)] and incubated at 65\degree C for 15 min; the sample volume was increased to 400\mu l with 100% ethanol and stored at -80\degree C. The purity and concentration of total RNA was determined by spectrophotometric analysis. The total RNA (2\mu l RNA in 998 \mu l DEPC water) absorbance was determined at 260 nm (nucleic acid concentration) and at 280 nm (protein concentration); RNA samples with an absorbance ratio (260nm:280 nm) greater than 1.2 were deemed acceptable for further analysis. The integrity of the RNA was also determined. Total RNA samples (10\mu g) were separated by electrophoresis in an agarose-formaldehyde gel (1% w/v). The gel was stained with ethidium bromide solution [0.13% v/v 2\beta-mercaptoethanol; 0.001% v/v ethidium bromide (Sigma Chemical Co.; St Louis, USA) in
DEPC water] overnight and visualized under ultra violet light. Intact RNA was viewed as two distinct bands corresponding to 18S and 28S ribosomal RNA.

Total RNA samples (30 μg) were air dried under speed vacuum, reconstituted with 13.2 μl loading buffer [0.5 x northern running buffer (5xNRB: 0.1 M 3-N-morpholino-propanesulfonic acid, 50 mM sodium acetate, 5mM ethylenediaminetetraacetic acid disodium salt dihydrate (Sigma Chemical Co.; St. Louis, USA), 6% deionized formaldehyde, 0.5% deionized formamide (BDH Inc.: Toronto, Canada) in DEPC treated water] and incubated at 65°C for 15 min. Samples were placed on ice and 2 μL loading dye [25% bromphenol blue, 25% xylene cyanol FF, 0.5M ethylenediaminetetraacetic acid disodium salt dihydrate, 50% glycerol (Sigma Chemical Co.; St. Louis, USA)] added to each sample. Samples were then separated by electrophoresis in an agarose-formaldehyde gel (1% w/v) with 1 x northern running buffer. The gel was then soaked in 0.05N sodium hydroxide for 20 min, rinsed with 3x5min DEPC treated water and soaked in 10X SCC [single strength 1xSSC: 150 mM sodium chloride, 15mM sodium citrate (Sigma Chemical Co.; St. Louis, USA)]. The gel was then placed in contact with a positively charged nylon membrane [Zeta Probe GT Genomic (Bio-Rad; Richmond, USA)] and the RNA was transferred to the membrane by capillary elution for 12-16h. The membrane was soaked in 2xSSC for 5 min and allowed to air dry. The RNA was crosslinked to the membrane by ultraviolet radiation (125 mJoule, 125 sec) in a UV chamber [Bio-Rad GS Gene Linker™ (Bio-Rad; Richmond, USA)]. The membrane was wrapped in plastic wrap and stored at −20°C until hybridization.

Prior to hybridization, the membrane was allowed to come to room temperature and then incubated in hybridization buffer [1M sodium phosphate, 20%w/v sodium dodecyl sulphate, 0.3% v/v deionized formamide, 1% w/v bovine serum albumin (Sigma Chemical
Co. St. Louis, USA) for ~1h at 48°C under rotation. The blot was then hybridized in the same buffer and under the same conditions in the presence of radiolabelled probe (see below) for 18-20h. Post hybridization, the blots were washed 2x15 min with 150mM anhydrous sodium diphosphate/0.1% sodium dodecyl sulphate at 55°C, under rotation and then 3x15 min with 30mM anhydrous disodium phosphate/0.1% sodium dodecyl sulphate at 55°C, under rotation. The membrane was allowed to air dry and then sealed in a plastic bag. The membrane was exposed to X-ray film -80°C (Kodak Eastman BioMax AR; Rochester, USA); serial exposure times were used to ensure that the signal was within the linear range of the film. After autoradiographic exposure, the blot was stripped (3x20 min washes with 0.01xSSC/0.5% sodium dodecyl sulphate at 85-90°C, under agitation), prehybridized as described and reprobed with 32P-labelled cDNA to mouse 18S ribosomal RNA (gift from Dr G. Hammond) and exposed to x-ray film. The relative optical density of the probed RNA signal and the 18S signal were determined using computerized image analysis. Results are expressed as a ratio of probed mRNA signal:18S rRNA signal to correct for differences in gel loading and transfer of each sample.

A cDNA probe based on the structure of the rat connexin 43 gene [gift from Dr D. Paul; (McNutt et al, 1994)] was used. The probe was labeled with Ready-To-Go™ DNA Labeling Beads (-dCTP) (Amersham Pharmacia Biotech Inc.; Buckinghamshire, UK). The cDNA probe (60 ng) was denatured in boiling water for 10 min and then cooled on ice. The cDNA was added to the reaction mix (a room temperature stable bead containing buffer, dATP, dGTP, dTTP, FPLCpure™, Klenow Fragment (7-12 units) and random oligodeoxyribonucleotides) with [32P]dCTP [(5 μL: 3400 Ci/mmol); NEN, Dupont Canada Inc.; Mississauga, Canada]. The reaction mix was incubated at 37°C for 1h. The labeled
probe was eluted through a spin column (ProbeQuant G50 Micro Column; Amersham Pharmacia Biotech Inc.; Buckinghamshire, UK). The calculated specific activity of the connexin 43 probe was 0.92 dpm/µg. The labeled probe was then added to 6 ml of the hybridization buffer and incubated with the membrane as described in the previous section.

4.2.5. Statistical Analysis

All data were analyzed using parametric statistical tests after the data was submitted to both normality and equal variance testing. The mRNA and protein data were analyzed by one way analyses of variance followed by a post hoc, all pairwise multiple comparison Fisher's Test of Least Significant Difference determine significant interactions. Significance was set at $p < 0.05$. Data are presented as the mean +/- standard error of the mean (SEM) for n=5 per group.

RESULTS

4.3.1. Placental PGHS-II, Steroid Hormone Receptor and P450c17 Expression

The placental expression of total GC receptor immunoreactive protein (Ir-GRtotal) did not differ significantly amongst the four groups of animals (Figure 4-1a). However, the placental expression of the GC receptor alpha isoform (Ir-GRα) was increased significantly by both cortisol and cortisol+4OHA infusion ($p < 0.05$; Fisher’s Test of Least Significant Difference) (Figure 4-1b). Ir-GRα expression was localized to the uninucleate fetal trophoblast cells of the placenta and was excluded from the maternal syncytiun and outer capsule as well as the binucleate trophoblast cells (Figure 4-2). Immunoreactive PGHS-II was similarly localized to the uninucleate fetal trophoblast cells and excluded from the maternal tissue of the placentome and the binucleate trophoblast cells (Figure 4-2).
immunoreactive expression of estradiol receptor α (ir-ERα) and estradiol receptor β (ir-ERβ) was excluded from uninucleate fetal trophoblast tissue (Figure 4-2). Placental P450C17 immunoreactive protein expression was increased significantly by both cortisol and cortisol+4OHA infusion (p<0.05: Fisher’s Test of Least Significant Difference) (Figure 4-3).

4.3.2. Endometrial PGHS-II and Steroid Hormone Receptor Expression

The immunoreactive expression ERα and ERβ within the intercotytedonary endometrium did not differ amongst the four groups of animals (Figure 4-4). Ir-ERα and Ir-ERβ were localized to the luminal endometrial epithelium and scattered glandular epithelial cells with the intercotytedonary endometium (Figure 4-5). Immunoreactive PGHS-II was similarly expressed within the luminal endometrial epithelium (Figure 4-5).

4.3.3. Intrauterine PGDH Immunoreactive Protein Expression

Placental trophoblast PGDH immunoreactive protein expression was increased significantly animals (p<0.05: Fisher’s Test of Least Significant Difference) by both cortisol and cortisol+4OHA infusion (Figure 4-6). The level of PGDH immunoreactive protein expression in the intercotytedonary endometrium and myometrium was not significantly different amongst the four groups of animals (Figure 4-7a; Figure 4-7b).

4.3.4. Uterine PGHS-II, Estradiol Receptor and Connexin 43 Expression

Myometrial PGHS-I and PGHS-II immunoreactive protein expression were not increased by either cortisol or cortisol+4OHA infusion (Figure 4-8a; 4-8b.). The level of immunoreactive ERα and ERβ expression in the myometrium also was not significantly different amongst the four groups of animals (Figure 4-9a; Figure 4-9b). Myometrial
connexin 43 mRNA and protein expression were not altered significantly by either cortisol or cortisol + 4-OHA infusion (Figure 4-10a; 4-10b).
Figure 4-1a

Figure 4-1b

Figure 4-1: Placental Immunoreactive Glucocorticoid Receptor Expression

Values are presented as mean +/- SEM over for n=5 animals in each group. Statistical analysis was performed using a one way analysis of variance followed by a post hoc Fisher's Test of Least Significant Difference (p=0.05). The placental expression of total glucocorticoid receptor immunoreactive protein (GR<sub>total</sub>) did not differ significantly amongst the four groups of animals (Figure 4-1a. Expression of the glucocorticoid receptor alpha isoform (GR<sub>α</sub>) was increased significantly by both cortisol and cortisol+4OHA infusion (a), there were no statistical difference between the cortisol and cortisol+4-OHA groups (Figure 4-1b). 4-OHA = 4-hydroxyandrostenedione
Figure 4-2: Localization of Immunoreactive PGHS-II, GR α and ER Expression in the Ovine Placentome

Representative immunohistochemical staining of immunoreactive GR α and PGHS-II within the ovine placentome. GR α is localized to the uninucleate fetal trophoblast cells and excluded from the maternal syncytiunm and the binucleate trophoblast cells. PGHS-II is similarly localized to the uninucleate fetal trophoblast tissue and excluded from the maternal tissue of the placentome and the binucleate trophoblast cells. Representative immunohistochemical staining of immunoreactive ERα (A) and ERβ (B); both receptors are excluded from the uninucleate fetal trophoblast tissue.

4-OHA = 4-hydroxyandrostenedione  \( a = \) uninucleate fetal trophoblast tissue

b = maternal syncytial tissue  \( c = \) binucleate trophoblast cell
**Figure 4-3: Placental P450c17hydroxylase Immunoreactive Protein Expression**

Values are presented as mean +/- SEM over for n=4 animals in each group. Statistical analysis was performed using a one way analysis of variance followed by a post hoc Fisher's Test of Least Significant Difference; significance was set at p=0.05. The placental immunoreactive protein expression of P450C17hydroxylase (ir-P450c17) was increased significantly by both cortisol and cortisol +4-OHA infusion (a); there was no statistical difference between the the cortisol and cortisol +4-OHA treated animals.
**Figure 4-4 a**

<table>
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<th>ERα Expression</th>
<th>Arbitrary Optical Density Units</th>
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<tr>
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</tr>
<tr>
<td>Cortisol + 4-OHA</td>
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<td>3</td>
</tr>
<tr>
<td>Saline</td>
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</tr>
<tr>
<td>Saline + 4-OHA</td>
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**Figure 4-4 b**

<table>
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</tr>
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</tr>
<tr>
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</tr>
</tbody>
</table>

**Figure 4-4: Intercotyledonary Endometrial Immunoreactive Estradiol Receptor Expression**

Values are presented as mean +/- SEM over n=5 animals in each group. Statistical analysis was performed using a one way analysis of variance (p=0.05). The intercotyledonary endometrial expression of estradiol receptor α and β immunoreactive protein (ERα and ERβ) did not differ significantly amongst the four groups of animals.

4-OHA = 4-hydroxyandrostenedione
Figure 4-5: Localization of Immunoreactive PGHS-II and ER Expression in the Intercotyledonary Endometrium

Representative immunohistochemical staining of immunoreactive PGHS-II, ERα and ERβ within the intercotyledonary endometrium. PGHS-II, ERα and ERβ are localized with the luminal epithelium of the endometrium.

4-OHA = 4-hydroxyandrostendione

le: luminal epithelium
ge: glandular epithelium
Figure 4-6: Placental Immunoreactive PGDH Expression

Values are presented as mean +/- SEM over for n=5 animals in each group. Statistical analysis was performed using a one way analysis of variance followed by a post hoc Fisher’s Test of Least Significant Difference (p=0.05). The placental immunoreactive PGDH expression was increased significantly by both cortisol and cortisol +4-OHA infusion (a); there was no statistical difference between the the cortisol and cortisol+4-OHA treated animals.

4-OHA= 4-hydroxyandrostenedione
Figure 4-7: Maternal Intrauterine Tissue Immunoreactive PGDH Expression

Values are presented as mean +/- SEM over n=5 animals in each group. Statistical analysis was performed using a one-way analysis of variance (p=0.05). The immunoreactive protein expression of PGDH within the intercotyledonary endometrium and the myometrium did not differ statistically amongst the four groups of animals.

4-OHA= 4-hydroxyandrostenedione
Figure 4-8: Myometrial Immunoreactive PGHS Protein Expression

Values are presented as mean +/- SEM over for n=5 animals in each group. Statistical analysis was performed using a one way analysis of variance (p=0.05). The myometrial immunoreactive protein expression of PGHS-II and PGHS-I did not differ significantly amongst the four groups of animals.

4-OHA = 4-hydroxyandrostenedione
Immunoreactive 3

ERα Expression

Figure 4.9a: 4-Hydroxyandrostenedione

Values are presented as mean +/- SEM over n=5 animals in each group. Statistical analysis was performed using a one-way analysis of variance (p=0.05). The immunoreactive endometrial expression of cytoskeletal receptor α and β did not differ significantly amongst the four groups of animals.

