The Effects of Cortisol on the Development of the Fetal Hypothalamic-Pituitary-Adrenal Axis

by

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This thesis examines the effects of glucocorticoids on corticosteroid-binding globulin (CBG) and adrenocorticotrophic hormone (ACTH) in the fetus during late pregnancy. In fetal sheep, plasma corticosteroid-binding capacity (CBC) levels increased concomitantly with incremental cortisol infusion, and were abolished by fetal adrenalectomy. Therefore, CBG appears to be regulated by cortisol in the late-gestation ovine fetus. Conversely, in the human fetus at 0.75 of gestation, maternal glucocorticoid administration did not alter fetal plasma CBC levels.

Cortisol infusion (96h) to the ovine fetus at 0.8 of gestation elicited an increase in plasma ACTH and CBC levels similar to that seen at term. The cortisol-induced increase in CBC maintained a low percentage of free cortisol in circulation (7%), but the absolute concentration of free cortisol increased. Therefore cortisol negative feedback at the pituitary was not attenuated and POMC mRNA levels were reduced in both the pars distalis by 96% and pars intermedia by 38%. However, there was no change in concentration of $ACTH_{1-39}$, ACTH+related peptides (RP), or the ratio of $ACTH_{1-39}$:ACTH+RP in the pars distalis. This may reflect a continued production and secretion of $ACTH_{1-39}$ thereby contributing to the rise in plasma $ACTH_{1-39}$, or secretion may cease such that the tissue peptide levels remain unchanged. In the latter case, the rise in plasma $ACTH_{1-39}$ may be derived from a source outside the pars distalis. Potential sources include fetal lung and placenta. Cortisol did not suppress $ACTH_{1-39}$ concentrations in the fetal lung or POMC mRNA

and $ACTH_{1-39}$ levels in the placenta. Therefore the lung and the placenta are potential sources of ACTH in the presence of elevated plasma cortisol concentrations. Cortisol treatment did not alter prohormone convertase (PC) 1 mRNA levels in the fetal pituitary, lung or placenta. PC2 mRNA levels increased in the fetal pituitary, and placenta, but tissue and plasma α-melanocyte stimulating hormone (αMSH) concentrations did not change. Conversely, in the fetal lung PC2 mRNA levels and immunoreactive αMSH in the bronchiolar epithelium and alveoli were decreased after cortisol administration, which is consistent with a decrease in ACTH processing to αMSH by PC2 in the fetal lung. Therefore, intra-fetal administration of cortisol during late-gestation stimulates a rise in plasma $ACTH_{1-39}$ that does not appear to be due to a decrease in cortisol feedback on the fetal pituitary, but may be due to a combined increase in $ACTH_{1-39}$ output from the fetal pituitary, lung and placenta.

To my parents, who taught me that everything is possible.

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Peer review papers arising from research contained within this thesis

1. Jeffray TM, Marinoni E, Ramirez MM, Bocking AD, and Challis JRG. Effect of prenatal betamethasone administration on maternal and fetal corticosteroid-binding globulin. (submitted to Am J Obstet Gynecol)

2. Jeffray TM, Matthews SG, Hammond GL, and Challis JRG. Divergent changes in plasma ACTH and pituitary POMC mRNA after cortisol administration to late-gestation ovine fetus. Am J Physiol 274:E417-425 (1998).

3. Jeffray TM, Berdusco ETM, Wallace MJ, Fowden A, and Challis JRG. Effects of incremental cortisol and adrenalectomy on plasma corticosteroid binding capacity in fetal sheep. Can J Physiol Pharmacol 73:1568-1573 (1995).

Presented abstracts of research contained within this thesis

1. Jeffray TM, Rice GE, and Challis JRG. Differential effects of cortisol on POMC-derived peptides in the fetal pars distalis, lung and placenta. **The Society for Gynecologic Investigations.** Atlanta, Georgia, USA. 10-13 March 1999.

2. Jeffray TM, Matthews SG, and Challis JRG. Glucocorticoid effects on ovine placental POMC mRNA levels and processing during late gestation. **The Endocrine Society.** New Orleans, LA, USA. 24-27 June 1998. Abstract P3-485.

3. Jeffray TM, Marinoni E. Ramirez MM, Bocking AD, and Challis JRG. Effect of betamethasone on maternal corticosteroid-binding globulin in pregnancy. **The Society for Gynecologic Investigations.** Atlanta, Georgia, USA. 11-14 March 1998. Abstract 422.

4. Jeffray TM, Matthews SG, and Challis JRG. Differential regulation of proconvertase 1 and 2 in the pituitary and lung of the late gestation ovine fetus. **The Society for Gynecologic Investigations.** Atlanta, Georgia, USA. 11-14 March 1998. Abstract 465.

5. Jeffray TM, and Challis JRG. The effect of cortisol on function of the hypothalamic-pituitary-adrenal axis in the ovine fetus. **The Society for Gynecologic Investigations.** Chicago, Illinois, USA. 15-18 March 1995. Abstract P256.

6. Jeffray TM, Berdusco ETM, Wallace MJ, Fowden A, and Challis JRG. Effects of incremental cortisol and adrenalectomy (ADX) on plasma corticosteroid binding capacity (CBC) in fetal sheep. **The Society of Gynecologic Investigations.** Chicago, Illinois, USA. 15-18 March 1995. Abstract P254.

7. Challis JRG, Matthews SG, Yang K, Jeffray TM, Fraser M, Han X, Deol H, Wang S, Braems GA, Murotsuki J, Gagnon R, and Carter A. Fetal maturation, and responses to hypoxemia. **Society for the Study of Fetal Physiology,** Cambridge, England. 1995.

8. Challis JRG, Berdusco ETM, Jeffray TM, and Hammond GL. CBG during development. **IX International Congress on Hormonal Steroids**. Dallas, Texas, USA, 24-29 September 1994.

Table of Contents

List of Figures

Chapter Six

List of Tables

Chapter Four

Abbreviations

11βHSD	11β-hydroxysteroid dehydrogenase
3βHSD	3β-hydroxysteroid dehydrogenase
ACTH	adrenocorticotrophic hormone
ACTH-R	ACTH receptor
ACTH+RP	$ACTH_{1-39}$ plus larger ACTH-related POMC derived peptides (RP)
ADX	adrenalectomy
AVP	arginine vasopressin
cAMP	adenosine 3',5'-cyclic monophosphate
CBC	corticosteroid-binding capacity
CBG	corticosteroid-binding globulin
cDNA	complementary DNA
CLIP	corticotrophin-like intermediate lobe peptide
cpm	counts per minute
CRH	corticotrophin releasing hormone
DHEA	dehydroepiandrosterone
DNA	deoxyribonucleic acid
ER	endoplasmic recticulum
GR	glucocorticoid receptor
GRAb	ACTH antibody donated by Prof Greg Rice (Greg Rice Antibody)
HPA	hypothalamic-pituitary-adrenal
ir	immunoreactive
JP	joining peptide
kb	kilobase
kDa	kilodalton
LPH	lipotrophin
mRNA	messenger ribonucleic acid
MSH	melanocyte stimulating hormone
MW	molecular weight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	prohormone convertase
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
RIA	radioimmunoassay
ROD	relative optical density
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SON	supraoptic nucleus
TGN	trans Golgi network

Chapter One

General Introduction

Preterm delivery is the most common problem in clinical obstetrical practice and a major contributor to neonatal morbidity and mortality. In the United States of America preterm birth (birth prior to 37 weeks of gestation) accounts for approximately 10% of all deliveries (Creasy, 1994), and 83% of neonatal mortality (Copper *et al.* 1993). In Canada the incidence of preterm delivery was 6.8% of all live births in 1992-1994, an increase of 0.5% over the previous decade (Joseph *et al* 1998). The etiology of most preterm deliveries remains unknown and there is no effective treatment for the prevention of preterm labour. Therefore, it is necessary to understand the mechanisms that precede and stimulate the onset and progression of parturition in the hope of improving treatment of the patient in preterm labour, and developing methods to prevent preterm labour and delivery.

Three decades ago Sir Graham Liggins conducted a series of elegant studies which clearly implicated the fetal hypothalamic-pituitary-adrenal (HPA) axis in the timing of parturition in the sheep (Liggins *et al.* 1967; Liggins, 1968; Liggins and Kennedy, 1968). This literature review will outline the development and function of the HPA axis in parturition in the sheep and human fetus, with emphasis on the known effects of glucocorticoids on fetal HPA axis function.

