EXPRESSION AND REGULATION OF PROSTAGLANDIN RECEPTORS AND CYTOCHROME P450c17 HYDROXYLASE IN THE LATE GESTATION OVINE PLACENTA

By

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Previous studies in sheep reported that fetal cortisol produced in late gestation causes increased placental PGHS-2 expression and PGE_2 synthesis. It has been suggested that PGE_2, acting through prostaglandin receptors, upregulates placental P450c17 resulting in altered placental steroid synthesis at term. Using a glucocorticoid-induced ovine model of preterm labour, we demonstrated significant increases in placental PGHS-2 protein expression and fetal plasma PGE_2 which were associated with P450c17 mRNA upregulation after intrafetal cortisol infusion. Co-infusion with meloxicam increased P450c17 expression despite blocking PGE_2 synthesis. We also localized EP1, EP3, and FP receptors to uninucleate cells of the fetal trophoblast. However, increased exogenous and endogenous (by periconceptional undernutrition) cortisol had no effect on altering prostaglandin receptor expression. Therefore, we suggest that increased PGE_2 output in late gestation may not be the major pathway in regulating P450c17 and that cortisol and/or other glucocorticoid-stimulated intermediates could play a role in regulation of placental P450c17.

"Ideals are like stars: you will not succeed in touching them with your hands, but like the seafaring man on the ocean desert of waters, you choose them as your guides, and following them, you reach your destiny."

~ Carl Schurz

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List of Abbreviations

4-OHA	4-hydroxyandrostenedione
11β-HSD	11-beta-hydroxysteroid dehydrogenase
AA	arachidonic acid
ACTH	adrenocorticotropin releasing hormone
ANOVA	analysis of variance
AVP	arginine vasopressin
BLAST	basic local alignment search tool
C	cortisol
cAMP	cyclic adenosine monophosphate
CAP	contractile associated protein
CBG	corticosteroid binding globulin
cDNA	complementary deoxyribonucleic acid
CM	cortisol + meloxicam
cPGES	cytosolic prostaglandin E synthase
cPLA$_2$	cytosolic phospholipase A_2
CRH	corticotropin releasing hormone
CRH-R1	corticotropin releasing hormone receptor subtype 1
Cx-43	connexin 43
Cys	cysteine
D	day (of gestation)
DAB	diaminobenzadine
E_2:P_4	estrogen/progesterone ratio
ELISA	enzyme-linked immunosorbent assay
EP	prostaglandin E_2 receptor
ER	estrogen receptor
FP	prostaglandin $F_{2\alpha}$ receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HPA	hypothalamic-pituitary-adrenal
IL-1β	interleukin 1β
IL-4	interleukin 4
IL-6	interleukin 6
IM	intramuscular
IP3	inositol triphosphate
IV	intravenous

kDa	kilodalton
LSB	Laemmli sample buffer
mPGES	membrane-bound prostaglandin E synthase
mRNA	messenger ribonucleic acid
N	nourished
OATP	organic anion transporter protein
OT	oxytocin receptor
P450c17	cytochrome P450 C17/C21 hydroxylase
P450scc	cytochrome P450 side chain cleavage enzyme
PBS	phosphate buffered saline
PC1	prohormone convertase 1
PG	prostaglandin
PGD_2	prostaglandin D_2
PGDH	prostaglandin dehydrogenase
PGE_2	prostaglandin E_2
PGES	prostaglandin E synthase
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGFM	13,14-dihydro-15-keto-$PGF_{2\alpha}$
PGFS	prostaglandin F synthase
PGHS-1	prostaglandin synthase type 1
PGHS-2	prostaglandin synthase type 2
PGI_2	prostacyclin
PGT	prostaglandin transporter
PLA_2	phospholipase A_2
POMC	proopiomelanocortin
PTB	preterm birth
RIA	radioimmunoassay
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
ROD	relative optical density units
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
S	saline
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
$sPLA_2$	secretory phospholipase A_2
TP	thromboxane receptor
TX	thromboxane

TXA_2	thromboxane A_2
UN	undernutrition
V1b	arginine vasopressin receptor V1b

Chapter One

General Introduction

1.1. Preterm Birth

1.1.1. Clinical Significance of Preterm Birth

Preterm birth (PTB), which is defined as birth before 37 weeks gestation, is a major clinical problem in obstetrics. It occurs in about 5-10% of all pregnancies and accounts for approximately 70% of neonatal mortality and morbidity (Challis et al., 2002; Lumley, 2003). Despite recent advances in the medical field, the incidence of PTB continues to rise, affecting women in both developed and underdeveloped countries (Gibb, 1998; Gilbert, 2006). Although infant mortality is decreasing, morbidity related to PTB is significant as survivors are more likely to suffer from adverse health outcomes, which contribute to a poor quality of life (Robertson et al., 1992). A large number of studies have focused on the impact of premature birth of less than 32 weeks gestation, as compared to 32-36 weeks, due to the greater prevalence of adverse perinatal outcomes such as acute respiratory distress syndrome, necrotizing enterocolitis and intraventricular hemorrhage (Gilbert et al., 2003; Luig and Lui, 2005; Moutquin, 2003; Robertson et al., 1992; Vergani et al., 2000). However, independent of the time of delivery, individuals born prematurely are at an increased risk of developing a variety of health problems that begin in early childhood and can continue through into adulthood such as cerebral palsy, cognitive impairment, deafness, blindness, respiratory conditions, and

complications from hospitalization in a neonatal intensive care unit (Elovitz et al., 2006; Takahashi et al., 2005). Furthermore, some may suffer from neurosensory impairments and face educational setbacks due to learning difficulties.

There is no single cause accounting for all cases of PTB. Instead, the causes are diverse, encompassing a variety of physiological, psychological, and social factors which place a woman at greater risk for delivering prematurely (Neggers et al., 2006; Steer et al., 2006). Previous preterm delivery, multi-fetal pregnancies, placental anomalies, cervical or uterine disorders, infection (in approximately 30% of cases), premature rupture of the membranes, stress, smoking, and low socio-economic status have all been identified as factors contributing to PTB (Challis et al., 2000; Steer, 2006). However, 50% of all preterm births are deemed idiopathic with no identified cause (Challis et al., 2002). Moreover, PTB imparts significant emotional stress on the affected family members and treatment of preterm infants comes at a major cost to the healthcare community and to society in general (Gilbert, 2006). Presently, there are no effective treatments or diagnostic indicators of preterm delivery; thus, current research in this area aims to investigate and understand the molecular and physiological processes involved in labour onset in order to identify new markers of preterm birth.

1.2. Parturition

1.2.1. General Characteristics of Parturition

Parturition involves a complex series of endocrine and mechanical interactions that occur between the fetus, placenta, and mother. Its timing requires the synchronous coordination of maternal uterine contractility and cervical dilation with complete maturation of fetal organ systems, which culminates in the expulsion of the fetus from the uterus to the extrauterine environment (Challis et al., 2000). The process of parturition has been studied in a variety of mammalian species, with a considerable amount of information being derived from studies utilizing the sheep model. Combined, these studies provide evidence to suggest that it is the fetus itself that triggers the onset of labour through maturation and sustained activation of the fetal hypothalamic-pituitary-adrenal (HPA) axis and subsequent increased cortisol production.

The course of pregnancy, up to and including parturition, is divided into four distinct phases with respect to uterine activity (**Figure 1.1**). The first phase, designated Phase 0, accounts for a majority of gestation and is defined by uterine quiescence. During this phase, myometrial activity is inhibited by a variety of molecules such as progesterone, prostacyclin, relaxin, nitrous oxide and parathyroid hormone-related peptide (Challis et al., 2000; Chwalisz and Garfield, 1997; Downing and Sherwood, 1985; Fergusen et al., 1992; Lye and Challis, 1982; Porter et al., 1981; Sakbun et al., 1990; Thiede et al., 1990). Despite the overall characteristic of uterine quiescence in Phase 0, some myometrial contractions can occur (i.e. Braxton-Hicks); however, they are

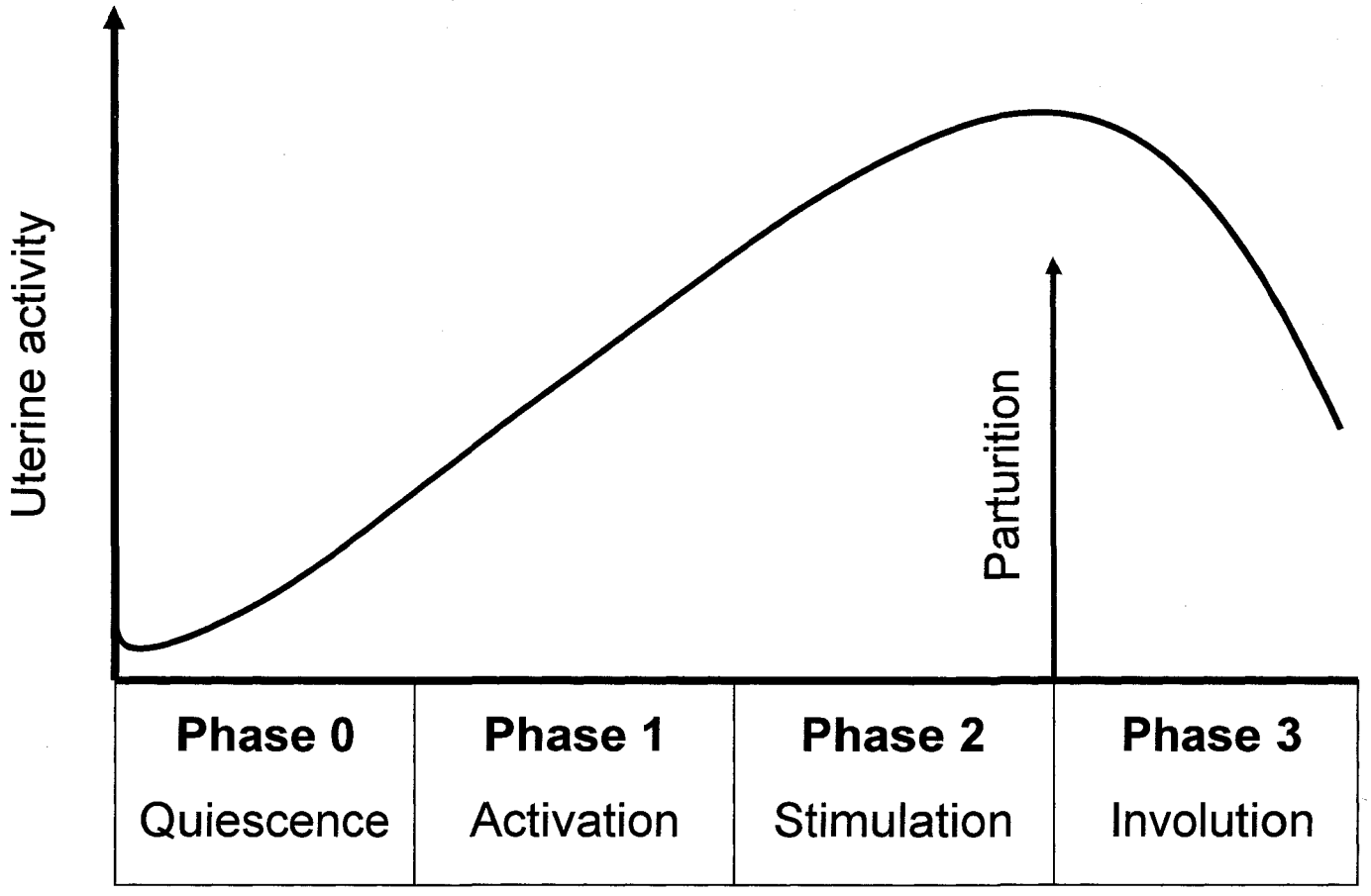


Figure 1.1 Phases of uterine activity during pregnancy and parturition (Adapted from Challis et al., 2002).

inadequately synchronized and are of low amplitude, resulting in minor increases in uterine pressure (Harding et al., 1982).

The next phase, Phase 1, is marked by the transition from uterine quiescence to uterine activation, which marks the beginning of parturition. The timing of the activation phase, and thus the length of gestation, is determined by the genotype of the fetus (Challis et al., 2000; Kitts et al., 1984, 1985). Evidence suggests that the timing of uterine activation can be influenced by both an endocrine pathway, in which maturation of the fetal HPA axis causes an increase in circulating cortisol, and a mechanical pathway involving uterine stretch due to the growing fetus (**Figure 1.2**) (Challis et al., 2000; Ou and Lye, 1997). Particularly, genes for the contractile-associated proteins (CAPs), which include connexin 43 (Cx-43), oxytocin receptor (OT), PG receptors, and ion channels, specifically Ca^{2+} channels, are known to be upregulated in this phase (Challis et al., 2000). Furthermore, this increase in CAP gene expression is associated with a decrease in progesterone and an increase in estrogen levels, thereby contributing to myometrial contractility and preparing the uterus to respond to contractile stimuli (Challis et al., 2000; Lye and Porter, 1978; Whittle et al., 2000).

Phase 2, which is the second phase of parturition, is referred to as the stimulation phase (Challis et al., 2000). Uterotonins, such as PGs (PGE_2 and $PGF_{2\alpha}$) and oxytocin, act on their receptors to stimulate myometrial activity, causing it to fully contract and result in the delivery of the baby, thereby marking the end of Phase 2 (Challis et al., 2000; Chard, 1989; Fuchs et al., 1982). During

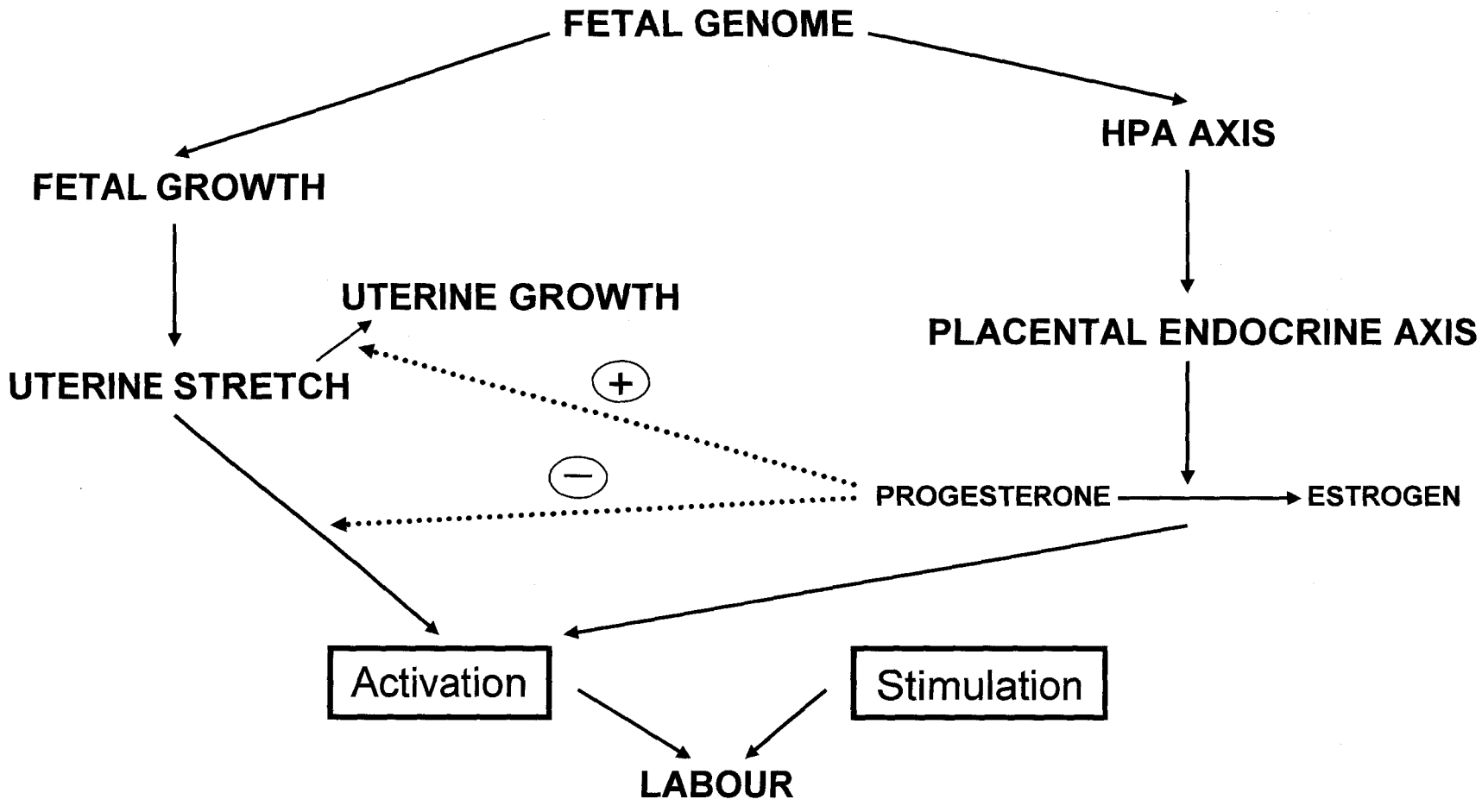


Figure 1.2 Schematic of the onset of labour

The onset of labour is dictated by the fetal genome proceeding through either a fetal growth pathway with increases in uterine stretch or a fetal endocrine pathway involving fetal HPA axis activation. Both pathways are interdependent in regulating parturition. (Adapted from Challis et al., 2000).

Phase 3, the uterus undergoes involution and the placenta is expelled; both processes being regulated by oxytocin (Challis et al., 2000).

A disturbance in the inhibition mechanism(s) critical in Phase 0 of pregnancy or a response to overwhelming stimuli may precipitate early activation of the endocrine and mechanical pathways, resulting in onset of myometrial contractions and spontaneous preterm labour (Norwitz et al., 1999).

1.2.2. Ovine Parturition

1.2.2.1. Role of the Fetal HPA Axis

Past and current research studies using sheep have lead to a greater understanding of the relationship between fetal HPA function and the process of parturition. Its critical role in the timing of labour has been demonstrated by experiments involving intrafetal administration of exogenous cortisol, synthetic glucocorticoids, or adrenocorticotropic hormone (ACTH) which resulted in premature delivery of the fetus (Liggins, 1968, 1969; McLaren et al., 1996; Thorburn et al., 1991). Similarly, fetal hypophysectomy, adrenalectomy, and lesions to the hypothalamic-pituitary stalk and paraventricular nuclei of the hypothalamus all resulted in a prolongation of gestation (Gluckman et al., 1991; McDonald et al., 1991).

In late gestation (term in sheep = 145 days), maturation of the fetal HPA axis occurs in which corticotropin-releasing hormone (CRH), produced by the hypothalamus, is secreted into the hypophyseal portal system to drive synthesis of proopiomelanocortin (POMC) and subsequently, ACTH from the anterior

pituitary (Liggins et al., 1973; Whittle et al., 2001). Protein and mRNA expression of CRH is present at Day (d) 60 of gestation and continues to rise to d120 where its expression is greatly increased over the remainder of gestation (Matthews et al., 1991, 1996). At the level of the pituitary, CRH exerts its effect by binding to the CRH receptor subtype I (CRH-R1), thereby stimulating the production of ACTH (Young and Rose, 2002). CRH-R1 expression increases from d100-135 of gestation and then, as term approaches, its levels decline due to receptor down-regulation and decreased ligand binding by CRH and cortisol (Lu et al., 1991). Consistent with these findings, responsiveness of the fetal pituitary to intrafetal CRH infusion decreases from d125 until term (Norman et al., 1985).

The hypothalamic hormone, arginine vasopressin (AVP), has also been identified as contributing to the stimulation of ACTH synthesis in the fetal sheep (Matthews & Challis, 1995). In cultured fetal pituitary cells, both CRH and AVP were found to increase POMC mRNA expression and stimulate ACTH output in a dose-dependent manner (Durand et al., 1986; Matthews & Challis, 1995). Moreover, in late gestation, both CRH and AVP have a synergistic effect on the production of ACTH (Carr et al., 1995). Despite the decreased responsiveness of the fetal pituitary to CRH, AVP has been found to have a greater stimulatory effect (using an *in vitro* system) on ACTH production in late gestation and thus it has been suggested that AVP may be responsible for stimulating the rise in ACTH over the last 20 days of gestation (Durand et al., 1986; Whittle et al., 2001). Although a decrease in AVP receptor (V1b) mRNA has recently been reported in late gestation, these levels may not correlate with AVP binding

(Rabadan-Diehl and Aguilera, 1998; Young and Rose, 2002). Thus, together, both of these hypothalamic hormones play a role in stimulating ACTH production in late gestation.

ACTH is produced within the anterior pituitary (pars distalis and pars intermedia) from cleavage of its precursor, POMC, by prohormone convertase 1 (PC1). In the pars distalis, POMC is expressed from d60 and continues to rise to d120 of gestation where it rapidly increases its expression levels to term, with no further changes during labour onset (Matthews et al., 1994; Hennessy, 1982; Holloway et al., 2001; Norman et al., 1985). These data are consistent with the plasma data in which the concentration of ACTH gradually increases from d110 of gestation to term and undergoes a further surge in output with the progression of labour (Holloway et al., 2001; Norman et al.,1985). Additionally, ACTH receptor mRNA increases after d130 of gestation and ACTH, itself, can increase its own receptor signal transduction by coupling its receptor to adenylate cyclase, thus increasing adrenal responsiveness through to the end of gestation (Durand et al., 1981; Fraser et al., 2001). Furthermore, Farrand et al. (2006) identified three major subpopulations of corticotrophs in the fetal pituitary whose differential expression of ACTH, POMC, and CRH-R1 may play a key role in HPA activity during normal parturition and during periods of intrauterine stress. Finally, PC1 expression increases toward the end of gestation suggesting that increased ACTH production could partially be due to increased cleavage of POMC (Holloway et al., 2001).

Subsequent to this rise in ACTH, there is a sustained increase in fetal plasma cortisol concentrations (Magyar et al., 1980). At the level of the adrenal glands, ACTH induces expression of major steroidogenic enzymes such as P450 side chain cleavage enzyme (P450scc) and P450 C17/C21 hydroxylase (P450c17), which are involved in *de novo* synthesis of cortisol (Whittle et al., 2001). Therefore, ACTH plays a key role in stimulating cortisol production in the fetal adrenals, as observed in late gestation (Conley et al., 1997; Durand et al., 1982).

In late gestation, there are several mechanisms which work to sustain acitivity of the HPA axis. First, there appears to be an attenuation of the normal negative feedback regulation of HPA function (Challis & Brooks, 1989). Cortisol exerts its effect on the fetal pituitary in late gestation through glucocorticoid receptors (GR). Fetal pituitary GR mRNA and cortisol binding has been observed to increase from d135 until term and subsequently decrease with the progression of labour (Yang et al., 1992). GR expression in the hypothalamus also decreases in late pregnancy (Matthews & Challis, 1996). Second, corticosteroid binding globulin (CBG), a high-affinity cortisol binding protein, increases in expression and concentration in the fetal pituitary and plasma through gestation (Ballard et al., 1982; Berdusco et al., 1993; Challis et al., 1995). CBG may act to bind cortisol and limit the amount of free cortisol available that could potentially exert a negative feedback. Third, the enzyme 11-β-hydroxysteroid dehydrogenase (11β-HSD), which interconverts cortisol and its inactive metabolite cortisone, is expressed in the fetal pituitary (Yang et al.,

1995). Within the pituitary, 11β-HSD has been reported to preferentially metabolize cortisol to cortisone, thereby reducing the negative feedback on pituitary ACTH production. Thus, these data together suggest that ACTH production from the pituitary may be incompletely inhibited by the increase in cortisol levels due to an attenuation of the normal negative feedback loop (Whittle et al., 2001).

Maintenance of HPA axis activation was also originally believed to be positively regulated by placental PGs, particularly PGE_2, in late gestation. Evidence to support this idea came from a number of studies among which intrafetal infusion of PGE_2 had been shown to stimulate fetal plasma ACTH and cortisol production (Louis et al., 1976; Thorburn & Challis, 1979) or inhibition of PGE_2 production resulted in a decrease in fetal plasma ACTH and cortisol levels (McKeown et al., 2000; Unno et al., 1998; Young et al., 1996). However, Reimsnider et al. (2005) have recently shown that PGHS-1 and -2 selective inhibition with resveratrol and nimesulide, respectively, during late gestation did not significantly alter plasma ACTH or cortisol concentrations, suggesting that PGE_2 does not tonically stimulate fetal ACTH secretion and thus does not positively regulate the fetal HPA axis. Despite these findings, further investigation into the regulation of the fetal HPA axis in late gestation is warranted. Thus, the mechanisms regulating the sustained activation of the fetal HPA axis through the end of gestation are complex and multi-factorial.

1.2.2.2. The Placental Endocrine Axis in Parturition

The 'classical' model of ovine parturition predicts that altered placental steroid synthesis at term is controlled by the direct regulation of P450c17 activity by cortisol, produced from the fetal adrenal glands (Liggins et al., 1977; Mason et al., 1989). Consequently, the activation of P450c17, which diverts pregnenolone away from the production of progesterone and toward the production of C19 steroid precursors, causes a significant increase in estrogen synthesis and a decline in progesterone secretion (Anderson et al., 1975; Mason et al., 1989; Whittle et al., 2001). Estrogen was then thought to cause an increase in PGHS-2 expression and activity within the intrauterine tissues leading to the production of PGE_2 and $PGF_{2\alpha}$ and triggering the expression of a cassette of CAPs (Whittle et al., 2001). As a result, the CAPs would activate the myometrium resulting in increased uterine contractility and labour onset. However, both past and recent studies have provided evidence against this classical mechanism. First, there is an increase in placental PGE_2 output over the last 15-20 days of ovine gestation, before the observed rise in P450c17 activity (Challis et al., 1976). Also, Whittle et al. (2000) reported increased expression of PGHS-2 mRNA and protein in placental trophoblast and a rise in fetal plasma PGE_2 after intrafetal infusion of cortisol and an aromatase inhibitor, 4-hydroxyandrostenedione (4-OHA). Thus, PG biosynthesis increased independent of a rise in estradiol levels. Furthermore, PGHS-2 mRNA and protein expression preceded P450c17 expression and both enzymes were co-localized to uninucleate trophoblast cells (Gyomorey et al., 2000a). In *in vitro* studies, direct regulation of P450c17 by

PGE_2 was observed in bovine adrenocortical cells (Rainey et al., 1991), and in preliminary studies, a dose- and time-dependent upregulation of P450c17 mRNA and protein in cultured ovine placental trophoblasts treated with exogenous PGE_2 has been reported (D. Rurak, personal communication).

Therefore, the 'current' model of ovine parturition has been proposed which suggests that two separate pathways of PG production exist (**Figure 1.3**). First, fetal cortisol induces placental PGHS-2 expression and subsequently causes an increase in PGE_2 production. Placental PGE_2 acts in an autocrine, paracrine, or intracrine manner through PG receptors to upregulate placental P450c17 expression, thus driving the surge in estradiol production and fall in progesterone, observed at term. Second, the increased placental estradiol output induces endometrial PGHS-2 expression and $PGF_{2\alpha}$ synthesis, thereby contributing to the increased uterine activity observed at term. Therefore, these observations preclude a direct effect of estradiol on stimulation of placental PGHS-2 expression and are consistent with glucocorticoid regulation of PGHS-2 within the placenta. However, the mechanism by which PGE_2 increases P450c17 is not understood and there is a lack of information concerning PG receptor expression and regulation in term and preterm ovine tissues.