Figure 4.9b: 4-Hydroxyandrostenedione

Immunoreactive 3

ERβ Expression

Arbitrary Optical Density Units

0

1

2

3

4

5

0

1

2

Arbitrary Optical Density Units

Figure 4.9b

Immunoreactive 3

ERα Expression

Arbitrary Optical Density Units

0

1

2

Arbitrary Optical Density Units

Figure 4.9a
Connexin 43 mRNA and Immuno-reactive Protein Expression

**Figure 4-10a:** Myocardial Connexin 43 mRNA and Immuno-reactive Protein Expression

- **4-OHA + 4-OHA + Saline**
- **Saline**
- **4-OHA + Saline**
- **Control**

<table>
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<th>Immuno-reactive Protein Expression</th>
<th>Arbitrary Optical Density Units</th>
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</tr>
<tr>
<td>8</td>
<td>0</td>
<td>16</td>
</tr>
</tbody>
</table>

Connexin 43 Expression

- **Cx43 mRNA : 18 S mRNA**

**Figure 4-10b**

- **CX43 mRNA**
- **0.0000**
- **0.04**
- **0.12**
- **0.16**

Immuno-reactive

- **4-OHA + 4-OHA + Saline**
- **Saline**
- **4-OHA + Saline**
- **Control**
4.4. Discussion

We have demonstrated that intrafetal cortisol infusion in the presence and absence of increased placental estradiol output leads to simultaneous increases in the expression of GRα, PGHS-II and PGDH within the fetal trophoblast tissue. We determined that trophoblast cells express both PGHS-II and GRα and the expression of both ERα and ERβ is excluded from this tissue. We suggest that the cortisol-dependent/estradiol independent increase in trophoblast PGHS-II expression may be mediated by a receptor-dependent, positive feedback mechanism. The exclusion of the ER from this tissue precludes an effect of estradiol on trophoblast PGHS-II expression or activity. Paradoxically, the increased trophoblast expression of GRα was also correlated with an increase in trophoblast PGDH expression suggesting that cortisol may increase PGDH in a receptor mediated manner. We have demonstrated that placental trophoblast P450C17 expression was increased by cortisol independent of a feedback effect of placental estradiol synthesis; these observations confirm the role of GC or a GC-stimulated intermediate in the induction of trophoblast P450C17 expression. Endometrial epithelial cells were found to express PGHS-II, ERα and ERβ suggesting that the estradiol-dependent increase in endometrial PGHS-II expression may be mediated in a receptor-dependent manner. The findings of the present study suggest that cortisol induced increases in intrauterine PG production occur independently of any change in the maternal intrauterine tissue expression of PGDH. And finally, we were unable to demonstrate alterations in the myometrial expression of PGHS-II or connexin 43; these findings may reflect differences in the mechanism of cortisol induced preterm labor compared with term labor.
We have previously demonstrated (Chapter 3) that intrafetal cortisol infusion in the presence or absence of increased placental estradiol synthesis increases fetal trophoblast PGHS-II expression. The trophoblast cell also expresses GRα and the findings presented in this chapter show that the expression of this receptor increases with cortisol infusion regardless of changes in placental estrogen output. A similar increase in PGHS-II and GR expression was observed with spontaneous term labor (Gupta et al, 2000). These observations suggest that cortisol can increase the expression of its own active receptor isoform, GRα, that in turn mediates an increase in PGHS-II gene expression. A direct correlation between receptor number and cellular responsiveness has been demonstrated in a variety of cell lines leading to the concept of GR autoregulation (Schmidt et al, 1994). Although autoregulation has generally been exhibited in the form of receptor down-regulation (negative auto-regulation) hormone mediated up-regulation has been reported in a variety of tissues including the ovine fetal hippocampus and pituitary, a human leukemic T cell line and a human B-cell line (Ramdas et al, 1999; Matthews et al, 1995; Denton et al, 1993). In particular, we found a similar increase in the fetal pituitary expression of GR mRNA in the fetuses treated with cortisol+4-OHA suggesting that cortisol, independent of increases in placental estradiol synthesis, upregulated the expression of its own receptor in the pituitary (Holloway et al, 2000). A change in the receptor transcription rate, an alteration in the receptor mRNA stability and/or post-translational modifications may mediate the increase in GR expression. In addition, both PGE₂ and cAMP have been found to increase GR expression in human articular chondrocytes suggesting that PGE₂ could act in an autocrine/paracrine manner to increase trophoblast GRα expression (DiBattista et al, 1991). The expression of GRtotal reflects the levels of both the active GRα isoform and the inactive
GRβ isoform which are derived from alternate splicing of the GR mRNA transcript. The regions of the GR gene cloned from the sheep reflect the common sequences between GRα and GRβ cloned in the human and the rodent. In the present study we have assumed that GRα and GRβ isoforms expressed in the sheep are similar to that described in the human and the rodent. Given that the level of GRtotal protein was not altered but the GRα level did increase, cortisol and/or PGE₂ may have mediated a shift in GR mRNA splicing towards the active form of the receptor; however mechanisms regulating alternative splicing remain unclear. In addition, the immunohistochemical technique used in this study did not allow us to detect a translocation of the GRα from the cytoplasm to the nucleus; however, we assume that cortisol binding to GRα in the trophoblast cell cytoplasm mediated a shift of the ligand bound receptor to the nucleus. Therefore, we conclude that cortisol increased in fetal trophoblast PGHS-II gene expression in a receptor-mediated, positive feed-back manner.

We and others have demonstrated that P450c₁₇ expression and activity in the ovine placenta increases immediately prepartum; it has been suggested that this increase may be mediated by fetal adrenal GC (Mason et al, 1989, Mason et al, 1988; Anderson et al, 1975). However, the in vivo studies examining cortisol effect on P450c₁₇ expression/activity failed to demonstrate a direct, receptor mediated effect of GC on P450c₁₇ expression and did not consider the possibility of an indirect stimulatory effect mediated by a cortisol-induced intermediate. Gyomorey et al (2000) demonstrated that trophoblast PGHS-II expression and PGE₂ production preceded the trophoblast expression of P450c₁₇ and the rise in plasma estradiol concentration. Thus, we hypothesized that PGE₂ could act as a GC-stimulated intermediate to elicit an autocrine/paracrine upregulation of P450c₁₇ within fetal trophoblast cells of the placenta and lead to the surge in placental estradiol synthesis observed in the final
days of gestation. In the present study we demonstrated that P450C17 expression increases in the presence of both increased fetal plasma cortisol concentration and placental trophoblast PGE₂ production; this increase occurred independent of a feedback effect of placental estradiol synthesis. Although we do not provide direct evidence of PG effects on P450C17 expression our findings support the hypothesis that PGE₂ or another GC-stimulated intermediate may increase P450C17 expression at the end of gestation. Further examination of this hypothesis is warranted.

We have demonstrated previously that maternal endometrial PGHS-II expression and PGF₂α output were dependent upon cortisol-induced increases in placental estradiol output (Chapter 3). Previous studies have localized ER expression, in particular ERα, to the luminal endometrial epithelium (Wu et al, 1997; Leung et al, 1999; Riley et al, 2000). An increase in ER mRNA expression and the mRNA expression of its associated heat shock proteins 70 and 90 has been reported with the onset of labor at term (Wu et al, 1997; Leung et al. 1999). Further, inhibition of intrauterine PG production by nimesulide, a specific PGHS-II inhibitor, during term labor led to a decrease in endometrial ER mRNA expression suggesting that PGs may play a role in the up-regulation of ER at term (Wu et al, 1998). In the present study we characterized the expression of the ERα and ERβ within the intercotyledonary endometrium and the exclusion of these receptors from the fetal trophoblast tissue. The level of expression of either receptor was not altered by cortisol infusion in the presence or absence of increased placental estradiol production. ERα and ERβ are not isoforms but distinct proteins encoded by separate genes on separate chromosomes; both are functional receptors. The basal level of ER expression in the endometrium may have been sufficient to elicit the changes in PGHS-II expression during cortisol induced preterm
labor. As discussed in Chapter 3 estradiol mediates an increase in PGHS-II expression in a variety of tissues including non-pregnant ovine endometrium and myometrium. Although the PGHS-II gene does not express an estrogen response element, it has been suggested that estradiol mediates an increase in PGHS-II gene expression through activation of the AP-1 site within the 5' promoter (MacKay et al, 1999). Therefore, we conclude that the estradiol-dependent increase in endometrial PGHS-II expression and PGF2α expression is mediated by the expression of the ER within this tissue.

We have determined that the increase in PG output by the maternal intrauterine tissues (endometrium and myometrium) occurs independently of altered expression of PGDH. Paradoxically, fetal trophoblast PGDH mRNA and protein expression was increased by intrafetal cortisol infusion irrespective of increased placental estradiol output and decreased placental progesterone secretion (see Chapter 3). Our findings are consistent with those of Riley et al (2000) who demonstrated that ovine trophoblast PG metabolism remained constant from day 70 to 135 of gestation but increased by approximately 3 fold with the spontaneous onset of term labor and appeared to be independent of changes in intrauterine estrogen concentration. These studies are in direct contrast to reports of decreased intrauterine PGDH expression and activity with the onset of labor at term or preterm in the human (Giannoulias and Challis, unpublished results; Patel et al, 1999). In addition, GCs inhibited PGDH expression and activity in cultured human trophoblast cells (Patel et al, 1999). In these studies, progesterone was found to maintain PGDH expression/activity and that cortisol was suggested to interfere with progesterone at the GR to cause a dose dependent suppression of PGDH expression/activity (Patel and Challis; unpublished results).

A similar inhibitory effect of GC on PGDH activity has been reported in human
promonocytic U937 cells (Tong et al, 2000). However, prenatal dexamethasone administration increased PGDH expression significantly in fetal rat lung tissue and GC increased PGDH expression in human erythroleukemia cells (Xun et al, 1991; Tsai et al, 1987). These observations suggest that GC effects on PGDH are both tissue and species dependent. The physiological significance of increasing placental trophoblast PGDH expression and activity at the onset of ovine parturition is not yet understood, nor is the significance of expressing both PG synthesizing and metabolizing enzymes within the same cell type. PGDH may act to limit the intracellular activity of PGE2 within the ovine trophoblast cell. Alternatively, PGDH may limit the transfer of bio-active prostaglandins into the fetal and/or maternal circulation. Regardless of the increased expression/activity PGDH in the trophoblast tissue and sustained PGDH expression/activity within the maternal intrauterine tissues, the net intrauterine output of PGE2 and PGF2α increases through the end of gestation and the progression of labor. Further investigation into the role and regulation of trophoblast PGDH expression at the end of gestation and the onset of labor is required.

We did not observe changes in myometrial PGHS-II and ER expression in the four experimental groups of animals. Several studies have reported increased uterine PGHS-II and ER expression with the spontaneous term labor; however, these increased occur late in the progression of labor events (Gyomori et al, 2000; Wu et al, 1999). We terminated our experimental protocol at the early onset of active labor. Therefore, we may have missed increases in the expression ER and PGHS-II due to the relative short exposure to increased plasma estradiol concentration, the residual suppressive effects of placental progesterone production or the short duration of uterine activity. Wu et al (1999) reported that the myometrial sample was taken from the mid-ventral uterine body, an area much closer to the
level of the cervix relative to where our sample was taken. It has previously been demonstrated that PG play an important role in the induction of cervical compliance with ovine labor. PGE₂ and prostacyclin produced by the cervix mediate these changes. Increased expression of PGHS-II within the uterine body and not the uterine horn may play a role in the production of these PGs and may be an example of functional regionalization within the ovine uterus. The precise role and regulation of myometrial PGHS-II expression remains to be examined.

We also were unable to demonstrate a change in the myometrial expression of the gap junction protein connexin 43. Our findings are in contrast to other studies describing an increase in the size and number of myometrial gap junctions with the spontaneous onset of labor at term and an increase in connexin 43 gene expression with the onset of ACTH induced preterm labor (Verhoeff et al, 1985; McNutt et al, 1994). However, there are several differences between the study by McNutt et al (1994) and our own which may, in part, account for the differing results. In our study, the myometrial sample was collected from the lower region of the pregnant horn, towards the junction of the uterine horn and body as compared to the mid ventral horn region collected by McNutt et al (1994). The difference in expression of connexin 43 may reflect regional differences in myometrial expression of this CAP similar to regional differences observed in the human pregnant uterus (Sparey et al, 1999). Secondly, McNutt et al (1994) used the intrafetal administration of ACTH to prematurely induce labor. We infused a high concentration of cortisol in order to achieve dramatic increases in fetal and maternal plasma estradiol concentrations as well as a dramatic decrease in the maternal plasma progesterone concentration. These rapid changes in the plasma steroid profile may have not been sufficient to elicit increases in connexin 43 mRNA
or protein expression. Harding et al (1982) reported that the time interval between increased uterine activity and delivery was shorter by ~50% in preterm induced labor as compared to spontaneous term labor and speculated that this effect was due to dramatic fetal steroid changes. The maternal plasma level of progesterone in our study did not decrease until the final 8h of the cortisol infusion. Progesterone effects have been found to remain up to 48h after withdrawal of the steroid suggesting that progesterone may still be exerting an inhibitory effect to limit connexin 43 expression. Furthermore, alterations in post-translational processing, intracellular trafficking of connexin 43 and/or gap junction assembly may have occurred in response to cortisol and/or estradiol to increase the gap junction density. Thus, by measuring connexin 43 expression in whole tissue homogenate rather than gap junction size or number we were unable to distinguish any changes at these levels of gap junction expression. Finally, a change in connexin 43 expression may not have been required to mediate the increase in uterine activity observed prior to sacrifice. We examined the level of connexin 43 at three points in gestation (d100, d130 and term) and found that connexin 43 expression was present prior to term and increased with gestational age (data not shown). Verhoeff et al (1985) demonstrated that gap junctions were found at all times in gestation at low levels; the percentage of myometrial cell membrane occupied by gap junctions was ~0.1% between days 110-124 of gestation, decreased to 0.048% 2-3 days before delivery and then dramatically increased to ~0.2% during labor. We demonstrated that at ~d130-5 gestation, in the presence of increased concentration of the uterotonic PGF2α, uterine motility can be initiated even though the amount of myometrial cell membrane occupied by gap junctions is 50% less than at term labor. These findings suggest that a gap junction membrane density lower than observed at term labor may be sufficient to mediate
the propagation of signals required for polarized uterine activity. This observation supports the hypothesis that there may be different mechanisms of uterine motility mediating preterm induced labor as compared with term labor. The role and regulation of connexin 43 expression in the ovine myometrium at term and preterm labor requires further investigation.