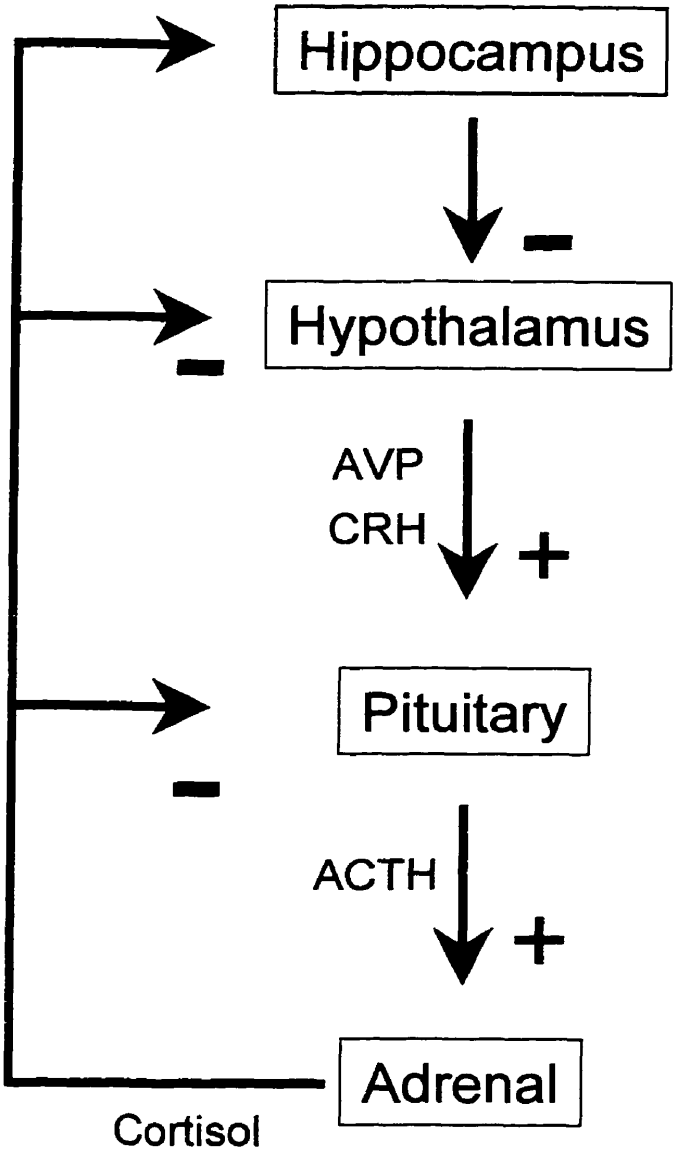
1.1 The Hypothalamic-Pituitary-Adrenal Axis

In overview, corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus are secreted into the hypophyseal portal vasculature. CRH and AVP stimulate synthesis of pro-opiomelanocortin (POMC), the precursor to adrenocorticotrophic hormone (ACTH), as well as ACTH secretion from

the pars distalis region of the pituitary (or hypophysis). Plasma ACTH acts directly upon the adrenal cortex to stimulate corticosteroid production. Glucocorticoids have multiple effects including regulation of the sympathetic and parasympathetic nervous systems, metabolism, and cardiovascular function. In addition, glucocorticoids feedback on both the hippocampus, which regulates hypothalamic function, and at the hypothalamus to inhibit CRH. Glucocorticoids also act directly on the pituitary to inhibit ACTH output, thus regulating HPA axis function (Figure 1.1). However, in the fetus toward the end of gestation, the negative feedback effects of cortisol on HPA axis function appear to be attenuated such that there is a continual rise in plasma levels of ACTH and cortisol. The increase in circulating fetal cortisol concentrations provides the stimulus for organ maturation and in sheep cortisol is the trigger for parturition.

In the sheep, fetal hypophysectomy (Liggins and Kennedy, 1968) or adrenalectomy (Drost and Holm, 1968) significantly increased gestational length to >160 days (term~145-150 days), whereas intra-fetal administration of either ACTH or cortisol stimulated premature delivery (Liggins, 1968). Interestingly, maternal hypophysectomy (Denamur and Martinet, 1961), or ACTH or cortisol administration to the ewe had no effect upon gestational length (Liggins, 1968). These studies established that the fetal, and not the maternal HPA axis was critical for the timing of parturition. Utilizing the chronic fetal catheterization model, documented by Kraner in 1965, it became possible to continuously monitor endocrine parameters of the sheep fetus throughout gestation. Several investigators have documented the concomitant

Figure 1.1 Schematic diagram showing the regulation of the hypothalamic-pituitary-adrenal axis. CRH, corticotrophin releasing hormone; AVP, arginine vasopressin; ACTH, adrenocorticotrophic hormone. Stimulatory (+) and inhibitory (-) actions are shown.


Hippocampus
-
Hypothalamus
-
AVP
CRH
+
Pituitary
-
ACTH
+
Adrenal
Cortisol

rise in plasma cortisol and ACTH concentrations in the fetal sheep during the last third of gestation (Bassett and Thorburn, 1969; Norman *et al.* 1985). These observations suggest that the fetal HPA axis becomes more active and less responsive to glucocorticoid negative feedback as gestation progresses. However, the underlying mechanisms of HPA axis activation and the role it plays in the onset and progression of labour are not completely understood.

Cortisol, the product of HPA activation, has been shown to induce 17α-hydroxylase, C_{17-21} lyase activity (Steele *et al.* 1976) and aromatase activity (Challis *et al.* 1991) in the ovine placenta, promoting a shift from progesterone to estrogen production (Steele *et al.* 1976). In both sheep and humans it is the increase in local estrogen action in conjunction with increasing uterine stretch that is associated with myometrial activation (Challis and Lye, 1993). Myometrial activation consists of increased: 1) number of oxytocin and prostaglandin receptors, 2) L-type Ca^{2+} and Na^{+} channels and 3) connexin 43 gap junctional protein. In the activated state the uterus responds to uterotonins such as oxytocin and prostaglandins with the synchronous, coordinated contractions of labour. The increase in the estrogen:progesterone ratio also stimulates cervical ripening by remodeling the cervical matrix with new collagen and proteoglycan synthesis (Challis and Lye, 1993).

The role of cortisol in the initiation of labour in humans is less clear. Plasma cortisol and ACTH levels in the human fetus rise with advancing gestational age (Lockwood *et al.* 1996), similar to the endocrine profile in the sheep fetus (Norman *et al.* 1985). However, in anencephalic human fetuses, the absence of a pituitary

appears to alter gestational length with a greater proportion of fetuses born preterm and post-term (Swaab and Honnebier, 1973), unlike the consistent prolongation of gestation seen in the ovine and bovine species (Kennedy *et al.* 1957; Binns *et al.* 1964). This difference in the control of length of gestation in the human and sheep is not understood, however it may suggest that development of the HPA axis in the human fetus is not the only trigger of parturition. One major difference between the HPA axis of the sheep and human fetus is the function of the adrenal gland. The primary steroid produced by the human fetal adrenal is dehydroepiandrosterone (DHEA), not cortisol as in the sheep (Mesiano and Jaffe, 1997). DHEA production in the fetal and maternal adrenal increases with advancing gestation, and is the substrate for estradiol biosynthesis in the human placenta. In the placenta, 3β-hydroxysteroid dehydrogenase (3βHSD) converts DHEA to androstenedione. Androstenedione is converted by 17-ketosteroid reductase (also called 17β-hydroxysteroid dehydrogenase) to testosterone, which is aromatized to produce estradiol. In the sheep, increasing adrenal cortisol late in gestation stimulates an increase in the conversion of pregnenolone, via the Δ^5 pathway to estrogen, resulting in decreased progesterone accompanied by increased estrogen (Challis and Lye, 1993). Conversely, in the human, there is no indication of a fall in progesterone or an acute rise in estrogen, either in plasma or within intrauterine tissues at the time of labour (Challis, 1998). However, there does appear to be an increase in estrogen receptor expression within intrauterine tissues and a change in the ratio of estrogen:progesterone within intrauterine tissues. Therefore in humans

the mechanism of "activating" the myometrium appears to occur at a paracrine rather than endocrine level (Challis, 1998).

The prepartum elevation in glucocorticoids has additional functions associated with preparing the fetus for extra-uterine existence in both the human and sheep (Liggins *et al.* 1973). Cortisol has been associated with maturation of the lung (Froh and Ballard, 1994), the switch from a fetal to an adult type of hemoglobin (Wood *et al.* 1972), and maturational changes in the gut, pancreas and brain (Liggins, 1976; Challis *et al.* 1977). Therefore, cortisol is critical in the prepartum maturation of fetal organ systems necessary for independent life, in addition to its role in initiating parturition.

1.1.1 Hypothalamus

The hypothalamus is located in the anterior region of the diencephalon, which lies below the hypothalamic sulcus and in front of the interpeduncular nuclei. It is composed of a variety of nuclei and nuclear areas and is regulated by serotinergic inputs from the limbic system and particularly the hippocampus. The two hypothalamic nuclei associated with the production of the trophic hormones corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) that regulate pituitary function are the paraventricular nucleus (PVN) and the supraoptic nucleus (SON). CRH containing neurons are localized primarily in the parvocellular region of the PVN, whereas AVP is present in the parvocellular and magnocellular PVN and the exclusively magnocellular SON (Everitt and Hökfelt, 1986). The axons of the parvocellular neurons project to the external lamina of the median eminence

and neuropeptides are released into the hypothalamic-hypophyseal portal circulation (Antoni *et al.* 1983). Axons of magnocellular neurons of the PVN and SON project through the internal lamina of the median eminence and the nerve terminals reside within the pars nervosa (Reichlin, 1992). Therefore the parvocellular neurons modulate anterior pituitary function, and magnocellular neurons regulate systemic arginine vasopressin (AVP) and oxytocin (Swanson *et al.* 1983).

In sheep fetuses in which the PVN has been lesioned, at approximately 100 days of gestation, there is a prolongation of pregnancy, suggesting that hypothalamic function is necessary for the correct timing of parturition (Gluckman *et al.* 1991; McDonald and Nathanielsz, 1991). However, the lesions inflicted in these studies were quite extensive and other regions of the brain were also ablated, therefore the contribution of the hypothalamus to changes in gestational length is unclear. The importance of hypothalamic influences on the pituitary is evident in studies in which the hypothalamus and pituitary were disconnected by severing the stalk of the pituitary gland and then inserting a silastic membrane to prevent any further communication that may occur by diffusion (Liggins *et al.* 1973). There was no change in the histology of the anterior pituitary, but, if the disconnection occurred prior to 110 days of gestation, the pregnancy was prolonged to and terminated at 160 days of gestation (normal term ~145-150d). Interestingly, if the disconnection did not occur until after 120 days of gestation, fetuses delivered at normal term (Liggins *et al.* 1973). These studies indicate that the hypothalamus is very important in early maturation or development of pituitary function but has a less critical role during late gestation.