1.2.2.3. Myometrial Events in Parturition

At labour onset, the pattern of uterine activity changes from long duration, low amplitude, and low frequency contractures to high frequency, high amplitude, and short duration contractions associated with increased intrauterine pressure

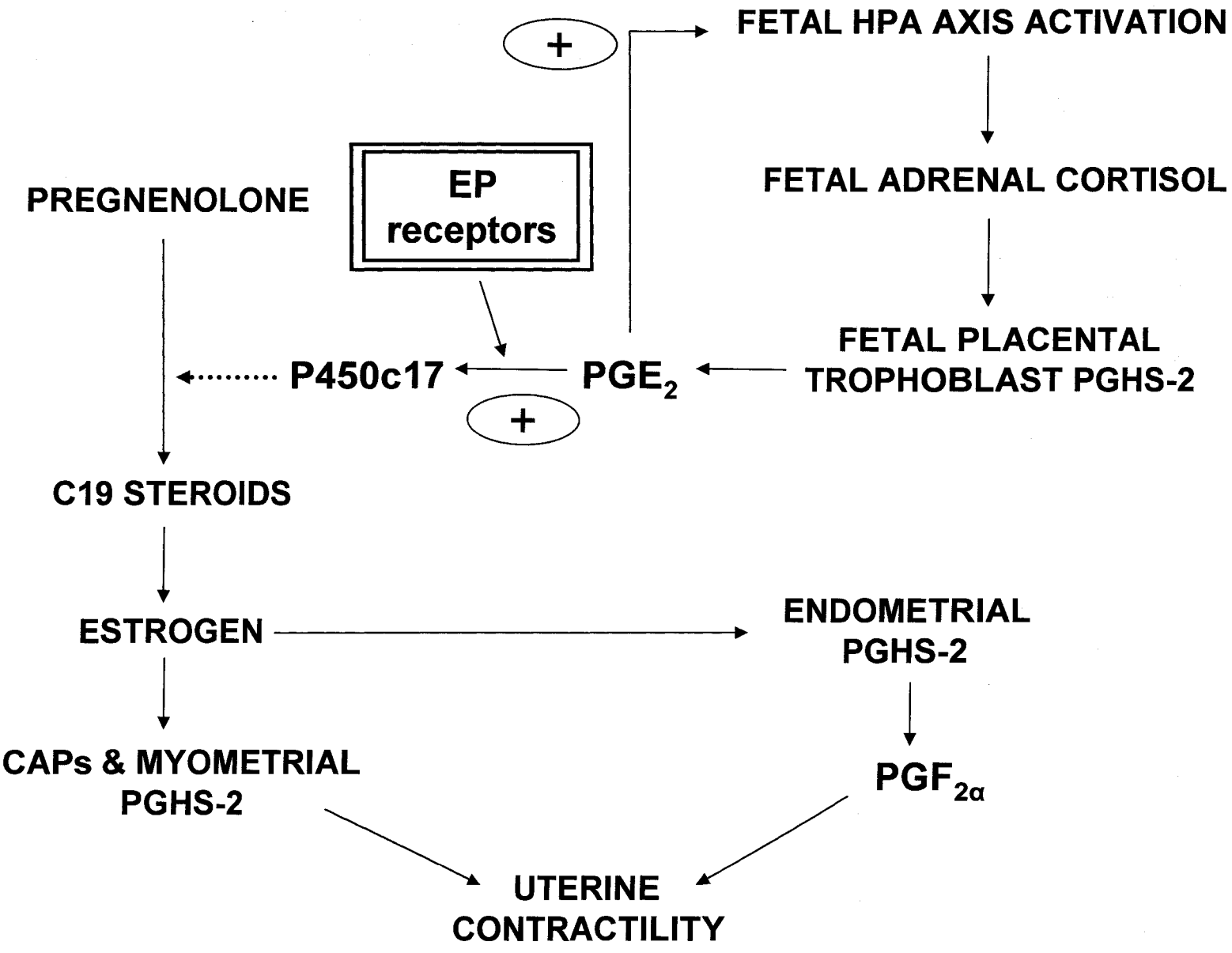


Figure 1.3 Endocrine events in the onset of ovine parturition (Adapted from Whittle et al., 2001).

(Whittle et al., 2001). Thus two distinct, yet overlapping phases for myometrial contractility have been defined: uterine activation and uterine stimulation, which have previously been described (Challis et al., 2000). Briefly, uterine activation involves the upregulation of myometrial CAPs which allow the myometrium to respond to contractile stimulants in a coordinated manner. On the other hand, uterine stimulation refers to the synchronous, contractile response of the uterus to uterotonins such as PGs and oxytocin. In sheep, expression of myometrial Cx-43 increases with the onset and progression of labour and regulation of CAP expression and myometrial contractility has been linked with the increase in the estrogen:progesterone (E_2:P_4) ratio (Petrocelli and Lye, 1993). In turn, the steroid effects on CAP gene expression are dependent on mechanical signals such as myometrial stretch (Garfield et al., 1979; Ou et al., 1998, 2000; Petrocelli & Lye, 1993). Thus, the final stage of labour involves communication between fetal and placental endocrine signals, leading to a maternal contractile response.

1.3. Prostaglandins

1.3.1. Discovery of Prostaglandins

Prostanoids, which are comprised of prostaglandins (PGs) and thromboxanes (TXs), are oxygenated metabolites of C-20 polyunsaturated fatty acids, such as arachidonic acid and eicosapentaenoic acid, that are liberated from membrane phospholipids (Cha et al., 2006; Narumiya et al., 1999).

The discovery of PGs and understanding their biosynthesis initially came from Goldblatt and von Euler in 1935 who, respectively, demonstrated that a lipid

soluble factor contained in semen could stimulate smooth muscle contraction and decrease blood pressure (Cha et al., 2006; von Euler, 1935; Goldblatt, 1935). Later, two major PGs (PGE_2 and $PGF_{2\alpha}$), which play important roles in reproductive physiology, particularly during pregnancy and parturition, were isolated by Bergstrom and colleagues (Bergstrom, 1967). Furthermore, they determined that PGs are formed from unsaturated fatty acids, primarily arachidonic acid (Bergstrom, 1967; Cha et al., 2006), and additional studies conducted by Bergstroem and Samuelsson (1965) revealed the process by which PGs (and leukotrienes) are synthesized and metabolized (Bergstroem and Samuelsson, 1965).

1.3.2. Structure of Prostaglandins

PGs, which are produced from the activity of prostaglandin synthases, are derived from C-20 unsaturated fatty acids (Narumiya et al., 1999). They contain a cyclopentane ring with two side chains attached to this ring, denoted as α and ω (Narumiya et al., 1999). Modifications to the cyclopentane ring differentiate PGs into different types, classified A through I. Types A, B, and C do not occur naturally and can only be artificially produced via extraction procedures. PG types G and H, which are intermediate PGs in the biosynthetic pathway of prostanoids (**Figure 1.4**), share the same ring structure but differ at C-15, where PGG contains a hydroperoxy group and PGH contains a hydroxyl group (Narumiya et al., 1999). A further classification system of PGs divides them into three series (1, 2, or 3) based upon the number of double bonds that exist in their

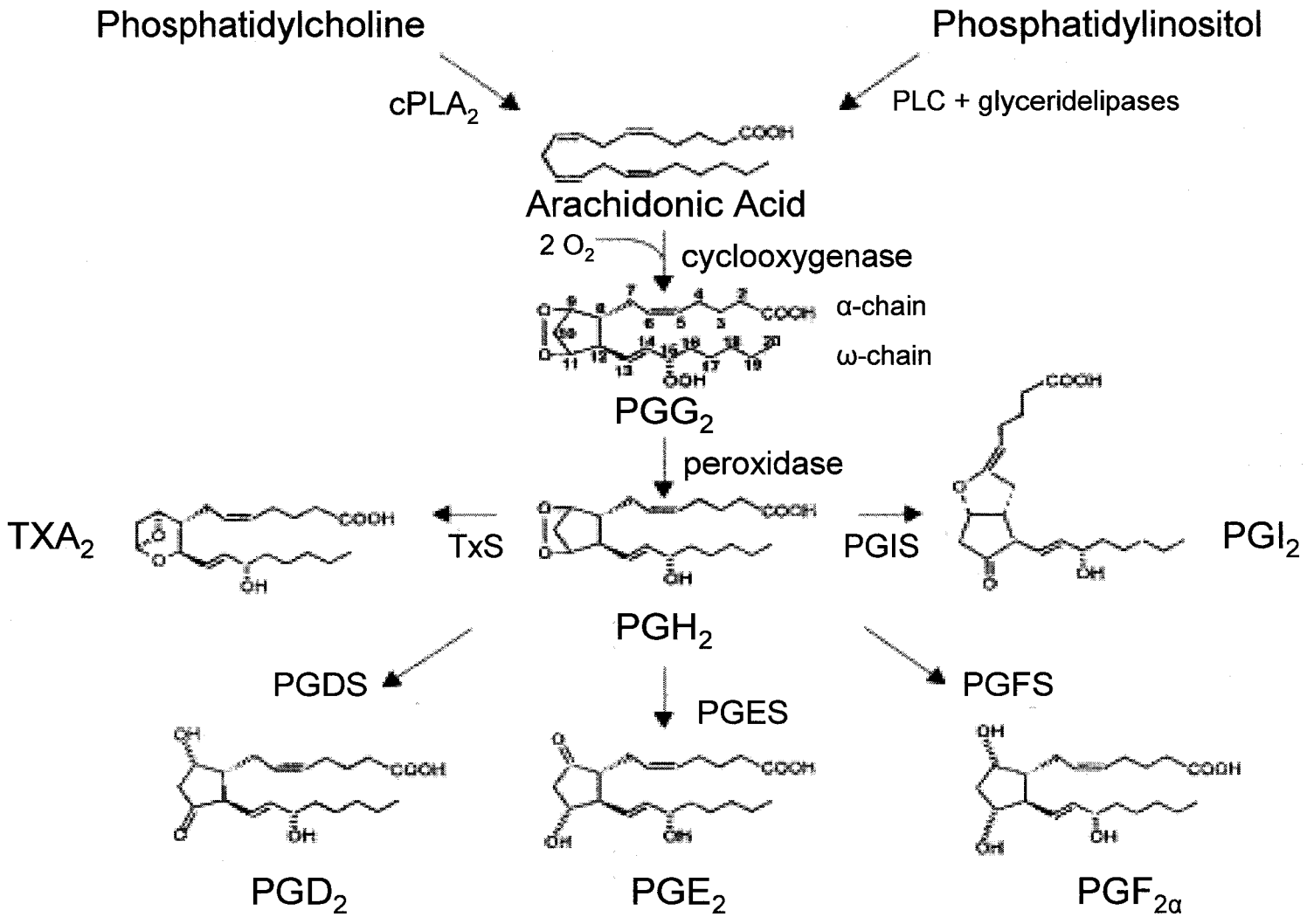


Figure 1.4 Prostanoid biosynthetic pathway (Adapted from Bos et al., 2004).

side chains: series 1 contain a 13-trans double bond; series 2 contain 5-cis and 13-trans double bonds; and series 3 contain 5-cis, 13-trans, and 17-cis double bonds (Narumiya et al., 1999).

1.3.3. Synthesis of Prostaglandins

PGs are synthesized in response to various physiological and pathological stimuli. The initial step in their synthesis is the liberation of fatty acids from membrane phospholipids. Fatty acid precursors for the synthesis of PGs differ between each of the three series of PGs. Series 1 PGs are produced from γ-homolinolenic acid (8, 11, 14-eicosatrienoic acid), series 2 from arachidonic acid (5, 8, 11, 14-eicosa tetraenoic acid), and series 3 from 5, 8, 11, 14-eicosapentaenoic acid (Narumiya et al., 1999). Of these precursors, arachidonic acid is the most abundant fatty acid in mammals; thus, series 2 prostanoids are the most predominantly formed in biological systems. Therefore, in order to begin synthesis of PGs, mobilization of arachidonic acid is required, which is achieved by the action of phospholipases, including phospholipase A_2 (PLA_2) and phospholipase C enzymes (Clark et al., 1991; Dennis, 1987). It is this initial step that is referred to as the rate limiting step in the production of PGs.

PLA_2 can exist as a cytosolic form, $cPLA_2$ (85-110 kDa), or as the extracellular secretory types, $sPLA_2$ I, II, and III (14 kDa forms) (Challis et al., 2000; Smith, 1992; Zhang et al., 1996). Their enzyme activities are distinguished biochemically by virtue of their preference for hydrolyzing the acyl bond in the sn-2 position of the glycerol backbone of specific fatty acids (Bingham and Austen,

1999; Rice, 1998; Uozumi et al., 1997). $cPLA_2$ has been shown to preferentially mobilize arachidonic acid as compared to $sPLA_2$, which exhibits little preference for the fatty acid (Clark et al., 1991). Under basal conditions, arachidonic acid predominantly exists as an esterified form in the plasma membrane; however, after phospholipase-mediated mobilization, arachidonic acid becomes unesterified (Clark et al., 1991). In intrauterine tissues, such as the fetal membranes and myometrium, PLA_2 isozymes have been localized (Skannal et al., 1997a, b); however, species-specific differences in their expression levels have been reported. In sheep, PLA_2 activity was present in all intrauterine tissues, exhibiting highest levels within the amnion (Grieves and Liggins, 1976). In a more recent study, $cPLA_2$ mRNA detected in the endometrium increased significantly with spontaneous and glucocorticoid (GC)-induced preterm labour but could not be detected in the fetal membranes (Zhang et al., 1996), whereas in human amnion, PLA_2 expression gradually increased to the time of labour, with no further increases in expression (Olson et al., 1995).

In the next step, arachidonic acid is converted to PGG_2, a prostanoid precursor, which is subsequently peroxidized to PGH_2. Both of these enzymatic reactions are catalyzed by prostaglandin endoperoxide H synthases (PGHS; also known as cyclooxygenases) (Schuster, 1998). Two isozymes exist, denoted as PGHS-1 and PGHS-2, which are encoded by different genes (Kraemer et al., 1992; Kujubu et al., 1992). The molecular weights of PGHS-1 and PGHS-2 are 70 kDa and 72 kDa, respectively, and the proteins share 60-65% sequence homology, differing primarily in their membrane binding domains (Slater et al.,

1999; Spencer et al., 1999). PGHS-1, which is involved in important biological functions such as renal water reabsorption and platelet aggregation, is present in many tissues where it is constitutively expressed and developmentally regulated (Bos et al., 2004; Brannon et al., 1994; Morita et al., 1995). In contrast to this, PGHS-2 expression is inducible in response to hormones, mitogens, and/or various pro-inflammatory mediators (Morita et al., 1995). Both isozymes have been immunolocalized to the lumen of the endoplasmic reticulum, and the inner and outer membranes of the nuclear envelope (Morita et al., 1995; Spencer et al., 1998). Immunolocalization and expression patterns of the PGHS enzymes over the course of pregnancy and parturition have been well described in many species. Briefly, over the course of gestation in the sheep, PGHS immunoreactivity shifts from the maternal syncytium in early pregnancy to the fetal trophoblast cells in late pregnancy (Boshier et al., 1991). A more detailed description of their expression profiles within sheep intrauterine tissues will be discussed in Section 1.5.1.

After PGHS activity, the production of PGs is completed by cell-specific synthases. In this final stage, the PG intermediate, PGH_2, is converted into PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, or thromboxanes, which are then either transported out of the cell or within the nucleus to elicit their intended physiological effect (Jabbour and Sales, 2004; Narumiya et al., 1999). More specifically, PGES, which mediates PGE_2 production, exists in at least two different major forms: cytosolic (cPGES) and membrane-bound (mPGES) (Helliwell et al., 2004a). On the other hand, the $PGF_{2\alpha}$ synthase, PGFS, has not been found to exist as

separate cytosolic or membrane-bound forms. Both PGES and PGFS expression have been reported by various groups in the intrauterine tissues of both human and sheep (Helliwell et al., 2004a). In ovine placental and endometrial tissues, increased expression of mPGES has been reported in late gestation (Zhang et al., 2006). Also, PGES protein was localized to the fetal trophoblast in sheep from d65 of gestation and continued to increase to term, where its protein expression levels increased significantly (Martin et al., 2002). Furthermore, PGFS mRNA has been detected in ovine placenta, endometrium and myometrium during late gestation; however, its expression was found to be predominantly expressed in the endometrium (Palliser et al., 2004) and there were no labour-associated changes in its expression levels (Wu et al., 2001).

1.3.4. Metabolism of Prostaglandins

The regulation of PGs is governed by the balance between PG synthesis and metabolism. As previously described, PGHS enzymes play an important role in the production of PGs, where NAD+-dependent 15-hydroxyprostaglandin dehydrogenase (PGDH), also known as type 1 PGDH, is recognized as the key enzyme responsible for PG catabolism (Okita and Okita, 1996). PGDH has been purified from cell extracts obtained from various organs of the body (i.e. lung and kidneys) and in gestational tissues such as the placenta, from which human PGDH has been cloned (Ensor et al., 1990; Okita and Okita, 1996; Riley et al., 2000). The two predominant PGs associated with parturition, PGE_2 and $PGF_{2\alpha}$, are inactivated by type 1 PGDH through the reversible initial oxidation of 15(S)-

hydroxyl groups into 15-keto metabolites which possess diminished PG activity (Anggard et al., 1971; Keirse et al., 1975, 1976). Other PGs, such as PGE_1 and PGI_2, may also serve as substrates for this enzyme (Ensor and Tai, 1995).

Another enzyme capable of PG catabolism is NADP-dependent PGDH, otherwise known as type 2 PGDH or carbonyl reductase (Okita and Okita, 1996). It uses NADP+ as a cofactor and shares 20% sequence homology with type 1 PGDH (Okita and Okita, 1996; Wermuth, 1992). Although type 2 PGDH possesses a wider range of substrate affinity, it requires higher concentrations of PGs for optimal activity, as compared to type 1 PGDH, suggesting it is not the major enzyme responsible for PG metabolism (Chang and Tai, 1981; Hansen, 1976; Okita and Okita, 1996). Furthermore, the NADP+ cofactor usually exists at a reduced state in living cells as compared to NAD+ (Mibe et al., 1992).

The expression and regulation of PGDH during pregnancy and parturition has been well-described from studies using human intrauterine tissues. Its expression and activity levels were measured in both the placenta and, more predominantly, in the chorion of the fetal membranes (Sangha et al., 1994). These findings suggest that the chorion may act as a metabolic barrier preventing the passage of PGs, produced by the fetal membranes, from reaching the myometrium and disturbing uterine quiescence (Challis et al.,1999; Sangha et al., 1994). In the case of preterm labour, increased PGHS-2 activity and decreased chorionic PGDH expression have been demonstrated, thereby increasing PG output (Challis et al., 2000).

Similarly, in the sheep, PGDH has been immunolocalized to the placenta, fetal membranes, endometrium and myometrium over the course of gestation (Keirse et al., 1976, 1977; Riley et al., 2000). In the fetal membranes, PGDH is primarily localized to the chorion, and in the placentomes, it is principally expressed by the uninucleate trophoblast cells on the fetal side, whereas the binucleate trophoblast cells do not express PGDH (Riley et al., 2000). Also, in late gestation, PGHS-2 and PGDH are localized to the uninucleate cells of the fetal trophoblast, thus there may be autocrine regulation of PGs by PGDH within the cell (Boshier et al., 1991; Gibb et al., 1996; Riley et al., 2000). Resultantly, PGDH may have a central role in determining the concentration of biologically active PGs within the placenta and fetal membranes at the time of both term and preterm labour.

1.3.5. Prostaglandin Transporters

At physiologic pH, PGs predominate as charged organic anions and diffuse poorly through the lipid bilayer due to the low intrinsic permeability of the plasma membrane to PGs (Baroody and Bito, 1981; Bito and Baroody, 1975; Chan et al., 1998). In order to exert their biological effects and bind to membrane-bound PG receptors, PGs need to be exported outside of cells. Moreover, the cellular uptake of PGs is essential to facilitate PG inactivation and metabolic clearance by the action of intracellular catabolic enzymes (Bito, 1975; Chan et al., 1998). Therefore, it has been demonstrated in a variety of tissues, including the vagina and uterus, that PGs undergo facilitated, carrier-mediated

transport by the PG transporter (PGT) (Bito and Spellane, 1974; Chan et al., 1998).

The PGT is a broadly expressed, 12 membrane-spanning domain integral membrane protein belonging to the family of solute-carrier organic anion transporter proteins (OATPs), specifically the OATP 2A1 family (Banu et al., 2005; Chan et al., 1998; Schuster, 1998). Apart from containing 12 transmembrane domains, it shares other common structural features with OATPs such as clusters of cysteine residues in the extracellular loops and multiple glycosylation and phosphorylation sites (Banu et al., 2008; Zhou and You, 2007). It has been cloned in a variety of species such as rat (Kanai et al., 1995), mouse (Pucci et al., 1999), human (Lu et al., 1996), cow (Banu et al., 2003) and, most recently, sheep (Banu et al., 2008). With respect to reproductive processes such as pregnancy, a majority of studies have examined the role of PGT using bovine tissues. Banu et al. (2003) have reported that bovine PGT is expressed in the endometrium and myometrium throughout the estrous cycle and bovine PGT transports both PGE_2 and $PGF_{2\alpha}$ with equal affinity. Furthermore, during pregnancy in the cow, PGT mRNA and protein demonstrate higher levels of expression in the maternal caruncles versus the fetal membranes, particularly over the last 100 days of gestation (Banu et al., 2005). Thus, the higher expression of PGT in the maternal caruncle may favour the transport of PGs from the fetal side to the maternal side within the placentome as this is where the maternal caruncle and fetal cotyledons closely oppose each other and allow the exchange of nutrients and metabolites (Banu et al., 2005; Challis et al., 2002).

In the sheep, PGT mRNA and protein were found to be abundantly expressed in the endometrium and its expression levels were regulated in a spatial and temporal pattern during the estrous cycle, in which expression levels increased to the time of luteolysis (Banu et al., 2008). Additionally, silencing of the PGT gene in an ovine endometrial epithelial cell line has been reported to impair transport of both PGE_2 and $PGF_{2\alpha}$, suggesting that PG transport, both in and out of endometrial cells, requires a PGT-mediated mechanism (Banu et al., 2008; Schuster, 1998). Further studies into the expression pattern of PGT during ovine pregnancy still remains to be elucidated.

The ovine PGT cDNA contains an open reading frame consisting of 1935 nucleotides, encoding 644 amino acids (Banu et al., 2008). The predicted amino acid sequence of ovine PGT shares high sequence homology with other species, particularly 92% and 83% with cow and human, respectively (Banu et al., 2008). Also, both the amino- and carboxy-terminal domains are identified as being present intracellularly and ovine PGT has been determined to be cationic due to the net positive charge of the amino acids that comprise it (Banu et al., 2008). Furthermore, through hydropathy and structural analyses, Banu et al. (2008) have suggested that arginine (R561) and lysine (K614) in the 11^{th} and 12^{th} transmembrane domains, respectively, are involved in ligand binding, particularly for PG transport. Finally, multiple serine, threonine, and tyrosine phosphorylation sites for various kinases have been predicted to be present in the transmembrane domains, and in the extracellular and intracellular loops (Banu et al., 2008; Schuster, 1998).

1.4. Prostaglandin Receptors

1.4.1. History of Prostaglandin Receptors: Identification and Classification

The first lines of evidence suggesting the existence of distinct receptors for prostanoids came from the work of Pickles in 1967 who demonstrated that different prostanoids elicited distinct activity patterns on a variety of isolated preparations of smooth muscle (Pickles, 1969). Subsequent studies, over the next 10 – 15 years supported the existence of multiple prostanoid receptor types (Andersen and Ramwell, 1974; Gardner and Collier, 1980). However, a working classification for the PG receptors did not exist until Kennedy et al. (1982) designated them according to functional data as DP, EP, FP, IP, and TP for the respective naturally occurring prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2. Later studies with PGE_2 demonstrated multiple activities to specific agonists and antagonists, therefore prompting a further subdivision of the EP receptors into four different subtypes denoted as EP1, EP2, EP3, and EP4 (Coleman et al., 1994).

1.4.2. Molecular Biology and Structure of Prostaglandin Receptors

PG receptors belong to the family of G protein-coupled rhodopsin-type receptors (Narumiya et al., 1999; Zhu et al., 2006). They contain seven putative transmembrane domains, an extracellular amino terminus, an intracellular carboxy terminus, three extracellular and three intracellular loops (Coleman et al., 1994; Narumiya et al., 1999). The most conserved regions of the prostanoid receptors are in the seventh transmembrane domain and the second

extracellular loop where both regions play fundamental roles in ligand recognition and binding (Coleman et al., 1994; Narumiya et al., 1999; Savarese and Fraser, 1992). In the cloned prostanoid receptors of the mouse, there are 28 amino acid residues conserved near the transmembrane domains; among which 8 residues are shared by other families of rhodopsin-type receptors and are believed to be associated with maintaining structure and/or function (Narumiya et al., 1999; Savarese and Fraser, 1992). Furthermore, there are two cysteine (Cys) residues conserved within the first and second extracellular loops that are believed to be critical for stabilization of receptor conformation and ligand binding through the formation of a disulphide bond (Dohlman et al., 1991). In addition, there is a wide distribution of serine and threonine residues in the cytoplasmic portion of the prostanoid receptors allowing for multiple sites of phosphorylation (Narumiya et al., 1999). Similar to other rhodopsin-type receptors, phosphorylation of these residues is thought to function in receptor desensitization (Hausdorff et al., 1990).

Since the cloning of the TXA_2 receptor by Hirata et al. in 1991, homology screening based on its sequence was performed in various species and all eight types of prostanoid receptors, originally defined through pharmacological studies, have now been identified (Narumiya et al., 1999). Among the functionally distinct PG receptors (i.e. contractile or relaxatory functions), overall homology ranges from 20-30% (Narumiya et al., 1999). Also, there is limited homology among receptor subtypes as reported for the EP receptors (Sugimoto and Narumiya, 2007). For example, the amino acid identity of EP1 to EP2, EP3, and EP4 is 30%, 33%, and 28%, respectively (Toh et al., 1995). However, homology of a

particular type or subtype of receptor among different species is much higher. For instance, 84% and 89% sequence homology exists between human and mouse EP1 and FP, respectively (Narumiya et al., 1999; Sugimoto and Narumiya, 2007).

At the molecular level, an intron-exon relationship is conserved both among the different prostanoid receptors and across a range of species. Based on genetic studies of the human TP receptor, a gene organization consisting of three exons separated by two introns (one in the 5'-non-coding region and the other at the end of the sixth transmembrane domain) exists among all prostanoid receptors (Batshake et al., 1995; Foord et al., 1996; Funk et al., 2001; Hasumoto et al., 1997; Katsuyama et al., 1998; Nusing et al., 1993; Regan et al., 1994). Additional exons encoding the carboxy-terminal tails in some of the prostanoid receptors have been identified (Namba et al., 1993; Regan et al., 1994; Sugimoto, 1993). Thus, multiple isoforms are generated through alternative mRNA splicing, as reported for human EP3 and ovine FP, which differ in their carboxy terminus (Pierce et al., 1998; Namba et al., 1993). Since it is the carboxy terminus that determines G-protein specificity, one receptor might elicit opposing actions and different signal transduction pathways depending on the specific isoform expressed and the G-protein it associates with (Narumiya et al., 1999; Sugimoto et al., 2007).

1.4.3. Signal Transduction in Prostaglandin Receptors

PGs mediate their effects through specific G-protein coupled receptors present in the plasma membrane and/or nucleus of target cells. Molecular cloning and agonist-induced stimulation studies have uncovered information regarding their signal transduction pathways by examining the levels of downstream second messengers and by identifying coupling to heterotrimeric G-proteins. G-proteins are composed of three structural subunits designated α, β, and γ (Hamm, 1998). Functionally, two subunits exist upon receptor activation that can act as effectors in signal transduction due to the dissociation of the G_{α} subunit from the $G_{\beta\gamma}$ subunits (Rens-Domiano and Hamm, 1995). Based on the signal transduction pathways that are activated upon receptor ligation and resultant biological effect(s), two categories have been defined for the prostanoid receptors: 1.) contractile receptors (including EP1, EP3, FP, and TP) and 2.) relaxatory receptors (EP2, EP4, DP, and IP). The two significant PGs involved in pregnancy and parturition, PGE_2 and $PGF_{2\alpha}$, and their respective receptors, will be discussed below.

Among the contractile receptors, FP has been reported to generate increases in intracellular Ca^{2+} concentrations and/or inositol triphosphate (IP3) due to the activation of phospholipase C coupled to $G_{\alpha q}$ (Narumiya et al., 1999). EP1 also results in an increase in free Ca^{2+} concentrations within target cells, similarly associating with the $G_{\alpha q}$ protein (Jabbour and Sales, 2004; Watabe et al., 1993). Interestingly, EP3 possesses the ability to elicit a number of different signal transduction cascades depending on the splice variant that is expressed

(Kotani et al., 2000). Predominantly, EP3 is known to cause a decrease in cyclic adenosine monophosphate (cAMP) production by the inhibition of adenylate cyclase through the $G_{\alpha i}$ subunit (Negishi et al., 1988). However, it can also elicit a relaxatory response, which results in an increase in cAMP via receptor coupling to the $G_{\alpha s}$ protein, depending on the splice variant expressed within a particular tissue (Narumiya, et al., 1999).

On the other hand, the relaxatory receptors, EP2 and EP4, are coupled to $G_{\alpha s}$ and mediate increases in cAMP by stimulation of adenylate cyclase. They are both referred to as relaxatory receptors as they are known to promote vasodilation (Jabbour and Sales, 2004). The expression of PG receptors in intrauterine tissues will be discussed in Section 1.5.2.

1.4.4. Nuclear Prostaglandin Receptors

Although PG receptors are well characterized as being present on the plasma membrane, there is increasing evidence which suggests the existence of a functional intracellular PG receptor population. The first clear evidence for this comes from studies conducted by Bhattacharya et al. (1998) who demonstrated localization of EP receptors to the nuclear envelope of porcine brain and myometrial fractions. Further support for this came with the identification of EP receptors, particularly EP3 and EP4, in the nuclear membrane of cerebral endothelial cells (Bhattacharya et al., 1999; Gobeil et al., 2002). Moreover, Gobeil et al. (2002) were the first to have demonstrated a distinct functional role for nuclear EP3 receptors versus plasma membrane EP3 receptors within

endothelial cells of the brain. Gobeil et al. (2002) reported that EP3 receptors bound to the plasma membrane function to elicit immediate physiological actions where nuclear EP3 plays a role in gene regulation (Zhu et al., 2006).

In support of the accumulating evidence for intracrine PG signalling due to the presence of nuclear PG receptors, biosynthetic enzymes have also been localized to the nuclear envelope. $cPLA_2$ has been demonstrated to be present in the nucleus (Neitcheva and Peeva, 1995; Schievella et al., 1995) and the nuclear membrane has been defined as an important site for metabolism of arachidonic acid (Capriotti et al., 1988). Furthermore, PGHS-1 and PGHS-2 have been identified in the nuclear and perinuclear regions of various cell types (Regier et al., 1995; Morita et al., 1995; Spencer et al., 1998; Marvin et al., 2000; and Parfenova et al., 2001). Specifically, Gyomorey et al. (2000a) have demonstrated PGHS-2 expression in the perinuclear region of ovine placental trophoblast cells (Gyomorey et al., 2000).