Based on the findings presented in Chapter 3 and the present chapter we propose a new model for the onset of ovine parturition (Figure 4-11). We suggest that towards the end of gestation there is a gradual and sustained increase in fetal adrenal cortisol production produced in response to maturation of the fetal hypothalamic-pituitary-adrenal axis and leading to a receptor-mediated, feed-forward increase in fetal trophoblast PGHS-II expression and PGE_2 output. Placental PGE_2 in turn mediates an autocrine/paracrine increase in trophoblast P450C17 expression/activity to promote placental estrogen production and also acts in a positive feedback manner to sustain fetal HPA axis activation. Estrogen, in a receptor-mediated manner, up-regulates the expression of maternal endometrial PGHS-II and perhaps myometrial PGHS-II leading to an increase in PGF_2α output. These changes in intrauterine PG production appear to occur in the presence of a paradoxical GC mediated increase in the expression of the PG metabolizing enzyme PGDH and in the absence of a change in maternal intrauterine tissue PG metabolism. Estrogen also increases the uterine expression of CAPs and the onset of uterine contractility. This hypothesis follows a tissue specific progression of parturition events from a fetal signal to a maternal labor response.
We suggest that towards the end of gestation there is a gradual and sustained increase in fetal adrenal cortisol production produced in response to maturation of the fetal HPA axis and leading to a receptor-mediated, feed-forward increase in fetal trophoblast PGHS-II expression and PGE₂ output. Placental PGE₂ in turn mediates an autocrine/paracrine increase in trophoblast P450<sub>C₁₇</sub> expression/activity to promote placental estrogen production and also acts in a positive feedback manner to sustain fetal hypothalamic-pituitary-adrenal axis activation. Estrogen, in a receptor-mediated manner, up-regulates the expression of maternal endometrial PGHS-II and perhaps myometrial PGHS-II leading to an increase in PGF₂α output. These changes in intrauterine PG production appear to occur in the presence of a paradoxical GR mediated increase in the expression of the PG metabolizing enzyme PGDH. Estrogen also increases the uterine expression of CAPs and the onset of uterine contractility. This hypothesis follows a tissue specific progression of parturition events from a fetal signal to a maternal labor response.

Figure 4-11: Proposed Hypothesis of Ovine Parturition
Chapter Five

The Characterization of Human Amnion Epithelial and Mesenchymal Cells; the Cellular Expression, Activity and Glucocorticoid Regulation of Prostaglandin Output.

The contents of this chapter have been published (Placenta; 21:394-401, 2000) and appear here with the permission of the journal (refer to attached authorization).

The experiments were designed, implemented and interpreted solely by Whittle WL. The co-authors Gibb W and Challis JRG were the principal investigators who supervised the experimental design, implementation and interpretation.
5.1. INTRODUCTION

The onset and progression of human parturition has been associated with elevated intrauterine tissue, amniotic fluid and maternal serum levels of prostaglandins, in particular PGE$_2$ and PGF$_{2\alpha}$ (Challis et al, 1988). These prostaglandins are likely involved in the mechanisms of cervical ripening, uterine contractility and the extracellular matrix remodeling involved in membrane rupture and placental separation (Challis et al, 1997). Prostaglandins are formed from the precursor arachidonic acid; phospholipase enzymes release arachidonic acid from phospholipid stores within the cell membrane. Arachidonate is metabolized by prostaglandin H synthase (PGHS) which catalyzes a cyclooxygenation and peroxidation reaction to form prostaglandin H$_2$ (PGH$_2$). PGH$_2$ is subsequently converted to a variety of prostaglandins including PGE$_2$ and PGF$_{2\alpha}$. Two isoforms of PGHS have been identified. PGHS type I (PGHS-I) has been described as a housekeeping gene with a wide tissue distribution in most mammalian species. PGHS type II (PGHS-II) has a limited distribution and expression but is highly inducible and upregulated by a variety of cytokines and growth factors (Goppelt-Streube et al, 1995; Inoue et al, 1995; Xu et al, 1995).

The amnion has been identified as a site of prostaglandin synthesis by the intrauterine tissues throughout gestation and parturition (Challis et al, 1988). The innermost layer of the amnion is a single layer of epithelial cells, which overlies the mesenchyme, an area of fibroblast cells embedded in an extracellular matrix (Bryant-Greenwood, 1998; Head et al, 1998). PGHS-I and II mRNA and protein have been identified within the amnion epithelium and the mesenchyme throughout gestation (Feuntes et al, 1996; Gibb et al, 1996, Hirst et al, 1995; Teixeria et al, 1994). The expression of PGHS-II increases at term and further with the onset of labour (Feuntes et al, 1996; Gibb et al, 1996; Hirst et al, 1995; Teixeria et al, 1994).
Quantitative PGHS-II mRNA expression was positively correlated with the increased PGHS activity observed within the whole amnion tissue (Hirst et al, 1995; Teixera et al, 1994). Thus, it was suggested that the surge of intrauterine prostaglandins observed at term and with onset of labour is due in part to the increased expression and activity of the inducible PGHS-II within the whole amnion.

Primary cell culture preparations have been used to study the regulation of amnion prostaglandin synthesis: GCs have been found to be positive regulators of cultured amnion cell prostaglandin production (Economopoulos et al, 1996; Smeija et al, 1993; Zakar et al, 1993; Potestio et al, 1988). It has recently been shown that amnion culture preparations may contain both epithelial and mesenchymal cells in varying proportions (Economopoulos et al, 1996). In a mixed cell culture preparation, GC exposure upregulated PGHS-II mRNA and PGHS-II immunoreactive protein expression in amnion mesenchymal cells but not in amnion epithelial cells (Economopoulos et al, 1996). These data suggest that the mesenchymal cells may be responsible for the increase in amnion PGE₂ biosynthesis in response to GC exposure; however, the possibility of an epithelial-derived, GC-stimulated intermediate affecting mesenchymal cell PGHS-II expression can not be excluded. Dexamethasone stimulated PGHS-II mRNA expression and PGE₂ production by isolated amnion mesenchymal cells but significantly inhibited PGE₂ output by isolated epithelial cells (Blumstein et al, 2000). These cultured mesenchymal cells did not express characteristic CD68 marker observed in vivo; these mesenchymal cultures underwent two passages and did not retain this response to dexamethasone beyond the third passage. The inhibitory effect of dexamethasone on epithelial cell PGE₂ output was observed following three days in culture whereas the stimulatory mesenchymal cell response to dexamethasone was observed
following six days in culture (Blumstein et al. 2000). Thus, the effects of dexamethasone may be modulated by the isolation procedure and culture conditions.

To address the apparent cell-specific characteristics of amnion epithelial and amnion mesenchymal cells, the objectives of the present study were: (1) to establish a simple technique for the isolation and culture of pure amnion epithelial and mesenchymal cell preparations not requiring serial enzyme digestion and passage; (2) to characterize the cellular expression of PGHS-II and PG output within these separated amnion cells; (3) to characterize the effect of GC treatment on PG output by these separated amnion cell types.

5.2. METHODS AND MATERIALS

5.2.1. Isolation of Amnion Cells

Term (38-40 weeks gestation) placenta with attached fetal membranes were collected immediately following labour and spontaneous vaginal delivery (n=3 patients) or prior to the onset of labour following elective Cesarean section (n=4 patients) from patients delivering at Mount Sinai Hospital (Toronto, Canada). Appropriate patient consent was obtained and ethical approval was received from the University of Toronto and Mount Sinai Hospital Ethics Approval Boards.

All manipulations were carried out under sterile technique. The amnion was peeled from the chorion and cut approximately 2 cm from the placental disc, washed in phosphate buffered saline (Dulbecco’s PBS, pH 7.5; Gibco/BRL; Burlington, Canada) and divided. To isolate amnion mesenchymal cells, approximately two thirds of the amnion was finely minced with scissors, transferred to 50 ml of 1mg/ml collagenase A (Boehringer Mannheim Canada; Dorval, Canada) and incubated at 37°C with gentle shaking for 2h. After digestion,
the suspension was filtered through 100-micron nylon mesh; cells were pelleted by centrifugation at 2500 g for 10 min. The pellet was re-suspended in 3 ml Dulbecco’s Modified Essential Medium (DMEM, pH 7.5; Gibco/BRL; Burlington, Canada) and layered on a discontinuous Percoll gradient [(5%/20%/40%/60% vol/vol) (Sigma Chemical Co.; St. Louis, USA)]. The gradient was then centrifuged at 800 g for 20 min. At approximately the 20% Percoll level, a single band of cells was aspirated. The cells were washed in DMEM, counted on a hemocytometer and suspended in DMEM supplemented with 10% fetal calf serum (FCS: ICN Canada; Montreal, Canada) and antibiotics [(1000U/ml penicillin/0.1mg/ml streptomycin/0.23μg/ml amphotericin B) (Sigma Chemical Co.; St. Louis, USA)]. The cells were plated in 8 well glass chamber slides (Labtek/Nunc; Naperville, USA) at a density of 250 000 cells per 500 μl medium per well. They were maintained in culture at 37°C with a water saturated atmosphere and 5% CO₂. After 48 or 96 h of culture, the medium was removed and the cells were washed with DMEM. DMEM (500 μl) with or without arachidonic acid (1 μM; ICN Canada; Montreal, Canada) was added (Potestio et al 1988). In addition, cells were treated with cortisol [10-1000 nM; (Steraloids; Wilton, USA)] either in the presence or absence of arachidonic acid. Also, cells were treated with dexamethasone [DEX 1μM; (Steraloids; Wilton, USA)] in the presence of arachidonic acid as a positive control. After 24 h incubation, the media were collected and stored at −80°C and the cells were fixed with 4% paraformaldehyde (Sigma Chemical Co.; St. Louis, USA) and stored in 70% ethanol for further analysis.

To isolate epithelial cells, the 1/3 of the dissected tissue was placed in a 250 ml tissue flask containing 0.25% trypsin (Gibco/BRL; Burlington, Canada) in DMEM (digestion media) and incubated for 20 min at 37°C with gentle shaking. This first digestion supernatant
was discarded: the tissue was then incubated with two subsequent 30 min washes of digestion media. The medium was collected and the cells were pelleted by centrifugation at 2,500 g for 10 min. The pellets were collected, cells purified over a Percoll gradient, re-suspended, plated and treated as described for the cells dispersed with collagenase. At the end of treatment cell viability was established by trypan blue (Gibco/BRL; Burlington, Canada) exclusion; all cultures demonstrated >90% viability.

5.2.2. Cell Characterization

Amnion cells were characterized using antibodies specific for two separate filament proteins expressed in epithelial and mesenchymal cells respectively and an antibody specific for CD68, a cluster determinant factor shown to be expressed by amnion mesenchymal cells (Whittle et al. 1999). Cytokeratin, an epithelial cell lineage marker, was detected using a mouse monoclonal antihuman antibody (Dako; Glostrup, Denmark) at 1:2,500 dilution. Vimentin, a mesenchymal cell lineage marker, was detected using a rabbit polyclonal antihuman antibody (Dako; Glostrup, Denmark) at 1:500 dilution. CD68 was detected with a rabbit monoclonal antihuman antibody (Dako; Glostrup, Denmark) at 1:500 dilution. Prostaglandin H synthase type II protein was localized with a polyclonal rabbit antihuman antibody (PG27-Oxford Biomedical Research Inc.; Oxford, USA) at 1:250 dilution. Glucocorticoid receptor protein was identified with a monoclonal mouse antihuman antibody (PA1-511: Affinity Bioreagents; Neshanic Station, USA) at a 1:50 dilution. Antibodies were diluted in 0.2% bovine serum albumin (Sigma Chemical Co.; St. Louis, USA) in phosphate buffered saline [PBS: 150 mM NaCl, 10 mM Na₂HPO₄, 1.5 mM NaH₂PO₄; pH 7.5; (Sigma Chemical Co.; St. Louis, USA)]. Cells were rehydrated with serial increasing dilutions of ethanol ending with two washes with PBS. Endogenous peroxidase activity was quenched by
pretreatment with 0.3% hydrogen peroxide (ACP Chemicals Inc.; Montreal, Canada) in PBS.