CRH immunoreactivity was detected in the parvocellular neurons of the PVN in the hypothalamus of the human fetus from 16 weeks (term = 37-40 weeks) (Bugnon *et al.* 1984), and the ovine fetus from 47 days of gestation (term = 150 days) (Levidiotis *et al.* 1987). Immunoreactive (ir) AVP was clearly visible in the SON and PVN of the human fetus at 23-28 weeks of gestation (Benirschke and McKay, 1953; Rinne, 1963), and in the sheep fetus from 42 days of gestation (similar to the appearance of CRH) (Levidiotis *et al.* 1987). However immunohistochemistry is not a sensitive method of detection therefore CRH and AVP may be synthesized at lower than detectable levels earlier in gestation.

In the hypothalamus of the human fetus CRH concentrations do not change throughout gestation (Ackland *et al.* 1986), and there is little information available regarding changes in the abundance of AVP. Conversely, in the sheep fetus the changes in hypothalamic CRH and AVP peptide and messenger ribonucleic acid (mRNA) levels throughout gestation have been examined extensively. However, because the hypothalamus is not an isolated organ but rather a collection of nuclei contained within the brain, the measurement of hypothalamic content is quite difficult, and has resulted in conflicting data.

The quantity of ir-CRH measured by radioimmunoassay in the fetal sheep hypothalamus was significantly greater at 125-130 days of gestation as compared to 100-110 days, but at 140 days of gestation ir-CRH levels fell to concentrations similar to that at 100-110 days of gestation (Brieu *et al.* 1988; Brooks and Challis, 1988). Additional studies reflected a similar pattern of hypothalamic ir-CRH expression however the timing was slightly different. Immunoreactive CRH

concentrations measured by radioimmunoassay and ACTH-releasing activity in the hypothalamus increased progressively from early gestation (63-88 days), was dramatically elevated at 138 days of gestation, and then fell prepartum (143 days) to levels similar to that seen in the 133 day old fetus (Brieu *et al.* 1989). The reported decrease in hypothalamic ir-CRH content at term may reflect a decrease in synthesis or an increase in secretion. CRH secretion from hypothalami collected from fetuses at 100 and 140 days of gestation was measured using a perifusion system. Although the concentration of hypothalamic ir-CRH was similar at both ages, the basal release of CRH at day 140 days of gestation was significantly greater than at day 100 (Brooks *et al.* 1989). Watabe *et al.* (1991) used several different CRH antibodies to examine CRH levels throughout gestation by both radioimmunoassay and immunhistochemistry, and reported an increase from early gestation (48-50 days) to mid-gestation (100-108 days), but no change between mid- (100 days) and late-gestation (138-140 days). CRH eluted from Sephadex G75 columns was identified as a single peak by the antibodies used by Watabe *et al.* (1991) and Brooks *et al.* (1989), thus demonstrating antibody specificity. Saoud and Wood (1996a) similarly reported significantly higher hypothalamic ir-CRH concentrations at term compared to early- and mid-gestation (74-101 days). However, the hypothalamic ir-CRH, separated on a Sephadex G75 column, in this study did not co-elute with ovine CRH. Western blot detection revealed the molecular weight of this hypothalamic ir-CRH was ~20kD which is consistent with the molecular weight of pro-CRH, the precursor peptide of CRH (Saoud and Wood, 1996a).

Using *in situ* hybridization techniques, Myers *et al.* (1993) reported an increase in CRH mRNA levels in the PVN from days 105-107 to days 128-130 of gestation, with mRNA levels on days 138-140 at an intermediate level compared to the previous two time points. In contrast, Matthews and Challis (1995), also using *in situ* hybridization reported CRH mRNA present in the ovine fetal hypothalamus from day 60 onward, with levels increasing progressively until term (140 days) and decreasing post-partum. In the previous study, Myers *et al.* (1993) examined the mid-rostral regions of the PVN exclusively, whereas Matthews and Challis (1995) assessed coronal sections of the entire PVN at three different depths. It is possible that the modest decrease seen by Myers *et al.* may reflect changes in a subpopulation of neurons, which may be differentially regulated and not representative of the changes within the PVN as a whole. Therefore, despite some conflicting data the pattern of the synthesis and release of CRH appears to be consistent with increasing production and secretion with advancing gestation.

In 1989 Brieu and Durand reported that the concentration of ir-AVP in the fetal sheep hypothalamus increased progressively between days 63 and 138 but decreased on day 143 (Brieu *et al.* 1989). The decrease in AVP seen at term in these studies reflects a decrease in ir-AVP in the PVN since the SON would not be included in the samples of hypothalamus collected. In contrast Saoud and Wood (1996a) reported that hypothalamic ir-AVP concentrations continued to increase throughout gestation and into neonatal life, however the authors did not specify how the hypothalami were dissected for this study. Using *in situ* hybridization, AVP mRNA was detected at 60 days of gestation in the fetal sheep PVN, and although

the distribution of AVP mRNA within the magnocellular and parvocellular nuclei changed, there was no change in the amount of AVP mRNA measured in the PVN as a whole until post-partum, when levels increased dramatically (Matthews and Challis, 1995). Therefore, it appears that AVP mRNA and peptide is abundant in the fetal sheep hypothalamus throughout gestation with even higher levels post-partum.

CRH and AVP are secreted into the hypothalamic-hypophyseal portal circulation, which is the major blood supply of the pars distalis (90%). The branches of the carotid arteries supplying the median eminence empty into a rich capillary network extending upward to the floor of the third ventricle and downward to the pars distalis, effectively connecting the hypothalamus and the pituitary (Everitt and Hökfelt, 1986).

This system develops early in gestation, and in humans was intact and presumed functional from 11.5 to 16.8 weeks of gestation (term = 37-40 weeks) (Thliveris and Currie, 1980). In the sheep, the development of the hypophyseal portal circulation was not examined until 1987 when Levidiotis *et al.* infused Indian ink to the ovine fetus beginning on day 45. The ink was distributed throughout the median eminence, pituitary stalk and pars distalis suggesting that the portal system is functional and therefore hypothalamic CRH and AVP may act on the pars distalis from day 45 onward (Levidiotis *et al.* 1987). The presence of hypothalamic CRH, AVP and a functional hypophyseal portal circulation suggests that trophic drive to the pituitary begins early in gestation.