In reproductive tissues, Astle et al. (2005) demonstrated EP receptor localization to the nuclear and perinuclear regions of smooth muscle cells in human myometrial samples. More specifically, EP1, EP2, and EP4 receptors were found to be predominantly localized to the nuclear and perinuclear regions of smooth muscle cells in both the upper and lower segments of the myometrium (Astle et al., 2005). Also, FP receptor expression has been localized to the nuclear region of myocytes in the rat myometrium throughout gestation and at postpartum (Al-Matubsi et al., 2001). Nuclear and/or perinuclear PG receptor immunolocalization have yet to be demonstrated in other reproductive tissues

such as the placenta, fetal membranes, and endometrium. The existence of functional nuclear PG receptors and PG biosynthetic enzymes contributes to the complexity of PG signalling pathways in gestational tissues and warrants further studies into their expression and function during pregnancy. Combined, these studies suggest nuclear PG synthesis, which can account for situations where there is increased PG output but no change in plasma membrane receptor levels.

1.5. Prostaglandins and Parturition

1.5.1. Prostaglandin Action in Ovine Pregnancy and Parturition

Prostaglandins (PGs), such as PGE_2 and $PGF_{2\alpha}$, are key effectors in the onset of parturition where they are known to play fundamental roles in cervical ripening (Keirse, 1993), uterine contractility, membrane rupture (McLaren et al., 2000), maintenance of uterine and placental blood flow (Rankin, 1976; Rankin and Phernetton, 1976a,b), fetal energy conservation (Thorburn, 1992) and in the endocrine pathway through stimulation via the HPA axis (Challis et al., 2000, 2002). Their effects on the mechanism of parturition have been illustrated through studies on both PG action and inhibition. For example, intrafetal administration of synthetic or natural PGs, at various times during gestation, results in premature activation of the fetal HPA axis and parturition (Hollingworth et al., 1995; Young et al., 1996). Conversely, inhibitors of PG synthesis, such as nimesulide and meloxicam, have been shown to lengthen gestation and prolong or attenuate the progression of labour in several species (Chester et al., 1972; Ma et al., 1999; McKeown et al., 2000; Unno et al., 1998).

In sheep, toward the end of gestation and at the onset of labour, increased concentrations of PGs can be measured in the fetal and maternal plasma and in amniotic fluid (Challis et al., 1976; Olson et al., 1985). PGE_2 levels rise gradually over the remaining 15-20 days of gestation whereas levels of $PGF_{2\alpha}$ increase sharply in the last 12-24 hours of term during active labour (Challis et al., 1976; Gyomorey et al., 2000b). Previous studies have revealed two distinct, hormone-regulated pathways of intrauterine PG synthesis: a cortisol-dependent, estrogen-independent mechanism within trophoblast tissue that leads to elevations in fetal plasma PGE_2, and an estrogen-dependent mechanism within maternal endometrium which leads to increased maternal plasma $PGF_{2\alpha}$, necessary for uterine activity and parturition (Whittle et al., 2000).

In the cortisol-dependent, estrogen-independent PGE_2 pathway, the placentome has been identified as the major source of circulating PGE_2 (Olson et al., 1986; Palliser et al., 2004), and over the course of gestation, there is an increase in PGHS-2 expression and activity (Rice et al., 1990; 1992) resulting in increased PGE_2 output (Evans et al., 1981; 1982a; Risbridger et al., 1985). As previously mentioned, PGHS-2 mRNA and protein have been localized predominantly to the uninucleate cells of the fetal trophoblast in ovine placentomes, and to a lesser extent, in the endometrium and myometrium (Gibb et al., 1996, 2000; Gyomorey et al., 2000b; Zhang et al., 1996). Consistent with these findings, PGES mRNA and protein also localized to all three tissues but was primarily found to be expressed by the fetal trophoblast (Martin et al., 2002; Palliser et al., 2004). Despite a lack of PGES mRNA upregulation at term, a

gradual rise in PGES protein expression was observed until term where its levels significantly increased with labour onset (Martin et al., 2002; Palliser et al., 2004). *In vivo* studies using exogenous, intrafetal administration of natural or synthetic GCs in sheep have also demonstrated similar increases in placental PGHS-2 and PGE_2 production (Challis et al., 1997; McLaren et al., 2000; Whittle et al., 2000). Furthermore, GR receptor has been localized within the uninucleate cells of the fetal trophoblast and its levels were increased with labour onset (Gupta et al., 2003). Therefore, activation of the fetal HPA axis and the subsequent increase in cortisol output, upregulates placental PGHS-2 and increases PGE_2 output, as observed in late gestation.

After the progressive rise in fetal plasma PGE_2, there is a pronounced increase in the circulating levels of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM), a $PGF_{2\alpha}$ metabolite, in the maternal plasma due to the estrogen-dependent mechanism of endometrial PG production (Whittle et al., 2000). Supporting evidence has demonstrated correlations between the rise in PGFM with elevated maternal estradiol concentrations and increased PGHS-2 expression in both the endometrium and myometrium (Gyomorey et al., 2000a; Wu et al., 1999a, b). Temporally, endometrial PGHS-2 expression precedes the increase in myometrial PGHS-2 expression (Gyomorey et al., 2000a; Wu et al., 1999a) and estradiol has been shown to significantly increase PGHS-2 expression in the endometrium and myometrium of non-pregnant ewes after progesterone priming (Wu et al., 1997). Similarly, maternal estradiol infusion in late gestation significantly increased PGHS-2 expression in the endometrium and myometrium

thereby causing an increase in uterine PG production and subsequent fetal delivery, despite the absence of a rise in cortisol (Wu et al., 2004). PGFS expression has been demonstrated in various intrauterine tissues; however, a significantly larger amount of PGFS mRNA was present in the endometrium versus the placenta in late gestation ewes (Palliser et al., 2004). Furthermore, the estrogen receptor (ER) has been localized exclusively to the endometrium and myometrium in late gestation and, its expression was found to increase with both glucocorticoid-induced preterm labour and term labour (Leung et al., 1999; Wu et al., 1997).

As previously mentioned, the current model of ovine parturition proposes that the increased concentrations of fetal plasma PGE_2, at the time of parturition, could regulate the expression and/or activity of placental P450c17 in an autocrine, paracrine, or intracrine manner (Whittle et al., 2000, 2006). Using cultured bovine adrenal cells and pre-ovulatory cells, treatment with PGE_2 or $PGF_{2\alpha}$, respectively, was shown to induce P450c17 expression (Rainey et al., 1991; Wijayagunawarde, 1999). In an *in vivo* sheep model, infusion of nimesulide after the spontaneous onset of term labour elicited a significant decrease in fetal plasma PGE_2 and placental P450c17 expression (Ma et al., 1999). Although a decrease in fetal plasma cortisol also occurred, the authors could not exclude the possibility that placental PGE_2 may act as an intermediate capable of the induction of P450c17 activity (Ma et al., 1999). Gyomorey et al. (2000a) also suggested that locally produced PGs could alter placental steroidogenesis by regulating P450c17 expression since both PGHS-2 and

P450c17 proteins are co-localized to uninucleate trophoblast cells. However, the mechanism(s) underlying regulation of altered placental steroid synthesis at term have yet to be determined. Therefore, one of the objectives of the studies described herein was to investigate the influence of PGs on regulating placental P450c17 expression using sheep models of preterm labour.

1.5.2. Intrauterine Prostaglandin Receptor Expression in Pregnancy and Parturition

A number of studies using different species such as humans and sheep have been conducted to assess the localization and abundance of PG receptors in term and preterm intrauterine tissues. However, the expression of PG receptors varies considerably, both between species and tissues, and within the same species and/or tissue.

Within the intrauterine tissues, a majority of studies have focused on PG receptor expression in the myometrium. Pharmacological studies demonstrated the presence of the contractile PG receptors, EP1, EP3, and FP, in the myometrium of non-pregnant ewes (Crankshaw et al., 1995). Ma et al. (1999) reported a labour-associated increase in ovine myometrial EP3, EP4, and FP mRNA expression but described lower levels of expression of EP3 in the endometrium. More recently, Gyomorey et al. (2000a) demonstrated EP1-4 and FP receptor mRNA expression in ovine myometrium, with no change in expression levels with spontaneous term labour.

With respect to the role of PG receptors in uterine quiescence and contractility, Matsumoto et al. (1997) reported down-regulation of the FP and EP3

contractile receptors in the myometrium of pregnant women as compared to non-pregnant women, suggesting that their decreased expression may facilitate uterine quiescence during pregnancy (Matsumoto et al., 1997). Similarly, Sooranna et al. (2005) also demonstrated a reduction in EP3 and FP receptor expression using an *in vitro* system of cultured human myocytes. Consistent with these observations, rat myometrial FP receptor protein expression increased over the course of gestation with a marked elevation at term (Al-Matubsi et al., 2001), whereas relaxatory EP2 receptor expression decreased as pregnancy advanced thereby mediating myometrial contractility (Al-Matubsi et al., 2001; Brodt-Eppley and Myatt, 1998). A similar pattern of PG receptor expression was observed with RU-486 (progesterone antagonist) treatment near term in the pregnant rat myometrium (Dong and Yallampalli, 2000). Further investigations have revealed the presence of relaxatory receptors (EP2, EP4) in the lower myometrial segment and contractile receptors (EP1, EP3, FP) in the upper segment, exhibiting regional variation in PG receptor expression in relation to uterine contraction patterns observed during labour (Astle et al., 2005; Brodt-Eppley et al., 1999; Leonhardt et al., 2003; Smith et al., 2001a).

Studies have also been performed assessing PG receptor expression in fetal membranes from term and preterm subjects. Fukai et al. (1984) were among the first to report PG receptor expression, specifically FP, in human amnion and decidua. Since then, *in vitro* studies using amnion WISH cells (an immortalized human cell line) have demonstrated the expression of EP1 and EP3 receptors in response to cytokine and hormone-induced stimulation (Spaziani et

al., 1997, 2000). In a primate *in vivo* study using pregnant baboons, all EP receptor subtypes (1-4) were detected in the chorion; however, only EP4 was found to significantly decrease its expression with advancing gestation (Smith et al., 2001b). More recently, Grigsby et al. (2006a) immunolocalized all EP receptor subtypes to the fetal membranes collected from women at term and preterm; however, EP3 protein expression was found to be the most abundant within these tissues. Also, there were no alterations in expression of EP receptor subtypes in the fetal membranes across gestation (Grigsby et al., 2006a). In the amnion from sheep, a labour-associated decrease in FP mRNA expression was observed (Palliser et al., 2006).

Despite the studies conducted to investigate PG receptor expression and regulation in various intrauterine tissues, there has been a lack of data addressing their expression and regulation in the placenta. Grigsby et al. (2006a) demonstrated EP1-4 receptor subtype localization in human placental villous tissue with no change in expression with respect to gestational age and labour. Of interest, in a recent study by Palliser et al. (2005), the mRNA expression patterns for the EP receptor subtypes and FP were investigated in placentomes of dexamethasone-induced or spontaneous term labour ewes. In their studies, they were unable to detect placental mRNA expression of EP1 and found varying expression profiles for EP receptors 2-4 and FP depending on the labouring group (dexamethasone-treated versus spontaneous labour). Between all intrauterine tissues studied, they demonstrated an overall lower expression of

EP2, EP3, EP4, and FP in placentomes versus their expression in endometrium and/or myometrium (Palliser et al., 2005).

The regulation of PG receptor expression in intrauterine tissues still remains to be definitively determined; however, various studies have identified molecules that could potentially play a role in their expression during pregnancy and parturition. First, hormones such as CRH and progesterone may influence PG receptor levels. In the rat uterus, exogenous administration of progesterone increased EP2 mRNA expression and had no effect on FP mRNA, whereas the opposite occurred in pregnant rats treated with estradiol (Dong and Yallampalli, 2000). Consistent with this data, Liang et al. (2008) also reported a progesterone-induced down-regulation of FP mRNA expression in cultured human myocytes. Second, PG receptors may be regulated by pro- and anti-inflammatory cytokines. Both interleukin-1β (IL-1β) and interleukin-4 (IL-4) caused EP1 receptor levels to increase in cultured human amnion cells (Spaziani et al., 1997), and in human myometrial cells, IL-1 induced an increase in EP4 mRNA expression (Erkinheimo et al., 2000). Furthermore, IL-1β stimulated an increase in FP mRNA and protein expression in human uterine myocytes, whereas interleukin-6 (IL-6) reduced FP mRNA levels (Liang et al., 2008). Finally, PG receptors may regulate their own expression through negative feedback mechanisms which result in receptor down-regulation or internalization (Olson et al., 2003; Srinivasan et al., 2002). In support of this, Yamamoto et al. (2005) have shown a dose-dependent decrease in FP mRNA levels in response

to increasing concentrations of exogenous $PGF_{2\alpha}$, suggesting an auto-regulatory mechanism by which PG receptors regulate their own expression levels.

1.6. Perinconceptional Undernutrition and Parturition

As previously outlined, maturation of the fetal HPA axis in late gestation provides the trigger for parturition. As a result, cortisol concentrations in the fetal plasma significantly increase resulting in the maturation of organ systems (i.e. lung) and metabolic pathways in order to prepare the fetus for its transition into extrauterine life (Liggins et al., 1994). As demonstrated in species such as the sheep and human, this prepartum surge in cortisol also plays a significant role in activating the placental endocrine axis, which is essential for labour onset, and precocious activation of this axis may result in preterm delivery (Bloomfield et al., 2003, 2004; Challis et al., 2000; Kumarasamy et al., 2004; Liggins et al., 1973).

In addition to the identified causes of premature birth (i.e. infection, multi-fetal pregnancy), increasing evidence has suggested that stress and severe maternal undernutrition (UN) during pregnancy may result in delivery at preterm (Bloomfield et al., 2003; Dole et al., 2003). In support of this, Fowden et al. (1983, 1987) suggested that marked variations in dietary intake, during late gestation in ovine pregnancy, could potentially result in premature delivery. In their studies, they demonstrated significant increases in fetal and maternal plasma concentrations of PGE_2 and PGFM after a period of fasting in late gestation; corresponding to increases in circulating free fatty acids, such as arachidonic acid, which are precursors of PG biosynthesis (Fowden et al., 1983,

1987). Similarly, in the horse, Stammers et al. (1995) reported that alterations in dietary fatty acid intake during late gestation have been associated with increased PG biosynthesis. Stammers et al. (1995) demonstrated increased production of essential fatty acids, such as arachidonic acid, in UN mares and suggested that this could enhance the likelihood of preterm delivery since arachidonic acid is one of the main precursor fatty acids for PGE_2 and/or $PGF_{2\alpha}$ synthesis.

In the sheep, it has been previously shown that a period of maternal UN prior to the time of conception and extending into the early part of gestation can alter HPA axis development in the fetus (Bloomfield et al., 2003; Edwards and McMillen, 2002). Bloomfield et al. (2003) reported that maternal UN from 60 days before until 30 days after conception, defined as the periconceptional period, can cause premature activation of the HPA axis resulting in a precocious rise in circulating fetal cortisol concentrations and resultant preterm birth (Bloomfield et al., 2003). In this study, preterm delivery occurred in approximately half of the ewes exposed to a period of moderate maternal UN, where the remaining ewes carried their fetus to term regardless of nutritional manipulation. Additionally, fetal ACTH levels were higher in all UN ewes regardless of the timing of birth (Bloomfield et al., 2003). Furthermore, using the periconceptional UN model, Kumarasamy et al. (2004) reported later increases in the levels of circulating PGs (fetal PGE_2 and maternal PGFM) and in the E_2:P_4 ratio, subsequent to the rise in cortisol. In this cohort of ewes, maternal PGFM levels were significantly elevated in the preterm UN group; however, no

alterations in the concentration of circulating fetal plasma PGE_2 between UN and nourished groups was reported, despite the earlier timing of the initial rise of fetal PGE_2 within the UN preterm group (Kumarasamy et al., 2004). Thus, continued investigation into the effect of maternal nutritional status on gestational length and pregnancy outcome will continue to offer new insight into the mechanisms underlying preterm birth.

Chapter 2

Rationale, Hypothesis, and Specific Aims

2.1. Rationale and Hypothesis

One of the major obstetrical problems at present is preterm birth (birth prior to 37 weeks gestation), which occurs in about 5 -10% of all pregnancies and accounts for approximately 70% of neonatal mortality and morbidity (Challis et al., 2002). It affects women in both developed and underdeveloped countries with a continuous rise in incidence (Gibb, 1998; Gilbert, 2006). Individuals born prematurely are at an increased risk of developing a variety of health problems such as deafness, blindness, respiratory, and neurological disorders, in early childhood that can continue into adulthood (Challis et al., 2002). Preterm birth imparts significant emotional stress on the affected family members and treatment of preterm infants comes at a major cost to the healthcare community and to society in general. Therefore, the objective of this thesis is to further our understanding of the molecular and endocrine events involved in parturition, both term and preterm. With this knowledge, new clinical strategies and/or medical technologies can be developed to identify and characterize new diagnostic indicators of preterm birth, with the potential to prevent its occurrence.

In late gestation within the sheep, maturation of the fetal HPA axis results in an increase in cortisol output from the fetal adrenals, triggering the onset of parturition. Subsequently, cortisol activates the placental endocrine axis, which results in a production of PGE_2 and increased activity of the enzyme P450c17,

thereby altering the pattern of steroid synthesis at term. This thesis will explore the hypothesis that in late pregnancy and at parturition, placental PGE_2, acting through PG receptors, would directly upregulate the steroidogenic enzyme, P450c17, in an autocrine, paracrine, or intracrine manner. In order to investigate this, we used two different ovine models of preterm labour: 1.) exogenous intrafetal glucocorticoid administration; 2.) endogenous elevation of internal glucocorticoid in response to maternal periconceptional undernutrition (UN).

The induction of labour, at term and preterm, involves a complex series of endocrine and mechanical interactions that occur between the fetus, placenta, and mother. PGs, such as PGE_2 and $PGF_{2\alpha}$, are key effectors in the onset of parturition. Referred to as the 'triggers' of labour, they are known to play key roles in cervical ripening (Keirse, 1993), uterine contractility, membrane rupture (McLaren et al., 2000), maintenance of uterine and placental blood flow (Challis et al., 2002), fetal energy conservation (Thorburn, 1992), and in the endocrine pathway through stimulation via the hypothalamic-pituitary-adrenal axis (HPA axis) (Challis et al., 2000, 2002). PGs mediate their effects through specific G-protein coupled receptors present in the plasma membrane and/or perinuclear region of target cells (Narumiya et al, 1999; Bhattacharya et al., 1998, 1999). There are four receptors characterized for PGE_2 (EP1-4) and one for $PGF_{2\alpha}$ (FP) which are functionally categorized by the signal transduction pathways that are activated upon ligand binding (Coleman et al., 1994; Narumiya et al., 1999). Stimulation of contractile receptors (EP1, EP3, FP) results in Ca^{2+} mobilization

and/or cAMP inhibition whereas activation of relaxatory receptors (EP2, EP4) promotes cAMP production.

Much of our current knowledge concerning the physiological mechanisms involved in parturition has come from studies utilizing various comparative animal models. In particular, the use of sheep has elucidated a wealth of information regarding our understanding of term and preterm labour. In a classical model of ovine parturition, it was believed the altered placental steroid synthesis, observed at term, was controlled by the direct regulation of P450c17 activity by cortisol produced in late gestation from the fetal adrenals, in response to HPA axis activation and maturation (Liggins et al., 1977; Mason et al., 1989). Consequently, the increase in estrogen output, from P450c17 activation, caused a decline in progesterone levels and stimulation of placental PG synthesis leading to myometrial contractility and labour onset. However, later studies from our laboratory provided evidence contrary to the classical sequence of events. First, there is an increase in placental PGE_2 output over the last 15-20 days of ovine gestation, before the observed rise in P450c17 activity (Challis et al., 1976). Whittle et al. (2000) also found an estrogen-independent increase in placental PGHS-2 mRNA expression and PGE_2 levels after intra-fetal cortisol infusion. Furthermore, ovine placental PGHS-2 mRNA and protein expression preceded P450c17 expression and both enzymes were co-localized to the uninucleate trophoblast cells (Gyomorey et al., 2000a). In preliminary studies, using an *in vitro* model, we have shown PGE_2-stimulated upregulation of P450c17 mRNA and protein in cultured ovine placental trophoblasts (D. Rurak,

personal communication). Thus, the current model of ovine parturition suggests that cortisol, produced in response to fetal HPA axis maturation in late gestation, increases placental PGHS-2 expression thereby increasing PGE_2 output. This rise in PGE_2, acting through PG receptors, induces placental P450c17 resulting in altered placental steroid synthesis at term. However, the mechanism by which PGE_2 increases P450c17 is not understood and there is a lack of data concerning PG receptor expression and regulation in term and preterm ovine placentomes.

2.2. Specific Aims

2.2.1. Chapter 3: The Expression and Regulation of Prostaglandin Receptors and Cytochrome P450c17 Hydroxylase by Cortisol and PGE_2 in Late Gestation Ovine Placentomes

In this chapter, we determined the effect of exogenous glucocorticoid administration with or without intrafetal meloxicam (PGHS-2 specific inhibitor) on the ovine placental endocrine axis. We hypothesized that exogenous cortisol administration would upregulate placental PGHS-2 enzyme expression thereby causing an increase in PGE_2 production. PGE_2, in turn, acting through PG receptors, would cause a later increase in P450c17 expression in placental trophoblast resulting in a rise in estrogen and decline in progesterone.

Use of the PGHS-2 specific inhibitor, meloxicam, allowed us to investigate the role of PGs in regulating P450c17 expression. Previous studies in sheep utilizing maternal meloxicam administration, after RU486 (progesterone receptor antagonist)-induced preterm labour, demonstrated decreases in uterine

contractility and circulating PG levels (McKeown et al., 2000; Rac et al., 2006). Therefore, we predicted animals treated with meloxicam should exhibit elevated levels of PGHS-2 but not PGE_2, and P450c17. The specific aims for this study were:

1. To determine the effect of cortisol +/- meloxicam administration on the expression of PGHS-2 and P450c17 in late gestation ovine placentomes.
2. To characterize the expression of PG receptors in late gestation ovine placentomes and to determine the effect of cortisol +/- meloxicam administration on regulation of PG receptor expression.

2.2.2. Chapter 4: The Effect of Periconceptional Undernutrition on Prostaglandin Receptor Localization and Expression in Late Gestation Ovine Placentomes

In this chapter, in a set of experiments run in tandem with those in Chapter 3, we investigated the effect of elevated endogenous glucocorticoid on PG receptor expression in late gestation ovine placentomes using a periconceptional UN model. In previous studies, we demonstrated that periconceptional UN results in a precocious rise in fetal cortisol levels and subsequent increases in PG concentrations and estrogen synthesis, resulting in preterm delivery (Bloomfield et al., 2003). Therefore, we hypothesized that periconceptional UN might alter the expression pattern and levels of PG receptors in late gestation ovine placentomes. The specific aims for this study were:

1. To characterize the localization of PG receptors in late gestation ovine placentomes.

2. To determine the effect of perinconceptional UN on changes in placental PG receptor expression and regulation.

Chapter 3

The Expression and Regulation of Prostaglandin Receptors and Cytochrome P450c17 Hydroxylase by Cortisol and PGE_2 in Late Gestation Ovine Placentomes

3.1. Introduction

The preparation for labour, at term and preterm, involves a complex interplay of endocrine and mechanical processes that occur between the fetus, placenta, and mother. PGs, such as PGE_2 and $PGF_{2\alpha}$, are key effectors in the onset of parturition and mediate their effects through specific G-protein coupled receptors present in the plasma or nuclear membrane of target cells (Narumiya et al., 1999; Bhattacharya et al., 1998, 1999). There are four receptors characterized for PGE_2 (EP1-4) and one for $PGF_{2\alpha}$ (FP) which are functionally categorized by the signal transduction pathways that are activated upon receptor ligation (Coleman et al., 1994; Narumiya et al., 1999). Stimulation of contractile receptors (EP1, EP3, FP) causes inhibition of cAMP (EP3) and/or an increase in inositol triphosphate (IP_3) and Ca^{2+} mobilization, with resultant increases in intracellular Ca^{2+} concentrations (EP1 and FP). Activation of relaxatory receptors (EP2, EP4) stimulates adenylate cyclase and promotes cAMP production (Narumiya et al., 1999; Sugimoto et al., 2007).

A number of studies using different species such as humans and sheep have been conducted to assess the localization and abundance of PG receptors in term and preterm intrauterine tissues. Pharmacological studies demonstrated

the presence of EP1, EP3, and FP in the myometrium of non-pregnant ewes (Crankshaw et al., 1995). Ma et al. (1999) reported a labour-associated increase in ovine myometrial EP3, EP4, and FP mRNA expression but described lower expression of EP3 in the endometrium. More recently, Gyomorey et al. (2000a) demonstrated that EP1-4 and FP receptor mRNA expression in ovine myometrium did not change with spontaneous term labour. Further investigations have revealed the presence of relaxatory receptors (EP2, EP4) in the lower myometrial segment and contractile receptors (EP1, EP3, FP) in the upper segment, exhibiting regional variation in PG receptor expression in relation to uterine contraction patterns observed during labour (Astle et al., 2005; Brodt-Eppley et al., 1999; Leonhardt et al., 2003). PG receptor expression has also been investigated in the fetal membranes from term and preterm subjects (Grigsby et al., 2006a, b; Palliser et al., 2006; Smith et al., 2001).

Despite the studies conducted to investigate PG receptor expression and regulation in various intrauterine tissues, there has been a lack of information addressing their expression and regulation in the placenta. Grigsby et al. (2006a) demonstrated EP1-4 receptor subtype localization in human placental villous tissue with no change in expression with respect to gestational age and labour. Of interest, in a recent study by Palliser et al. (2005), the mRNA expression patterns for the EP receptor subtypes and FP were investigated in term and preterm ovine tissues. They demonstrated an overall lower expression of EP2, EP3, EP4, and FP in placentomes versus their expression in endometrium and/or myometrium.

In sheep, sustained activation of the fetal HPA axis and the resultant increase in fetal cortisol concentrations provides the trigger for placental PG synthesis at term (Challis et al., 2000). Several studies have demonstrated increases in placental PGHS-2 expression and PGE_2 production after spontaneous term labour and intrafetal cortisol administration (Gyomorey et al., 2000a, 2000b; Whittle et al., 2000). Results from these studies have suggested that the increased levels of PGE_2, in late gestation, may directly increase the expression of the steroidogenic enzyme P450c17 in the placenta, which results in a fall in progesterone and sharp rise in estrogen concentrations observed during labour (Gyomorey et al., 2000a; Whittle et al., 2000, 2006). In support of this, it has been reported that placental PGE_2 production increases over the last 15-20 days of gestation, before the observed rise in P450c17 activity and that PGHS-2 mRNA and protein expression precedes P450c17 expression in ovine uninucleate trophoblast cells (Challis et al., 1976; Gyomorey et al., 2000a). Also, in preliminary *in vitro* studies from our laboratory, PGE_2 caused a dose- and time-dependent increase in P450c17 mRNA and protein within primary cultures of ovine trophoblast cells (D. Rurak, personal communication). Therefore, we hypothesized that in late pregnancy and parturition, placental PGE_2, acting through PG receptors, would directly upregulate P450c17 in an autocrine, paracrine, or intracrine manner. However, the mechanism by which PGE_2 increases P450c17 is not understood and there is a lack of information concerning PG receptor expression and regulation in term and preterm ovine tissues. Thus, in the present study, we examined the role of PGs in regulating

levels of the contractile PG receptors EP1, EP3, and FP, and placental P450c17 expression, after exogenous glucocorticoid administration, with or without the PGHS-2 specific inhibitor, meloxicam.

3.2. Materials and Methods

3.2.1. Animal Care and Maintenance

This project was approved by the University of British Columbia (UBC) Committee on Animal Care and conformed to the guidelines of the Canadian Council on Animal Care. Fifteen pregnant sheep (Dorset-Suffolk cross) with a mean weight of 70.3 $\pm$ 3.4 kg (range 46-86 kg) were used in the study. Six of the ewes carried single lambs and the remaining nine carried twins. To achieve time-dated pregnancies, groups of animals (2-4) were brought into synchronous estrous by implantation of vaginal pessaries (Veramix®, Tuco Products, Orangeville, ON) containing medroxyprogesterone acetate, which inhibits ovulation. Two weeks later, the pessaries were removed, and ovarian follicular development was induced by intramuscular (IM) injection of 250 – 500 IU of pregnant mare's serum gonadotropin (Equinex®, Ayerst Laboratories, Montreal, QC) and the ewes were placed with a ram for the next 72 – 96 hrs for mating. Pregnancy was checked by measurement of maternal plasma progesterone concentration (UBC Division of Reproductive Endocrinology and Fertility Endocrine Service Laboratory) 17 – 19 days post-ovulation and confirmed 50 – 90 days later by real-time ultrasound observation of the fetus and/or placenta. Each group of ewes were brought to the Child and Family Research Institute

(Vancouver, BC) 1 – 2 weeks prior to surgery where they were kept in large pens in view of flock mates. The animals had free access to water, food, and hay.