Cells were then washed in PBS and incubated with immune serum that served as a blocking agent for nonspecific binding. Primary antibody was applied and the tissues were incubated at 4°C for 18-24h. Primary antibody binding was visualized as per the Vectastain ABC Kit (Vector Laboratories; Burlingame, USA). Cells were washed, incubated with biotinylated secondary antibody for 60 min, washed again and incubated with the ABC (avidin biotin-peroxidase complex) for 60 min. After a final washing, the immunoreactive proteins were visualized after the addition of 3,3′diaminobenzidine (Fast DAB tablets, Sigma Chemical Co.; St. Louis, USA) for 2 min. Cells were counterstained with hematoxylin and eosin, dehydrated in graded ethanol, cleared and cover slips applied. Control cells were treated identically but with the omission of primary antibody.

5.2.3. Radioimmunoassay of PGE₂

Culture media was assayed for PGE₂ by standard radioimmunoassay technique described in section 3.2.6.4. Briefly, aliquots (100-300μL) of culture media were incubated with rabbit anti-PGE₂ antiserum (100μL; final dilution 1:4000; titrated to give a B/Bo of ~35-40%) and [5.6.8.11.12.14.15(n)-³H] PGE₂ [(100μL; ~10 000 cpm); (NEN Dupont Canada Inc.; Mississauga, Canada)] at 4°C for ~18h. The antiserum bound PGE₂ was separated from the unbound PGE₂ using neutral dextran-coated charcoal and centrifugation at 15 000 g at 4°C for 10 min. The supernatant containing the bound PGE₂ was decanted to scintillation vials containing 4 mL CytoScint™ (ICN Biomedicals Inc.; Costa Mesa, USA) and counted on a beta counter (Tri-Carb 2100OTR Liquid Scintillation Analyzer; Camberra Packard Instrument Co.; Meriden, USA). The intra and interassay coefficients of variation were 5% and 7% respectively.
5.2.4. *Data Analysis*

Results are expressed as the mean +/- SEM of averages from individual cultures. There were 8 replicates of each treatment per culture. All data were submitted to normality and equal variance testing. Statistical comparisons were made using a Students’ t-Test or an One-Way Analysis of Variance (ANOVA) followed by post hoc, all pairwise multiple comparison Tukey’s test determine significant interactions. Significance was accepted at \( p \leq 0.05 \).

5.3. **RESULTS**

5.3.1. *Amnion Cell Characterization*

Separate amnion epithelial and amnion mesenchymal cell primary cultures (n=7 cultures of each cell type) were established. Staining of typical control cultures is presented in *Figure 5-1a* and *5-1b*. Cells isolated using trypsin digestion were identified as epithelial cells. These cells stained positively for cytokeratin (*Figure 5-1c*) but were negative for vimentin and CD68 (*Figure 5-1e, 5-1g*). Epithelial cells were circular in shape and formed clusters that were scattered across the culture plate. Cells dispersed using mechanical digestion and collagenase treatment were identified as mesenchymal cells. The cells stained positively for vimentin and CD68 (*Figure 5-1f, 5-1h*) but were negative for cytokeratin (*Figure 5-1d*). Mesenchymal cells formed pseudopod cytoplasmic extensions, which spread to form a layer across the culture plate. There was 13.0 +/- 1.0 % contamination of the mesenchymal cultures by epithelial cells. There was 3.0 +/- 1.0 % contamination of the epithelial cultures by mesenchymal cells.
5.3.2. **Immunoreactive PGHS-II Expression and PGHS Activity**

Immunoreactive PGHS-II protein (ir-PGHS-II) and PGHS activity were found in both epithelial and mesenchymal cells (*Figure 5-2, 5-3*). Basal PGE₂ output by amnion mesenchymal cells at 72h was 22 fold greater (p<0.05) and at 120h was 32 fold greater (p<0.05) than PGE₂ output by amnion epithelial cells (*Figure 5-2*). Arachidonic acid stimulated a 5.0 fold increase in PGE₂ output (p<0.05) by the epithelial cells after 72h in culture and a 3.6 fold increase (p<0.05) after 120h but did not alter PGE₂ output by the mesenchymal cells (*Figure 5-4*). Despite the addition of substrate, epithelial cell PGE₂ output remained significantly less than basal mesenchymal cell PGE₂ output; there was a 5-fold difference at 72h (p<0.05) and an 8-fold difference at 120h (p<0.05) (*Figure 5-4*). For both cell types, there was no difference in the mean PGE₂ output between cells isolated from tissues collected either prior to the onset of labour (elective Cesarean section) or following spontaneous vaginal delivery (*Table 5-1*).

5.3.3. **Effects of Glucocorticoid Treatment on PGE₂ Output**

Immunoreactive GR protein (ir-GR) was present in both cell types (*Figure 5-5*). GC increased epithelial cell PGE₂ output in the absence or presence of arachidonic acid (p<0.05). Mesenchymal cell PGE₂ output was not altered by cortisol in the absence or presence of arachidonic acid but was stimulated by dexamethasone in the presence of arachidonic acid (p<0.05) (*Figure 5-6*). Despite GC and substrate stimulation, epithelial cell PG output (1153.0 +/- 359 pg/5x10⁵ cells/24h; n=4) remained significantly less than basal mesenchymal PG output (4892.0 +/- 1431 pg/5x10⁵ cells /24h; n=4; p<0.05).
Figure 5-1: Amnion Cell Culture Identification

Cells dispersed by trypsin are shown in the upper panels; cells dispersed with collagenase in the lower panels. Cells dispersed with trypsin stained positively for cytokeratin and negatively for CD 68 and vimentin. Cell dispersed with collagenase stained negatively for cytokeratin and positively for CD 68 and vimentin. a) Buffer control trypsin dispersed cells stained with hematoxylin and eosin b) Buffer control collagenase dispersed cells stained with hematoxylin and eosin c) Trypsin dispersed cells stained positively for cytokeratin d) Collagenase dispersed cells did not stain for cytokeratin e) Trypsin dispersed cells did not stain for CD68 f) Collagenase dispersed cells stained positively for CD68 g) Trypsin dispersed cells did not stain for vimentin h) Collagenase dispersed cells stained positively for vimentin. Magnification 400X
Values are mean +/- standard error of the mean for separate cultures (amnion epithelial culture n=7; amnion mesenchymal culture n=6). *p<0.05 versus epithelial cell culture. Statistical comparisons were made using Unpaired Students' t-Test.

PGE₂ output by amnion mesenchymal cells was significantly greater than that of amnion epithelial cells following 72 and 120h in culture.
Figure 5-3: Cellular Distribution of Immunoreactive PGHS-II Protein

Amnion epithelial and mesenchymal cells stained positively for ir-PGHS-II.

a) Buffer control epithelial cells stained with hematoxylin
b) Buffer control mesenchymal cells stained with hematoxylin
c) Epithelial cells stained positively for ir-PGHS-II and counterstained with hematoxylin
d) Mesenchymal cells stained positively for ir-PGHS-II and counterstained with hematoxylin.

Magnification 400X
Administration of 1 μM arachidonic acid (aa) increased PGE₂ output by the amnion epithelial cells but did not alter PGE₂ output by the mesenchymal cells at either time point in culture.

Figure 5-4: Effects of Substrate on PGE₂ Output by Cultured Amnion Cells

Values are mean +/- standard error of the mean for separate cultures (amnion epithelial culture n= 6; amnion mesenchymal culture n= 7). * p<0.05 versus epithelial cultures. no aa : ** p<0.05 versus epithelial cultures.
Figure 5-5: Cellular Distribution of Immunoreactive Glucocorticoid Receptor Protein

Amnion epithelial and mesenchymal cells stained positively for immunoreactive glucocorticoid receptor (ir-GR).

a) Buffer control treated epithelial cells stained with hematoxylin
b) Buffer control treated mesenchymal cells stained with hematoxylin
c) Epithelial cells stained positively for ir-GR and counterstained with hematoxylin
d) Mesenchymal cells stained positively for ir-GR and counterstained with hematoxylin.

Magnification 400X
Figure 6-6: Effects of Glucocorticoids on PGE$_2$ Output by Amnion Epithelial and Mesenchymal Cells

Values are mean +/- standard error of the mean for separate cultures (amnion epithelial and mesenchymal cultures n=4).

Amnion epithelial cell PGE$_2$ output was stimulated by increasing concentrations of cortisol and dexamethasone in the absence (* p<0.05) and presence of arachidonic acid (aa, ** p<0.05). Amnion mesenchymal cell PGE$_2$ output was not stimulated by increasing concentrations of cortisol but was stimulated by dexamethasone (1 μM DEX; ***p<0.05) in the presence of 1 μM arachidonic acid.
Table 5.1.

Amnion Cell Culture PGE₂ Production Before and After the Onset of Labour

Mean Basal PGE₂ Production (pg/5 x 10⁵ cells/24 hours)

<table>
<thead>
<tr>
<th></th>
<th>Cesarean Section</th>
<th>Vaginal Delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial Cells</td>
<td>171.0 +/- 87.0 (n=4)</td>
<td>210.3 +/- 48.5 (n=3)</td>
</tr>
<tr>
<td>Mesenchymal Cells</td>
<td>8872.0 +/- 2644.0 (n=3)</td>
<td>4341.0 +/- 2117 (n=3)</td>
</tr>
</tbody>
</table>
5.4. DISCUSSION

This study has shown that amnion epithelial and mesenchymal cells can be isolated separately and established as relatively pure primary cultures and that these two cell types have distinct basal and substrate stimulated PGE_2 output. Further, these two cell types responded to GC stimulation, however, the pattern of response was different for each cell type.

The method used to establish these cell culture preparations is modified from that of Okita et al (1983). The trypsin digestion breaks the protein connections between the cells without interrupting the basement membrane and the extracellular matrix of the mesenchymal layer, permitting the release of the amnion epithelial cells. The mechanical digestion increases the tissue surface area exposed to the collagenase and allows the amnion mesenchymal cells embedded within this layer to be liberated. Collagenase treatment digests the membrane similar to the action of endogenous metalloproteinase enzymes that digest the major components of the basement membrane and the extra-cellular matrix (including type IV collagen, laminin and fibronectin) to cause membrane rupture at term. The Percoll gradient removes any cellular debris and red blood cells from the preparation. Given the striking and consistent differences in the round shape of the epithelial cells and the stretched shape of the mesenchymal cells, morphological criteria as well as cell markers can be used to identify these cell types within an amnion culture preparation.

Our cell isolation technique differs from that reported by Keelan et al (1997) and Blumstein et al (2000). In these studies, the amnion tissue underwent a mechanical digestion followed by serial trypsin dispersions. Cells were plated for 2h and then the non-adherent "epithelial" cells were removed and cultured independently. The remaining, adherent,
purified "mesenchymal" cells were cultured until confluence, dispersed with trypsin and replated. We obtained a great enough yield of amnion mesenchymal cells by collagenase dispersion that we did not need to submit the cells to a second trypsin digestion and passage which could affect mesenchymal cell viability, cell function or cell marker expression. In contrast to our study, Blumstein et al (2000) reported that the amnion mesenchymal cells did not express CD68, a cluster determinant factor shown to be expressed by amnion mesenchymal cells (Whittle et al, 1999). Secondly, Blumstein et al (2000) reported the mesenchymal cells did not retain their functional characteristics beyond the third passage. These data suggest that the isolation/purification technique used and the length of time in culture significantly influences mesenchymal cell character and function representing a confounding variable in the interpretation of amnion function.

Our technique effectively and consistently isolated and identified highly enriched culture preparations; epithelial cells were contaminated by <5% mesenchymal cells and mesenchymal cells were contaminated by <14% epithelial cells. Both amnion epithelial and mesenchymal cells expressed ir-PGHS-II and produced PGE₂ in culture; however, mesenchymal cells, at both time points in culture, had significantly greater basal PG output than the epithelial cells. Given that PGHS-I and PGHS-II are expressed in both of these cell types (Hirst et al, 1995), PG production may represent the activity of both PGHS isoforms in varying proportion. However, while the contamination of mesenchymal culture by epithelial cells is unlikely to account for the measured PGE₂ output, the reverse contamination of epithelial culture by mesenchymal cells could account for much of the basal PGE₂ output by these preparations. These findings are in contrast to those of Keelan et al (1997) and Blumstein et al (2000) who reported greater basal PGE₂ output by epithelial cells. However,
in these studies the epithelial cell output was measured following three days in culture and
the mesenchymal cell output was measured following six days in culture; it has been
previously documented that mixed amnion cell PGE\textsubscript{2} output decreases as a function of
culture. Thus, the lower level of PGE\textsubscript{2} output by mesenchymal cells as compared to epithelial
cells may reflect the greater length of time these cells spent in culture prior to the
determination of basal PGE\textsubscript{2} output. As well, the differences in basal PGE\textsubscript{2} output between
our study and that of Blumstein et al (2000) and Keelan et al (1997) may reflect the different
isolation/purification techniques. However, it is not truly valid to compare the results of those
studies with our own because the data are not expressed using a common denominator. Our
results represent PGE\textsubscript{2} output per well with comparable number of viable cells per well
between the two cell types: Blumstein et al (2000) and Keelan et al (1997) have reported PG
production as PGE\textsubscript{2} output per \( \mu \)g protein content of the culture well. Since the PG
production rates between the two studies have been standardized to different reference values
these data can not be compared. We have measured the protein content per million cells and
determined that the amnion mesenchymal cells have a greater protein concentration as
compared to the amnion epithelial cells (data not shown). Thus, we believe that evaluating
PGE\textsubscript{2} output per million cells is a more accurate method of comparing PGE\textsubscript{2} production by
the two cell types.