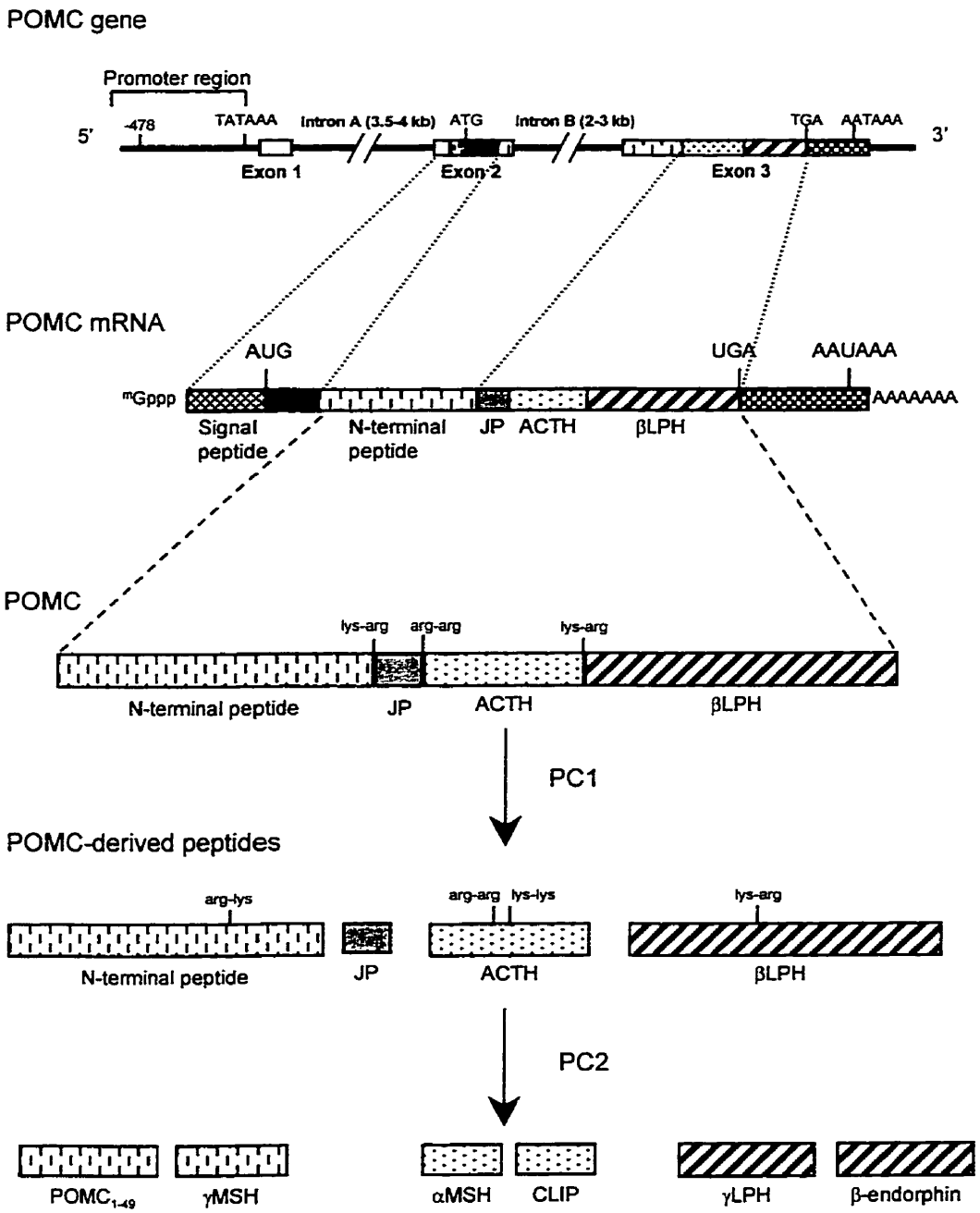
1.1.2 The Fetal Pituitary Gland

The pituitary or hypophysis is a relatively small endocrine gland situated within the sella turcica at the base of the brain. The pituitary is comprised of three morphologically different regions, which originate from two separate embryonic sources. The pars nervosa (neurohypophysis or neural lobe) is a down-growth from the floor of the diencephalon, and the pars distalis (adenohypophysis or anterior lobe) is an upgrowth from the roof of the primitive foregut, named Rathke's pouch (McLachlan, 1998). These two tissues fuse and the wall of Rathke's pouch adjacent to the pars nervosa forms the pars intermedia (intermediate lobe) at approximately 5 weeks of gestation in the human (Ikeda *et al.* 1988) and 26 days of gestation in sheep (Perry *et al.* 1982). The human pituitary is distinguishable and the floor of the sella turcicia is in place by 7 weeks (Mulchahey *et al.* 1987), and the sheep pituitary is formed by 40 days of gestation (Perry *et al.* 1982). The pars distalis and pars intermedia regions of the pituitary synthesize the precursor prohormone peptide, pro-opiomelanocortin (POMC).

1.1.2.1 Pars distalis

There are at least five different types of secretory cells within the pars distalis (Guyton and Hall, 1996), including corticotroph cells. Corticotrophs synthesize POMC, which is enzymatically cleaved into smaller peptides including N-terminal peptide, β-lipotrophin (βLPH), $ACTH_{1\text{-}39}$, β-endorphin, and α-melanocyte stimulating hormone (αMSH; Figure 1.2; Holm and Majzoub, 1995).

Figure 1.2 Diagrammatic representation of the structure of the pro-opiomelanocortin (POMC) gene and the processing of POMC by prohormone convertase 1 (PC1) and 2 (PC2). POMC is cleaved by the actions of PC1 to produce N-terminal peptide, joining peptide (JP), adrenocorticotrophic hormone (ACTH), and β-lipotrophin (βLPH). These peptides may be cleaved further by PC2 to produce γ-melanocyte stimulating hormone (γMSH), α-melanocyte stimulating hormone (αMSH), corticotrophin-like intermediate lobe peptide (CLIP), γ-lipotrophin (γLPH), and β-endorphin. lys-arg, arg-arg, arg-lys, and lys-lys are the dibasic pairs of amino acids that are sites of prohormone convertase cleavage. (Adapted from Lundblad and Roberts, 1988; Zhou *et al.* 1993; Holm and Majzoub, 1995).


POMC gene
Promoter region
5'
-478
TATAAA
Intron A (3.5-4 kb)
ATG
Intron B (2-3 kb)
TGA
AATAAA
3'
Exon 1
Exon 2
Exon 3
POMC mRNA
AUG
UGA
AAUAAA
mGppp
AAAAAAA
Signal peptide
N-terminal peptide
JP
ACTH
βLPH
POMC
lys-arg
arg-arg
lys-arg
N-terminal peptide
JP
ACTH
βLPH
PC1
POMC-derived peptides
arg-lys
arg-arg
lys-lys
lys-arg
N-terminal peptide
JP
ACTH
βLPH
PC2
POMC1-49
γMSH
αMSH
CLIP
γLPH
β-endorphin

Corticotroph cells are immunopositive for ACTH at 7-8 weeks of human gestation (Baker and Jaffe, 1975; Begeot *et al.* 1978), however, ir-αMSH is not visible until 14 weeks (Baker and Jaffe, 1975). Immunoreactive ACTH and αMSH were separated from human fetal pituitary extracts using high pressure liquid chromatography, and the concentrations of each did not change between weeks 11 and 19 of gestation (Brubaker *et al.* 1982), however the ratio of ACTH to large molecular weight POMC-derived peptides was not examined. Silman *et al.* (1976) showed that there was a maturation of corticotroph function in the human pituitary that was reflected by a change in the relative abundance of POMC-derived peptides in the pars distalis. That is, there was more αMSH and C-terminal ACTH peptides present in the human fetal pituitary during mid-gestation, and these peptides decreased as the production of $ACTH_{1-39}$ increased in late-gestation (Silman *et al.* 1976).

Maturation of corticotrophs within the pars distalis is well documented in the sheep fetus. ACTH positive corticotrophs are arranged in cords and clusters, and are detectable in the fetal sheep pars distalis as early as day 38 of gestation (Perry *et al.* 1985). By 90 days of gestation there are three morphologically distinct types of corticotrophs present, the "fetal", "intermediate" and "adult" cell types (Perry *et al.* 1985). The "fetal" corticotrophs are large, columnar, weakly ACTH positive and are only present during fetal life. The number of "fetal" corticotrophs is greatest at day 90 of gestation, declines by day 130 of gestation and the "fetal"-type corticotroph is virtually non-existent in the term pituitary (Perry *et al.* 1985). The "adult" corticotrophs are small, stellate and strongly ACTH immunopositive, similar to the

corticotrophs seen in adulthood. These cells are scattered throughout the pars distalis, and increase in number as gestation progresses (Perry *et al.* 1985). The "intermediate" corticotrophs stain ACTH-positive but do not fit into either the "fetal" or the "adult" category; however they do possess characteristics of both cell types, and therefore may be a transitional cell type (Perry *et al.* 1985).

Corticotroph morphological maturation is consistent with a change in corticotroph function in the ovine fetus. The ratio of bioactive to ir-ACTH increases with advancing gestation in both fetal sheep plasma and within the pars distalis (Silman *et al.* 1979; Perry *et al.* 1985; Brieu and Durand, 1987; Saphier *et al.* 1993). Functional maturation was induced *in vitro* by culturing fetal sheep corticotrophs in the presence of cortisol or AVP for four days, resulting in an increase in the ratio of bioactive to ir-ACTH secreted after AVP stimulation (Brieu and Durand, 1987). Fetal adrenalectomy at 120 days of gestation prevented the maturation of the "fetal" to "adult" corticotroph, however the maturation could be induced *in vivo* by cortisol infusion at 109-115 days of gestation prior to the expected rise in endogenous fetal plasma cortisol concentrations (Antolovich *et al.* 1989). These data suggest that cortisol and AVP are important in corticotroph maturation.

1.1.2.2 Pars intermedia

The intermediate lobe also contains corticotrophs, commonly referred to as melanotroph cells, which are much smaller and more dense than their pars distalis counterparts. The functioning of the pars intermedia is influenced by dopaminergic inputs from the arcuate nucleus. The pars intermedia stains intensely

immunopositive for ACTH and αMSH in the sheep during fetal life, but in the adult pars intermedia ACTH staining is only weak, while ir-αMSH remains abundant (Perry *et al.* 1985). Although, the human fetus has a rudimentary pars intermedia (Silman *et al.* 1976), there is little information available regarding fetal pars intermedia development or function. However, the adult pars intermedia does exhibit intense immunostaining for ACTH (Asa *et al.* 1995).

In the fetal sheep, melanotrophs are ACTH immunopositive at 60 days of gestation (Mulvogue *et al.* 1986). *In vitro* studies have shown that fetal sheep melanotrophs maintain a basal level of ACTH secretion throughout gestation, and respond to CRH and AVP stimulation (Fora *et al.* 1996). In addition, there appears to be a maturation of melanotroph responsiveness similar to that reported for corticotrophs such that cells collected from fetuses at 138-145 days of gestation have greater CRH and CRH + AVP responsiveness than melanotrophs from fetuses at 100-115 days of gestation (Fora *et al.* 1996). These data indicate that the pars intermedia may contribute to plasma ACTH concentrations in response to increased CRH or AVP stimulation. However, pars intermedia function is predominantly regulated by neural inputs, specifically the neurotransmitter dopamine acting via the dopamine type-2 receptor. Mice that are dopamine type-2 receptor deficient have a Cushing's-like syndrome which is characterized by increased number of melanotrophs, POMC and prohormone convertase 1 (PC1) mRNA, plasma ACTH and βendorphin, accompanied by adrenal hypertrophy and elevated plasma corticosterone concentrations (Saiardi and Borrelli, 1998). These data provide compelling evidence for a role of the pars intermedia in contributing to circulating

ACTH concentrations. It has also been postulated that the pars intermedia is the major source of large molecular weight POMC-derived peptides (Roebuck *et al.* 1980; Saphier *et al.* 1993). Large molecular weight POMC-derived peptides in circulation may act to inhibit $ACTH_{1-39}$ action at the level of the fetal adrenal (Roebuck *et al.* 1980; Schwartz *et al.* 1995) or they may be converted to bioactive ACTH in the plasma or at peripheral sites (Roebuck *et al.* 1980). Therefore the fetal sheep pars intermedia may be a source of ACTH production during fetal life; however its role remains uncertain and requires further investigation.

1.1.2.3 Pro-opiomelanocortin (POMC)

Pro-opiomelanocortin (POMC) is a common precursor molecule, which is cleaved into many smaller peptide hormones (Eipper and Mains, 1977). POMC has been localized to many tissues including the adrenal, thymus, testes, spleen, kidney, ovary, lung, thyroid, and gastrointestinal tract (Smith and Funder, 1988). However, the pars distalis corticotroph and the pars intermedia melanotroph are considered the predominant sites of POMC production in mammals (Lundblad and Roberts, 1988).