3.2.2. Surgical Preparation

Surgery was conducted at 116 – 123 days gestation (mean = 119.5 $\pm$ 0.5 days, term ~145 days). Food was withdrawn for ~18 hrs prior to surgery but ewes were given ad libitum access to water. Approximately 30 min before surgery, a 6 mg intravenous (IV) dose of Atropine (Glaxo Laboratories, Montreal, QC) was administered via the jugular vein to control salivation. Anaesthesia was induced by IV sodium pentothal (1 g). The ewe was then placed in a supine position on the surgical table, intubated with a cuffed endotracheal tube (Mallinckrodt, St.Louis, MO) and maintained on a ventilator for the duration of the surgery. Anaesthesia was maintained with isofluorane (1 – 2% Arrane®, Baxter Corporation, Mississauga, ON) and nitrous oxide (60%; balance O_2). During surgery, 500 ml of 5% dextrose solution (Baxter Corporation, Mississauga, ON) was given at a rate of ~2 ml/min via the maternal jugular vein. Aseptic technique was utilized throughout the surgical procedure. The ewe's abdomen was shaved and the surgical area (ventral abdomen) was washed and swabbed with 7.5% povidin-iodine (Betadine® surgical scrub, Purdue Frederick, Pickering, ON). A sterile, disposable Caesarean birth drape (Source Medical, Burnaby, BC) was placed over the surgical area.

A midline abdominal incision was made in the ewe to expose the uterus. An incision was made in the uterus at a site free of cotyledons and major blood

vessels and the fetal head was exteriorized. The edge of the uterine incision was secured to the skin of the fetal neck to limit the loss of amniotic and allantoic fluid. Silicone rubber catheters (Silastic®, Dow Corning Corporation, Midland, MI) were placed in the fetal trachea and amniotic cavity. The tracheal catheter was inserted through a small incision made between 2 adjacent cartilaginous rings of the trachea just below the larynx. It was distally advanced approximately 4 cm into the trachea and was anchored to the skin overlying the incision. The amniotic catheter was anchored to the skin of the fetal neck. Silicone rubber catheters were then implanted occlusively in a fetal carotid artery and jugular vein and each catheter was anchored to the skin overlying the incision site. The fetal skin incision was then closed and the fetal head was returned to the amniotic cavity. Amniotic fluid was then replaced with ~1500 ml of warmed, sterile 0.9% irrigation saline (Baxter Corporation, Mississauga, ON) and the uterine incision was then closed. In ewes carrying twins, only one fetus was instrumented.

In ten of the animals, uterine electromyographic (EMG) electrodes were implanted into the myometrium. The fetal catheters and electrodes were then exteriorized through a small incision in the maternal flank. The abdominal muscle layer was closed and silicone rubber catheters were placed into the maternal femoral artery and vein through a small incision in the groin. The maternal abdominal, flank, and groin incisions were then closed.

All catheters were filled with heparinized saline (12 U/ml, Hepalean, Organanon Teknoka, Toronto, ON), capped and stored in a plastic bag tied to the

wool on the ewe's back. Elastic crepe bandages were wrapped around the ewe's midsection to keep the bag and incision clean. Post-surgical analgesia was provided by IM injection of buprenorphine (Temgesic®, 0.3 mg, Schering-Plough, Hertfordshire, UK) towards the end of surgery.

Following completion of surgery, the ewe was removed from the ventilator and observed carefully until spontaneous breathing resumed. The endotracheal tube was then removed and the ewe was transported back to the holding room. Food was offered and the animal was observed continuously until it was standing and eating (approximately 1 -2 hrs post-surgery). The patency of the catheters was maintained by flushing daily with sterile heparinized saline. Prophylactic antibiotic consisting of 500 mg Ampicillin (Novopharm, Scarborough, ON) was administered to the ewe on the day of surgery and for 3 days post-operatively. Ampicillin (250 mg) was injected daily into the amniotic cavity for the duration of the preparation. In order to monitor fetal well-being, fetal arterial blood samples (0.4 ml) were taken daily for determination of blood gas status, oxygen saturation, and haemoglobin, glucose, lactate, sodium, potassium, and chloride concentrations. The animals were allowed to recover from surgery for a minimum of 5 days before the start of the protocol.

3.2.3. Experimental Protocol

The protocol involved fetal IV infusion of saline (n=6), cortisol (n=4), and cortisol + meloxicam (n=5) for 80 hrs, beginning at ~126.7 days (123-132d) gestation and lasting for 80 hrs (**Figure 3.1**). This protocol is based on previous

studies conducted by Whittle et al. (2000). Saline infusion consisted of 0.9% saline at 3 ml/hr. The cortisol infusion consisted of 0.45 mg/hr cortisol (Sigma Chemical, St.Louis, MO) made up in 0.9% saline with a volume rate of 3 ml/hr. For these experiments, a lower dosage of cortisol was chosen than that used by Whittle et al. (2000) as we did not want the animals to be in established labour before or at the end of the infusion period as observed in the previous study by Whittle et al. (2000). The cortisol + meloxicam infusions consisted of cortisol (0.45 mg/hr) and meloxicam (0.4125 mg/hr; Metacam®, Boehringer Ingelheim, Burlington, ON) at an infusion rate of 3 ml/hr. The amount of meloxicam administered was determined based on the pharmacokinetics of the drug and previous experiments using indomethacin, a non-selective PGHS-1/-2 inhibitor, which has similar kinetic properties as meloxicam (D. Rurak, personal communication). The cortisol and cortisol + meloxicam infusions were sterilized by filtering through a 0.22 μm nylon syringe filter (MSI, Westboro, MA). During the experiments, fetal (2.5 ml) and maternal (3.0 ml) arterial blood samples were collected starting at 24 hrs before the start of the infusion period and at 8 hr intervals after infusion start until the end of the 80 hr period.

One half of the fetal blood sample was placed in chilled vacutainer tubes containing EDTA immediately after collection (Becton-Dickinson, Rutherford, NJ) for analysis of plasma cortisol and PGE_2. The maternal sample was placed in a chilled vacutainer tube containing heparin for analysis of plasma estradiol and progesterone. All samples were then centrifuged at 3000 g for 15 min at 4^0C in a

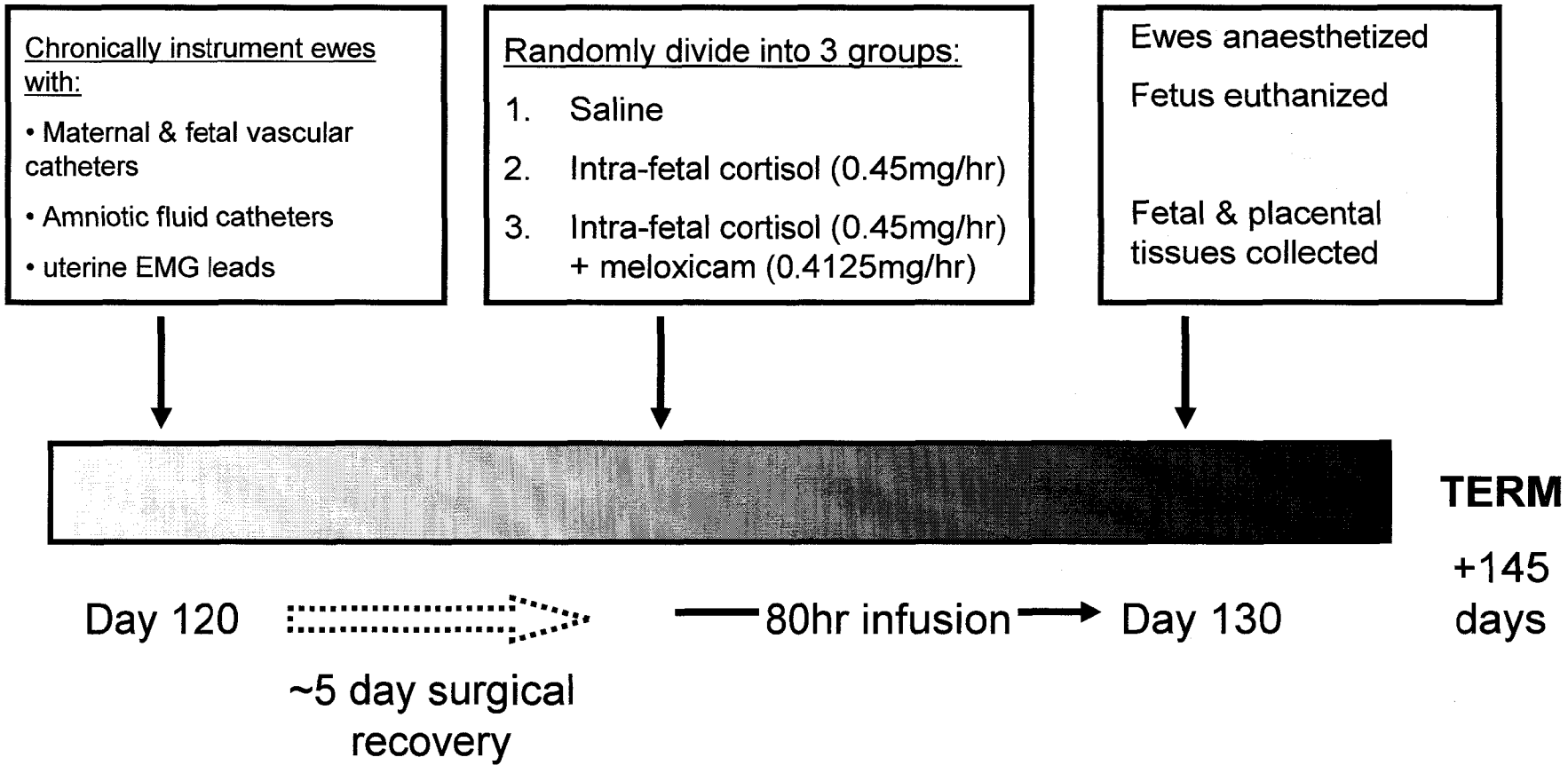


Figure 3.1 Schematic representation of the experimental design

refrigerated centrifuge (Eppendorf-Brinkman Instruments, Westbury, NY). The plasma was then transferred to 1.5 ml tubes and stored at -80^{0}C until analysis.

3.2.4. Physiological Measurements

During the experiments, fetal arterial, tracheal, and amniotic pressures were measured using disposable strain-gauge manometers (Ohmeda Inc., Madison, WI). In all animals, fetal heart rate was determined with a cardiotachometer (Astro-Med, West Warwick, RI). All variables were recorded on a Grass K2G polygraph (Astro-Med, West Warwick, RI) coupled to a computerized data acquisition system (Chart® v 4.2, ADInstruments, Colorado Springs, CO). In the animals with uterine EMG leads, contracture patterns were also processed and recorded. Fetal and maternal blood gas and acid-base status, and glucose, lactate, sodium, potassium, and chloride concentrations were measured with a combined Radiometer automated blood gas analyzer (AB500), oximeter (OSM3), and electrolyte analyzer (System 620, Radiometer, Copenhagen).

3.2.5. Tissue Collection

At the end of the 80 hr infusion period, the animals were moved to the surgical theatre. Using the same anaesthetic and surgical procedure as previously described (Section 3.2.2.), access to the instrumented fetus was once again gained. The infused fetus was removed rapidly from the uterus, the umbilical cord was tied and 5 ml of sodium pentobarbitone (Euthanyl®, MTC

Pharmaceuticals, Cambridge, ON) was administered intravenously to euthanize the fetus. Tissues and organs were then removed from the fetus and stored at -80^{0}C. Four to five cotyledons were removed from the uterus, along with samples of myometrium, endometrium, and fetal membranes (amnion and allantois). These samples were weighed, placed in small plastic bags on dry ice and transferred to -80^{0}C for storage. The uterine and abdominal incisions were closed and the ewes were subject to the same post-surgical procedures as previously described (Section 3.2.2.). Following recovery from anaesthesia, the ewes were returned to the holding pens. Those still carrying fetuses were kept until delivery. All ewes and surviving lambs were returned to the UBC farm, according to policies at the University of British Columbia.

3.2.6. Plasma Collection, Hormone and Eicosanoid Analysis

Maternal and fetal blood samples were collected 24 hours prior to the start of infusion and every 8 hours after the start of infusion. Fetal cortisol concentrations were determined by radioimmunoassay (RIA) using the ^{125}I-labelled Cortisol Coat-a-Count® RIA kit (Diagnostic Products Corporation (DPC), Siemens Medical Solutions, Los Angeles, CA). Plasma samples (50 µl) were incubated with 1 ml of ^{125}I-labelled cortisol tracer in polypropylene cortisol antibody-coated tubes for 45 min in a water bath set at 37^{0}C. Samples were decanted overnight and counted for 1 min in a gamma counter (Packard Cobra II Auto-Gamma, Canberra Packard, Meriden, CT).

Maternal plasma concentrations of estradiol and progesterone were determined by RIA using the ^{125}I-labelled Estradiol Coat-a-Count® and ^{125}I-labelled Progesterone Coat-a-Count® (DPC, Siemens Medical Solutions, Los Angeles, CA) kits, respectively. Plasma samples (100 µl) were transferred to polypropylene antibody-coated tubes (estradiol or progesterone) and incubated with 1 ml of ^{125}I-labelled estradiol or progesterone tracer at room temperature (RT) for 3 hrs. Samples were decanted overnight and counted for 1 min in a gamma counter. All RIA runs included a standard curve with corresponding calibrators supplied in the kit. Sensitivities for each kit, as stated by the manufacturer, are as follows: 2 ng/ml (cortisol); 0.02 ng/ml (progesterone); 8 pg/ml (estradiol). Intra-assay and inter-assay coefficients of variation, as determined by the manufacturer, are as follows: 4.8% and 5.2% for cortisol; 2.7% and 3.9% for progesterone; 7.0% and 8.1% for estradiol. Control serum used in all RIA analyses was a tri-level human-based serum (DPC, Siemens Medical Solutions, Los Angeles, CA) and concentrations were validated for use in cortisol, estradiol, and progesterone kits, respectively.

Fetal plasma concentration of PGE_2 was determined using a polyclonal PGE_2 enzyme-linked immunosorbent assay (ELISA) kit (Neogen, Lexington, KY). Plasma samples underwent extraction to purify PGE_2 prior to ELISA analysis. Methanol (200 µl) was added to 1 ml of plasma and subsequently vortexed and centrifuged at 12 000 rpm for 1 min. The supernatant was collected, diluted 1:3 in phosphate buffered saline (PBS) without $CaCl_2$ and $MgCl_2$ (Gibco, Burlington, ON), and applied to a 5 ml syringe (Becton Dickinson, Mississauga, ON)

attached to a C_{18} Sep-Pak© column (Waters Corporation, Milford, MA). Prior to sample application, the column was preconditioned with 2 ml of methanol followed by 2 ml of Ultra-Pure water (Gibco, Burlington, ON). Once the sample was applied, the flow rate was adjusted to ~ 1 ml/min and the column was subsequently washed with 2 ml of 15% methanol (diluted in Ultra-Pure water) followed by 2 ml of petroleum ether (Fisher Scientific, Fair Lawn, NJ). PGE_2 was eluted with 2 ml of methyl formate (Acros Organics, Geel, Belgium) and the eluate was evaporated under a stream of nitrogen gas for ~ 25 min. The residue was then stored at -20^{0}C until assayed. The day of the ELISA, the residue was reconstituted in 1.2 ml of diluted extraction buffer (supplied by kit) and 50 µl of each sample, as well as known standards (supplied within the kit), were added to a 96-well PGE_2 antibody pre-coated plate in duplicate. At the end of the procedure, the reaction was stopped with 50ul of 1N HCl (BioShop, Burlington, ON) and the plate was read at 450nm (Packard SpectraCount™, Canberra Industries Inc., Meriden, CT). The sensitivity of the assay, as stated by the manufacturer, is 0.1 ng/ml and the intra-assay and inter-assay coefficients of variation are $\leq$10% (as determined by manufacturer).

3.2.7. Western Blotting and Antibody Characteristics

Frozen cotyledons (500 mg) were crushed with a pestle over ice and homogenized with radioimmunoprecipitation assay (RIPA) lysis buffer (15mM TRIS-HCl, pH 8; 150mM NaCl; 0.5% deoxycholatic acid; 0.1% sodium dodecyl sulphate; 1% (vol/vol) Triton X-100) containing Complete Mini EDTA-free

protease inhibitors (Roche, Laval, QC). Homogenates were centrifuged at 10000 rpm for 15 min to remove tissue debris and the remaining supernatant was stored at -80^{0}C until further use. Protein concentrations were measured using the Bradford protein assay (Bradford, 1976).

Ovine placental protein samples (20 µg) were mixed with RIPA buffer and Laemmli sample buffer (LSB) (Laemmli, 1970) and incubated at 55^{0}C for 10 min to denature the protein. Samples were subsequently separated by 11% sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 80 V. Proteins were electrophoretically transferred at 4^{0}C onto a 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) at 110 V for 1.5 hrs. Membranes were blocked overnight at 4^{0}C in a 3% normal goat serum (NGS) and 0.1% PBS-Tween 20 (Sigma, Oakville, ON) solution (for EP receptor subtypes 1-3 and FP). PGHS-2 and β–actin blots were blocked in a 5% (w/v) non-fat powdered milk in 0.1% PBS-Tween 20 solution. After blocking, membranes were incubated at RT for 1 hr with the following primary antibodies (all from Cayman Chemical, Ann Arbor, MI): rabbit anti-human polyclonal EP1 receptor (1:500); rabbit anti-human polyclonal EP2 receptor (1:500); rabbit anti-human polyclonal EP3 receptor (1:600); rabbit anti-mouse FP receptor (1:400); and mouse anti-human PGHS-2 (1:1000). After washing with 0.1% PBS-Tween 20 (5 x 5 min, RT), membranes were incubated with either anti-rabbit IgG or anti-mouse IgG coupled to horseradish peroxidase (1:3000) (Amersham, Piscataway, NJ) for 1 hr at RT. At the end of the secondary antibody incubation, membranes were washed in 0.1% PBS-Tween 20 (6 x 5 min, RT), proteins were visualized

using chemiluminescence (Western Lightning, Perkin Elmer, Waltham, MS) and exposed to X-OMAT blue autoradiography film (Eastman Kodak Co., Rochester, NY). For preabsorption controls, the primary antibodies were incubated overnight at 4^0C with their respective blocking peptides: EP1 (catalogue #101740; sequence: GLTPSAWEASSLRSSRHSGLSHF), EP2 (catalogue #101750; sequence: SLRTQDATQTSCSTQSDASKQADL); EP3 (catalogue #101760; sequence: NQTSVEHCKTHTEKQKECNF), and FP (catalogue #101802; sequence: SMNSSKQPVSPAAGL) (Cayman Chemical, Ann Arbor, MI) at an antibody:antigen ratio of 1:10 in a 3% NGS - 0.1% PBS-Tween 20 solution as this was an adequate ratio to see a reduction or disappearance in the protein band.

All blots were stripped for 30 min at RT (0.1M glycine solution, pH 2.7 – 2.9) and reprobed with an anti-mouse β-actin antibody (1:10000) (Sigma, Oakville, ON) as an internal control to verify similar protein loading in each lane. Scion Image analysis software (Frederick, MD) was employed to quantify protein band density for EP1, EP3, FP, PGHS-2, and β-actin by densitometry. Results are presented as the ratio of respective protein of interest to β-actin in relative optical density (ROD) units.

Protein expression data for EP2 is presented in Appendix A, only as preliminary data. BLAST searches of the PG receptor antigen sequences used to raise the receptor antibodies did not result in a match for ovine EP2 and EP4 sequences. BLAST searches for the EP1, EP3, and FP antigens resulted in the following identity scores, respectively: 88%, 80%, and 66%.

3.2.8. RNA isolation, Real-time PCR and Primer Characteristics

To determine mRNA expression of PGHS-2 and P450c17, total RNA was isolated from frozen placentomes (100 mg) which were pulverized on dry ice and transferred into polypropylene tubes. Samples were homogenized over ice in 1 ml of Trizol reagent (Invitrogen, Burlington, ON) and RNA was extracted following the manufacturer's protocol. Isolated RNA subsequently underwent DNase treatment (Ambion, Austin, TX) to remove contaminating DNA and RNA integrity was assessed on a 1% agarose gel. Purity of the RNA was determined from the OD_{260}:OD_{280} measurements and concentrations were calculated from the OD_{260} readings. RNA (2 μg) was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen, Burlington, ON) and RNase H (Invitrogen, Burlington, ON) was used at the end of the protocol to remove remaining RNA.

Real-time quantitative RT-PCR was used to measure expression of PGHS-2, P450c17, and β–actin mRNA. PCR conditions for each primer set were optimized using a gradient PCR machine (DNA Engine DYAD, MJ Research, Reno, NV) prior to quantitative RT-PCR. Real-time PCR reaction mixtures contained Platinum Taq Supermix (Invitrogen, Burlington, ON), SYBR Green (0.0032% v/v; Molecular Probes, Invitrogen, Burlington, ON) and specific forward and reverse primers for the gene of interest (100 pmol/μl; Invitrogen-Illumina, San Diego, CA). Real time quantitative PCR reactions were performed using the Rotor-Gene RG-3000 system (Corbett Research, New South Wales, Australia). PCR cycles for PGHS-2 and β-actin consisted of an initial denaturation step at 95^0C for 5 min, followed by 45 cycles of denaturation at 95^0C for 20 sec,

annealing at 60^{0}C for 20 sec, and extension at 72^{0}C for 20 sec. PCR cycles for P450c17 consisted of an initial denaturation step at 95^{0}C for 5 min, followed by 45 cycles of denaturation at 95^{0}C for 45 sec, annealing at 60^{0}C for 30 sec, and extension at 72^{0}C for 90 sec. At the end of each cycle, a further 15 sec step was included in order to melt any primer-dimers present and to allow measurement of fluorescence released only by the specific amplicon. Amplification of β-actin was measured in each sample and was used as a housekeeping gene for normalization. Details of forward and reverse primers and product sizes are shown in **Table 3.1**.

Messenger RNA expression levels for PGHS-2, P450c17, and β-actin were each determined using relative quantitation by comparison to a standard curve. Standard curves were generated by serial dilutions of a reference sample and were included in each PCR run. Standards and samples were measured in duplicate and a non-template control and internal standard were included in all runs. Expression levels of the genes of interest in each sample were normalized to β-actin.

3.2.9. Statistical Analysis

All data are represented as the mean +/- standard error of the mean (SEM) throughout. Western blot and real-time PCR data were analyzed using one-way ANOVA followed by a Tukey multiple comparison test. Plasma hormone and eicosanoid data was analyzed using repeated measures two-way ANOVA followed by a Holm-Sidak multiple comparisons post-hoc test. Where

Gene	Forward primer (5' - 3') Reverse primer (5' - 3')	Accession #	Product Size (base pairs)
Ovine PGHS-2	CTCTTCCTCCTGTGCCTGATG GCGACGGTTATGCTGTCTCTC	U68486	284
Ovine P450c17	TGATGATTGGACACCACCAGTTG AGAGAGAGAGGCTCGGACAGATC	M14564	298
Ovine β-actin	CGGGATCCATCCTGCGTCTGGACCTG GGAATTCGGAAGGAAGGCTGGAAGAG	AF129289	279

Table 3.1 Primer sequences and characteristics for ovine PGHS-2, P450c17, and β–actin used for real-time quantitative RT-PCR

the normality test failed, raw data was log transformed. All statistical measurements and plots were performed using SigmaStat® version 3.1 and SigmaPlot® version 9.0 (Systat Software, San Jose, CA). A value of $p \leq 0.05$ was considered significant.

3.3. Results

3.3.1. Plasma Concentrations of Cortisol, Estradiol, and Progesterone

Radioimmunoassay was utilized to determine concentrations of cortisol (fetal) and estradiol and progesterone (maternal) in plasma over the course of the 80 hr infusion period. After 8 hrs post-infusion, fetal cortisol concentrations were significantly increased in both C and CM treated animals versus S control ($p<0.001$; **Figure 3.2A**). Mean fetal cortisol concentrations increased in the C group from 19.4 $\pm$ 8.4 ng/ml at infusion start to 101.1 $\pm$ 6.7 ng/ml at the end of 80 hrs. Similarly, in the CM group, cortisol rose from 23.9 $\pm$ 13.4 ng/ml at infusion start to 99.3 $\pm$ 16.7 ng/ml at the end of the infusion period.

Maternal concentrations of estradiol were significantly higher in the C (beginning at 64 hrs) and CM (beginning at 56 hrs) groups compared to the S control during the last 24 hrs of the infusion period ($p<0.05$; **Figure 3.2B**). Mean concentrations in the C and CM groups, respectively, at the beginning of the infusion period were 26.1 $\pm$ 7.7 pg/ml and 30.2 $\pm$ 7.5 pg/ml. At the end of 80 hrs, mean estradiol levels in the C group increased to 100.0 $\pm$ 38.7 pg/ml and in the CM group, 131.9 $\pm$ 32.1 pg/ml. Progesterone concentrations in maternal plasma

in all groups remained relatively constant throughout the 80 hr infusion period with no significant changes in either the S, C, or CM groups (**Figure 3.2C**).

3.3.2. Regulation of PGHS-2 and P450c17 in Late Gestation Ovine Placentomes

Expression of PGHS-2 mRNA and protein in placentomes from ewes treated with cortisol +/- meloxicam was detected by real-time quantitative RT-PCR and western blot. There was a trend for PGHS-2 mRNA to increase in both cortisol (C) and cortisol + meloxicam (CM) groups compared to the saline (S) control ($p=0.079$; **Figure 3.3A**). PGHS-2 protein, represented as a 72 kDa immunoreactive band, was expressed in all experimental groups (**Figure 3.4A**) and administration of exogenous glucocorticoid, in the presence or absence of meloxicam treatment, significantly increased PGHS-2 protein expression ($p<0.05$; **Figure 3.4B**).

Analysis of PGE_2 concentrations in the fetal plasma over the 80hr infusion period was determined using ELISA. PGE_2 levels were significantly elevated in the C group versus the S and CM groups at both 40hrs and 80hrs post-infusion ($p<0.05$; **Figure 3.5**). Also, within the C group, PGE_2 levels were significantly greater in the last 40hrs of infusion versus the first 40hrs ($p<0.05$; **Figure 3.5**).

P450c17 mRNA expression in d130 ovine placentomes was significantly upregulated in both C and CM groups versus the S control ($p<0.05$) in response to glucocorticoid administration (**Figure 3.3B**).

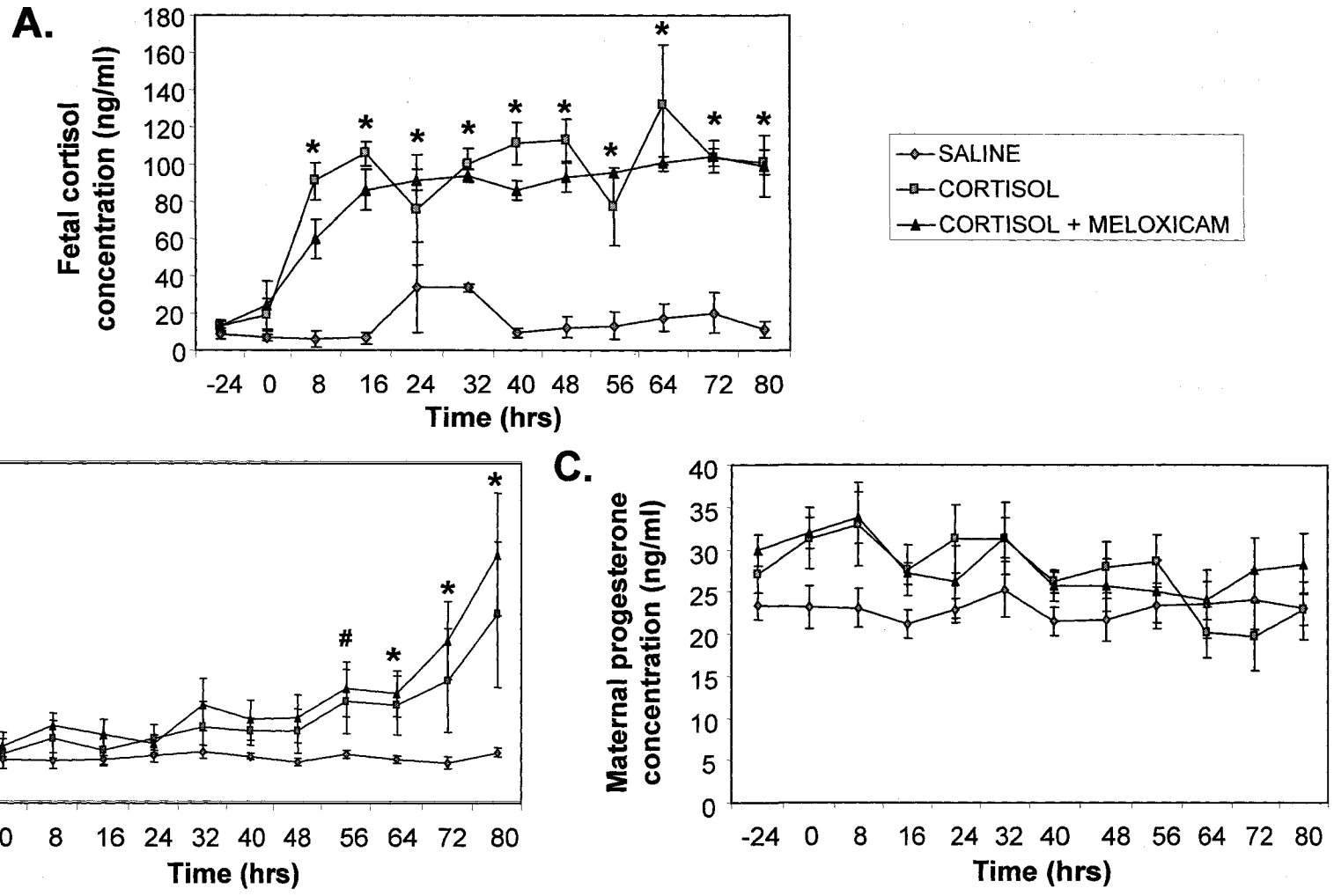


Figure 3.2 Fetal cortisol and maternal estradiol and progesterone plasma concentrations after exogenous glucocorticoid administration +/- meloxicam in d130 ovine placentomes

A.) Fetal cortisol concentration was significantly increased in both cortisol (C; n=4) and cortisol + meloxicam (CM; n=5) groups versus saline control (S; n=6) starting at 8 hrs post-infusion (*$p<0.001$). **B.)** Maternal estradiol concentration was significantly increased in the C (n=3) and CM (n=4) groups (*$p<0.05$) versus the S (n=5) control from 64 hrs until infusion end. CM group had significantly elevated levels of estradiol at 56 hrs (#$p<0.05$). **C.)** Maternal progesterone concentrations in S (n=6), C (n=4), and CM (n=5) groups remained relatively constant throughout the 80 hr period. All values are represented as the mean +/- SEM. Statistical analyses were performed using repeated measures two-way ANOVA followed by a Holm-Sidak multiple comparisons test. Significance was set at $p \leq 0.05$.