Our data suggest that mesenchymal cells may make a significant contribution to the
net PG output by whole amnion at term. However, \textit{in vivo} these cells do not exist in
comparable numbers. There are 7-10 times more epithelial cells than mesenchymal cells
within the intact amnion membrane (Head \textit{et al}, 1998). \textit{In vivo}, these two cell types do not
exist in isolation but are intimately juxtaposed, and the regulation of prostaglandin synthesis
may reflect paracrine interactions between them. In addition, PGHS-II mRNA and ir-PGHS-II protein at term is consistently expressed in the amnion epithelial cells but PGHS-II expression at term within the mesenchymal cells is variable (Gibb et al, 1998). Therefore, despite the differences we and others report, it remains difficult to extrapolate from \textit{in vitro} information to the relative contribution of each cell type to the increased levels of prostaglandins \textit{in vivo}.

We found no significant difference in PG output by either cell type from tissues collected before or after the onset of labour. This is in contrast to the results of Olson et al (1983) showing that freshly dispersed amnion cells from tissues collected following labour and vaginal delivery had significantly greater PG output than tissues collected following elective Cesarean section prior to the onset of labour. Brown et al (1998) have described fetal membranes collected prior to the onset of labour \textit{in vitro} as either "activated" with upregulated membrane PG synthesis prior to the onset of clinical labour or "non-activated" with lower basal PG synthesis. It is possible that the tissues we collected following elective Cesarean section were "activated" thus precluding the detection of any differences between labour and non-labour-non-activated tissues.

Substrate availability was not a significant factor regulating PG output in the amnion mesenchymal cells although it was in the amnion epithelial cells. PGHS activity within the mesenchymal cells may be at a level such that further increases in substrate do not affect prostaglandin output. Brown et al (1998) described activated fetal membranes as having high basal prostaglandin production, unresponsive to stimuli. They suggested that in such tissues PGHS-II was maximally induced and incapable of responding to further stimulation. Our data suggest that PGHS-II activity within the amnion mesenchyme undergoes a sustained
induction at term prior to the onset of clinical labour and that substrate availability is not a rate-limiting step in PG output within the mesenchyme.

Substrate availability did appear to limit PG output within the amnion epithelial cells. These data are consistent with the finding that confluent amnion cells treated with mouse epidermal growth factor only produced PG in the presence of nonesterified arachidonic acid (Casey et al. 1987). Arachidonic acid is liberated from membrane phospholipid stores by a variety of phospholipase enzymes, in particular phospholipase A₂ and C. Increased phospholipase activity within the intrauterine tissues has been observed at term and with the onset of labour (Aitken et al. 1996; Skannal et al, 1997). The arachidonic acid content of the amniotic fluid also increases 6-10 fold at this time (MacDonald et al. 1974). These data suggest that increased substrate for PG synthesis within the fetal membranes is available at term. Within the system used in this study, it is possible that there is greater phospholipase activity in the mesenchyme cell culture producing more arachidonic acid substrate for prostaglandin production as compared to the epithelial cell culture.

GCs have been identified as positive regulators of prostaglandin synthesis in cultured amnion cells in contrast with the negative regulatory effect of GCs on prostaglandin output observed in other tissues including freshly dispersed amnion cells (Gibb et al, 1993). This stimulatory effect is receptor dependent, linked to the activity of protein kinases and involves increased PGHS-II expression (Economopoulos et al, 1996; Zakar et al, 1995; Smeija et al, 1993; Potestio et al, 1988). Economopoulos et al (1996) found that dexamethasone increased mRNA and protein expression of PGHS-II in the amnion mesenchymal cells only and not the epithelial cells. We found PG output by both cell types was increased by dexamethasone: both cell types expressed ir-GR consistent with observations in intact fetal membranes (Sun
et al. 1996). PGHS-II is an early response gene and studies using an amnion cell line (WISH cells) have described the induction of PGHS-II mRNA, protein and PG synthesis within 30 minutes of exposure to epidermal growth factor with a rapid decline to near baseline within 2-3h (Perkins et al, 1996). In the present study, cells were treated for 24h. It is possible that an early induction and subsequent inhibition pattern of PGHS-II expression and activity within the epithelium occurred but was not detected in the study by Economopoulos et al (1996). The prostaglandin we measured could reflect prostaglandin produced immediately following GC exposure and released into the culture media where they remained unmetabolized for the culture period since there is little prostaglandin metabolizing activity in the amnion (Cheung et al, 1990). Alternatively, Economopoulos et al (1996) can not rule out the possibility that a GC-induced factor produced by the mesenchymal cells affected epithelial PGHS-II expression. Our finding also differ from those of Blumstein et al (2000) who reported a 40% inhibition of epithelial cell PGE2 output in response to dexamethasone treatment; their cultures were contaminated by ~13% mesenchymal cells and again the possibility GC-stimulated, inhibitory factor produced by the mesenchymal cells can not be excluded. For example, cytokines are produced by amnion epithelial and mesenchymal cells; mixed amnion cell PGE2 output reflects a balance between pro and anti-inflammatory cytokine autocrine/paracrine action. Dexamethasone has been found to alter this production and may have caused a shift in the balance towards an anti-inflammatory effect. In addition, Blumstein et al (2000) reported that their cultures were treated for 16h and our cultures were treated for 24h: the difference in PGE2 output may reflect the different culture time and a dynamic balance between PGE2, GC exposure and pro-and anti-inflammatory cytokines action.
Both Blumstein et al (2000) and our study demonstrated a significant increase in PGE$_2$ output by amnion mesenchymal cells in response to dexamethasone likely due to an increase in PGHS-II expression. However, we did not observe this effect when the cells were treated with cortisol. 11βhydroxysteroid dehydrogenase type I (11βHSD-I), the enzyme capable of the interconversion of cortisol and its in active metabolite cortisone, has been localized to the amnion epithelium and mesenchyme of intact fetal membranes at term. It is possible that 11βHSD-I is expressed by the mesenchymal cells in culture and is acting as a dehydrogenase to metabolize cortisol and thereby limit its effect on PG production. Dexamethasone is a poor substrate for 11βHSD-I and thus would be capable of inducing PGHS-II expression and activity in the mesenchymal cells. Future studies regarding the distinct patterns of GC action and the activity of 11βHSD on PGHS-II expression and activity within these cell types and possible paracrine interactions between the two amnion cell types are required to resolve these issues.

In summary, we have shown that basal output and substrate regulation of prostaglandin synthesis differs between amnion cell types. Given the differences and potential interactions between these cells, we suggest that useful information can be obtained regarding the regulation of amnion prostaglandin synthesis in each cell type studied separately and in reconstituted cell culture systems. Such experiments would characterize the potential and nature of paracrine interrelationships that might exist between these cells in vivo.
Chapter Six

The Role of 11ß Hydroxysteroid Dehydrogenase Type 1,

Corticotropin Releasing Hormone and Paracrine Interactions in the

Regulation of Human Amnion Epithelial and Mesenchymal Prostaglandin Output.
6.1. Introduction

The amnion, composed of both epithelial and mesenchymal cells, has been identified as the main site of intrauterine PG production at term and with the onset of labor (Bryant-Greenwood, 1998; Challis et al, 1997). In vitro, GCs have been previously demonstrated to have dose dependent, receptor mediated positive effect on amnion PGHS-II expression and PGE₂ output (Economopoulos et al, 1996; Zakar et al, 1995; Smeija et al, 1993; Potestio et al, 1988). Economopoulos et al (1996) reported that the stimulatory effect of GCs on PGHS-II occurred exclusively in the mesenchymal cells of a mixed amnion cell culture. However, in Chapter 5 we demonstrated that amnion epithelial cells prepared and cultured separately from amnion mesenchymal cells had increased PGE₂ output in response to both cortisol and dexamethasone treatment. Amnion mesenchymal cell PGE₂ production was increased only in response to dexamethasone treatment. We had speculated that 11βhydroxysteroid dehydrogenase type I (11βHSD-I) which interconverts cortisol and its inactive metabolite cortisone, may play a role in the differential effects of GCs on these two isolated cell types. 11βHSD-I is expressed in both amnion epithelial and mesenchymal cells in intact fetal membrane at term (Sun et al, 1997). In vivo, 11βHSD-I generally acts in the reductase direction to produce cortisol from cortisone reflecting a lower Km value for cortisone as compared with cortisol (Stewart and Krozowski, 1999). However, it is possible that 11βHSD-I expressed by the mesenchymal cells in culture acts as a dehydrogenase to metabolize cortisol and thereby limit its effect on PG production. Dexamethasone is poor a substrate for 11βHSD-I and thus would be capable of inducing PGHS-II expression and activity in the mesenchymal cells (Stewart and Krozowski, 1999). 11βHSD-I may play a stimulatory role in the regulation of amnion PG production. Patel et al (1999) demonstrated
that the 11\(\beta\)HSD-I activity in cultured chorion trophoblast cells led to cortisone decreasing PGDH expression and activity. This effect was attenuated by carbenoxolone, an 11\(\beta\)HSD-I inhibitor, suggesting that 11\(\beta\)HSD-I reductase activity converted cortisone to cortisol that in turn affected PG metabolism. To examine the role of 11\(\beta\)HSD-I in the GCs regulation of amnion PG output we will treat separate amnion epithelial and mesenchymal cell preparations with cortisol or cortisone in the presence and absence of carbenoxolone, the 11\(\beta\)HSD-I inhibitor.

Corticotropin releasing hormone (CRH) is a 41 amino acid hypothalamic peptide present within the intrauterine tissues from the first trimester onwards (MacLean and Smith, 1999). Placental and maternal plasma CRH levels increase through gestation and further exponential increases are observed in the weeks prior to delivery at term and preterm (MacLean and Smith, 1999). During that time there is concomitant decrease in the plasma concentration of the CRH binding protein that leads to increased CRH bio-activity (MacLean and Smith, 1999). The timing of these events has suggested that CRH may act as a trigger for the onset of the labor cascade. Immunoreactive CRH expression is also present in amnion epithelial and scattered amnion mesenchymal cells in intact fetal membranes (Riley et al, 1991). In vitro, mixed amnion cell cultures produce immunoreactive CRH and mixed amnion cultures treated with CRH had increased PGE\(_2\) output (Jones et al, 1990; Jones et al, 1989). However, the amnion cell type responsible for CRH output and PGE\(_2\) production in response to CRH treatment has not yet been identified. To determine which amnion cell type responds to CRH we will treat separate amnion epithelial and mesenchymal cell preparations with CRH and measure PG output.
CRH acts through membrane bound receptors; these receptors have been characterized within the amnion epithelium but not the amnion mesenchyme (Florio et al., 2000; Kareteris et al., 2000; Karteris et al., 1998). Thus, CRH may be produced by the mesenchyme and in turn act as a paracrine mediator of epithelial cell PGE$_2$ production. However, paracrine interactions between the two cell types has not yet been documented. The intimate juxtaposition of the epithelial and mesenchymal cells within intact fetal membranes suggests that such interactions may be possible. In a mixed amnion cell preparation, both IL-1$\beta$ and dexamethasone upregulated PGHS-II mRNA and protein expression amnion mesenchymal cells but not amnion epithelial cells (Gibb et al., 1999; Economopoulos et al., 1996). One possible explanation for this differential effect is that a cytokine or a GC stimulated intermediate produced by the mesenchymal cells inhibited epithelial cell PGHS-II expression. Alternatively, a cytokine or a GC stimulated intermediate produced by the epithelial cells increased mesenchymal cell PGHS-II expression. Thus, these observations raise the possibility that paracrine interactions between the two cell types may regulate amnion PG production. To investigate the possible role of paracrine interactions between amnion epithelial and mesenchymal cells in the regulation of PG output we will treat separate amnion epithelial cell preparations with media conditioned by amnion mesenchymal cells and vice versa.

Therefore objectives of the present study were to further define the cell specific regulation of amnion PG production. We hypothesize that 11$\beta$HSD-I expression within the amnion epithelial and mesenchymal cells will play a role in the GC regulation of amnion PG output. We suggest that CRH will increase PG output by amnion epithelial cells and may act
as a paracrine mediator between the epithelial and mesenchymal cells supporting the hypothesis that paracrine interactions between these cells regulates amnion PG production.

6.2. METHODS AND MATERIALS

6.2.1. Isolation and Culture of Amnion Cells

Term (38-40 weeks gestation) placentae with attached fetal membranes were collected immediately following labour/spontaneous vaginal delivery or prior to the onset of labour following elective Cesarean section (n=5 patients) from consenting patients delivering at Mount Sinai Hospital (Toronto, Canada). The isolation and culture of amnion cells were conducted as described in section 5.2.1. Cells were plated at a density of 250 000 cells/500 μL media/per well and cultured at 37°C in a water saturated atmosphere with 5% CO₂ for 72h in DMEM supplemented with 10% fetal calf serum and antibiotics. After 72h of culture, the medium was removed and the cells were washed with DMEM.