McMillen *et al* (1988) was the first to report the presence of POMC mRNA in the pars distalis of fetal sheep. Using dot blot analysis they reported POMC mRNA abundance was high at 100-135 days of gestation and decreased at 141-144 days of gestation (McMillen *et al.* 1988). The authors proposed, from these findings, that the increase in plasma ACTH in the near term ovine fetus was a result of increased post-translational processing of POMC to ACTH. However, several independent

reports have shown, using both Northern blot analysis and *in situ* hybridization, that pars distalis POMC mRNA levels rise with advancing gestation in the ovine fetus to peak levels during labour (Myers *et al.* 1991; Yang *et al.* 1991; Matthews *et al.* 1994). POMC mRNA levels in the pars intermedia increased from 60 days to 100 days of gestation and remained at this level throughout gestation and into adulthood (Matthews *et al.* 1994). Pars intermedia POMC mRNA levels were consistently 5-fold greater than pars distalis POMC mRNA levels (Matthews *et al.* 1994). In light of these observations, it appears that the increase in abundance of POMC mRNA in the fetal sheep pars distalis with advancing gestation may be the source of the late gestation increase in fetal plasma ACTH. The discrepancy between the study of McMillen *et al.* (1988) and the later studies (Myers *et al.* 1991; Yang *et al.*1991; Matthews *et al.* 1994) may be due to the limitations of the dot blot technique, such as the lack of an internal control to correct for variation in the amount of RNA loaded onto the dot blot. In addition, there may be a disparity with the pituitary tissue examined, such that the pars intermedia may have been included with the pars distalis at earlier gestational ages but not at later gestational ages, when the pars intermedia is much easier to separate from the pars distalis.

The POMC gene consists of a promoter of approximately 400 base pairs (bp) at the 5' end of the gene followed by 3 exons and 2 introns (Fig. 1.2). Exon 1 is not translated and exhibits less than 50% homology between species. Exon 2 encodes the initiator methionine and a 26 amino acid (aa) hydrophobic signal peptide which is ~90% homologous among mammals (Eberwine and Roberts, 1983). Except for this signal peptide and 18aa of the N-terminal peptide the remainder of the POMC

precursor molecule is encoded by exon 3. The regions encoding the N-terminal peptide, ACTH, αMSH, and βendorphin are >95% homologous within mammals. The region between the N-terminal peptide and ACTH, called the joining peptide (JP), is poorly conserved among species and therefore it has been suggested to not have any biologically important function (Holm and Majzoub, 1995). The promoter of human POMC gene contains both TATA and CAAT boxes, which lie 28 and 62bp upstream from the transcription start site respectively. This promoter has nine identified regulatory elements that include cAMP and glucocorticoid regulatory elements, which mediate increased transcription by cAMP and decreased transcription by glucocorticoids (Therrien and Drouin, 1991; Jacobson and Drouin, 1994). Tryspin-like prohormone convertase endopeptidase enzymes, entitled prohormone convertases perform the proteolytic processing of POMC to smaller peptides.

1.1.2.4 Prohomone convertase enzymes

The family of seven subtilisin/kexin-like enzymes furin, PC1 (or PC3), PC2, PACE4, PC4, PC5 (or PC6), and PC7 (or PC8) are collectively called prohormone convertases. The members of this family cleave peptides at basic residues and typically at pairs of basic amino acid sites. In general, although each convertase exhibits unique sequence characteristics, each convertase has conserved regions. These include: 1) the "prosegment", which is thought to act as an intramolecular chaperone assisting in the folding of the zymogen within the ER, 2) a catalytic segment, which is the most conserved domain; 3) a second chaperone-like domain,

called the P-domain; also thought to serve an important role in zymogen folding; and 4) the C-terminal segment, which is the most variable domain, and has been shown to contain a transmembrane domain in both furin and PC6 (Seidah *et al.* 1994).

Each of the convertase enzymes has a unique distribution. Furin and PACE4 are ubiquitously present in most tissues and cells. PC5/PC6 is found in a subset of endocrine and non-endocrine cells, and PC4 has only been found within testicular-germ cells. PC7/PC8 expression is widespread with the most abundant sources being the colon and lymphoid-associated tissues. PC1/PC3 (herein called PC1), and PC2 are present only within endocrine and neuroendocrine cells, including the corticotrophs (Seidah *et al.* 1991). It is PC1 and PC2 that are attributed to cleaving POMC into smaller peptide hormones. PC1 cleaves POMC to produce N-terminal ACTH, $ACTH_{1-39}$, and βLPH, while PC2 cleaves these peptides further to produce γMSH, αMSH, CLIP, γLPH and βendorphin (Figure 1.2).

The information available to date on PC1 and PC2 has been collected predominantly from *in vitro* studies and from the mouse and rat *in vivo*. PC2 was originally isolated and characterized from a human insulinoma cDNA library by homology to Kex2, a known yeast endoprotease, and from a mouse pituitary cDNA library by homology to human furin (Seidah *et al.* 1990). PC1 and PC3 were simultaneously identified from cDNA libraries from mouse insulinoma and AtT20 cells (Seidah *et al.* 1991; Smeekens *et al.* 1991).

In the adult mouse PC1 mRNA expression is present in all melanotrophs and in 20% of pars distalis cells, PC2 mRNA is similarly expressed in all melanotrophs

but levels were not detected in the pars distalis (Seidah *et al.* 1991). This pattern of expression was also seen in the adult rat (Bloomquist *et al.* 1991). Using Northern blot analysis, the ratio of PC1:PC2 was reported as 3-5:1 within the diverse cell population of the pars distalis, and 1:15 within the neurointermediate lobe of adult mouse and rat pituitaries (Benjannet *et al.* 1991). This pattern and level of mRNA expression corresponds to the processing of POMC in the adult pituitary with the pars distalis producing predominately ACTH and β-lipotrophin (βLPH), and the pars intermedia predominantly producing the shorter peptides αMSH, corticotrophin-like intermediate lobe peptide (CLIP), γ-LPH, and β-endorphin.

PC1 and PC2 are first synthesized as pro-enzymes from which the N-terminal pro-segment is removed in order to generate the active proteinase. Zymogen activation was reported to be an autocatalytic step for PC2 (Bloomquist *et al.* 1991). The rate of N-terminal pro-segment cleavage could potentially represent a mechanism by which the cell could control the efficiency of precursor processing. Pro-PC1 processing begins in the endoplasmic reticulum (ER). In contrast, PC2 processing begins in the trans-Golgi network (TGN). Cleavage of pro-PC1 to PC1 occurs quickly and PC1 transits rapidly from the ER to the TGN. However, pro-PC2 slowly exits from the ER and only a small fraction of the newly synthesized pro-PC2 reaches the TGN at any given time (Seidah, 1993). The rate of conversion of pro-PC2 to PC2 in the TGN is modulated by a specific binding protein 7B2 (Seidah *et al.*1994). Within the TGN, both enzymes are tyrosine-sulphated. In addition, PC1 has complex N-linked carbohydrate glycosylation, which is endoglycosidase H resistant whereas, PC2 has high mannose glycoslyation and is sensitive to

endoglucosidase H. The N-linked sugars are critical in directing the correct folding of the enzymes and/or in defining their rate of exit from the ER (Seidah, 1993). Biosynthetic studies on the rate of processing of pro-PC2 to PC2 invariably showed that it is much slower than that of pro-PC1 to PC1 (Seidah, 1993).

Several studies have further explored the relationship between PC1, PC2 and POMC-derived peptides. The AtT-20 cell line is a corticotroph cell line derived from a mouse anterior pituitary tumor, which express PC1 mRNA but PC2 mRNA levels are undetectable (Bloomquist *et al.* 1991). The stable expression of antisense RNA to PC1 in AtT-20 cells inhibits PC1 expression and hence normal POMC processing is halted at the early stages such that POMC is the predominant product in cell extracts and media (Bloomquist *et al.* 1991). Co-expression experiments have also been used to study the function of PC1 and PC2 in POMC processing. Co-injection of POMC and PC1 RNA into *Xenopus* oocytes resulted in the production of mature ACTH (Korner *et al.* 1991). In addition, PC1 and POMC were co-expressed in the constitutively secreting BSC-40 cell line, and in endocrine-tissue derived cell lines PC12 and AtT-20, which exhibit regulated secretion. In all of these studies, POMC was cleaved into ACTH and β-LPH, showing that PC1 alone is capable of cleaving POMC to ACTH. When PC2 and POMC were co-expressed in the same cell lines the cleavage products were β-endorphin, an N-terminally extended ACTH containing the JP, and either αMSH or desacetyl-αMSH. Therefore, it was determined that PC2 cleaves at all five pairs of basic residues whereas PC1 cleaves two sites preferentially, releasing ACTH from N-terminal peptide + JP and βLPH (Benjannet *et al.* 1991; Thomas *et al.* 1991). PC1 and PC2 function has also been examined using

stable transfection models and the cell line AtT-20 (Zhou and Mains, 1994). Overexpression of PC1 in AtT-20 cells (27-fold increase in PC1 enzyme level), resulted in an increase in the rate of the first several cleavages of POMC, and these cells contained primarily ACTH and βLPH, however, β-endorphin was also detected (Zhou and Mains, 1994). Overexpression of PC2 led to the production of γMSH and βendorphin (Zhou and Mains, 1994). However, others have previously suggested that *in vitro* studies and overexpression models may not be representative of enzyme behaviour *in vivo* (Steiner *et al.* 1974; Steiner, 1991). Therefore PC1 cleavage of POMC into β-endorphin may be due to aberrant cleavage in the overexpression model of PC1, since there is no evidence of this PC1 activity *in vivo*.