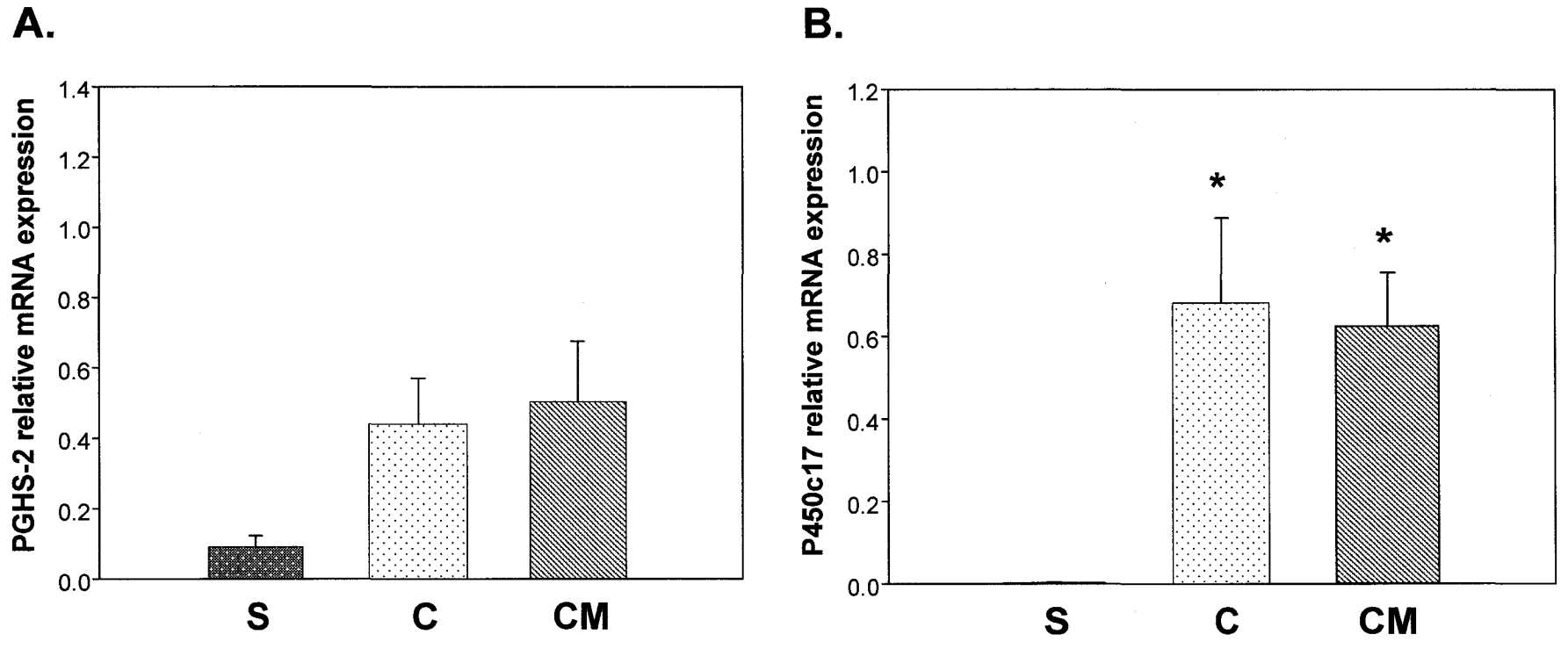


Figure 3.3 PGHS-2 and P450c17 mRNA expression in cortisol +/- meloxicam treated d130 ovine placentomes

A.) There was a trend for PGHS-2 mRNA expression to increase in the cortisol (C; n=4) and cortisol + meloxicam (CM; n=5) treated d130 placentomes versus the saline control (S; n=5). **B.)** P450c17 mRNA expression was significantly upregulated in C and CM groups versus S control (*$p<0.05$). All values are represented as the mean +/- SEM. Statistical analysis was performed using one-way ANOVA followed by a Tukey multiple comparison post-hoc test. Significance was set at $p \leq 0.05$.

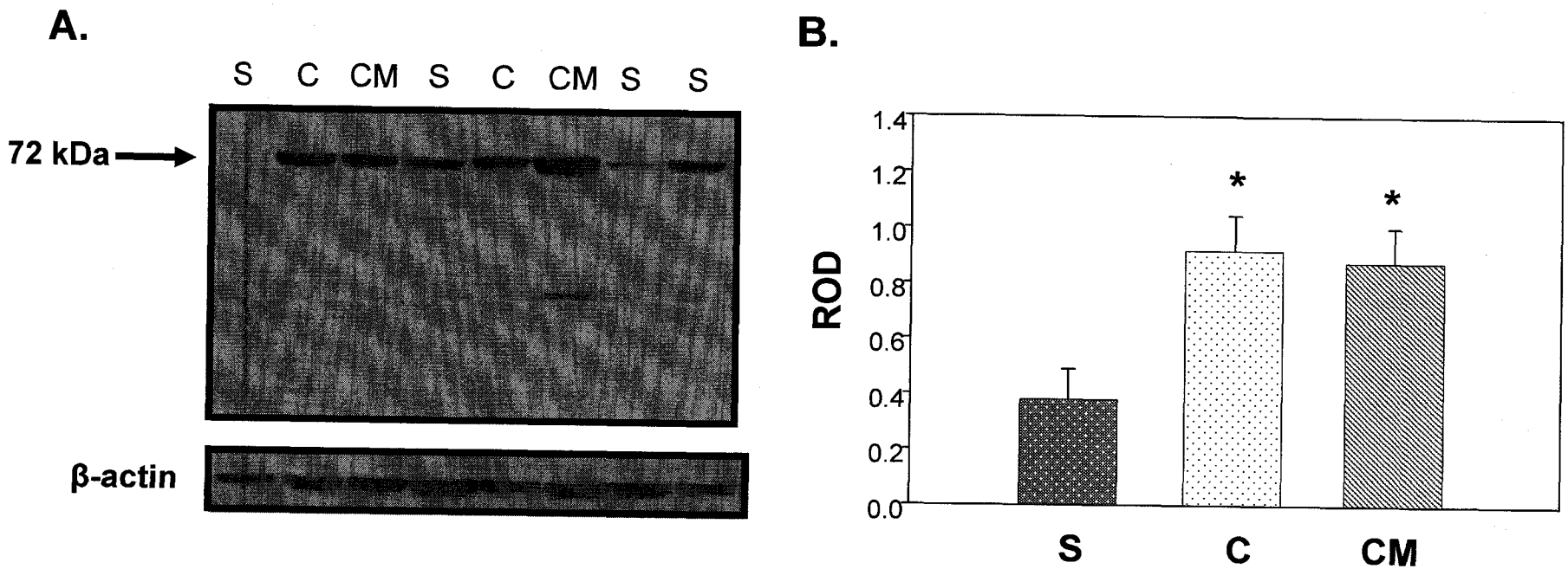


Figure 3.4 PGHS-2 protein expression in d130 ovine placentomes treated with cortisol +/- *meloxicam*

A.) A representative western blot demonstrating a 72 kDa band representing PGHS-2 protein in placentomes of saline (S; n=6), cortisol (C; n=4), cortisol + meloxicam (CM; n=5) treated groups. β-actin was used to normalize protein expression levels. **B.)** PGHS-2 protein expression was statistically significant in the C and CM groups versus S control (*$p<0.05$). Values are expressed in relative optical density (ROD) units. Bars represent the mean +/- SEM. Significant differences were determined by one-way ANOVA followed by a Tukey multiple comparison post-hoc test. Significance was set at $p \leq 0.05$.

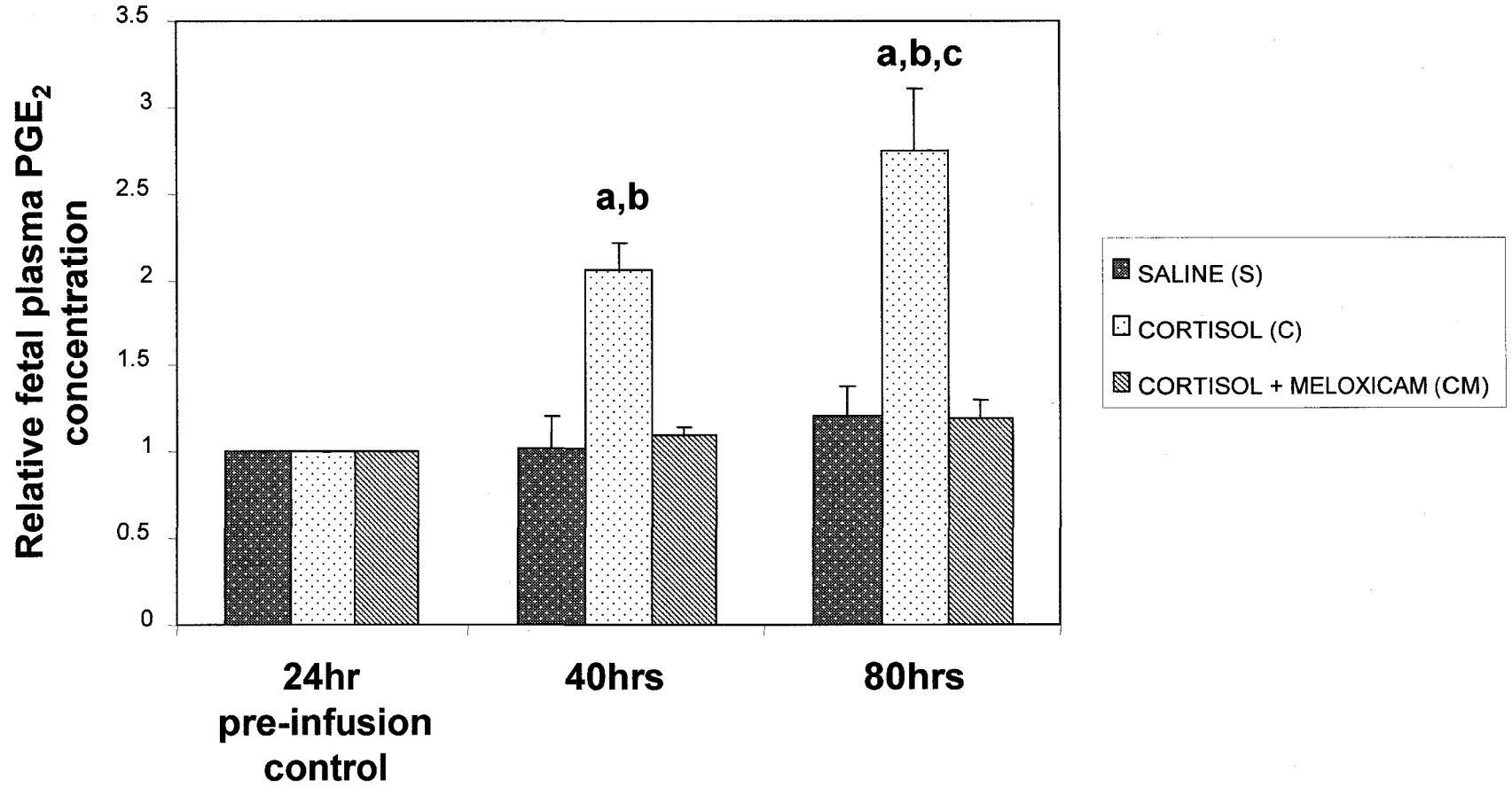


Figure 3.5 Fetal plasma PGE_2 concentration after exogenous glucocorticoid administration +/- meloxicam in d130 ovine placentomes

Fetal PGE_2 concentration was significantly increased in the cortisol group (C; n=3) versus the saline control (S; n=5 ($^{a}p<0.05$) and cortisol + meloxicam (CM; n=4) groups ($^{b}p<0.05$). Within the C group, PGE_2 was significantly increased at the end of infusion (80hrs) versus the first 40hrs of the infusion period ($^{c}p<0.05$). PGE_2 concentrations were normalized to their 24hr pre-infusion controls. All values are represented as the mean +/- SEM. Statistical analyses were performed using repeated measures two-way ANOVA followed by a Holm-Sidak multiple comparisons test. Significance was set at $p \leq 0.05$.

3.3.3. Expression of EP1, EP3, and FP Receptor Protein in Late Gestation Ovine Placentomes

Western blotting was used to characterize and assess protein expression levels of PG receptors EP1, EP3, and FP in d130 ovine placentomes. PGE_2 receptor subtypes EP1 and EP3 were both expressed in d130 ovine placentomes. A 33 kDa band representing EP1 was detected in all groups (**Figure 3.6A**) however glucocorticoid treatment did not have an effect on EP1 protein expression levels (**Figure 3.6C**). Similarly, EP3 was expressed in all groups and exogenous cortisol infusion had no effect on EP3 protein levels (**Figure 3.7A & C**). Preabsorption of the primary EP1 and EP3 antibodies with their respective blocking peptides eliminated the 33 kDa (**Figure 3.6B**) and 53 kDa (**Figure 3.7B**) signals, respectively.

The $PGF_{2\alpha}$ receptor, FP, was detected in all experimental groups (represented as a 70 kDa band) however exogenous glucocorticoid administration with or without meloxicam treatment had no effect on FP protein expression levels (**Figure 3.8A & C**). Preabsorption of the FP primary antibody with its respective blocking peptide abolished the 70 kDa signal (**Figure 3.8B**).

3.4. Discussion

In this study, we demonstrated the expression of PGHS-2, the contractile PG receptors, EP1, EP3, and FP, and the steroidogenic enzyme P450c17 in late gestation ovine placentomes following exogenous glucocorticoid administration. Intrafetal infusion of cortisol with or without meloxicam (PGHS-2 specific inhibitor) resulted in a trend for increased placental PGHS-2 mRNA expression and a

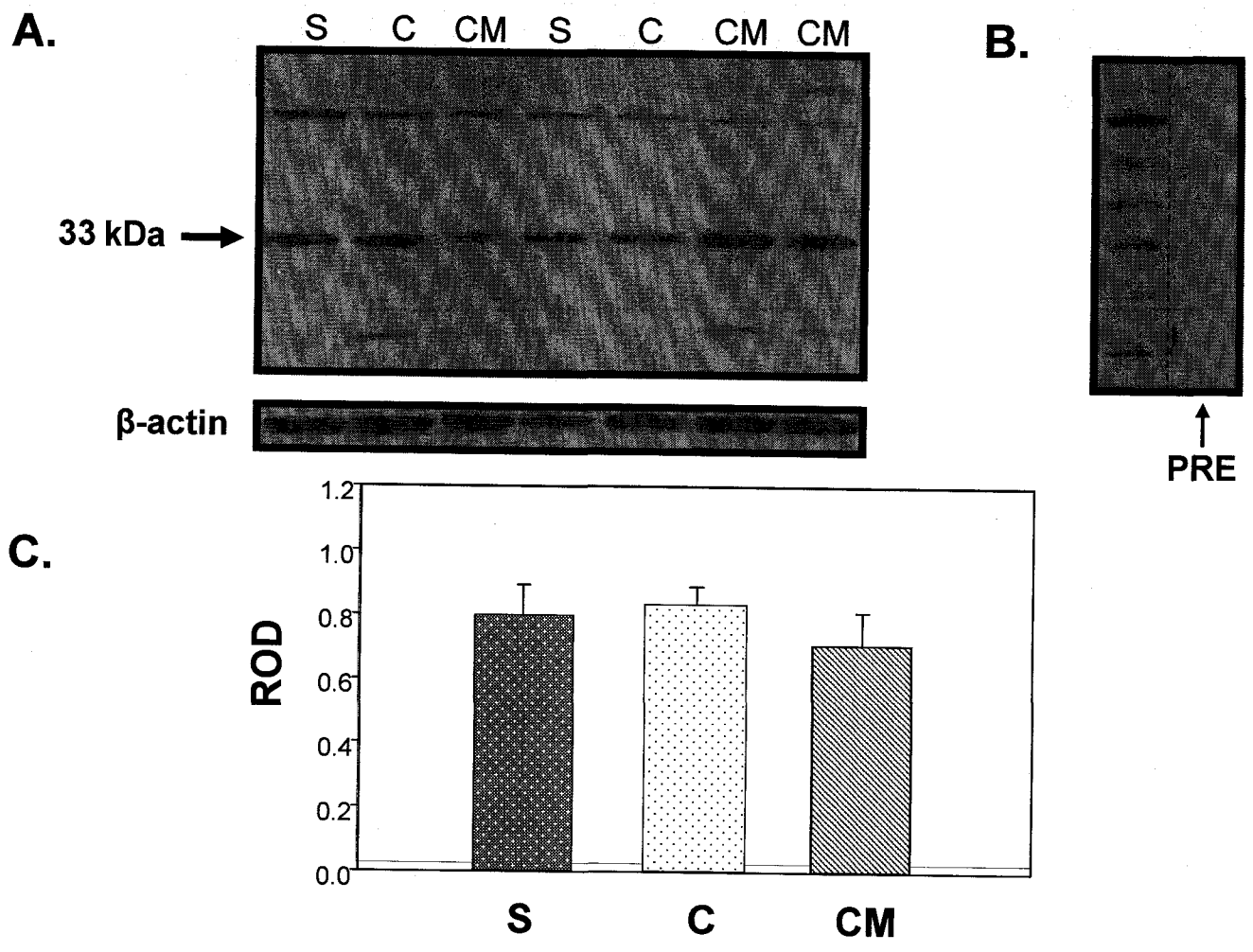


Figure 3.6 EP1 receptor protein expression in d130 ovine placentomes with cortisol +/- meloxicam treatment

A.) Western blotting revealed a 33 kDa band representing EP1 in saline (S; n=6), cortisol (C; n=4), and cortisol + meloxicam (CM; n=5) treated d130 placentomes. A representative blot is shown. β-actin was used to normalize protein expression levels. **B.)** Preabsorption (PRE) with an EP1 antigen eliminated the 33 kDa signal. **C.)** There was no difference in EP1 receptor protein expression between S, C, and CM groups. Values are expressed in relative optical density (ROD) units. Bars represent the mean +/- SEM. Statistical analysis was performed using one-way ANOVA.

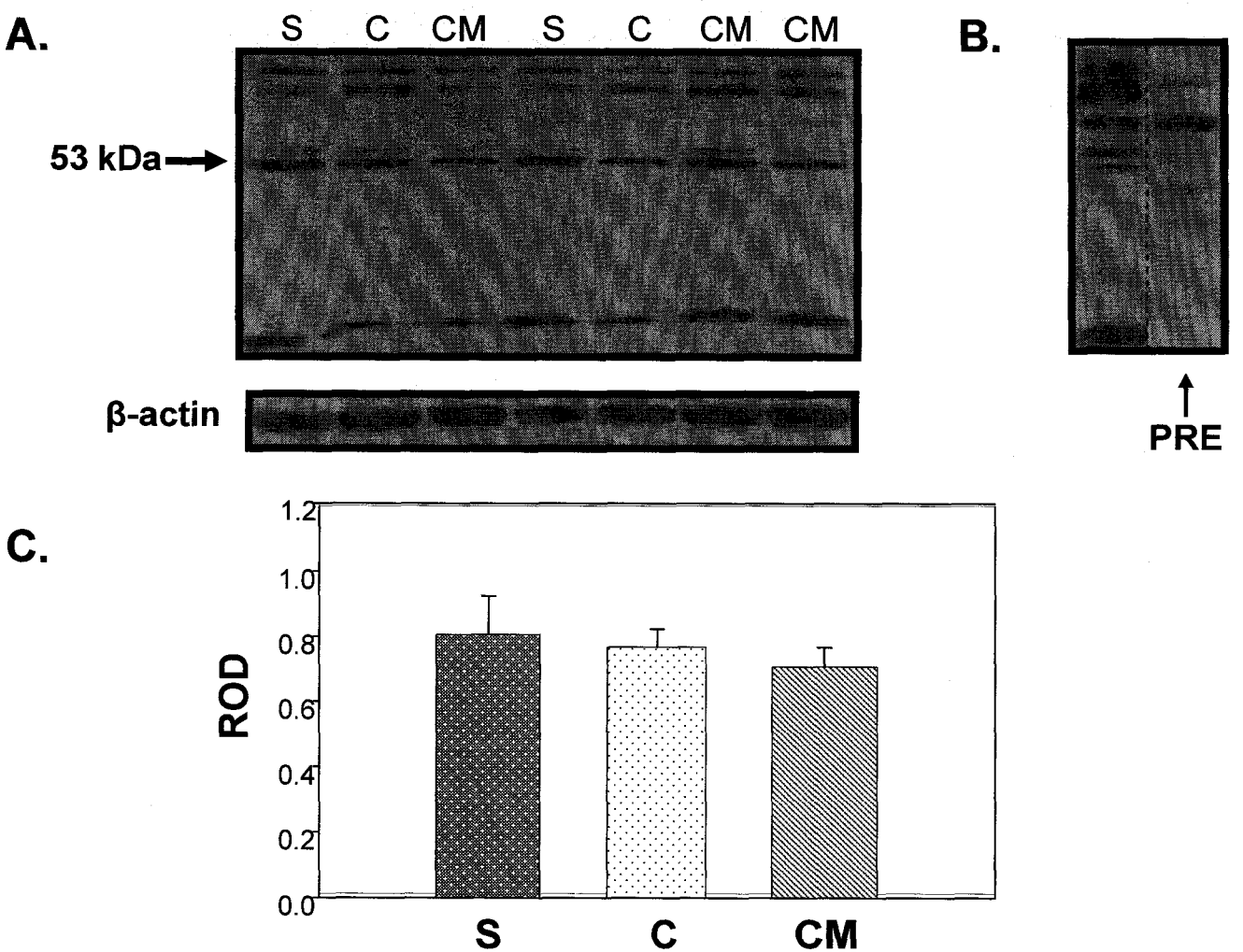


Figure 3.7 EP3 receptor protein expression in cortisol +/- meloxicam treated d130 ovine placentomes

A.) EP3 receptor protein expression (53 kDa band) was demonstrated in placentomes from saline (S; n=6), cortisol (C; n=4), and cortisol + meloxicam (CM; n=5) treated animals. A representative blot is shown. β-actin was used to normalize protein expression levels. **B.)** The 53 kDa band was diminished by preabsorption (PRE) with an EP3 antigen. **C.)** There was no difference in EP3 receptor protein levels between all 3 groups. Values are expressed in relative optical denisty (ROD) units. Bars represent the mean +/- SEM. Statistical analysis was performed using one-way ANOVA.

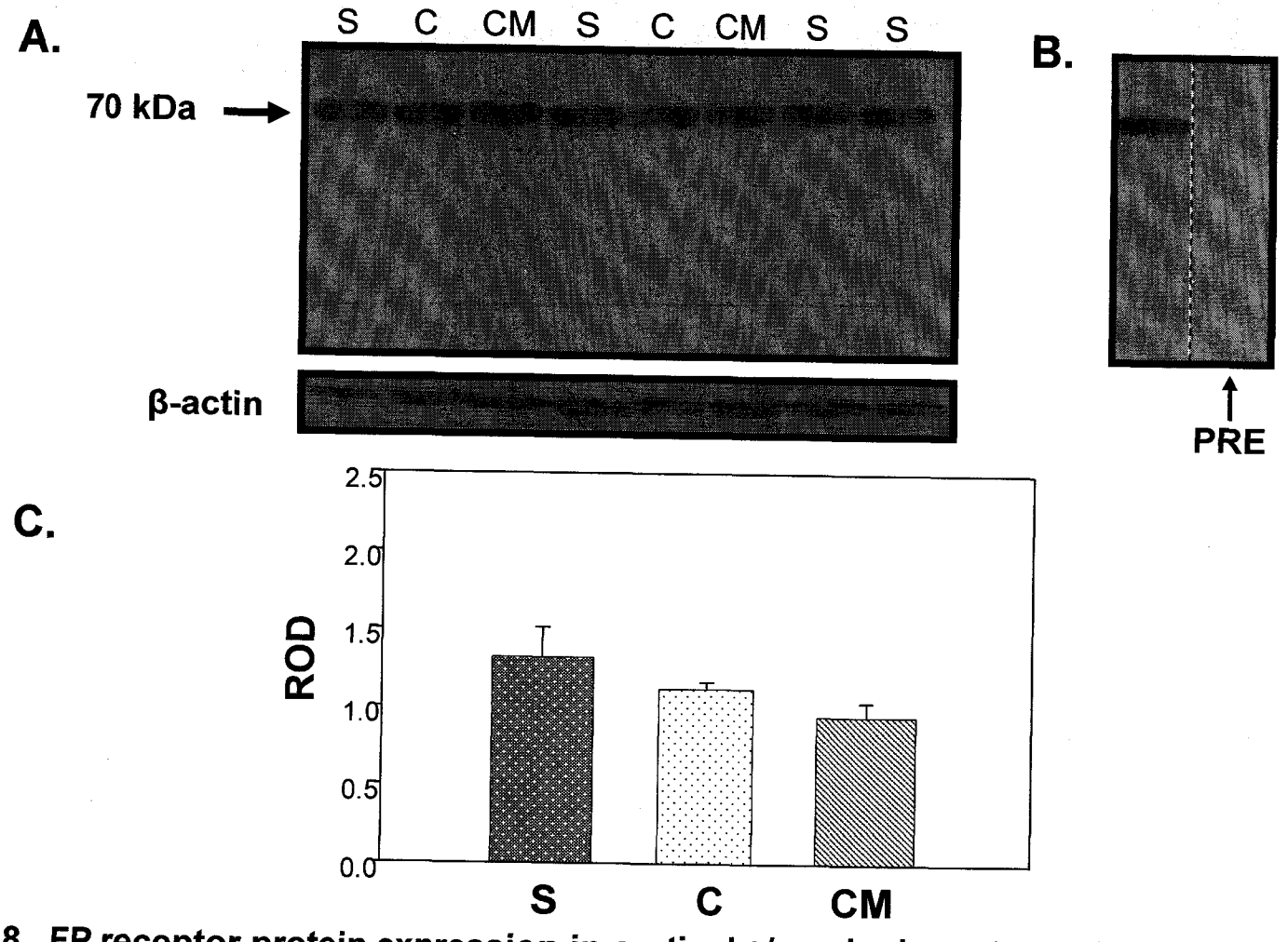


Figure 3.8 FP receptor protein expression in cortisol +/- meloxicam treated d130 ovine placentomes

A.) Western blotting revealed a 70 kDa band representing FP in saline (S; n=6), cortisol (C; n=4), and cortisol + meloxicam (CM; n=5) treated d130 placentomes. A representative blot is shown. β-actin was used to normalize FP protein expression levels. **B.)** Preabsorption (PRE) with an FP antigen eliminated the 70 kDa band. **C.)** There was no difference in FP receptor protein expression between S, C, and CM groups. Values are expressed in relative optical density (ROD) units. Bars represent the mean +/- SEM. Statistical analysis was performed using one-way ANOVA.

significant increase in PGHS-2 protein expression. PGE_2 concentrations in the fetal plasma were significantly elevated after cortisol administration but remained at baseline levels in ewes co-treated with meloxicam. Although meloxicam blocked PGE_2 synthesis, it did not prevent an increase in placental P450c17 mRNA expression, as observed after exogenous glucocorticoid administration. Furthermore, cortisol treatment had no effect on altering the expression levels of the PG receptors, EP1 (33 kDa), EP3, (53 kDa), and FP (70 kDa), despite the increased PGE_2 concentrations. Therefore, it appears that the late gestation increase in placental PGE_2 is not an obligatory step in upregulating the expression of P450c17.