6.2.2. Cell Culture Treatments

Amnion epithelial and mesenchymal cells were treated with cortisol (1000 nM), cortisone (1000nM) (Steraloids; Wilton, USA) in the presence or absence of carbenoxolone (800 nM) (Sigma: Sigma Chemical Co.; St. Louis, USA) for 24h. Cells were also treated with dexamethasone [(1000 nM) (Steraloids; Wilton, USA)] as a positive control. Based on the findings presented in Chapter 5 all treatments were supplemented with 1μM arachidonic acid. Following 24h incubation, the media were collected and stored at −20°C. The cells were fixed with 4% paraformaldehyde and stored in 95% ethanol.

Amnion epithelial and mesenchymal cells were treated with corticotropin releasing hormone (1000 ng/ml) (Sigma Chemical Co.; St. Louis, USA) (Jones et al, 1989). All
treatments were conducted in the presence of arachidonic acid (1µM). After 24h incubation, the media were collected and stored at -20°C. The cells were fixed with 4% paraformaldehyde and stored in 95% ethanol.

Amnion epithelial and mesenchymal cells were treated with 500 µL DMEM for 24 h; after the incubation period media from the individual cell types were collected, and mixed with a vortex. Mesenchymal cells were then treated with media conditioned by epithelial cells and epithelial cells were treated with media conditioned by mesenchymal cells. After a second 24h incubation, the media were collected and stored at -20°C and the cells were fixed with 4%paraformaldehyde and stored in 95% ethanol.

6.2.3. Immunocytochemical Localization of 11βHydroxysteroid Dehydrogenase Type I

11βHSD-I protein was localized using immunocytochemistry (antibody was a gift from Dr. K. Yang). This antibody had been previously used to characterize the expression of 11βHSD-I within intact fetal membranes at term (Sun et al, 1997). The immunocytochemical technique used was described in section 5.2.2. Briefly, cells were rehydrated with serial increasing dilutions of ethanol ending with two washes with PBS. Endogenous peroxidase activity was quenched by pretreatment with 0.3% hydrogen peroxide in PBS. Cells were incubated with immune serum that served as a blocking agent for nonspecific binding. Primary antibody was applied and the tissues were incubated at 4°C for 18-24h. Primary antibody binding was visualized as per the Vectastain ABC Kit. After a final washing, the immunoreactive proteins were visualized after the addition of 3,3'diaminobenzidine (Fast DAB tablets, Sigma Chemical Co.; St. Louis, USA) for 2 min. Cells were counterstained with hematoxylin and eosin, dehydrated in graded ethanol, cleared and cover slips applied. Control cells were treated identically but with the omission of primary antibody.
6.2.4. Radioimmunoassay of PGE$_2$

Culture media was assayed for prostaglandin E$_2$ by the standard radioimmunoassay technique described in section 5.2.3. The inter and intra assay coefficients of variation were 6% and 8% respectively.

6.2.5. Data Analysis

Results are expressed as the mean +/- SEM of averages from individual cultures (n=4 unless otherwise indicated). There were 6 replicates of each treatment per culture. Results are expressed as the mean +/- SEM of averages from individual cultures. All data were submitted to normality and equal variance testing. Statistical comparisons were made using a Students’ t-Test or an One-Way Analysis of Variance (ANOVA) followed by post hoc, all pairwise multiple comparison Tukey’s test determine significant interactions. Significance was accepted at p≤0.05.

6.3. RESULTS

6.3.1. Effects of Cortisol and Cortisone on Amnion Cell PGE$_2$ Output

Separate amnion epithelial and amnion mesenchymal cell primary cultures were established and characterized as described in Chapter 5. Cortisol in the presence or absence of carbenoxolone, increased epithelial cell PGE$_2$ output by ~35% (p<0.05) (Figure 6-1). Cortisone caused a similar increase in epithelial cell PGE$_2$ output (p<0.05) however, this effect was attenuated in the presence of carbenoxolone (Figure 6-1). Dexamethasone also increased significantly amnion epithelial cell PGE$_2$ output (p<0.05) (Figure 6-1). Mesenchymal cell PGE$_2$ output was not altered by cortisol or cortisone in the presence or absence of carbenoxolone but was stimulated by dexamethasone (p<0.05) (Figure 6-2). As
reported previously (*Chapter 5*) epithelial cell PGE$_2$ output remained significantly less than that of the mesenchymal cells despite the addition of exogenous substrate and GC stimulation (*data not shown*).

6.3.2. *Expression of Immunoreactive 11βHSD-I in Cultured Amnion Cells*

Amnion epithelial cells expressed immunoreactive 11βHSD-I in both control and GC treated cells (*Figure 6-3*); there was not change in the expression of 11βHSD-I with GC treatment (*data not shown*). Amnion mesenchymal cells did not express immunoreactive 11βHSD-I (*Figure 6-3*). GC treatment did not induce the expression of 11βHSD-I with the mesenchymal cells (*data not shown*).

6.3.3. *Effect of Corticotropin Releasing Hormone on Amnion Cell PGE$_2$ Output*

Corticotropin releasing hormone caused a modest increase in PGE$_2$ output by amnion epithelial cells (p=0.08) but increased significantly PGE$_2$ output amnion mesenchymal cells by ~97% (p<0.05) (*Figure 6-4*). Despite stimulation by CRH, PGE$_2$ output by the cells remained significantly less than that of the mesenchymal cells (p<0.05).

6.3.4. *Paracrine Interactions Between Amnion Epithelial and Mesenchymal Cells*

Media conditioned by amnion mesenchymal cells had no effect on PGE$_2$ output by amnion epithelial cells (*Figure 6-5*) but media conditioned by amnion epithelial cells caused an ~46% decrease in PGE$_2$ output by amnion mesenchymal cell (p=0.08) (*Figure 6-5*).
Figure 6-1: Effects of Cortisol and Cortisone on PGE$_2$ Output by Amnion Epithelial Cells

Values are mean +/- standard error of the mean for separate cultures (n=4); Statistical analysis was performed using a one way analysis of variance followed by a post hoc Dunn's test (p=0.05).

Amnion epithelial cell PGE$_2$ output was stimulated significantly by cortisol (1000nM) in the presence and absence of carbenoxolone (CBX: 800nM). PGE$_2$ output was also stimulated significantly by cortisone (1000nM); this stimulatory effect was attenuated in the presence of carbenoxolone. Dexamethasone (1000nM) also increased PGE$_2$ output significantly.
Figure 6-2: Effects of Cortisol and Cortisone on PGE$_2$ Output by Amnion Mesenchymal Cells

Values are mean +/- standard error of the mean for separate cultures (n=4); Statistical analysis was performed using a one way analysis of variance followed by a post hoc Dunn's test (p=0.05).

Amnion mesenchymal cell PGE$_2$ output was not stimulated by cortisol (1000nM) or cortisone (1000nM) in the presence of absence of carbenoxolone (CBX: 800nM). PGE$_2$ output was stimulated significantly by dexamethasone (1000nM).
Figure 6-4: Effects of Corticotropin Releasing Hormone on PGE$_2$ Output by Amnion Epithelial and Mesenchymal Cells

Values are mean +/- standard error of the mean for separate cultures (n=4 or 5). Statistical analysis was performed using Students t-test (α=0.05).

Corticotropin releasing hormone (1000 ng/ml) stimulated a non-significant 1.35 fold increase in amnion epithelial cell PGE$_2$ output and stimulated a significant 1.97 fold increase in amnion mesenchymal cell PGE$_2$ output. Despite CRH stimulation PGE$_2$ production by amnion epithelial cells remained less than that of the mesenchymal cells.
Figure 6-5: Effect of Conditioned Media on PGE$_2$ Output by Amnion Epithelial and Mesenchymal Cells

Values are mean +/- standard error of the mean for separate cultures (n=3 or 4).

Amnion mesenchymal cell media had no effect on amnion epithelial cell PGE$_2$ output. Amnion epithelial cell media elicited a non-significant decrease in amnion mesenchymal cell PGE$_2$ output.
Figure 6-3: Characterization of Distribution of Ir-11βHSD-I

Amnion epithelial but not mesenchymal cells stained positively for ir-11βHSD-I.

a. Buffer control epithelial cells stained with hematoxylin.
b. Epithelial cells stained positive for ir-11βHSD-I and counterstained with hematoxylin.
c. Buffer control mesenchymal cells stained with hematoxylin.
d. Mesenchymal cells stained negative for ir-11βHSD-I and counterstained with hematoxylin.

Magnification 400X
6.4. Discussion

In the present study we have demonstrated that both cortisol and cortisone increased PG output by amnion epithelial cells; these cells expressed 11βHSD-I. These observations suggest that 11βHSD-I activity may modulate the effects of GC on amnion epithelial cell PG production. In contrast, amnion mesenchymal cells did not express ir-11βHSD-I and did not increase PGE₂ output in response to respond to cortisol or cortisone treatment. However, mesenchymal cell PGE₂ output was increased by dexamethasone. CRH stimulated PGE₂ output by both epithelial and mesenchymal cells; however, CRH had a more potent effect on mesenchymal cell PGE₂ output as compared to the epithelial cells. Despite stimulation by either CRH or GCs, amnion epithelial cell PGE₂ output remained less than that of mesenchymal cells. Interestingly, epithelial cell conditioned media decreased mesenchymal cell PGE₂ production suggesting the possibility of an epithelial derived paracrine mediator affecting amnion mesenchymal PGE₂ output. These finding suggest that in vivo amnion PG production is the consequence of PG output from both epithelial and mesenchymal cells under complex autocrine/paracrine regulation.

We have demonstrated that GCs increase amnion epithelial cell PG output; we have demonstrated previously that these cells express PGHS-II and the GR (Chapter 5). Several investigators have reported that GCs have a dose dependent, receptor mediated effect on amnion PGHS-II expression (Smeija et al, 1997; Economopoulos et al. 1996; Zakar et al, 1993: Potestio et al, 1988). In addition, Cheung et al (1990) demonstrated that PGDH expression was excluded from amnion epithelium in intact fetal membranes. Therefore, the stimulatory effect of GCs on cultured amnion epithelial cell PG output is due exclusively to an increase in PG synthesis mediated by PGHS-II. As previously discussed (Chapter 3) the
PGHS-II gene expresses a glucocorticoid response element (GRE) within its 5' promoter region. The stimulatory effect of GCs is likely due to direct increase in gene expression mediated by the GRE or the interaction of the GR with other transcription factors at a different promoter site. Alternatively, GCs may increase PGHS-II mRNA stability or PGHS-II phosphorylation leading to an increase in PGHS activity.

Interestingly cultured amnion epithelial cells expressed 11βHSD-I; cortisone was as effective as cortisol in the stimulation of amnion epithelial cell PGE2 output and this effect was attenuated by the 11βHSD inhibitor carbenoxolone. These observations provide indirect evidence that 11βHSD expressed in the amnion epithelial cells acts in a reductase direction to produce cortisol from cortisone that in turn affects PGHS activity. These finding are consistent with those of Tanswell et al (1977) who reported that 11βHSD-I activity within the amnion contributes to the amniotic fluid concentration of cortisol. However, the specific reductase activity of 11βHSD within the amnion epithelial cells remains to be quantified. 11βHSD reductase activity has been reported in cultured chorion trophoblast cells (Sun et al, 1997). Patel et al (1999) demonstrated that this reductase activity produced cortisol from cortisone that in turn decreased PGDH expression and activity leading to an increase in chorion cell PG production. Recently it has been demonstrated that both PGE2 and PGF2α can increase 11βHSD-I reductase activity in cultured chorion trophoblast cells (Alfayid et al, 2000) suggesting that a positive feed-forward loop between cortisol bio-availability and PG production exists within the chorion. It is tempting to speculate that a similar positive feed-forward loop between cortisol bio-availability and PG production may exist within the amnion epithelium.
In contrast to the epithelial cells, cultured amnion mesenchymal cells did not express detectable ir-11βHSD-I; both cortisol and cortisone did not alter PGE_2 output by these cells. Paradoxically, although mesenchymal cell PGE_2 output was unresponsive to cortisol, PGE_2 output did increase when the cells were treated with dexamethasone. It is unlikely that low levels of 11βHSD-I are present but acting in a dehydrogenase direction given that cortisol in the presence of carbenoxolone, the 11βHSD-I inhibitor, did not alter PG output by the cells. It is also unlikely the PGs produced are being rapidly metabolized because PGDH is not expressed by the amnion mesenchyme (Cheung et al., 1990). It is possible that the cultured mesenchymal cells express 11βHSD-II, which functions exclusively as a dehydrogenase; however, the cultured mesenchymal cells did not express detectable ir-11βHSD-II (data not shown). In addition, carbenoxolone does inhibit both 11βHSD-II and 11βHSD-I (Stewart and Krozowski, 1999); if 11βHSD-II were present, cells treated with cortisol in the presence of carbenoxolone would to have been expected to have increased PGE_2 output. There is increasing evidence in support of a third NADP dependent 11βHSD isoform with exclusive dehydrogenase activity (Stewart and Krozowski, 1999). To date, this enzyme has been characterized in ovine tissues, rat Leydig cells and human granulosa-lutein cells (Stewart and Krozowski, 1999). It is possible that cultured amnion mesenchymal cells express this 11βHSD isoform which metabolizes cortisol such that cortisol in the presence and absence of carbenoxolone has no effect on PGE_2 output. The expression of 11βHSD and the direction of its activity within the amnion mesenchymal cells remain to be documented. Alternatively, the effects of dexamethasone may reflect a different pattern of receptor activity when the GR is bound to dexamethasone as compared to cortisol. The potency of dexamethasone binding to the GR may be greater than that of cortisol allowing the ligand-receptor complex to be
retained in the nucleus longer and permitting a genomic effect on PGHS-II expression. Dexamethaone-bound GR may form a heterodimer with another transcription factor that does not form when GR is bound to cortisol; this unique heterodimer could lead to an increase in PGHS-II expression. The role of GCs and 11βHSD in the regulation of amnion mesenchymal PG production requires further investigation.