In the fetal mouse pituitary, PC1 and PC2 immunoreactivity corresponds to the presence of PC1 and PC2 mRNA respectively. Furthermore, PC2 is co-localized with ir-αMSH containing cells, providing further support that PC2 cleaves POMC into the smaller POMC products *in vivo* (Marcinkiewicz *et al.* 1993). During fetal rat development PC1 and PC2 expression appears at a similar time and location during embryogenesis as POMC mRNA, while changes in PC1 and PC2 mRNA levels parallel changes in POMC processing (Zheng *et al.* 1994). PC1 expression in the fetal mouse pituitary increases progressively throughout gestation to a maximum level in adulthood, whereas PC2 expression is highest in the post-natal pituitary (Marcinkiewicz *et al.* 1993).

Haloperidol, a dopamine antagonist, given to adult rats increased pars intermedia PC1, PC2 and POMC mRNA levels. Within the pars distalis, POMC and PC2 mRNA abundance increased with haloperidol however, there was no effect on

PC1 mRNA (Day *et al.* 1992). Conversely, treatment with the dopamine agonist bromocriptine decreases pars intermedia PC1, PC2 and POMC mRNA levels but has no effect on pars distalis PC1, PC2 and POMC mRNA levels (Day *et al.* 1992). In adult rats, adrenalectomy increased, and glucocorticoids inhibited pars distalis POMC mRNA levels, however, neither of these paradigms altered PC1 mRNA levels (Day *et al.* 1992). This study examined the whole pars distalis, and not corticotrophs alone, therefore, changes in PC1 expression within the corticotroph may be masked by what is occurring within the other pars distalis cell types. In an attempt to resolve this issue, Day *et al.* (1992) also examined the effects of dexamethasone on a pituitary cell line (AtT20) which expresses POMC, PC1 and PC2 in relative amounts similar to that in pars distalis corticotrophs. Cells treated with dexamethasone (1μM), for a period of 30 minutes had a 50% reduction in PC1, PC2 and POMC mRNA levels, but after a 60-minute incubation PC1, PC2 and POMC mRNA levels had returned to control values (Day *et al.* 1992). This indicates that PC1, PC2 and POMC may be similarly regulated by glucocorticoids *in vitro*, however this was not true in the previous *in vivo* studies conducted in the adult rat. This difference may be a reflection of the cell populations, such that *in vitro* studies examined a corticotroph cell line and *in vivo* experiments measured POMC, PC1 and PC2 mRNA levels in mixed pars distalis cells. Alternatively, *in vivo* the regulation of PC1 and PC2 may be influenced by additional endocrine or paracrine factors that are not present in an isolated population of corticotroph cells. To date there is no information available on the effects of cortisol on PC1 and PC2 within the fetal pituitary, in particular during late gestation when fetal plasma cortisol concentrations are elevated.

1.1.2.5 Adrenocorticotrophic hormone (ACTH)

In fetal sheep ACTH has been detected in plasma at day 59 of gestation (Alexander *et al.* 1973) and the concentration rises progressively during the final 25 days of gestation to peak concentrations at term (Norman *et al.* 1985). Similarly, plasma ACTH concentrations in the human fetus also rise with advancing gestation peaking at term with concentrations 4- to 5-fold greater than those seen in normal adults (Allen, 1973). However, measuring ACTH is a complicated task for several reasons. First ACTH is secreted in a pulsatile manner such that single point measurements are only representative of plasma concentrations at that moment in time. Secondly, as we have discussed previously, ACTH is the product of prohormone cleavage, therefore antibody specificity becomes an important issue. The ACTH antibody characteristics determine the ability to measure $ACTH_{1-39}$, the bioactive moiety, or $ACTH_{1-39}$ plus additional peptides that share amino acid sequences but not necessarily bioactivity. The $ACTH_{1-39}$ containing peptides are called ACTH-related peptides or large molecular weight POMC-derived peptides and are reported as 31 and 22 kDa peptides (Eipper and Mains, 1980). Several independent investigators have shown a decrease in the ratio of large molecular weight POMC-derived peptides to $ACTH_{1-39}$ in fetal sheep plasma from 110 days and 120 days compared to term fetuses (Silman et al. 1979; Saphier *et al.* 1993). At 63 days of gestation the ratio of bioactive:immunoreactive ACTH was 0.3, and this increased to 0.7 at 144 days of gestation (Brieu and Durand, 1987).

1.1.2.6 Stimulation of ACTH secretion

The primary stimulators of ACTH secretion that have been identified are CRH and AVP. It is well established that CRH stimulates ACTH secretion from corticotrophs both *in vivo* (Wintour, 1984; Norman *et al.* 1985; Hargrave and Rose, 1986; MacIsaac *et al.* 1989) and *in vitro* (Durand *et al.* 1986; McMillen and Merei, 1993; Lu and Challis, 1994; Matthews and Challis, 1997). AVP stimulation of ACTH secretion and a possible synergistic action with CRH is also documented *in vivo* (Norman and Challis, 1987; MacIsaac *et al.* 1989) and *in vitro* (Durand and Cathiard, 1986; Matthews and Challis, 1997).

CRH and AVP act via independent receptors that are associated with different signal transduction pathways. CRH acts via the CRH type one receptor (CRH-R1) and adenylyl cyclase, and AVP acts through the V_3 receptor - phospholipase C pathway. CRH-R1 binding sites on the anterior pituitary of the sheep fetus have been shown to increase from day 65 of gestation to maximal numbers at day 125-130, and then decrease towards term, with no change in binding affinity throughout gestation (Lu *et al.* 1991). *In vitro*, CRH-stimulated ACTH-release was greater at 63 days of gestation than at 115 days of gestation, and the response was not different in term fetuses or lambs compared to that at 115 days (Durand, 1986). This suggests that CRH may be a more potent stimulus to ACTH release at term, since CRH binding sites are decreased at term compared to mid-gestation however, CRH stimulation *in vitro* elicited a similar ACTH response at these two ages in the ovine fetal pituitary.

AVP-stimulated ACTH output was greatest from ovine corticotrophs collected at 115 days of gestation and the responsiveness decreased progressively to term (144 days of gestation, Durand *et al.* 1986). It is interesting to note that the ratio of ir-AVP:ir-CRH in the fetal sheep hypothalamus was about 5:1 between days 63 and 123 of gestation, and this ratio decreased steadily to about 1.2:1 near term (Brieu *et al.* 1989). The authors suggest that the change in the ratio of hypothalamic AVP:CRH is consistent with AVP being of greater significance in younger fetuses and that CRH becomes more important during late gestation (Brieu *et al.* 1989). However, fetal plasma AVP concentrations rise during the last 12-24 hours before birth (Stark *et al.* 1979; Rose *et al.* 1981; Stark *et al.* 1981; Stark *et al.* 1982; Leffler *et al.* 1985), and the rise in systemic AVP near term may reflect an increase in ACTH stimulation. It has been shown previously that intravenous administration of AVP and CRH elicited an increase in plasma ACTH (Norman and Challis, 1987). Intra-fetal AVP (200ng) administration stimulates a greater rise in fetal plasma ACTH concentrations in the 110 –115 day ovine fetus than did CRH (1μg) however, the magnitude of response to both stimuli decreases towards term (Norman and Challis, 1987). These studies show that the fetal pituitary is sensitive to AVP and CRH stimulation early in gestation, however, as plasma ACTH concentrations rise during late gestation there appears to be a reduction in CRH and AVP stimulated ACTH output.

1.1.3 The Fetal Adrenal Cortex

The morphology and function of the fetal adrenal gland are quite different in the sheep compared to the human (or primate) therefore, I shall discuss them independently.

1.1.3.1 The primate fetal adrenal cortex

In the human fetus the primordium of the adrenal cortex appears at about 25 days of gestation. By 6-8 weeks' gestation the cells of the inner cortex have differentiated to form a distinct fetal zone, and the outer sub-capsular rim of immature cells is called the definitive or adult zone. (Winter, 1992). Mitotic activity in the fetal adrenal is thereafter, limited to the outer subcapsular layer (definitive zone) (Mesiano and Jaffe, 1997). The cells of the definitive zone divide rapidly and contribute centripetally via an indistinct transitional zone to an enlarging fetal zone that eventually occupies more than 80% of the gland (Johannison, 1968).

The adrenal cortex of the fetus and the adult produces cortisol, however 3β-hydroxysteroid-dehydrogenase (3βHSD) levels are low. Therefore, the conversion of pregnenolone to progesterone via the Δ^4 pathway to produce cortisol is similarly low, and pregnenolone is predominantly converted by $P450_{C17}$ (Δ^5 pathway) to produce large amounts of the 19-carbon steroids such as dehydroepiandrosterone (DHEA). DHEA is metabolized in the placenta to biologically active androgen or estrogen. The human placenta does not express cytochrome $P450_{C17}$ (17-hydroxylase and 17,20-desmolase) activity, therefore the placenta is dependent upon preformed C_{19}

steroids of either maternal or fetal adrenal origin as substrate for estrogen biosynthesis.