Early studies in both dexamethasone-induced and natural term labour ovine placentomes described this enhanced, pre-partum rise in P450c17 expression and activity (France et al., 1988; Mason et al., 1989). However, these studies did not investigate the direct relationship between cortisol and P450c17. Thus, we cannot rule out the possibility of a direct interaction between these two labour-associated molecules. In support of this, Nason et al. (1992) reported the presence of a glucocorticoid response element (GRE) consensus sequence in the promoter region of the rat P450c17 gene; a gene whose upstream region shares significant homology to both the bovine and human genes (Nason et al., 1992). Therefore, it is possible that a similar GRE sequence exists in the ovine P450c17 gene.

In preliminary studies from our laboratory and consistent with our initial hypothesis, we have shown a dose- and time-dependent upregulation of

P450c17 mRNA and protein in cultured ovine placental trophoblast cells treated with exogenous PGE_2. However, in the current study, our results indicate that the increased synthesis of PGE_2, in late gestation, is not the major pathway leading to upregulation of placental P450c17 expression. The discrepancy between these two studies is likely due to differences in experimental design. The previous studies utilized an *in vitro* system with isolated trophoblast cells versus our current study which made use of an *in vivo* model, in which other physiological pathways and mediators remain intact and can influence the observed results. Therefore, there may be other glucocorticoid-stimulated intermediates or pathways in the placenta that play a role in P450c17 regulation. As a result, further studies are warranted to investigate the regulation of placental P450c17 expression.

Use of the PGHS-2 specific inhibitor, meloxicam, allowed us to investigate the role of PGs in regulating P450c17 expression. Meloxicam, which has a plasma half-life of approximately 20 hours (Davies & Skjodt, 1999), has recently been studied in order to determine its efficacy and safety as a potential tocolytic (McKeown et al., 2000; Rac et al., 2006). Both McKeown et al. (2000) and Rac et al. (2006), demonstrated decreased uterine contractility after meloxicam administration in an RU-486-induced (a progesterone antagonist) ovine model of preterm labour. Furthermore, McKeown et al. (2000) reported attenuation of the fetal HPA axis after infusion of meloxicam. In late gestation, the increase in placental PGE_2 output has been identified in sustaining fetal HPA axis activation via a positive feed-forward mechanism (Whittle et al., 2001). In the study by

McKeown et al. (2000), the reported decrease in plasma PGE_2 concentrations, after meloxicam administration, were believed to contribute to the observed decrease in circulating cortisol levels, thereby indicating decreased HPA axis activation. Similarly, in a study by Ma et al. (1999), infusion of nimesulide (a PGHS inhibitor which exhibits preferential PGHS-2 inhibition) caused a significant decrease in circulating levels of PGE_2, cortisol, and mRNA abundance of P450c17. However, in our current study, fetal cortisol levels continued to rise despite infusion of meloxicam. Therefore, this difference could be due to the initially higher basal levels of PGE_2 in the cortisol + meloxicam treated animals, which may be elevated enough to sustain HPA axis activation.

In the current study, we observed no changes in the expression levels of the PG receptors in response to exogenous cortisol administration. However, we cannot exclude that ovine placental trophoblast cells might express a specific isoform(s) of the EP and FP receptor proteins that are unable to be detected by the antibodies used. Through alternative mRNA splicing, it has been reported that different isoforms exist for PG receptors, such as human EP3 and ovine FP (Namba et al., 1993; Pierce et al., 1998). Since we used antibodies targeted against human peptide sequences, there is the possibility that these may not be able to detect other isoforms expressed in sheep placentomes. Additionally, utilizing an ovine model of term and preterm labour, Palliser et al. (2005) reported mRNA expression of PG receptors in placentomes of dexamethasone-induced or spontaneous term labour ewes. In this study, they were unable to detect placental mRNA expression of EP1 and found varying expression profiles for EP

receptors 2-4 and FP depending on the labouring group (dexamethasone-treated versus spontaneous labour). In contrast, in our studies, we were able to detect expression of EP1 protein in late gestation ovine placentomes but found no change in the expression levels of EP1, EP3, and FP in response to exogenous glucocorticoid administration. This lack of consistency between our observations and those published by Palliser et al. (2005) could be attributed to differences in experimental design. In the current study, we concentrated on investigating PG receptor expression at the functional level by looking at protein expression versus mRNA expression. It is possible that by utilizing quantitative RT-PCR for the PG receptors, we may have also seen significant changes in the transcriptional regulation of EP receptor subtypes and FP, as observed by Palliser et al. (2005).

Initial studies describing PG receptor expression and regulation focused on their levels in the myometrium of non-pregnant and pregnant (term and preterm) subjects in an attempt to elucidate the mechanisms involved in uterine quiescence and activity (Astle et al., 2005; Crankshaw et al., 1995; Gyomorey et al., 2000; Leonhardt et al., 2003). Recently, a greater number of studies have investigated PG receptor expression in intrauterine tissues, other than the myometrium, such as the fetal membranes and placenta (Grigsby et al., 2006a; Palliser et al., 2006). However, to our knowledge, there have been no studies published that examined PG receptor protein expression in late gestation ovine placentomes in response to exogenous glucocorticoid administration. Thus, our studies are the first to describe their protein expression and regulation in the

ovine placenta utilizing an exogenous glucocorticoid-induced model of preterm labour.

Consistent with our findings in which we observed no alterations in the expression profiles of PG receptors in response to glucocorticoid infusion, some studies have suggested that PG receptor levels might reach a threshold prior to labour onset and no longer undergo changes in their expression levels (Palliser et al., 2005). In support of this, studies investigating the myometrium in non-labouring and labouring subjects have shown no change in sensitivity to exogenous PG administration (Baguma-Nibasheka et al., 1998) or in mRNA expression levels of EP1-4 and FP (Gyomorey et al., 2000b). Additionally, Yamamoto et al. (2005) have recently proposed a negative feedback mechanism in regulating FP receptor expression levels in a human myometrial cell line. In this study, they have shown a dose-dependent decrease in FP mRNA levels in response to increasing concentrations of exogenous $PGF_{2\alpha}$, suggesting an auto-regulatory mechanism by which PG receptors regulate their own expression levels.

Intrafetal administration of cortisol has been implicated in altering the frequency of ovine placentome distribution. A recent study by Ward et al. (2006) reported a greater number of A-type placentomes and a decrease in the number of D-types after intra-fetal delivery of cortisol. In addition, exogenous cortisol treatment has also been shown to influence the population and distribution of particular cell types in the fetal trophoblast, such as reducing the binucleate cell population (Ward et al., 2002). Therefore, it is possible that PG receptor

expression can vary depending on the type of placentome (A-D) and that the distribution of certain cell types expressing PG receptors may also be altered. Extending these observations to our findings might explain why there is no observed alteration in PG receptor expression levels as we did not distinguish what types of placentomes were used in our studies.

It has been well established that maturation of the fetal HPA axis in late gestation leads to an increase in cortisol concentrations which in turn stimulates placental PGE_2 output through enhancement of PGHS-2 activity (Challis et al., 2000; Whittle et al., 2001). Subsequent to the increased production of PGE_2 is an upregulation of placental P450c17 activity, which alters the pattern of placental steroid synthesis, as observed at term. In support of this, Gyomorey et al. (2000a, b) demonstrated an increase in placental PGHS-2 mRNA in both early labour and labouring groups and a later significant rise in P450c17 mRNA levels. The findings from our studies confirm the results described by Gyomorey et al. (2000a) in which increased fetal cortisol concentrations (via exogenous glucocorticoid administration) tended to increase PGHS-2 mRNA expression and significantly upregulated placental P450c17 mRNA expression. Furthermore, Gyomorey et al. (2000a) revealed co-localization of PGHS-2 and P450c17 proteins to the uninucleate trophoblast cells of sheep placentomes. Although increased PGE_2 output may not be the major pathway in regulating P450c17 expression, it could still contribute to its regulation; therefore, the immunohistochemical data reported by Gyomorey et al. (2000a) support the possibility that placental PGE_2 could act locally to regulate levels of P450c17.

This also suggests that EP receptor subtypes 1-4 and FP are also localized to the plasma membrane and/or nucleus of uninucleate trophoblast cells thereby mediating PGE_2 action on P450c17 expression and activity. In the current study, we were unable to determine immunolocalization of the PG receptors and P450c17 due to the lack of availability of fixed tissue samples. For this reason, immunohistochemical studies are required to determine cellular PG receptor localization in ovine placentomes.

In summary, we have shown the expression and regulation of EP1, EP3, and FP prostanoid receptors and P450c17 in response to exogenous glucocorticoid administration using an ovine model of preterm labour. Intrafetal cortisol infusion, with or without meloxicam, significantly increased placental PGHS-2 protein and P450c17 mRNA levels in late gestation ovine placentomes. The increase in PGHS-2 enzyme, after cortisol administration, resulted in increased synthesis of PGE_2 in the fetal plasma. However, in ewes co-infused with meloxicam, placental P450c17 mRNA expression was increased despite the effective block of PGE_2 synthesis. Thus, it appears that the increased PGE_2 output over the last 15-20 days of gestation may not be the only major pathway in regulating placental P450c17 expression. As a result, we suggest that cortisol and/or glucocorticoid-stimulated intermediates and/or pathways may have a role in regulating placental P450c17 expression levels. Also, since we observed no changes in the expression profiles of EP1, EP3, and FP in response to cortisol or cortisol + meloxicam infusion, alterations in their expression levels may not be as important as the increased PGE_2 output or the action of other mediators involved

in labour onset. Further studies are required in order to determine the cellular localization of placental PG receptors and the mechanisms that are involved in regulating P450c17 enzymatic activity in late gestation and labour onset.

Chapter 4

The Effect of Periconceptional Undernutrition on Prostaglandin Receptor Localization and Expression in Late Gestation Ovine Placentomes

4.1. Introduction

There have been a number of studies conducted, utilizing different species (i.e. humans, sheep, and rats), in order to investigate PG receptor expression and regulation in various intrauterine tissues such as the myometrium and fetal membranes (Astle et al., 2005; Brodt-Eppley et al., 1999; Grigsby et al., 2006; Leonhardt et al., 2003; Ma et al., 1999; Palliser et al., 2006). However, there has been a lack of information addressing their expression and regulation in the placenta. In a recent study, using human tissue, Grigsby et al. (2006a) demonstrated EP1-4 receptor subtype localization in human placental villous tissue with no change in expression with respect to gestational age and labour. Of interest, in a recent study by Palliser et al. (2005), the mRNA expression patterns for the EP receptor subtypes and FP were investigated in term and preterm ovine tissues. They demonstrated an overall lower expression of EP2, EP3, EP4, and FP in placentomes versus their expression in endometrium and/or myometrium.

In sheep, sustained activation of the fetal HPA axis and the resultant increase in fetal cortisol concentrations provides the trigger for placental PG synthesis at term (Challis et al., 2000). It has been suggested that the increased

levels of cortisol and/or PGE_2 induce the expression of the steroidogenic enzyme P450c17, which results in the altered pattern of placental steroid synthesis observed during labour (France et al., 1988; Mason et al., 1989; Gyomorey et al., 2000a; Whittle et al., 2000). Recent studies have demonstrated that maternal undernutrition during the periconceptional period could lead to premature delivery and we have reported accelerated maturation of the fetal HPA axis during late gestation in response to a period of moderate maternal undernutrition during the periconceptional period (Bloomfield et al., 2003). In this study, approximately 50% of ewes exposed to a period of maternal undernutrition, 60 days before until 30 days after conception, had a precocious rise in fetal cortisol concentrations and subsequently delivered prematurely (Bloomfield et al., 2003). Furthermore, the rise in circulating fetal cortisol concentrations occurred prior to observed increases in fetal PGE_2, maternal PGFM, and maternal estradiol concentrations (Kumarasamy et al., 2004). However, there are limited data describing PG receptor subtype expression and localization in term and preterm ovine placentomes and the mechanisms regulating their expression levels in the placenta remain unclear. Therefore, we aimed to investigate expression and localization of the contractile PG receptors EP1, EP3, and FP in late gestation ovine placentomes and to determine the effects periconceptional UN has on PG receptor protein levels. We hypothesized that preterm labour after perinconceptional UN would be associated with altered localization and/or expression of PG receptors in the placenta.

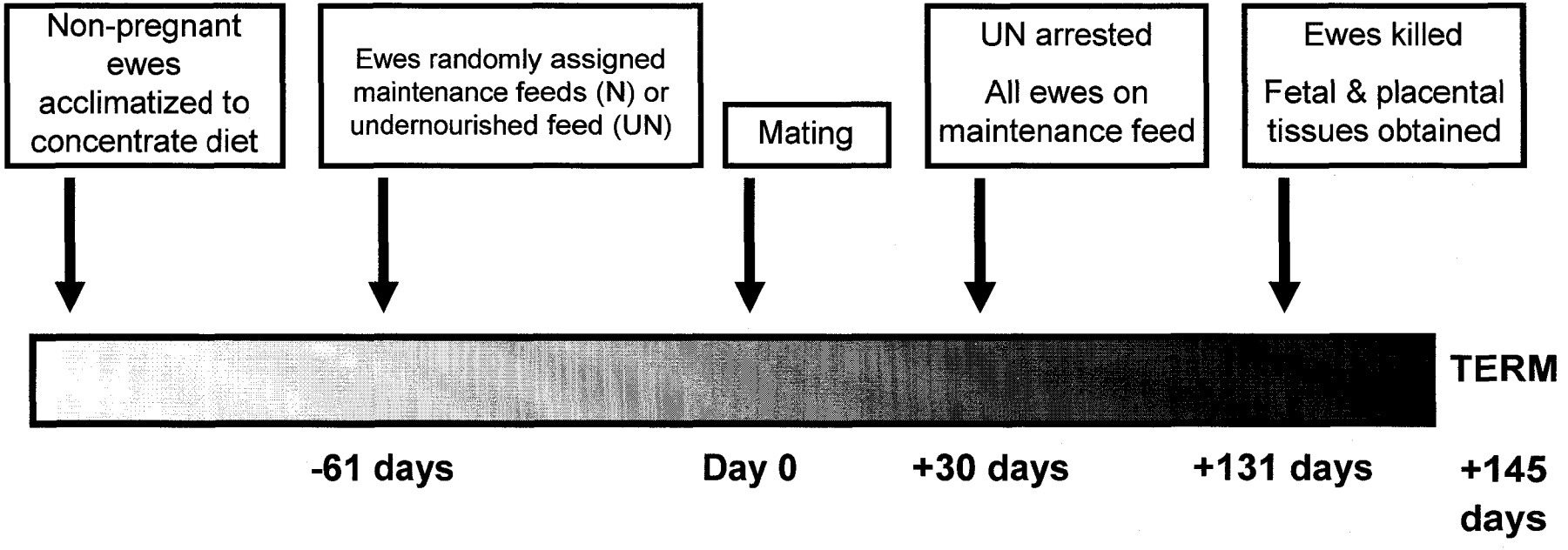
4.2. Materials and Methods

4.2.1. Animal Care

Experiments were approved by the Animal Ethics Committee at the University of Auckland, New Zealand. Prior to nutritional manipulation, 4-5 year old Romney ewes were housed in group pens in a photoperiod controlled feedlot and allowed to eat, at will, feed concentrates at a daily rate of 3-4% body weight. The concentrate feed used consisted of 65% lucerne, 30% barley, limestone, molasses, myocurb and essential trace elements (Oliver et al., 2005). All ewes were weighed on arrival to the feedlot and twice weekly thereafter for the duration of the study and then again at post mortem. Two weeks prior to mating with Dorset rams, estrous cycles were synchronized by means of progesterone-containing intravaginal devices (Wheaton et al., 1993). Intramuscular serum gonadotropin (200 IU chorionic gonadotropin) was given on intravaginal device withdrawal two days before mating to stimulate ovulation. Ewes were scanned by 35 and 49 days after mating for confirmation of pregnancy and to determine fetal number.

4.2.2. Experimental Design: Nutritional Manipulation

At 61 days before mating, ewes were weighed and randomly assigned to *ad libitum* concentrates throughout gestation (control, N) or to undernutrition (UN) from 60 days before until 30 days after mating (**Figure 4.1**). The periconceptional period was studied in order to determine the effects of maternal UN around the time of conception on fetal HPA axis maturation and the timing of



The UN regimen reduced maternal body weight by 15% and this was recovered upon re-feeding.

Figure 4.1 Experimental design for the periconceptional undernutrition regimen

birth (Bloomfield et al., 2003). Ewes in the control *ad libitum* group (N) were kept in group pens and allowed to eat at will from feed concentrates at a daily rate of 3-4% body weight to maintain body weight. Ewes in the undernourished (UN) group were housed in individual pens and received concentrates at a daily rate of 1-2% body weight, in order to reduce and maintain maternal body weight by 10-15% from 60 days before until 30 days after mating, with *ad libitum* feeding thereafter. Feed amounts were individually adjusted to achieve and maintain this weight. At the end of the experiment, ewes were killed on gestational day 131 by a lethal intravenous dose of injectable pentobarbitone (term 147 days). Placentomes were rapidly dissected on day 131 of gestation, immediately flash frozen in liquid nitrogen and subsequently stored at -80^{0}C until analysis.

4.2.3. Immunohistochemistry

Fixed placentomes (7 µm sections) from nourished and undernourished animals were deparaffinized in Xylene (3 x 5 min each; Sigma, Oakville, ON) and rehydrated in a graded ethanol series (100%, 90%, 70%; 2 x 1 min each) followed by washing in 0.1% PBS (2 x 5 min each). Antigen retrieval was performed by microwaving sections for 10 min in 0.01M citrate buffer (pH 6) followed by a 0.1% PBS wash (2 x 5 min each). Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide (EMD Biosciences, Gibbstown, NJ) in methanol for 30 min at RT followed by washing in 0.1% PBS solution (2 x 5 min each). To eliminate non-specific binding, tissues were incubated with normal goat serum (NGS) in 0.1% PBS (non-immune blocking solution) for 1 hr at RT.

Sections were then incubated overnight at 4^0C in a humidified chamber with the following primary polyclonal antibodies diluted in non-immune blocking solution, respectively: rabbit anti-human EP1 (1:100); rabbit anti-human EP2 (1:100); rabbit anti-human EP3 (1:100); rabbit anti-human EP4 (1:100); and rabbit anti-mouse FP (1:50). Negative control sections were incubated with either a concentration-matched rabbit immunoglobulin (IgG) (Vector Laboratories, Burlingame, CA) or omission of the primary antibody.

For preabsorption controls, the primary antibodies were incubated overnight at 4^0C with their respective blocking peptides: EP1 (catalogue #101740; sequence: GLTPSAWEASSLRSSRHSGLSHF), EP2 (catalogue #101750; sequence: SLRTQDATQTSCSTQSDASKQADL), EP3 (catalogue #101760; sequence: NQTSVEHCKTHTEKQKECNF), EP4 (catalogue #101775; sequence: GSGRAGPAPKGSSLQVTFPSETLNLSEKCI), and FP (catalogue #101802; sequence: SMNSSKQPVSPAAGL) (Cayman Chemical, Ann Arbor, MI) in a non-immune blocking solution at the following antibody:antigen ratios: 1:10 (EP1 and EP3), 1:40 (EP2 and EP4); 1:50 (FP) as this was an adequate ratio to see a reduction in staining.

The following day, placental sections were washed in 0.1% PBS-Tween 20 solution (2 x 5 min) and subsequently incubated with a biotinylated secondary anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA) diluted in non-immune blocking serum followed by an avidin-biotin peroxidase detection system (Elite ABC; Vector Laboratories, Burlingame, CA), both for 1 hr at RT in a humidified chamber. Diaminobenzadine (SigmaFast DAB tablets; Sigma,

Oakville, ON) was used to identify positive staining in tissues and sections were exposed to the DAB substrate for approximately 30 sec. The DAB reaction was subsequently stopped by addition of double distilled H_2O.

Sections were counterstained with Mayer's haematoxylin (Sigma, Oakville, ON) for 1 min, washed in 0.1% PBS solution for 5 min to clear haematoxylin, dehydrated in an ascending ethanol series (70%, 90%, 100%; 2 x 1 min each) and cleared in xylene overnight. Sections were then mounted with Cytoseal 280 (Richard Allan Scientific, Kalamazoo, MI) before being viewed by light microscopy.

Immunolocalization of EP2 and EP4 are presented in Appendix B. BLAST searches of the PG receptor antigen sequences used to raise the receptor antibodies did not result in a match for ovine EP2 and EP4 sequences. Therefore, due to this concern, they are presented here only as preliminary data. BLAST searches for the EP1, EP3, and FP antigens resulted in the following identity scores, respectively: 88%, 80%, and 66%.

4.2.4. Western Blotting

Protein extraction and western blotting of placentomes from normally nourished (N) and UN animals were performed in the same manner as described in Chapter 3 Section 3.2.7.

Briefly, protein was extracted from frozen cotyledons (500 mg) and crushed with a pestle over ice and homogenized with RIPA lysis buffer. Protein concentrations were measured using the Bradford protein assay (Bradford,

1976). Ovine placental protein samples (20 µg) were denatured and subsequently separated by 11% SDS-PAGE at a constant voltage of 80 V. Proteins were electrophoretically transferred at 4^0C onto a 0.45 µm nitrocellulose membrane and membranes were blocked overnight at 4^0C in a 3% normal goat serum (NGS) and 0.1% PBS-Tween 20 (Sigma, Oakville, ON) solution (for EP receptor subtypes 1-4, and FP) or in a 5% (w/v) non-fat powdered milk in 0.1% PBS-Tween 20 solution (for β–actin). After blocking, membranes were incubated with the same primary antibodies as used in the immunohistochemistry studies (all from Cayman Chemical, Ann Arbor, MI): rabbit anti-human polyclonal EP1 receptor (1:500); rabbit anti-human polyclonal EP2 receptor (1:500); rabbit anti-human polyclonal EP3 receptor (1:600); rabbit anti-human polyclonal EP4 receptor (1:500); rabbit anti-mouse FP (1:500) receptor. After incubation with the respective primary antibody, membranes were washed and incubated with anti-rabbit IgG coupled to horseradish peroxidase (1:3000) (Amersham, Piscataway, NJ) for 1 hr at RT. At the end of the secondary antibody incubation, membranes were washed, proteins were visualized using chemiluminescence (Western Lightning, Perkin Elmer, Waltham, MS) and exposed to X-OMAT blue autoradiography film (Eastman Kodak Co., Rochester, NY).

For preabsorption controls, the primary antibodies were incubated overnight at 4^0C with their respective blocking peptides (EP1, EP3, and FP; Cayman Chemical, Ann Arbor, MI) at an antibody:antigen ratio of 1:10 in a 3% NGS - 0.1% PBS-Tween 20 solution as this was an adequate ratio to see a reduction or disappearance in the protein band.

All blots were stripped and reprobed with an anti-mouse β-actin antibody (1:10000) (Sigma, Oakville, ON) as an internal control to verify similar protein loading in each lane. Protein expression levels for EP1, EP3, FP, and β-actin were quantified by densitometry. Results are presented as the ratio of respective protein of interest to β-actin in relative optical density (ROD) units.

Protein expression data for EP2 and EP4 have been presented in Appendix B. BLAST searches of the amino acid sequences of the antigens used to raise the antibodies did not result in a match with ovine EP2 and EP4 sequences. Therefore, due to this concern, they are presented here only as preliminary data. See Section 4.2.3. for the percent sequence similarities for the EP1, EP3, and FP receptor antigen sequences against known sheep sequences.

4.2.5. Statistical Analysis

All data are represented as the mean +/- standard error of the mean (SEM) throughout. Western blot data were analyzed using unpaired Student's T-test. All statistical measurements and plots were performed using SigmaStat® version 3.1 and SigmaPlot® version 9.0 (Systat Software, San Jose, CA). A value of $p \leq 0.05$ was considered significant.

4.3. Results

4.3.1. Localization of PG Receptors in d131 Ovine Placentomes

Immunohistochemistry was utilized to assess the localization of EP1, EP3, and FP receptors in late gestation ovine placentomes. All receptors were

localized to the fetal trophoblast (T) layer, predominantly in the uninucleate cells. Absence of positive staining was observed in the maternal syncitium (M) (**Figure 4.2 panels i, iii, v**). Preabsorption of the EP1 and EP3 primary antibodies with their respective blocking peptides diminished the positive staining (**Figure 4.2 panels ii and iv**). Preabsorption of the FP primary antibody with its blocking peptide did not result in a reduction of the positive staining (**Figure 4.2 panel vi**).

4.3.2. The Effect of Periconceptional Undernutrition on PG Receptor Expression

Western blotting was used to assess protein expression levels of EP1, EP3, and FP receptors in response to periconceptional undernutrition. The PGE_2 receptor subtypes EP1 and EP3 were both expressed in placentomes of both N and UN animals. EP1 was demonstrated as a 33 kDa immunoreactive band (**Figure 4.3A**) however periconceptional undernutrition had no effect on EP1 protein expression levels (**Figure 4.3C**). Similarly, EP3 was expressed in both groups however there were no alterations in EP3 protein levels after periconceptional UN (**Figure 4.4A & C**). Preabsorption of the primary EP1 and EP3 antibodies with their corresponding blocking peptides eliminated the 33 kDa (**Figure 4.3B**) and 53 kDa signals (**Figure 4.4B**), respectively.

The $PGF_{2\alpha}$ receptor, FP, was detected in both experimental groups (represented as a 70 kDa band) however perinconceptional UN had no effect on the levels of FP protein expression (**Figure 4.5A and C**). Preabsorption of the FP primary antibody with its respective blocking peptide eliminated the 70 kDa signal (**Figure 4.5B**).

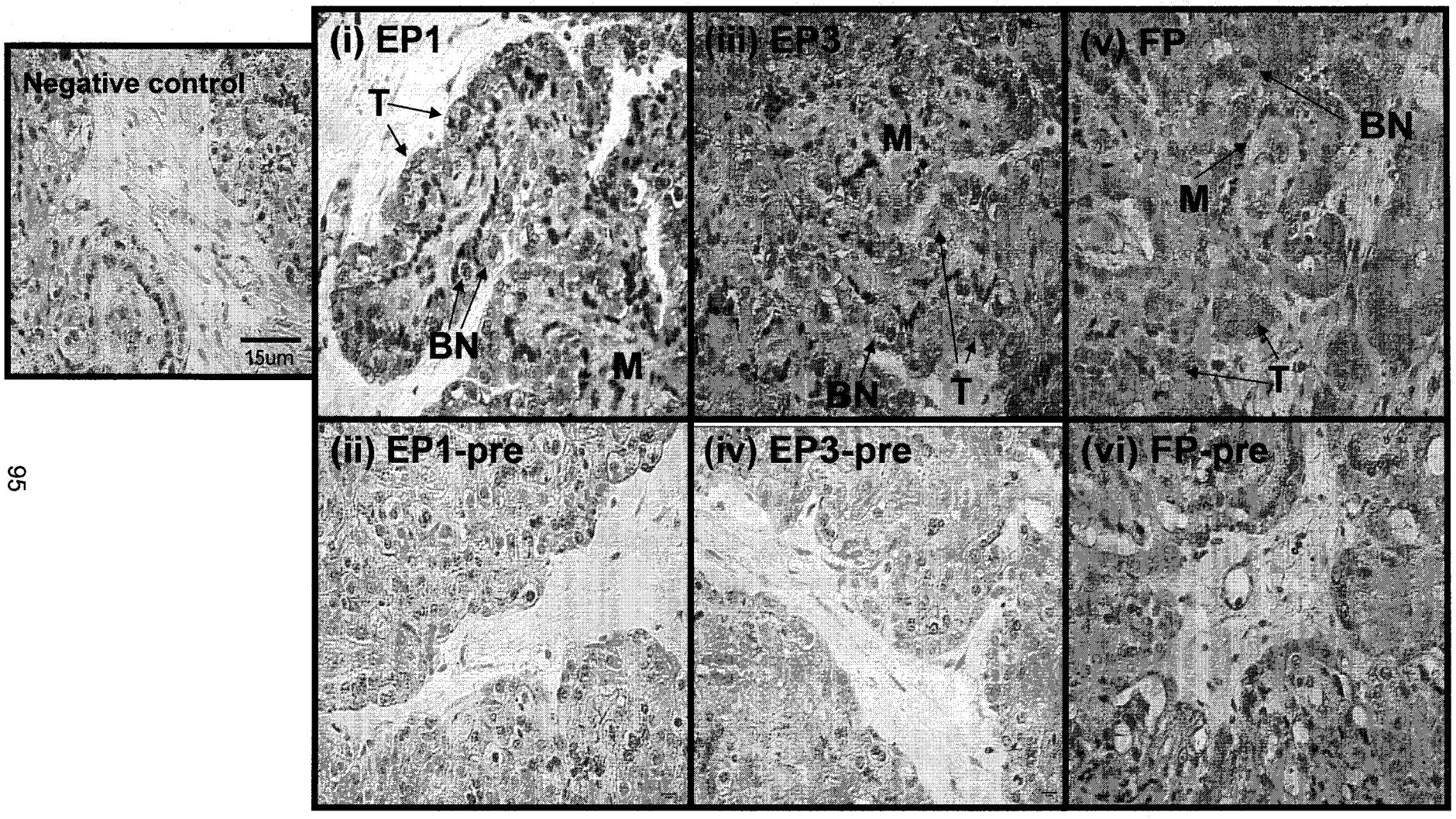


Figure 4.2 PG receptor protein localization in d131 ovine placentomes

Immunohistochemical analysis demonstrated EP1 (i), EP3 (ii), and FP (v) protein localization in the fetal trophoblast (T) of d131 ovine placentomes. Staining was absent in the maternal syncytium (M). Preabsorption with the EP1 (ii) and EP3 (iv) blocking peptides, respectively, diminished the positive staining. BN: binucleate cell; pre: preabsorption. Magnification 400x.