We have demonstrated that CRH elicits a non-significant increase in amnion epithelial cell PGE$_2$ output and a significant increase in amnion mesenchymal cell PGE$_2$ output. These findings are consistent with those of Jones et al (1990) who reported a receptor-mediated increase in PGE$_2$ output by mixed amnion cells treatment with the identical dose of CRH. CRH exerts its actions through specific, G protein coupled seven transmembrane domain receptors that exist in two types: CRH-R1 and CRH-R2 that arise from separate genes with multiple spliced variants (Karteris et al, 1998). Several investigators have reported the presence of the CRH-R1 and the exclusion of the CRH-R2 in term human amnion (Florio et al, 2000; Karteris et al, 2000; Karteris et al, 1998). In particular, CRH-R1α, R1c and R1d have been localized to the amnion epithelium; however, expression of any of the CRH receptor isoforms within the amnion mesenchyme was not detected by the techniques employed in these studies (Florio et al, 2000; Karteris et al, 2000: Karteris et al, 1998). CRH-1α is 415 amino acid protein structurally related to the calcitonin/vasoactive peptide/growth hormone releasing hormone family. The CRH-R1c variant has 40 amino acids deleted from the N terminus; as a result this receptor isoform has no signal transduction properties and its function remains unresolved (Karteris et al, 1998). CRH-R1d is identical to R1α except that it contains an exon deletion resulting in the absence of 14 amino acids in the seventh transmembrane domain (Grammatopoulos et al, 1999).
Binding studies in HEK-293 cells have revealed that CRH-R1α and R1d have similar binding characteristics however, R1d does not couple to the G protein subtypes that R1α can activate (Grammatopoulos et al., 1999). Recently, Karteris et al. (2000) determined that the CRH-R expressed within the fetal membranes does not lead to activation to adenylate cyclase and increased intracellular cAMP levels as previously hypothesized. Failure to stimulate adenylate cyclase was not due to CRH-R coupling to inhibitory G protein subunits but instead fetal membrane CRH-R was coupled to Go and Gq subunits. These subunits are linked with the IP4 signal transduction cascade and CRH-R ligand binding leads to a 5-fold increase in intracellular IP3 and diacylglycerol (DAG) concentration. Thus, it appears that CRH binding to the R1α isoform within the amnion epithelial and mesenchymal cells elicits an increase in the intracellular IP3 and DAG concentration that in turn increases PGE2 production. Our data suggests a more potent response of the mesenchymal cells to CRH as compared with the epithelial cells.

The IP3 signal transduction cascade leads to the activation of protein kinase C. Amnion tissue has been demonstrated previously to contain protein kinase C (Eyster et al., 1993). Moore et al. (1991) reported that protein kinase C (PKC) activation was required for oxytocin induced PG production by mixed amnion cells. Zakar et al. (1998 and 1994) and Smeijer et al. (1997) reported that the induction of mixed amnion cell PGHS-II was mediated by PKC: TPA (12-O-tetradecanoyl phorbol-13-acetate), a PKC activator, stimulated PGHS activity and the level of PGHS-II mRNA. In addition, PKC activation may lead to the phosphorylation and subsequent activation of an inactive pool of PGHS-II protein within the amnion cell. Bang et al. (2000) reported a similar modulation of inactive PGHS-II by phosphorylation within the ovine fetal brain. It appears that CRH receptor binding leads to a
signal transduction cascade involving increased IP3 and subsequent PKC activation. In turn, PKC can increase both PGHS-II expression and activity leading to increased PGE2 output by the amnion epithelial and mesenchymal cells. Furthermore, DAG, a byproduct of IP4 hydrolysis, contains arachidonic acid and can be used as a substrate for PG synthesis. Thus, CRH can increase substrate availability as well as PGHS activity.

To investigate the possibility of paracrine interactions between the two amnion cell types we treated epithelial cells with conditioned media collected from mesenchymal cells and vice versa. We had hypothesized that basal and glucocorticoid stimulated CRH production by the amnion epithelial and mesenchymal cells could be a paracrine mediator of amnion PG production and thus expected the conditioned media to stimulate PG production by both cell types in manner similar to the pattern of CRH stimulation. However, amnion epithelial cell PG production was not affected by mesenchymal cell conditioned media. Interestingly, epithelial cell conditioned media elicited an obvious yet non-significant decrease in PGE2 output by the mesenchymal cells. Given that PGE2 output by the mesenchymal cells was increased significantly by exogenous CRH it is unlikely that epithelial cell CRH is responsible for the suppression of mesenchymal cell PGE2 output. To date little information is known regarding specific cytokines, proteins, prostaglandins or other factors produced by the individual amnion cell types. Keelan et al (1997) reported that amnion epithelial but not amnion mesenchymal cells produced IL-6 and IL-8; IL-6 is a proinflammatory cytokine and IL-8 is a potent chemokine. High doses of IL-6 stimulated PGE2 production by mixed amnion cell cultures and amnion explant cultures; thus it is unlikely that epithelial cell IL-6 production suppressed mesenchymal cell PGE2 output (Furata et al, 2000). Amnion WISH cells, an immortalized amnion epithelial cell line,
produces IL-4: both IL-4 and IL-13 have been found to inhibit cytokine induced mixed amnion cell and amnion WISH cell PGE_2 production (Keelan et al., 1999; Keelan et al., 1998). These data suggest that amnion epithelial cells may produce an anti-inflammatory cytokine, capable of inhibiting basal or stimulated amnion mesenchymal PG output. Although it is tempting to speculate on the nature of the paracrine mediator(s) produced by the cultured amnion epithelial cells, further investigation is required.

In summary, we have demonstrated that both amnion epithelial and mesenchymal cells produce PGE_2 and respond to both CRH and GC stimulation. Cortisol has been previously demonstrated to increase intrauterine CRH concentration suggesting that cortisol may indirectly increase amniotic PGE_2 production through the stimulation of CRH. We speculate that 11βHSD-I reductase activity with the amnion epithelium increases epithelial cell PG output and may affect mesenchymal cell output. Amnion PG production may increase 11βHSD-I activity in a manner similar to that observed with the chorion suggesting that a positive feed-forward loop regulating local cortisol bio-availability and PG production may exist within the amnion. Paradoxically, amnion epithelial cells may produce a paracrine mediator that inhibits mesenchymal cell PG production. Further investigation of the paracrine interactions within the amnion is warranted to better understand the nature of the relationship between these two cell types in vivo.
General Discussion and Conclusion

A portion of this chapter has been accepted for publication in the journal Biology of Reproduction (in press; 2000) and appears here with the permission of the journal (refer to attached authorization).
At the end of gestation the fetus undergoes a sustained activation of its HPA axis leading to a progressive increase in fetal adrenal GC production. Concurrent with the rise in fetal GC levels there is a gradual increase in plasma, amniotic fluid and intrauterine tissue concentration of PGE$_2$ followed by an increase in PGF$_{2\alpha}$ and the onset of uterine activity (Challis et al., 2000). The goal of this thesis was to examine the relationship between fetal adrenal GC synthesis, intrauterine PG production and the onset of uterine activity.

We have determined that GCs increase intrauterine PG production both directly and indirectly through a GC-stimulated intermediate in the sheep and the human. Our hypothesis was that an increase in cortisol concentration would induce the expression of a critical CAP that in turn would lead to an increase in uterine contractility. However, we were unable to demonstrate an increase in myometrial connexin 43 expression in response to cortisol infusion in the presence or absence increased placental estradiol synthesis. An evolution towards uterine contractility did occur in the absence of cortisol-induced placental estradiol synthesis suggesting that cortisol may increase the expression of other CAP genes. We have demonstrated that cultured human amnion epithelial cells express 11βHSD-I and have provided indirect evidence that the reductase activity of this enzyme increases PGE$_2$ output by these cells. Preliminary studies have determined that paracrine interactions between amnion epithelial and mesenchymal cells may regulate the net PG production by this tissue.

We have established that at the end of gestation cortisol directly increases PGHS-II gene expression and activity within the trophoblast-derived intrauterine tissue. In the sheep, we demonstrated that an elevation in fetal plasma cortisol concentration, independent of increased placental estradiol production, leads to increased mRNA and protein expression PGHS-II within the fetal placental trophoblast tissue and a subsequent rise in fetal plasma
PGE₂ concentration. This stimulatory effect of cortisol appeared to be mediated by a positive feed-forward cascade involving the up-regulation of the active GC receptor isoform, GRα, within the trophoblast tissue. A similar stimulatory effect of GC occurred in the human cell preparation; cortisol and/or the synthetic GC dexamethasone stimulated PGE₂ output by separately cultured, trophoblast-derived amnion epithelial and mesenchymal cells. Both amnion cell types expressed PGHS-II and the GC receptor. Previous studies using mixed human amnion cell cultures demonstrated that the stimulatory effect of GC was receptor dependent, involved the activation of protein kinase and lead to increased PGHS-II mRNA, protein and activity (Economopoulos et al, 1996; Zakar et al, 1995; Smeija et al, 1993; Potestio et al, 1988). We have also provided indirect evidence that 11βHSD-I reductase activity within the amnion epithelial cells regulates PGE₂ production presumably through an increase in the amniotic concentration of bio-available GC. These in vivo and in vitro findings present evidence that GCs induce PGHS-II gene expression and PG production within the trophoblast-derived tissue at the end of gestation and onset of labor.

GCs freely diffuse across the cell membrane into the cytoplasm and subsequently translocate to the nucleus to exert genomic effects. The ligand-GRα complex becomes phosphorylated, dimerizes and subsequently binds to a GC response element (GRE) to induce transcription of the target gene through interaction with the basal transcription apparatus and/or to induce a conformational change in the chromatin structure increasing the accessibility of other regulatory factors (McKay et al, 1999; Bamberger et al, 1996). In addition to this classical mode of steroid hormone receptor action, a second mode of action based on protein-protein interactions between GR and other transcription factors has been documented (McKay et al, 1999; Gottlicher et al, 1998). This mode, referred to as
"crosstalk", involves the interaction of the GR-ligand complex with the AP-1 transcription factor family. The interaction between AP-1 factors and the GRα-ligand complex can be direct through protein-protein interaction, indirect through bridging proteins or through competition for a limiting co-factor (McKay et al, 1999; Gottlicher et al, 1998). The resultant interaction is either a non-DNA bound transcription factor linked to a DNA bound transcription factor or the sequestration of two transcription factors into a non-DNA binding complex. This transcription factor “crosstalk” can occur at either of the transcription factor’s DNA binding sites, at an adjacent non-related transcription factor DNA binding site or at a composite response element which is an overlap of the transcription factor’s DNA binding sites (McKay et al, 1999; Gottlicher et al, 1998). The net effect of “crosstalk” is either transcription factor synergism or negative interference of gene expression; the effect is dependent upon specific cell conditions including cell differentiation and stage of the cell cycle (McKay et al, 1999; Gottlicher et al, 1998). Recently, transcription factor “crosstalk” has been found to not only be limited to GRα and the AP-1 family but also occur between the nuclear hormone receptors and the NFκB transcription factors, CRE, C/EBP or the octamer binding factors. In addition, recent evidence suggests that GCs may also exert rapid, non-genomic effects through specific membrane bound receptors linked to a G protein signaling system. Thus, GCs can exercise genomic and non-genomic influences through a variety of mechanisms: the absence of a GC response element or negative GC response element on a given gene does not preclude GC regulation of that gene.

Traditionally, GCs have been classified as potent anti-inflammatory agents due to their effective inhibition of stimulated PGHS-II expression. The PGHS-II 5’ promoter region does not contain a negative GC response element; the mechanism of GC inhibition has been
postulated to be mediated through interference with an enhancer protein and/or and a decrease in PGHS-II mRNA stability (Goppelt-Streube, 1995). Consistent with the findings presented in this thesis, cortisol and the synthetic GC dexamethasone were found to increase PGE\textsubscript{2} output by cultured ovine trophoblast cells; this effect was inhibited by meloxicam, a specific PGHS-II inhibitor (Martin, Whittle and Challis; unpublished results). Preliminary studies have found a similar GC induced increase in PGHS-II activity in cultured human chorionic and placental trophoblast cells (Mirazi, Alfaidy and Challis; unpublished results). In addition, increased PG output and enhanced metabolism of arachidonic acid in response to GC stimulation has been reported in fetal rat lungs, rat gastric mucosa, murine fibroblasts, bone marrow derived myeloid leukemia cells and rat reno-medullary cells (Tsai et al, 1983; Avunduk et al. 1992; Honma et al. 1980; Russo-Marie Seillan et al. 1980; Chandabrose et al, 1978). This stimulatory effect was characterized as dependent upon concentration and cell differentiation. The 5' promoter of PGHS-II contains a full GRE at (−726-717) bp that could mediate an increase in gene expression (Xu et al, 1995). Alternatively, given the recent evidence supporting the synergistic interaction of transcription factors, GR could interact with a member of the AP-I, NFkB or C/EBP families at either of the respective transcription factor DNA binding sites or a composite response element. GR could interfere with a repressor transcription factor or synergize with a unique fetal trophoblast cell transcription factor to mediate a trophoblast cell specific PGHS response to GC. Zakar et al (1998) and Smeija et al (1993) demonstrated that the GC induction of PGHS-II in cultured amnion cells involved protein kinase C activity. GCs may also may act through a membrane bound receptor to initiate a signal transduction cascade that increases protein kinase activity leading to the phosphorylation and subsequent activation of an inactive, stored pool of PGHS-II.
Boon et al (2000) demonstrated a similar pattern of PGHS-II activation by phosphorylation in the brainstem of the ovine fetus.