Studies have shown that the fetal human adrenal produces cortisol in response to ACTH at 10 weeks of gestation (Seron-Ferre *et al.* 1978), and that responsiveness to ACTH is augmented by exposure to ACTH (Rainey *et al.* 1991). The increase in adrenal responsiveness with ACTH pretreatment has been shown to stimulate a 2.8-fold increase in ACTH-binding (Rainey *et al.* 1991), and an increase in ACTH-receptor mRNA levels (Mesiano *et al.* 1996). In addition, in vivo administration of metyrapone to the fetal rhesus monkey, to stimulate endogenous ACTH secretion, at 135-137 days (term ~155-172 days), significantly increased adrenal ir-3βHSD. The fetal human adrenal is the major source of cortisol production, as evidenced by the positive arteriovenous gradient in cord serum cortisol levels which persists throughout pregnancy (Beitins *et al.* 1973). Fetal plasma cortisol concentrations also rise with advancing gestation and at 38-40 weeks of gestation 60-75% of the cortisol in the fetal compartment represents direct fetal adrenal secretion, while the remainder derives from placental transfer or metabolic conversion of cortisone (Beitins *et al.* 1973). Maternal cortisol readily crosses the placenta, but during late gestation most cortisol transiting the placenta is oxidized by 11β-hydroxysteroid dehydrogenase (11βHSD) to cortisone, thereby protecting the fetus from high maternal cortisol. 11βHSD exists in at least two isoforms in the primate placenta, 11βHSD-1 and 11βHSD-2 (Pepe and Albrecht, 1998; Sun *et al.* 1998). In the human placenta, 11βHSD-1 operates predominately as a reductase converting cortisone to cortisol whereas, 11βHSD-2 acts essentially

as a unidirectional dehydrogenase inactivating cortisol to cortisone (Sun *et al.* 1997). Therefore in late gestation the activity of 11βHSD-2 (11βHSD-2), localized in human placental syncytiotrophoblasts is dominant. However, it has been reported in the baboon that 11βHSD reductase activity predominates in early- to mid-gestation (Pepe and Albrecht, 1984). The authors suggest that the placental conversion of cortisone to cortisol during early- and mid-gestation ensures that the fetal HPA axis remains inactive (Pepe *et al.* 1990). The change in 11βHSD activity appears to be attributable to an estrogen-mediated increase in 11βHSD-2 placental syncytiotrophoblasts (Pepe *et al.* 1990).

Cortisol concentrations in human fetal and maternal plasma rise with advancing gestation and provide the stimulus for fetal organ maturation (Pearson Murphy and Diez D'Aux, 1972; Carr *et al.* 1981). It has been suggested that in primates, unlike the sheep, the increase in cortisol is less important in the timing of parturition than the sharp rise in androgens over the last 25 days that provide the precursors for placenta estrogen production (Novy, 1977). Therefore a rise in plasma levels of adrenal steriods stimulates the increase in placental estrogen production which precedes parturition in both the human and the sheep.

1.1.3.2 The ovine fetal adrenal cortex

The adrenal gland can be recognized by day 28 of gestation in the fetal sheep (Wintour *et al.* 1975), and begins to develop two distinct zones within the cortex by day 60. The outer zone develops into the mature zona glomerulosa during late gestation, while the inner zone resembles the zona fasciculata. The zona reticularis

only becomes apparent in the 1-month-old lamb (Robinson *et al.* 1979; Webb, 1980). Despite the lack of a well-developed zona recticularis the fetal sheep adrenal has been shown to secrete androstenedione *in vitro*, and *in vivo* (Mitchell *et al.* 1986), and ACTH treatment enhanced fetal adrenal androstenedione secretion (Mitchell *et al.* 1986). In addition, it was shown that androstendione acts as a precursor for estrogen production (Mitchell *et al.* 1986). Therefore in the sheep the adrenal influences placental estrogen production by producing precursors and via the induction of $P450_{C17}$ and $P450_{aromatase}$ to increase placental estrogen synthesis.

The volume and weight of the ovine fetal adrenal gland increases linearly between day 53 and 130 of gestation and exponentially thereafter until term (Liggins, 1969; Boshier and Holloway, 1989). The late gestation change is due mainly to an increase in the size of the zona fasciculata (Boshier *et al.* 1991). It has been suggested that there are two periods of cellular hypertrophy between days 124-136 of gestation and again between day 143 and birth. DNA replication appeared to be highest between days 135 –143 (Durand *et al.* 1980). The growth rate of the zona fasciculata has also been examined using relative changes in total steroidogenic cell volume, total cell number and individual cell volume (Boshier and Holloway, 1989). These analyses defined three phases of growth within the zona fasciculata. The first phase occurred between day 53 and day 100 of gestation, and was characterized by marked cell hyperplasia. Phase two spanned from day 100 to day 130, and was a period of decreased cell proliferation (compared to phase 1), but a small increase in cellular hypertrophy. The final growth phase occurred between day 130 and 48

hours post-partum, and featured cell hypertrophy from day 130 to term, followed by maximal cell multiplication after birth (Boshier and Holloway, 1989).

1.1.3.3 Ovine adrenal responsiveness

The changes in fetal ovine adrenal proliferative activity are mirrored by changes in adrenocortical cell function. The fetal adrenal secretes the greatest amount of cortisol per gram wet weight, or per cell *in vitro*, at day 50-60 of gestation compared to all other times during pregnancy. At this time the adrenal responds to ACTH stimulation (Wintour *et al.* 1975; Glickman and Challis, 1980). However, the responsiveness is lost during mid-gestation, and reappears as term approaches (Wintour *et al.* 1975; Jones and Ritchie, 1977; Glickman and Challis, 1980; Challis *et al.* 1982; DeVane *et al.* 1982). It has been suggested that this loss of responsiveness during mid-gestation (d100-130) involves a functional block at G_s protein coupling (Manchester and Challis, 1982; Durand *et al.* 1984). Others have suggested that there is an inhibitory factor to steroidogenesis during mid-gestation, which is overcome near term. This hypothesis was based on the discrepancy between adrenal responses *in vivo* and *in vitro* during this time (Jones and Roebuck, 1980) and the evidence that cultured adrenocortical cells continue to secrete cortisol throughout mid-gestation (Durand *et al.* 1985; Brieu and Durand, 1987; Durand *et al.* 1982). The increase in adrenal responsiveness in late gestation has been attributed to: 1) the hyperplastic growth of the zona fasciculata, 2) enhanced sensitivity to ACTH, via increased adenylyl cyclase activity (Durand, 1981), and the development of stimulatory guanine nucleotide binding component (G_s) (Durand *et al.* 1985), and

the increase in the number of ACTH receptors between 140-144 days of gestation (Durand, 1979), 3) the development of cellular structures involved with steroidogenesis such as smooth endoplasmic recticulum and mitochondria (Durand, 1978; Robinson, 1979) and 4) enhanced steroidogenic enzyme expression and enzymatic activity (Anderson, 1972; Durand, 1982; Challis *et al.* 1986)

The pattern of adrenocortical responsiveness *in vitro* is reflected by changes in cortisol concentrations in fetal sheep plasma *in vivo*. Plasma cortisol concentrations in the sheep fetus are relatively high (~10ng/ml) in the first trimester (Wintour *et al.* 1975; Ali *et al.* 1992), low at day 100 (~2-5ng/ml) and rise progressively from day 125 (~10ng/ml) until term when plasma cortisol concentrations are 50-90ng/ml (Wintour *et al.* 1975; Norman *et al.* 1985). Isotope kinetic studies have shown that much of the cortisol present in fetal circulation between 100 and 120 days of gestation is of maternal origin and that only after 120 days does the fetus become a major contributor to plasma cortisol concentrations (Hennessy *et al.* 1982).

ACTH and adrenal maturation

ACTH does not cross either the sheep or human placenta (Jones *et al.* 1975; Mulchahey *et al.* 1987). Therefore fetal plasma ACTH concentrations depend entirely upon fetal ACTH production. Liggins and Kennedy (1968) were the first to demonstrate that hypophysectomy of ovine fetuses resulted in hypoplasia and atrophy of the fetal adrenal cortex, which was accompanied by prolongation of pregnancy. In addition, ACTH infusion to intact fetuses resulted in a precocious

increase in cortisol production and the onset of premature parturition (Liggins, 1968). *In vitro* studies confirmed that ACTH acts directly upon adrenocortical cells to stimulate cortisol secretion (Durand *et al.* 1984).

The mechanisms of ACTH action on adrenal steroidogenesis have been explored extensively. Infusion of synthetic ACTH ($ACTH_{1-24}$) to ovine fetuses for 72-100 hours increased $P450_{C17}$ mRNA levels, protein content and activity (Durand *et al.* 1982). Tangalakis *et al.* (1990) also found that $P450_{C17}$ and $P450_{SCC}$ mRNA levels increased significantly in response to a 24h ACTH infusion at 100 days of gestation. ACTH is also able to increase the sensitivity of adrenocortical cells to further ACTH stimulation. Cultured adrenocortical cells from ovine fetuses pretreated with ACTH *in vivo* have increased ACTH receptor numbers, enhanced coupling between the ACTH receptor, G_s protein and adenylyl cyclase, enhanced adenylyl cyclase activity and increased cAMP output when they are stimulated with ACTH *in vitro* (Durand *et al.* 1981; Manchester *et al.* 1983; Durand *et al.* 1985). In addition, intra-fetal administration of ACTH + metopirone, an 11β-hydroxylase inhibitor, blocked the ACTH-stimulated increase in adrenocortical responsiveness seen *in vitro* from fetuses that received ACTH alone (Lye and Challis, 1984). The authors suggested that cortisol may mediate the ACTH effects on adrenal responsiveness (Lye and Challis, 1984). Subsequent research has shown that the ACTH induced cAMP increase *in vitro* is enhanced by the presence of dexamethasone and significantly inhibited by the addition of the steroidogenesis inhibitor aminogluthetimide, which blocks $P450_{SCC}$ (Darbeida and Durand, 1987). These data

are consistent with a role of glucocorticoids in promoting the maturation of adrenal steroidogenesis.