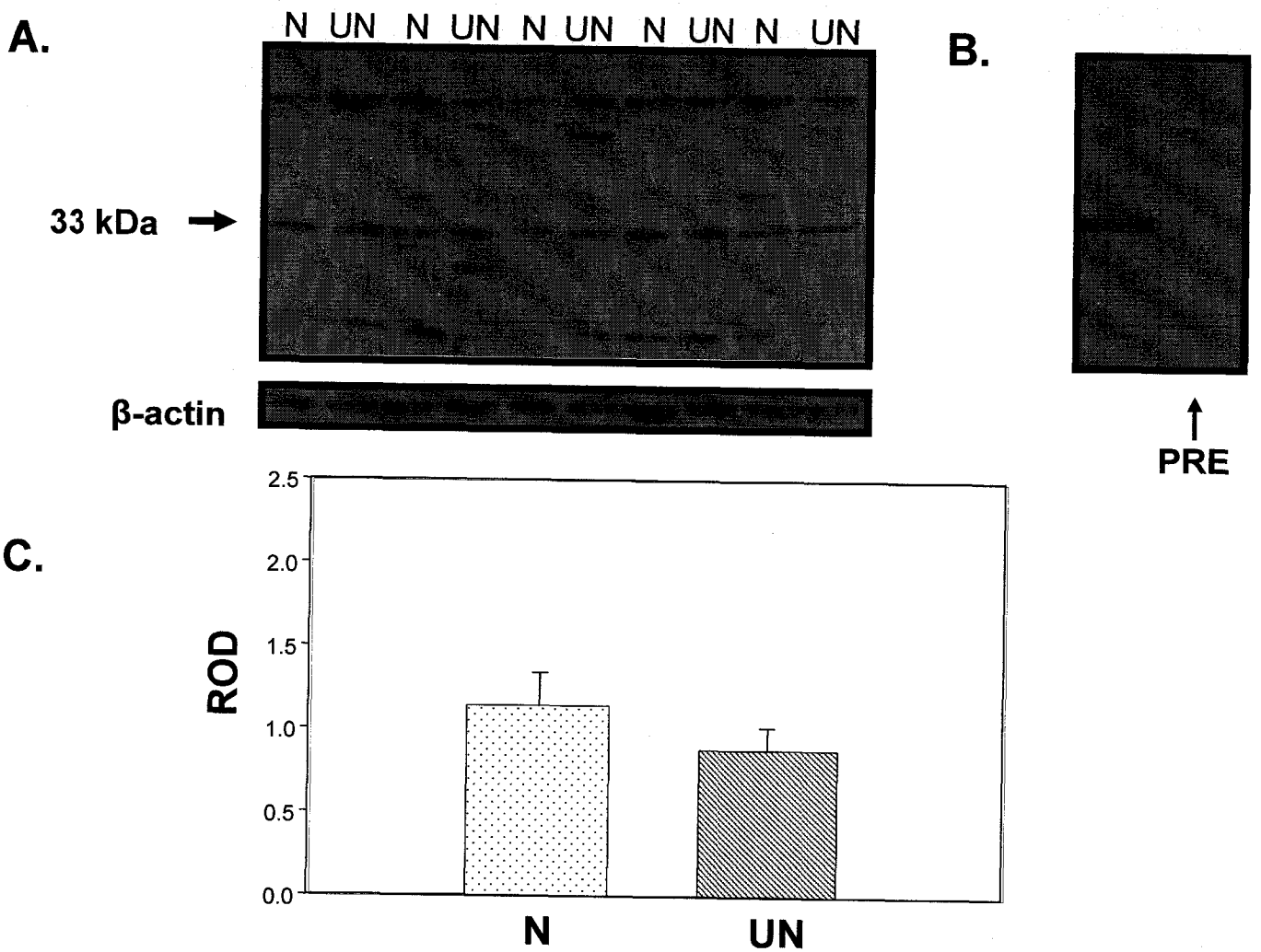


Figure 4.3 EP1 receptor protein expression in nourished and undernourished d131 ovine placentomes

A.) Western blotting revealed a 33 kDa band representing EP1 in nourished (N; n=7) and undernourished (UN; n=10) d131 placentomes. A representative blot is shown. β-actin was used to normalize protein expression levels. **B.)** Preabsorption (PRE) with an EP1 antigen eliminated the 33 kDa signal. **C.)** There was no difference in EP1 receptor protein expression between N and UN groups. Values are expressed in relative optical density (ROD) units. Bars represent the mean +/- SEM. Statistical analysis was performed using unpaired Student's T-test.

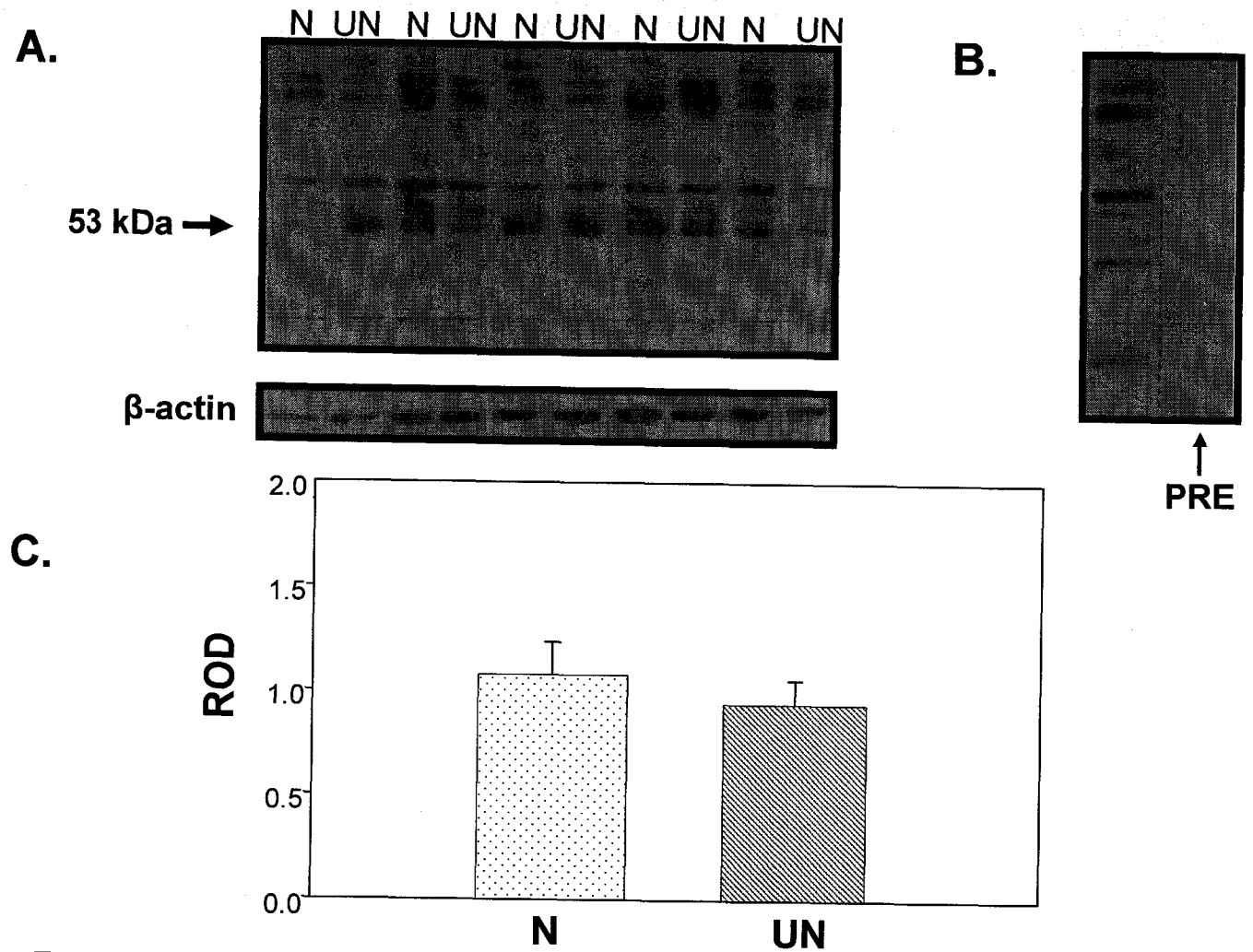


Figure 4.4 EP3 receptor protein expression in nourished and undernourished d131 ovine placentomes

A.) EP3 receptor protein expression (53 kDa band) was demonstrated in placentomes from nourished (N; n=7) and undernourished (UN; n=10) animals. A representative blot is shown. β-actin was used to normalize protein expression levels. **B.)** The 53 kDa band was diminished by preabsorption (PRE) with an EP3 antigen. **C.)** There was no difference in EP3 receptor protein levels between N and UN groups. Values are expressed in relative optical denisty (ROD) units. Bars represent the mean +/- SEM. Statistical analysis was performed using unpaired Student's T-test.

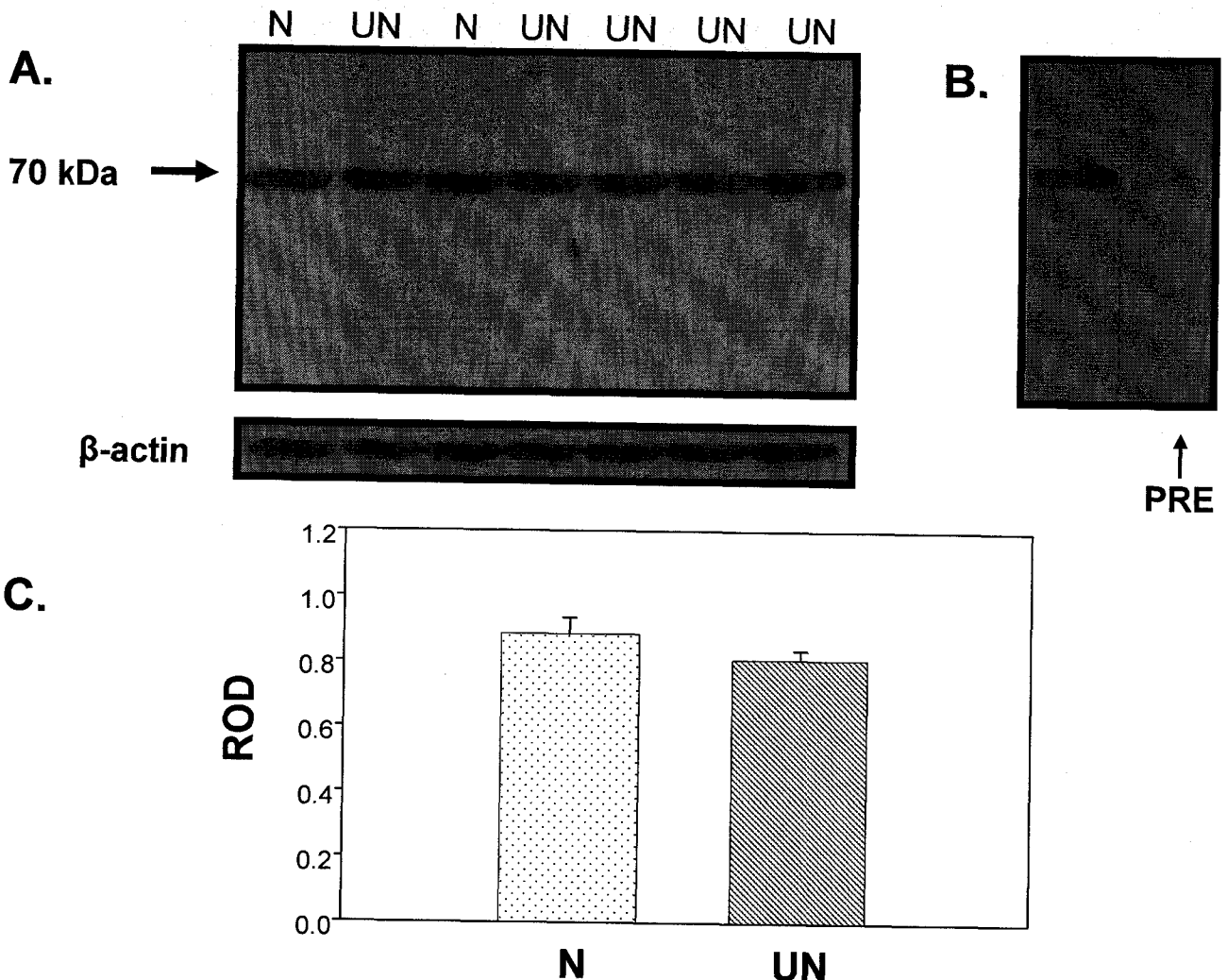


Figure 4.5 FP receptor protein expression in nourished and undernourished d131 ovine placentomes

A.) Western blotting revealed a 70 kDa band representing FP in nourished (N; n=7) and undernourished (UN; n=10) d131 placentomes. A representative blot is shown. β-actin was used to normalize FP protein expression levels. **B.)** Preabsorption (PRE) with an FP antigen eliminated the 70 kDa band. **C.)** There was no difference in FP receptor protein expression between N and UN groups. Values are expressed in relative optical density (ROD) units. Bars represent the mean +/- SEM. Statistical analysis was performed using unpaired Student's T-test.

4.4. Discussion

In this study, we determined the localization of contractile PG receptors EP1, EP3, and FP in late gestation ovine placentomes and determined the effect periconceptional undernutrition has on their protein expression levels. EP1, EP3, and FP receptors were all localized to the fetal trophoblast layer, predominantly in the uninucleate cells. However, periconceptional undernutrition had no effect on altering PG receptor protein expression levels or distribution.

It has been well documented in the literature that in late gestation of ovine pregnancy, maturation of the fetal HPA axis leads to increased cortisol concentrations which, in turn, stimulate placental PGE_2 production via enhancement of PGHS-2 enzyme activity (Challis et al., 2000; Whittle et al., 2001). Subsequently, there is an upregulation of placental P450c17 activity, following this rise in PGE_2, leading to a fall in progesterone levels and an increase in estrogen concentrations, as observed during labour onset. Using both a glucocorticoid-induced ovine model of preterm labour and term placentome samples, previous studies from our laboratory, and the results presented in Chapter 3, have shown pre-partum increases in fetal cortisol and placental PGHS-2 activity, PGE_2 levels, and P450c17 mRNA expression (Gyomorey et al., 2000a, b; Whittle et al., 2000).

Similarily, in a periconceptional undernutrition model, we have previously demonstrated a precocious rise in fetal cortisol concentrations and subsequent delivery at preterm (Bloomfield et al., 2003). Preterm delivery occurred in approximately half of the ewes exposed to a period of moderate maternal

undernutrition, where the remaining ewes carried their fetus to term regardless of nutritional manipulation. Additionally, we have reported an earlier increase in the timing of the initial rise of fetal PGE_2 within the undernourished preterm ewes; however, we found no alterations in the concentration of circulating PGE_2 in fetal plasma between undernourished and nourished groups (Kumarasamy et al., 2004). In the current study, we found no effect of periconceptional undernutrition on altering the protein expression levels of placental PG receptors. This could be attributed to the fact that there was no significant increase in PGE_2 levels in the undernourished group as described by Kumarasamy et al. (2004). Also, related to this observation, preliminary data from the same cohort of animals used in the current study have revealed no changes in placental PGHS-2 and P450c17 mRNA and protein expression (K Connor, personal communication). However, it is important to note that the time of tissue collection (d131) may account for the observations of the current study and those of previous studies from our laboratory. Therefore, PG receptor expression and regulation in the periconceptional undernutrition model appears to be similar to the glucocorticoid-induced model of preterm labour, as described in the previous results chapter, in which alterations in PG receptor expression levels may not be as significant as increased PGE_2 output or other mediators involved in the placental endocrine axis during parturition. However, further studies into the effect of periconceptional undernutrition on this axis are warranted.

In previous studies utilizing a sheep model, Fowden et al. (1983, 1987) suggested that marked variations in dietary intake, during late gestation, could

potentially result in premature delivery. In support of this, Fowden et al. (1983, 1987) have demonstrated significant increases in fetal and maternal plasma concentrations of PGE_2 and PGFM after a period of fasting in late gestation; corresponding to increases in circulating free fatty acids, such as arachidonic acid (AA), which are precursors of PG biosynthesis. In the horse, Stammers et al. (1995) reported that alterations in dietary fatty acid intake during late gestation were associated with increased PG biosynthesis. Stammers et al. (1995) demonstrated increased production of essential fatty acids, such as AA, in undernourished mares and suggested that this could enhance the likelihood of preterm delivery since AA is one of the main precursor fatty acids for PGE_2 and/or $PGF_{2\alpha}$ synthesis. However, since the undernourished ewes in our studies were maintained on maintenance feeds after 30 days post-conception until the end of the experiment, it is possible that there were enough dietary fatty acids available for PG biosynthesis, thereby reducing the need to increase fatty acid metabolism, as seen with the studies reported with undernourishment in late gestation (Fowden et al., 1983, 1987). Also, another reason which may help to explain the difference between our studies and those previously published could be due to the timing of the nutritional insult, in which we restricted feeds over the periconceptional period rather than in late gestation.

Our immunohistochemical data demonstrated PG receptor protein localization to the fetal trophoblast, predominantly in the uninucleate cells. In a previous study, Gyomorey et al. (2000a) revealed co-localization of PGHS-2 and P450c17 proteins to the uninucleate trophoblast cells of sheep placentomes.

Although increased PGE_2 output may not be the major pathway in regulating P450c17 expression as demonstrated in Chapter 3, it could still contribute to its regulation; therefore, our immunohistochemical data and the observations reported by Gyomorey et al. (2000a) support the possibility that placental PGE_2 could act locally to regulate levels of P450c17 during parturition.

Consistent with our findings in which we observed no alterations in the expression profiles of PG receptors in response to periconceptional UN, some studies have suggested different mechanisms that may influence PG receptor expression. First, Yamamoto et al. (2005) have recently proposed a negative feedback mechanism in regulating FP receptor expression in a human myometrial cell line in which they have shown a dose-dependent decrease in FP mRNA levels in response to increasing concentrations of exogenous $PGF_{2\alpha}$, suggesting an auto-regulatory mechanism by which PG receptors regulate their own expression levels. Also, Palliser et al. (2005) suggested that PG receptor levels might reach a threshold prior to labour onset and no longer undergo changes in their expression levels at the time of labour. Similarly, studies investigating the myometrium in non-labouring and labouring subjects have shown no change in sensitivity to exogenous PG administration or mRNA expression levels of EP1-4 and FP (Baguma-Nibasheka et al., 1998; Gyomorey et al., 2000b). Finally, since different splice variants exist for PG receptors as reported for human EP3 and ovine FP (Namba et al., 1993; Pierce et al., 1998), we cannot exclude that ovine placental trophoblast cells might express a specific isoform(s) of the EP and FP receptor proteins that are unable to be detected by

the antibodies used since these antibodies are targeted against human peptide sequences.

Undernourishment in early to mid-gestation has been implicated in altering the frequency of ovine placentome distribution and predominance of fetal tissue. Heasman et al. (1999) fed ewes a nutrient-restricted diet during the period of maximum placental growth (d30-80 gestation) followed by maintenance feeds thereafter and reported a greater number of placentomes, in which a majority were classified as B, C, or D-type placentomes, at term. It was also reported that placentomes of nutrient-restricted ewes contained a greater proportion of fetal tissue compared to controls (Heasman et al., 1999). Similarly, previous studies have reported increased growth on the fetal side of placentomes from ewes exposed to a period of undernutrition in early gestation (McMullen et al., 2005; Steyn et al., 2001). Also, Ward et al. (2002) demonstrated that the population and distribution of certain cell types in the fetal trophoblast can be influenced by increased concentrations of cortisol. Thus, it is possible that PG receptor expression can vary depending on the type of placentome (A-D) and that the distribution of certain cell types expressing PG receptors may also be altered. Therefore, in extending these observations to our current findings, we might have observed no alteration in PG receptor expression levels as we did not distinguish what types of placentomes were used in our studies. It is possible that periconceptional undernutrition could influence what type of placentome predominates, thereby potentially affecting PG receptor expression.

In summary, we have determined the localization and expression of PG receptors using an ovine model of preterm labour. EP1, EP3, and FP receptors were predominantly localized to the uninucleate cells of the fetal trophoblast layer of late gestation ovine placentomes. However, periconceptional undernutrition was found to have no effect on PG receptor protein expression levels. These results are consistent with our findings using a glucocorticoid-induced model of preterm labour, as described in the previous chapter, in which exogenous cortisol administration resulted in no alteration of PG receptor expression profiles. Therefore, it is possible that changes in PG receptor protein levels may not be as important as the increase in PGE_2 production or the action of cortisol or other glucocorticoid-stimulated intermediates and/or pathways involved in the placental endocrine axis during labour onset. Further studies are necessary in order to elucidate the mechanisms involved in the regulation of placental PG receptor expression during periconceptional undernutrition.

Chapter 5

General Discussion

5.1. Summary of Results and Physiological Implications

As previously described, the current model of ovine parturition proposes that in late gestation, maturation of the fetal HPA axis and the resultant increase in fetal cortisol concentrations provides the trigger for activation of the placental endocrine axis, resulting in increased PG synthesis at term (Challis et al., 2000). Subsequently, the increased levels of PGE_2 act as the stimulus to increase the expression of the steroidogenic enzyme P450c17 in the placenta, which results in a fall in progesterone and sharp rise in estrogen concentrations observed during labour (Gyomorey et al., 2000a; Whittle et al., 2000, 2006). In support of this, several studies have demonstrated increases in placental PGHS-2 expression and PGE_2 production after spontaneous term labour and intrafetal cortisol administration (Gyomorey et al., 2000a, 2000b; Whittle et al., 2000). Therefore, based on these previous findings, in our study, we hypothesized that in late pregnancy and at parturition, placental PGE_2, acting through PG receptors, would directly upregulate placental P450c17 in an autocrine, paracrine, or intracrine manner. However, the mechanism by which PGE_2 regulates P450c17 expression and activity is unknown, and there are limited data describing PG receptor expression in term and preterm ovine tissues, particularly in the placenta.

In order to investigate this, we used two different sheep models of preterm labour: 1.) a glucocorticoid-induced model; and, 2.) a maternal periconceptional UN model. Utilizing these two different models provided us with the opportunity to examine potential differences in the regulation of the placental endocrine axis, particularly with either an exogenous source of glucocorticoid through intrafetal administration, or an endogenous elevation of glucocorticoid in response to UN. Our overall objectives were to determine the following: 1.) the effect of exogenous cortisol administration on PGE_2 concentrations and P450c17 mRNA expression in late gestation ovine placentomes; and, 2.) to characterize the protein expression of PG receptors in late gestation ovine placentomes and to determine the effect of cortisol administration or perinconceptional UN on regulation of placental PG receptor expression and/or localization.

In the first set of experiments, we have shown the expression and regulation of the contractile PG receptors, EP1, EP3, and FP, and the enzyme P450c17 in response to exogenous intrafetal glucocorticoid administration. In addition to exogenous cortisol infusion, we administered the PGHS-2 specific inhibitor, meloxicam, which allowed us to investigate the role of PGs in regulating P450c17 expression. From our experiments, we demonstrated that intrafetal cortisol infusion in late gestation, with or without meloxicam, significantly increased placental PGHS-2 protein and P450c17 mRNA levels. The increase in PGHS-2 enzyme, after cortisol administration, resulted in increased synthesis of PGE_2 as measured in the fetal plasma. However, in ewes co-infused with meloxicam, there was an increase in P450c17 expression despite the effective

block of PGE_2 synthesis (Chapter 3). Thus, it appears that the increased PGE_2 output over the last 15-20 days of gestation may not be the major pathway in regulating placental P450c17 expression. Therefore, we suggest that cortisol and/or glucocorticoid-stimulated intermediates and/or pathways could have a role in regulating placental P450c17 expression levels. With respect to PG receptor expression, alterations in their expression levels may not be as important as the increased PGE_2 output or the action of cortisol and/or other glucocorticoid-induced mediators potentially involved in labour onset, as we observed no changes in the expression profiles of EP1, EP3, and FP.

In the second set of experiments, we determined the localization of EP1, EP3, and FP PG receptors in late gestation ovine placentomes and determined the effect periconceptional UN has on their protein expression levels. We determined that the EP1, EP3, and FP receptors were all localized predominantly to the uninucleate cells of the fetal trophoblast layer. However, periconceptional UN had no effect on altering PG receptor protein expression levels (Chapter 4). These results are consistent with our findings from the first set of experiments; therefore, this further supports the possibility that changes in PG receptor protein levels may not be as important as the increase in PGE_2 production or the action of cortisol and/or other glucocorticoid-induced intermediates/pathways involved in the placental endocrine axis during labour onset.

Taken together, our results do not demonstrate an exclusive role of PGE_2-induced P450c17 enzyme regulation; however, it is still possible that it contributes to its expression and activity in late gestation. As previously

demonstrated, both PGHS-2 protein and P450c17 have been localized to the uninucleate cells of the fetal trophoblast (Gibb et al., 1996; Gyomorey et al., 2000a, b). Consistent with these findings, PGES mRNA and protein was also found to be localized within the fetal trophoblast of ovine placentomes and its protein expression significantly increased with labour onset (Martin et al., 2002; Palliser et al., 2004). These *in vivo* data are further supported by preliminary *in vitro* studies in which PGE_2 caused a dose- and time-dependent increase in P450c17 mRNA and protein within primary cultures of ovine trophoblast cells (D. Rurak, personal communication). Thus, evidence still exists for PGE_2-induced regulation of P450c17; however, within the placental endocrine axis in late gestation, it may contribute to parturition in addition to other potential labour-associated molecules or pathways.

With respect to our PG receptor data, it appears, to our knowledge, that there are currently no published studies examining PG receptor protein expression in late gestation ovine placentomes in response to either exogenous glucocorticoid administration or undernutrition. Thus, our studies are the first to describe their protein expression and regulation in the ovine placenta using both an exogenous glucocorticoid-induced model of preterm labour and a perinconeptional UN model. However, in both of our preterm labour models, there was no observed change in PG receptor protein expression levels in response to the increased cortisol-induced PGE_2 output (Chapter 3 and 4). Consistent with our data, Grigsby et al. (2006) localized the EP1-4 receptor subtypes within human placental villous tissue and reported no change in

expression levels with respect to gestational age and labour (Grigsby et al., 2006a). Therefore, it appears that the increase in PGE_2 synthesis over the last 15-20 days of gestation or the action of cortisol and potential cortisol-induced intermediates involved in labour onset may play a more significant role in regulation of the placental endocrine axis versus alterations in PG receptor expression.

Finally, in the present study, we utilized *in vivo* sheep models to examine the mechanisms involved in the placental endocrine axis in parturition, whose advanced activation could result in preterm labour and delivery. The use of an *in vivo* animal model in studying pregnancy and parturition allows one to examine the maternal-fetal interactions that take place within the intact intrauterine environment. This is an advantage as compared to *in vitro* models, which, despite being very valuable systems to use, only look at cell populations in isolation and are thus limited as they do not mimic the complex tissue environment where multiple cell interactions occur (Barry and Anthony, 2008). Since many aspects of human pregnancy cannot be directly investigated due to both ethical and practical reasons, a variety of animal models have been used such as domestic ruminants (i.e. sheep) and laboratory rodents. However, of the models used, the pregnant sheep has been used extensively for over the past 40 years and has been critical in our current understanding of normal and complicated pregnancies (Barry and Anthony, 2008). For practical reasons, the sheep model allows for chronic catheterization in both fetal and maternal vasculature which allows for repetitive sampling and/or performing endocrine

manipulations to test for the regulation of hormones or effectors involved in the process. Also, sheep demonstrate many physiological similarities to humans concerning the basic endocrine and mechanical pathways that culminate in the initiation of labour. Thus, the findings from our current study within the *in vivo* sheep model of pregnancy help in expanding our knowledge regarding the underlying physiological mechanisms that result in term and/or preterm labour.

5.2. Limitations of the Present Study and Future Directions

One of the primary limitations that we faced within the studies undertaken concerned our PG receptor expression studies. First, the antibodies utilized in the studies are targeted against human PG receptor peptide sequences. After performing BLAST searches of the commercial antigen sequence against a database including known ovine peptide sequences for the EP and FP receptors, only the contractile group of receptors (EP1, EP3, FP) resulted in a close match with ovine sequences. Conversely, the EP2 and EP4 antigen sequences did not result in a match for those from the sheep. Therefore, the protein expression and localization results for EP2 and EP4 are presented as preliminary data within the attached appendices (A and B). Furthermore, the EP4 peptide sequence is targeted for the carboxy-terminal region which is the site that differs due to alternative mRNA splicing. As a result, this may further complicate the ability of detecting the EP4 protein within sheep tissues.