However, the trophoblast cells in vitro do retain an anti-inflammatory response to GC administration. Gibb et al (1993) found that GCs inhibited PGE2 production by freshly-dispersed mixed amnion cells yet with time in culture this inhibitory effect was reversed and PGE2 output was increased by GC treatment. DuVal et al (1998) reported that dexamethasone inhibited amnion WISH cell PG production. Blumstein et al (2000) reported that dexamethasone decreased amnion epithelial cell PGE2 production however, a decrease in the expression of PGHS-II was not observed. Several investigators have reported that GCs inhibit cytokine (IL-1β, TNFα, EGF) induced PGHS-II expression and this effect is mediated through negative interference with transcription factor binding at the two NFκB promoter sites, the CRE and a third as yet unidentified enhancer site (Wang et al., 1999; Wang et al., 1998). GCs have also been shown to increase the expression of IκB, the inhibitor protein of NFκB suggesting a possible dual mechanism of GC inhibition of NFκB activity (Wissink et al., 1998). We speculate that the pro-inflammatory effects of GC occur through the up regulation of basal state PGHS-II expression whereas the anti-inflammatory effects of GCs occur through the down regulation of the induced state of PGHS-II expression. Thus, GC can exert differential effects depending on the state of the cell activity.

This hypothesis may explain the differential effects of GCs on freshly dispersed human amnion cells versus cultured cells. Freshly dispersed amnion cells produce large quantities of pro-inflammatory cytokines as well as large quantities of PGE2. Administration of GCs at this time causes a decrease in PGE2 production (Gibb et al, 1993). As time in culture progresses the production of the proinflammatory cytokines decreases as does the
production of PGE₂. At this time the administration of GC causes an increase in PGHS-II expression and PGE₂ output (Gibb et al, 1993). Based on our hypothesis, we speculate that freshly dispersed cells have an increased level of PGHS-II expression and activity induced by autocrine/paracrine action of the pro-inflammatory cytokines and GCs cause a characteristic inhibition of induced PGHS-II expression. As time in culture progresses, the cytokine induction of PGHS-II is attenuated and PGHS-II expression decreases to a basal state capable of re-induction by GCs. Thus, depending upon the level of PGHS-II expression, GCs can exert a bi-phasic effect. We conclude that, at the end of gestation, fetal adrenal GC production directly increases basal PGHS-II expression and activity within intrauterine trophoblast-derived tissue; the precise molecular mechanism by which this induction occurs remains to be investigated.

We have established that in sheep increased maternal endometrial PGHS-II expression and PGF₂α output is dependent upon a cortisol-induced increase in placental estradiol production. Although we were unable to detect any change in the myometrial expression of PGHS-II previous studies have demonstrated that myometrial PGHS-II expression is correlated with placental estradiol production (Gyomorey et al, 2000). These findings suggest that estradiol directs PGHS-II expression within the maternally derived intrauterine tissues. This preferential stimulatory effect of estradiol appears to be mediated by the exclusive expression of the estradiol receptor, both α and β isoforms, within the maternal intrauterine tissues and the exclusion of these receptor isoforms from the fetal trophoblast tissue. It has been previously suggested that fetal cortisol directs the increase in placental trophoblast P450c17 expression leading to the shift in placental steroidogenesis towards increased estradiol production (Mason et al, 1989; France et al, 1988; Steele et al, 1976;
Anderson et al. 1975; Flint et al. 1975). The temporal pattern of trophoblast PGHS-II and P450c17 expression suggests that increased PGE2, produced as a result of GC stimulation, could induce P450c17. In support of this speculation, we have demonstrated that increased fetal plasma cortisol concentration, in the presence or absence of increased estradiol synthesis, leads to an increase in both trophoblast PGE2 production and P450c17 expression.

Several studies have shown that PGs can mediate the expression of P450c17; PGF2α increased P450c17 expression in bovine preovulatory cells and PGE2 increased P450c17 in cultured bovine adrenal cells (Wijayagunawarde et al. 1991; Rainey et al. 1991). Furthermore, the infusion of nimesulide (a potent PGHS-II inhibitor) following the onset of term ovine labor elicited a decrease in PGE2 plasma concentration and a significant decrease in placental trophoblast P450c17 mRNA expression. Preliminary in vitro studies have found that cultured ovine trophoblast cells treated with PGE2 have increased immunoactive P450c17 expression (Martin, Whittle and Challis, preliminary results). The 5' promoter region of the human, rat, porcine and bovine P450c17 genes contain specific cAMP responsive sequences (CRS-1, CRS-2) involved in the regulation of basal and stimulated gene transcription (Lund et al, 1998). In addition, P450c17 activity is regulated by phosphorylation of serine and threonine residues; this phosphorylation is rapidly induced by a cAMP dependent protein kinase (Biason-Lauber et al, 2000). PGE2, acting through either the EP2 or EP4 receptor, would increase intracellular and/or intranuclear cAMP concentration and lead to increased P450c17 gene expression activity. On the basis of these data we speculate that PGE2 may act as a GC-stimulated intermediate responsible for the induction of placental P450c17 and the consequent terminal surge in placental estrogen synthesis. Therefore, cortisol indirectly increases maternal intrauterine tissue PGHS-II
expression through the stimulation of trophoblast PGE$_2$ production, P450$_{C17}$ expression and the subsequent increase in estradiol synthesis.

In the human we have determined a similar indirect pattern of GC regulation of intrauterine PG production mediated by the cortisol-induced intrauterine expression of corticotropin releasing hormone. We demonstrated that CRH stimulates PGE$_2$ production in both amnion epithelial and mesenchymal cells. Immunohistochemical localization studies determined that CRH is expressed in the amnion epithelial and mesenchymal cells (Riley et al. 1991). Culture studies have shown that cortisol and/or dexamethasone increases CRH production by mixed amnion cells (Jones et al., 1989). In vivo, the antenatal administration of betamethasone increased the expression of CRH within amnion epithelial and mesenchymal cells (Marinoni et al. 1998). We speculate that cortisol-induced intrauterine CRH production, in particular by the amnion epithelial and mesenchymal cells, acts in an autocrine/paracrine manner to increase amnion PGE$_2$ production. Therefore, cortisol indirectly increases amnion PG production through the stimulation of intrauterine CRH production. We have also demonstrated that cultured amnion epithelial cells may produce a factor(s) which decrease amnion mesenchymal cell PG production suggesting the possibility that paracrine interactions between the two cell types may regulate net amnion PG production in vivo. The nature of these potential interactions and the mediators remains to be characterized.

Thus, despite the controversy regarding the use of the sheep as a suitable model for human parturition, this thesis highlights the remarkable similarity of intrauterine PG production and regulation between the two species and reinforces the use of the ovine model. Both species undergo activation of the fetal HPA axis through the end of gestation and onset of labor leading to increased adrenal GC production. As discussed, cortisol is a critical
mediator of intrauterine PG production. In both species, these PGs have been found to mediate the key events of labor including myometrial contractility, cervical ripening, membrane rupture and utero-placental blood flow. As well PGs have been found to act in a feed-forward manner to maintain intrauterine cortisol concentrations through the progression of labor. In the sheep, PG acts in an endocrine manner to maintain fetal HPA activation. Intrafetal PGE$_2$ infusion stimulated fetal plasma ACTH and cortisol production (Louis et al, 1976; Thorburn et al, 1979); inhibition of PGE$_2$ production at both term labor and RU486 induced preterm labor decreased fetal plasma ACTH and cortisol levels (McKeown et al, 2000; Unno et al, 1998; Young et al, 1996). In addition, PGE$_2$ increased adrenal cortical cell P450$_{c17}$ expression and activity suggesting that PGE$_2$ could directly increase de novo adrenal cortisol synthesis (Rainey et al, 1991). In the human, PG similarly acts to increase intrauterine cortisol concentration through paracrine/autocrine effects on intrauterine 11βHSD activity. Alfaidy et al (2000) has demonstrated that PGE$_2$ and PGF$_{2\alpha}$ increases the reductase activity of 11βHSD-I in the chorion trophoblast cells and decreases the dehydrogenase activity of 11βHSD-II in the placental trophoblast cells leading to a net increase in the intrauterine concentration of bio-available GC. Therefore, at the end of gestation a positive feed-back cascade between systemic and local GC production and intrauterine PG production drives the labor process in both species.

One important difference between sheep and human parturition is the source of androgen precursors used for intrauterine estrogen synthesis and subsequent PG production. In the sheep, at the end of gestation the intrauterine tissues express the enzyme P450$_{c17}$ to produce a local intrauterine source of androgen precursor from pregnenolone leading to a surge in estradiol production and a decline in placental progesterone secretion. In contrast,
the human intrauterine tissues do not express P450_{c17} at any time in gestation and must rely upon the maternal and fetal adrenal for androgen precursors; thus, a decline in intrauterine progesterone secretion does not occur with human parturition. However, in both species PGE_2 may play a role in the regulation of androgen precursor production. As discussed in the sheep, we speculate that placental PGE_2 production may mediate an autocrine/paracrine induction of P450_{c17}. In the human, we speculate that PGE_2 could increase fetal adrenal P450_{c17} expression leading to increased androgen precursor production.

A second difference in the regulation of PG production between the sheep and the human is the role of intrauterine PG metabolism. In the human, intrauterine progesterone production tonically maintains the expression and activity of PGDH. In effect, PGDH acts as a metabolic barrier to protect the target organs- cervix and myometrium- from the effects of PG. At term, an increase in the intrauterine concentration of cortisol directs a decrease in the expression and activity of PGDH leading to a net increase in intrauterine PG production. In the sheep, we had anticipated that a similar decrease intrauterine PGDH expression and activity would occur. However, the cortisol-dependent increase in trophoblast PGE_2 production and the estradiol-dependent increase in endometrial PGF_{2a} production appeared to occur independently of changes in the expression of PGDH by the maternal intrauterine tissues- the endometrium and the myometrium. Paradoxically, fetal trophoblast expression of PGDH was increased despite an increase in fetal plasma cortisol concentration and a decline in placental progesterone production. This increase could be mediated through the increased trophoblast expression of the active GC receptor isoform. The physiological significance of increased PGDH expression at the end of gestation and onset of parturition is not yet
understood nor is the significance of expressing both PG synthesizing and metabolizing enzymes within the same cell type. These parameters remain to be examined.

The findings presented in this thesis contribute to our understanding of the mechanisms involved in the onset and progression of labor at term and preterm. This critical information can be used for the development of an effective tocolytic therapy. Specific inhibition of intrauterine PGHS-II expression and activity would block the production of PGE\textsubscript{2} and PGF\textsubscript{2α} and the subsequent PG-mediated events—cervical ripening, myometrial contractility and membrane rupture. In addition, a decrease in intrauterine PG production would interrupt the positive feed-back effect of PGs on fetal HPA activity and intrauterine 11βHSD activity. In the sheep specific inhibition of PGHS-II at term labor and RU486 induced preterm labor has been found to effectively inhibit uterine contractility, intrauterine PG production and fetal HPA activity. In the human, the prophylactic administration of nimesulide, a PGHS-II inhibitor, has been reported to delay preterm delivery (Groome et al, 2000). However, the inhibition of intrauterine PG production may not be sufficient to interrupt preterm labor events. Recall that, despite the blockade of cortisol-induced placental estradiol production and endometrial PGF\textsubscript{2α} output, elevated fetal plasma cortisol concentration mediated an evolution towards increased uterine activity. These observations suggest that the possibility of other cortisol-induced uter tonic agents produced by the intrauterine tissues should not be excluded; there may be multiple mechanisms regulating uterine contractility. The redundancy of mechanisms regulating parturition suggests that PGHS-II inhibition in combination with another therapy may be a more effective tocolytic alternative.
In summary, sustained activation of the fetal HPA axis leads to elevated fetal adrenal GC production that increases intrauterine PG production. We conclude that this increase in GC concentration both directly increases fetal trophoblast derived PG production and indirectly increases maternal intrauterine tissue PG production through the stimulation of placental estradiol synthesis. Comparing the events of ovine parturition with those of human parturition, our conclusion is that GCs directly regulate PG production within human trophoblast-derived amnion epithelial and mesenchymal cells. We also conclude that a similar indirect effect of GC on PG production exists within this tissue but is mediated by GC-stimulated intrauterine corticotropin releasing hormone (CRH) synthesis. Furthermore, GC regulated PG production within the epithelial cells is influenced by 11βHSD activity. We conclude that paracrine interactions between the amnion epithelial and mesenchymal cells regulate the net production of PG by this tissue. GC induced PG production in turn stimulates the onset of labor events including cervical ripening, membrane rupture and uterine contractility. Thus, parturition follows a tissue specific progression of events from a fetal signal to a maternal labor response.
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