Second, with respect to our western blot experiments, we were able to demonstrate the presence of EP1, EP3, and FP receptor protein as the expected

immunoreactive bands, and therefore corresponding protein sizes, were detected. Since we did not observe any significant changes in their respective protein expression levels, we cannot exclude that ovine placental trophoblast cells might express a specific isoform(s) of the EP and FP receptor proteins that are unable to be detected by the antibodies used. Since the antibodies are targeted against human peptide sequences, there is the possibility that these may not be able to detect other isoforms expressed in sheep placentomes. Moreover, for some of the receptors (EP1 and EP3), additional immunoreactive bands appeared that subsequently disappeared with their respective preabsorption studies. Thus, it is possible that these additional bands could be other receptor isoforms that result from alternative splicing, as published for human EP3 and ovine FP (Namba et al., 1993; Pierce et al., 1998). However, since the respective protein size and expression profiles for the PG receptors have not been previously characterized, it is difficult to analyze the additional immunoreactive bands with confidence. Hence, densitometry analyses were not performed for the purpose of this project. In future, it would be beneficial to further investigate these bands and begin to ascertain placental PG receptor expression in pregnancy and parturition.

A third limitation with respect to our PG receptor expression studies relates to our experimental design, in which we examined functional protein levels versus mRNA transcript levels. Although we did not observe any significant change in the levels of PG receptor protein expression in response to exogenous glucocorticoid administration, it is possible that transcription for the

PG receptors may be significantly upregulated in late gestation ovine placentomes. In a previous study, Palliser et al. (2005) reported mRNA expression of PG receptors in placentomes of dexamethasone-induced or spontaneous term labour ewes. In this study, they found varying expression profiles for EP receptors 2-4 and FP depending on the labouring group (dexamethasone-treated versus spontaneous labour). Therefore, it is possible that by utilizing quantitative RT-PCR for the PG receptors, we may also witness significant changes in the transcriptional regulation of EP receptor subtypes and FP, as observed by Palliser et al. (2005).

Apart from the placental PG receptor changes, the possibility exists that the expression levels of PG receptors could be significantly altered in other intrauterine tissues in late gestation, such as the endometrium and myometrium. Within the myometrium, PG receptors have been reported to exhibit regional variation in their expression profiles, associated with uterine contraction patterns observed during labour (Astle et al., 2005; Brodt-Eppley et al., 1999; Leonhardt et al., 2003; Smith et al., 2001a). Furthermore, as previously described, in the estrogen-dependent pathway of parturition there is a pronounced increase in the circulating levels of PGFM in the last 12-24 hours of active labour and there are clear correlations between this rise in PGFM with elevated maternal estradiol concentrations and increased PGHS-2 expression in both the endometrium and myometrium (Gyomorey et al., 2000a; Wu et al., 1999a, b). Additionally, a significantly larger amount of PGFS mRNA was present in the endometrium versus the placenta in late gestation ewes (Palliser et al., 2004). Therefore, we

may have seen significant changes in the expression profiles of the PG receptors, specifically FP, within these tissues. As a result, further studies are required to determine the ontogeny of PG receptor expression in intrauterine tissues over the course of pregnancy and in parturition.

Lastly, in the second set of experiments using the maternal periconceptional UN model, and consistent with our glucocorticoid-induced preterm labour model, we did not observe any changes in placental PG receptor expression profiles. A potential limitation that may have affected our results could have been the timing of tissue collection. It is possible that at d131 of gestation, PG receptor protein levels have reached their maximum level of expression. In support of this, Palliser et al. (2005) suggested that PG receptor levels might reach a threshold prior to labour onset and studies investigating the myometrium in non-labouring and labouring subjects have shown no change in sensitivity to exogenous PG administration or mRNA expression levels of EP1-4 and FP (Baguma-Nibasheka et al., 1998; Gyomorey et al., 2000). Alternatively, the timing of tissue collection may have been too early to detect significant changes in PG receptor expression and therefore we cannot exclude the potential for receptor upregulation at a later time point in gestation or at the time of active labour.

5.3. Clinical Implications

Preterm birth (PTB), which occurs in about 5-10% of all pregnancies, is a major problem in obstetrics (Challis et al., 2002). Despite recent advances in the

medical field and decreases in infant mortality rates, the incidence of PTB continues to rise and morbidity related to prematurity is significant as survivors are more likely to suffer from adverse health outcomes such as deafness, blindness, respiratory and neurological disorders that can affect them for their lifetime (Robertson et al., 1992).

Potentially important mediators of PTB are PGs. PGs play a primary role in the mechanism of parturition, particularly in the placental endocrine axis. Thus, the findings from our current project expand our knowledge concerning the role of PGs in the labour axis and assist in determining the underlying physiological causes of PTB. From our studies, it appears that the increased PGE_2 output over the last 15-20 days of gestation may not be the major pathway in regulating placental P450c17 expression. Resultantly, we suggest that cortisol and/or cortisol-stimulated intermediates and/or pathways may function in regulating the expression levels and activity of P450c17 in the placenta. Also, since we observed no changes in the expression profiles of the PG receptors, EP1, EP3, and FP, both in response to intrafetal cortisol administration and maternal perinconceptional UN, we suggest that augmentation in their expression levels may not be as critical as the increased placental PGE_2 output or the action of glucocorticoids involved in labour onset.

Overall, these findings indicate the need to continue to investigate the role of PGs in regulating the cascade of events in parturition. Thus, by understanding the molecular and endocrine events involved in labour onset, tocolytic therapies,

new strategies, and/or medical technologies can be developed to identify new markers of preterm birth and to prevent its occurrence.

Appendix A

The Expression and Regulation of the Prostaglandin Receptor subtype EP2 by Cortisol and PGE_2 in Late Gestation Ovine Placentomes

A.1. Introduction

As previously outlined in Chapter 3, protein expression data for the PGE_2 relaxatory receptor subtype EP2, in response to exogenous cortisol +/- meloxicam administration, has been presented in this section. Results from a BLAST search of the EP2 receptor antigen sequence used to raise the antibody did not reveal a match for the ovine EP2 sequence. Due to this concern, the results are presented only as preliminary data within this appendix.

Similarly, a BLAST search of the EP4 receptor antibody did not come up with a match for the ovine EP4 sequence and repeated attempts of discerning EP4 protein expression by western blotting failed to demonstrate a 64 kDa band. Thus, EP4 protein expression has not been quantified in these samples.

A.2. Materials and Methods

A.2.1. Western Blot Analysis of EP2 Protein Expression

Please refer to Chapter 3 Section 3.2.7 for a detailed description of the Western blot procedure and EP2 antibody characteristics.

Briefly, protein was extracted from frozen cotyledons and concentrations were measured using the Bradford protein assay (Bradford, 1976). Ovine

placental protein samples (20 µg) were subsequently separated by 11% SDS-PAGE, electrophoretically transferred at 4^0C onto a nitrocellulose membrane, and blocked overnight as described in Chapter 3. After blocking, membranes were incubated with the following primary antibody: rabbit anti-human polyclonal EP2 receptor (1:500), followed by secondary antibody incubation with an anti-rabbit IgG antibody (1:3000). Protein bands were visualized using chemiluminescence and exposed to autoradiography film. For preabsorption controls, the EP2 primary antibody was incubated overnight at 4^0C with its respective blocking peptide at an antibody:antigen ratio of 1:10.

An anti-mouse β-actin antibody (1:10000) was used as an internal control to verify similar protein loading in each lane. Scion Image analysis software (Frederick, MD) was employed to quantify protein band density by densitometry. Results are presented as the ratio of respective protein of interest to β-actin in relative optical density (ROD) units.

A.3. Results

A.3.1. The Effect of Cortisol +/- Meloxicam Treatment on EP2 Protein Expression in d130 Ovine Placentomes

Western blot analysis revealed expression of the relaxatory PGE_2 receptor subtype EP2 in d130 ovine placentomes in all groups, as represented as a 64 kDa (**Figure A.1A**); however, exogenous glucocorticoid administration did not have an effect on EP2 protein expression levels (**Figure A.1C**). Preabsorption of the primary EP2 antibody with its respective blocking peptide eliminated the 64 kDa signal (**Figure A.1B**).

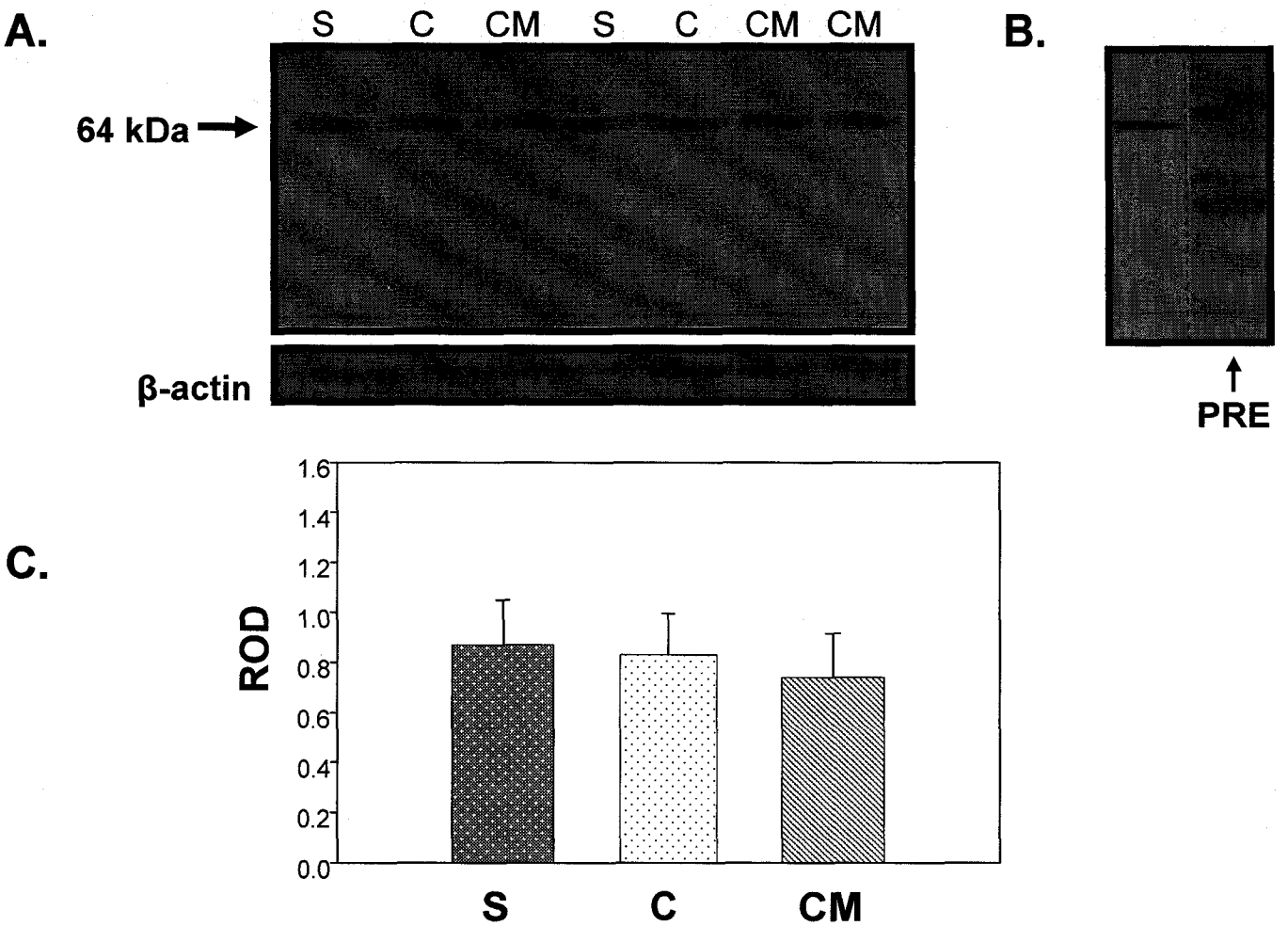


Figure A.1 EP2 receptor protein expression in d130 ovine placentomes with cortisol +/- meloxicam treatment

A.) Western blotting revealed a 64 kDa band representing EP2 in saline (S; n=6), cortisol (C; n=4), and cortisol + meloxicam (CM; n=5) treated d130 placentomes. A representative blot is shown. β-actin was used to normalize protein expression levels. **B.)** Preabsorption (PRE) with an EP2 antigen eliminated the 64 kDa signal. **C.)** There was no difference in EP2 receptor protein expression between S, C, and CM groups. Values are expressed in relative optical density (ROD) units. Bars represent the mean +/- SEM. Statistical analysis was performed using one-way ANOVA.

Appendix B

The Effect of Periconceptional Undernutrition on EP2 and EP4 Receptor Localization and Expression in Late Gestation Ovine Placentomes

B.1. Introduction

As previously outlined in Chapter 4, utilizing a periconceptional undernutrition model, immunohisotchemical localization and protein expression data for the placental PGE_2 receptor subtypes EP2 and EP4 have been presented in this section. Performance of BLAST searches of the amino acid sequences of the antigens used to raise the antibodies did not result in a match with ovine EP2 and EP4 sequences, respectively. Therefore, due to this concern, they are presented here only as preliminary data.

B.2. Materials and Methods

B.2.1. Immunohistochemistry

Please refer to Chapter 4 section 4.2.3 for a detailed description of the immunohistochemical procedure and EP2 and EP4 antibody characteristics.

Briefly, fixed placentomes (7 μm sections) from nourished and undernourished animals were deparaffinized and rehydrated. Antigen retrieval was performed as described in Chapter 4. Sections were blocked and then incubated overnight at 4^0C in a humidified chamber with the following primary polyclonal antibodies, respectively: rabbit anti-human EP2 (1:100) and rabbit anti-human EP4 (1:100). Negative control sections were incubated with either a

concentration-matched rabbit immunoglobulin (IgG) or omission of the primary antibody.

For preabsorption controls, the primary antibodies were incubated overnight at 4^0C with their respective blocking peptides (EP2 and EP4) at the following antibody:antigen ratios: 1:40 (EP2 and EP4), as this was an adequate ratio to see a reduction in staining.

The following day, placental sections were incubated with a biotinylated secondary anti-rabbit IgG antibody followed by an avidin-biotin peroxidase detection system. Diaminobenzadine was used to identify positive staining in tissues. Sections were counterstained with Mayer's haematoxylin, dehydrated in an ascending ethanol series and cleared in xylene overnight. Sections were then mounted before being viewed by light microscopy.

B.2.2. Western Blot Analysis of EP2 and EP4 Protein Expression

Please refer to Chapter 4 Section 4.2.4 for a detailed description of the western blot procedure.

Briefly, protein was extracted from frozen cotyledons of N and UN ewes and concentrations were measured using the Bradford protein assay (Bradford, 1976). Ovine placental protein samples (20 µg) were subsequently separated by 11% SDS-PAGE, electrophoretically transferred at 4^0C onto a nitrocellulose membrane, and blocked overnight as described in Chapter 4. After blocking, membranes were incubated with the following primary antibodies: rabbit anti-human polyclonal EP2 receptor (1:500) and rabbit anti-human polyclonal EP4

receptor (1:500) followed by secondary antibody incubation with an anti-rabbit IgG antibody (1:3000). Protein bands were visualized using chemiluminescence and exposed to autoradiography film. For preabsorption controls, the primary antibodies were incubated overnight at 4^0C with their respective blocking peptides (EP2 and EP4) at an antibody:antigen ratio of 1:10.

An anti-mouse β-actin antibody (1:10000) was used as an internal control to verify similar protein loading in each lane. Scion Image analysis software (Frederick, MD) was employed to quantify protein band density by densitometry. Results are presented as the ratio of respective protein of interest to β-actin in relative optical density (ROD) units.

B.3. Results

B.3.1. Immunohistochemical Localization of EP2 and EP4 in d131 Ovine Placentomes

Immunohistochemistry was utilized to assess the localization of EP2 and EP4 receptors in late gestation ovine placentomes. Both receptors were localized to the fetal trophoblast (T) layer, predominantly in the uninucleate cells. Positive staining was absent in the maternal syncitium (M) (**Figure B.1 panels i & iii**). Preabsorption of the EP2 and EP4 antibodies with their respective blocking peptides diminished the positive staining (**Figure B.1 panels ii & iv**).

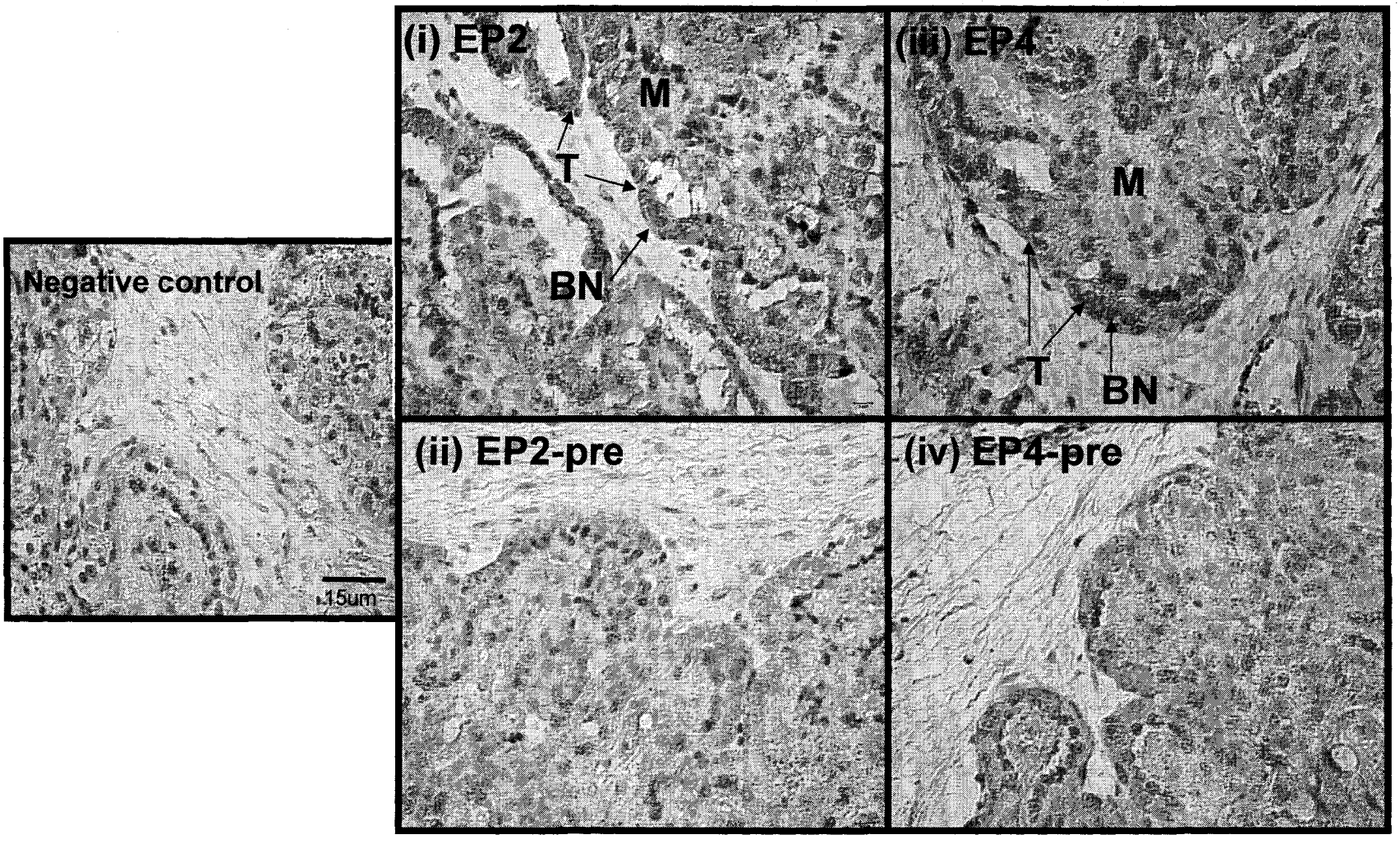


Figure B.1 PG receptor protein localization in d131 ovine placentomes

Immunohistochemical analysis demonstrated EP2 (i) and EP4 (iii) protein localization in the fetal trophoblast (T) of d131 ovine placentomes. Staining was absent in the maternal syncytium (M). Preabsorption with the EP2 (ii) and EP4 (iv) blocking peptides, respectively, diminished the positive staining. BN: binucleate cell; pre: preabsorption. Magnification 400x.

B.3.2. The Effect of Periconceptional Undernutrition on EP2 and EP4 Receptor Protein Expression

Western blotting was used to assess protein expression levels of the relaxatory PGE_2 receptors EP2 and EP4 in response to periconceptional undernutrition. EP2 and EP4 were expressed in placentomes of both N and UN animals. EP2 was demonstrated as a 53 kDa immunoreactive band (**Figure B.2A**); however, periconceptional undernutrition had no effect on EP2 protein expression levels (**Figure B.2C**). Similarly, EP4 was expressed in both groups; however, there were no alterations in EP4 protein levels after periconceptional UN (**Figure B.3A & C**). Preabsorption of the primary EP2 and EP4 antibodies with their corresponding blocking peptides eliminated the 53 kDa (**Figure B.2B**) and 64 kDa signals (**Figure B.3B**), respectively.

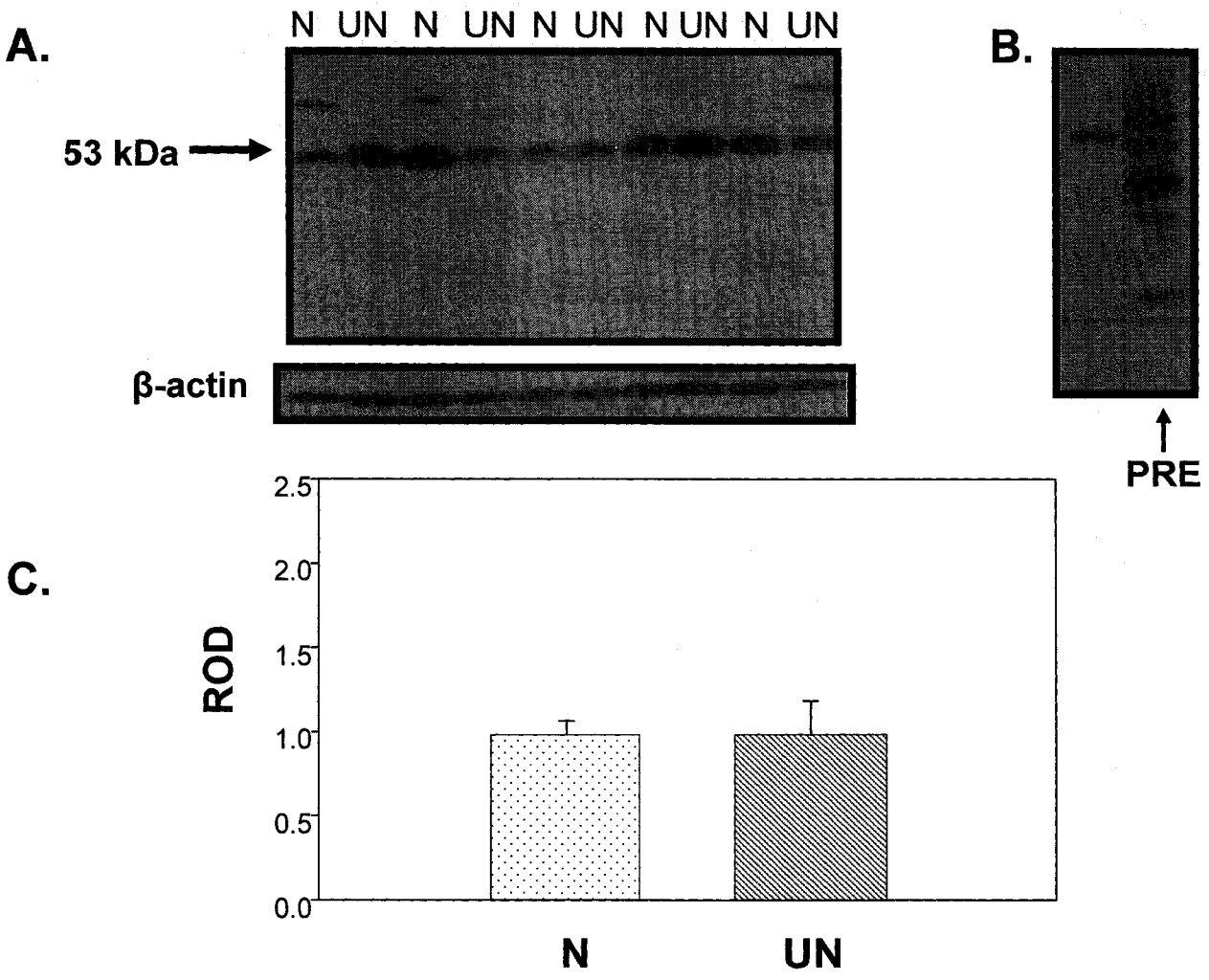


Figure B.2 EP2 receptor protein expression in nourished and undernourished d131 ovine placentomes

A.) Western blotting revealed a 53 kDa band representing EP2 in nourished (N) and undernourished (UN) d131 placentomes. A representative blot is shown. β-actin was used to normalize EP2 protein expression levels. **B.)** Preabsorption (PRE) with an EP2 antigen eliminated the 53 kDa band. **C.)** There was no difference in EP2 receptor protein expression between N and UN groups. Values are expressed in relative optical density (ROD) units. Bars represent the mean +/- SEM. Statistical analysis was performed using unpaired Student's T-test.

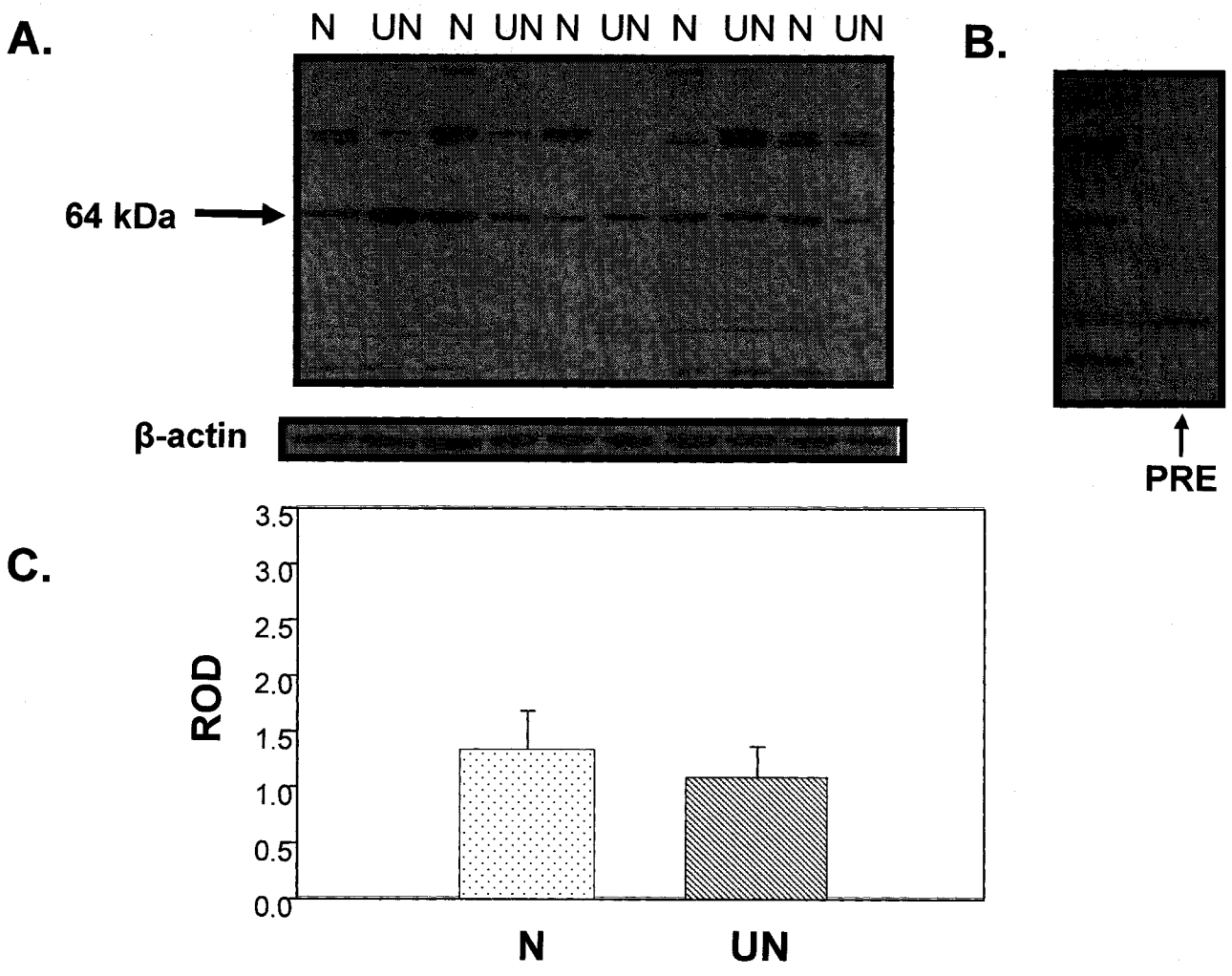


Figure B.3 EP4 receptor protein expression in nourished and undernourished d131 ovine placentomes

A.) EP4 receptor protein expression (64 kDa band) was demonstrated in placentomes from nourished (N; n=7) and undernourished (UN; n=10) animals. A representative blot is shown. β-actin was used to normalize protein expression levels. **B.)** The 64 kDa band was diminished by preabsorption (PRE) with an EP4 antigen. **C.)** There was no difference in EP4 receptor protein levels between N and UN groups. Values are expressed in relative optical denisty (ROD) units. Bars represent the mean +/- SEM. Statistical analysis was performed using unpaired Student's T-test.

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