An additional role of ACTH in adrenal maturation that has been postulated is the ACTH promotion of mitogenesis. Studies in the sheep have shown that fetal hypophysectomy-induced adrenal hypoplasia was reversed by ACTH supplementation (Boshier *et al.* 1981; Robinson, 1983) and parturition occurred at normal term (Liggins *et al.* 1967; Jacobs *et al.* 1994; Poore *et al.* 1997). Infusion of ACTH to intact ovine fetuses not only increased steroidogenesis, but also increased fetal adrenal weight by induction of hyperplasia and hypertrophy (Liggins, 1969; Robinson *et al.* 1983). However, the mitogenic effects of ACTH on adrenocortical cells could not be demonstrated *in vitro* (Challis and Brooks, 1989). Further studies have reported that ACTH has antimitogenic effects on adrenocortical cells (Masui and Garren, 1971; O'Hare and Neville, 1973; Hornsby and Gill, 1977). The reason for the contradictory *in vitro* and *in vivo* responses may be explained by a locally produced growth factor not present *in vitro*, or due to the destruction of the extracellular matrix in the *in vitro* system. In addition, it has been shown that intra-fetal ACTH administration selectively increases blood flow to the sheep fetal adrenal cortex, therefore potentially providing more nutrients, and growth factors to promote adrenal growth *in vivo* (Carter *et al.* 1993; Challis and Brooks, 1989).

ACTH receptor

ACTH binds to the melanocortin-2 receptor commonly called the ACTH receptor (ACTH-R). ACTH-R is a G_s-protein linked seven-transmembrane receptor,

which utilizes the adenylyl cyclase second messenger signaling pathway. ACTH receptors were identified in fetal sheep adrenal subcellular preparations (Durand, 1979). ACTH binding sites increase 5-fold from 123 days of gestation until birth, of which 80% of this increase occurs after day 140 (Durand, 1979). The rise in ACTH-R abundance was associated with adrenal cortical hyperplasia and increases in the number of receptors per cell (Durand, 1979). However, there was no change in the affinity of the ACTH-R throughout gestation (Durand, 1979). It has been postulated that during mid-gestation adrenal insensitivity may be due to a lack of GTP and low α_s activity (Durand *et al.* 1985b) since ACTH (10^{-6}M) added alone, to adrenocortical cells (120 days of gestation) in culture, did not elicit an increase in intracellular cAMP. However, ACTH in the presence of GTP or GTP analogues did elicit an increase in cAMP from cultured adrenocortical cells collected from fetuses at 120 days of gestation (Durand *et al.* 1981). Challis and Roberts (1988) have also reported that G_s activity was a relative rate-limiting step to ACTH-stimulated conversion of ATP to cAMP however, this did not appear to change as a result of treating fetuses with $ACTH_{1-24}$ *in vivo*. These data are consistent with a lack of GTP *in vivo* in mid-gestation contributing to the lack of adrenal responsiveness to ACTH at this time. Conversely, at 140d when the adrenal is responsive, ACTH alone did increase cAMP levels (Durand *et al.* 1984).

Intra-fetal $ACTH_{1-24}$ administered at 115 days of gestation, for 5 days, increased the number of adrenal ACTH receptors (Durand *et al.* 1981), and this was correlated with an increase in adenylyl cyclase responsiveness *in vitro* (Durand *et al.* 1981). Sheep fetuses treated with ACTH *in vivo* (100h) had increased adrenal 17α-

hydroxylase and 3β-HSD activity (Challis *et al.*1982; Manchester *et al.* 1983). In addition, Darbeida and Durand (1990) have shown enhanced adrenal cortical cell ACTH-R number and cAMP induced cholesterol translocation from cytoplasm into mitochondria after dexamethasone treatment. It has been recently shown that dexamethasone or ACTH treatment of adrenocortical cells in culture increased ACTH-R mRNA levels (Picard-Hagen *et al.* 1997). These data suggest that ACTH not only increases ACTH-R mRNA, ACTH-R number and steroidogenesis directly but also stimulates an intra-cortical feed-forward effect of glucocorticoids on ACTH-R number and steroidogenesis in the late-gestation ovine fetus.

High molecular weight POMC-derived peptides

It has been suggested previously that decreased adrenal responsiveness during mid-gestation may be due to an inhibitory substance preventing the adrenal from responding to ACTH (Roebuck *et al.* 1980). High molecular weight POMC-derived peptides are weakly steroidogenic at high concentrations, in the rat *in vitro* and *in vivo* (Estivariz *et al.* 1982; Gasson, 1979), and in the sheep fetus *in vitro* (Roebuck *et al.* 1985). However, at physiological levels high molecular weight POMC-derived peptides have been shown to inhibit ACTH-induced steroidogenesis in fetal sheep adrenocortical cells (Jones and Roebuck, 1980; Roebuck *et al.* 1980; Schwartz *et al.* 1995). Therefore it has been suggested that the balance between the proposed stimulatory action of $ACTH_{1-39}$, and the proposed inhibitory action of high molecular weight POMC-derived peptides, is involved in the regulation of fetal adrenal responsiveness and basal cortisol secretion (Roebuck *et al.* 1980; Durand *et*

al. 1982; Schwartz *et al.* 1995). This hypothesis correlates well with the pattern of decreasing large molecular weight POMC-derived peptides in the fetal circulation and increasing plasma $ACTH_{1-39}$ and adrenal responsiveness as gestation progresses (Saphier *et al.* 1993).

1.2 Extra-Pituitary Sites of ACTH Production

It has been postulated that during fetal life additional sources of ACTH production may contribute to the late gestation rise in plasma ACTH. Extra-pituitary ACTH production may be responsible for the detectable levels of plasma ir-ACTH concentrations in hypophysectomized fetuses with functional adrenal glands (Poore *et al.* 1997). Adrenal function was maintained in hypophysectomized fetuses (surgery performed at 115 days of gestation) with a low-dose ACTH infusion, which achieved plasma ir-ACTH concentrations of 60pg/ml. The infusion was stopped for a period of four hours (days 132-138 of gestation) and plasma ACTH concentrations remained at 30pg/ml throughout (Poore *et al.* 1997). Considering the short half-life of $ACTH_{1-24}$ (20 seconds) in fetal sheep plasma (Jones *et al.* 1978), the authors suggested that the maintenance of ir-ACTH in the plasma of hypophysectomized fetuses was due to the contribution of extra-pituitary sites of ACTH production. This is the first study to maintain adrenal function with an ACTH infusion in hypophysectomized fetuses and subsequently measure the level of endogenous ACTH during a break in the ACTH infusion. Therefore, the stimulus to extra-pituitary sites of ACTH production may be a functional adrenal gland or the product of a functional adrenal gland, cortisol. The ACTH concentrations reported in this study

are high because the ACTH antibody (raised by Prof. Greg Rice) used recognizes $ACTH_{1-39}$, $ACTH_{1-24}$, and additional high molecular weight ACTH-related peptides.

Many alternative sites containing POMC-derived peptides have been identified; these include the human brain, thyroid, pancreas, gastrointestinal tract, placenta, gonads, skin, thymus, leukocytes, spleen, kidney, lung, and adrenal glands (Smith and Funder, 1988). However, the transcript length of POMC in many of these extra-pituitary sites differs from that seen within the pituitary. Human adrenal, thymus, placenta, skin, leukocytes and testis, and rat testis, ovary, and reproductive tract POMC mRNA was reported to be 400 nucleotides (nt), shorter than pituitary POMC (1200 nt) (Lacaze-Masmonteil *et al.* 1987; Jacobson and Drouin, 1994; Bardin *et al.* 1987). It was determined that the smaller transcript size was not due to heterogeneity in the length of the poly(A) tails (Bardin *et al.* 1987). S1 nuclease mapping analysis revealed that the POMC mRNA isolated from rat pituitary, testes and ovary shared the identical nucleotide sequences encoding ACTH, βLPH, and the 3' untranslated region, therefore it was deduced that the heterogeneity occurred within the 5' portion of the mRNA (Bardin *et al.* 1987). In fact, the shorter transcript lacked exon 1 and exon 2 (Lacaze-Masmonteil *et al.* 1987), which included the primary transcriptional initiation site. However, at least six other start sites have been identified in several non-pituitary tissues (adrenal, thymus, testes, spleen, kidney, ovary, lung, thyroid, and GI tract), between 41 and 162 nucleotides downstream from the 5' end of exon 3. The truncated molecules that might be translated would be devoid of a signal peptide, suggesting that they could not be secreted (Lacaze-Masmonteil *et al.* 1987; Clark *et al.* 1990). However, these tissues have been shown

to contain ACTH, N-terminal glycopepide$_{1-61}$, and βendorphin (DeBold *et al.* 1988) indicating that POMC is cleaved. In heterologous cells transfected with the truncated human POMC gene, translation occurred but the peptide products were not secreted (Clark *et al.* 1990). However, the human placenta also produces the truncated gene product and in perifusion studies the placenta secretes ACTH under basal conditions (Margioris *et al.* 1988; Waddell *et al.* 1993) and increases ACTH-output in response to CRH stimulation (Petraglia *et al.* 1987). These data provide evidence for the contribution of extra-pituitary sites of ACTH production to circulating ACTH concentrations *in vivo*, despite the truncated form of POMC mRNA.

1.2.1 ACTH in the placenta

The detection of POMC-derived peptides in extra-pituitary tissues was initially thought to represent peptides sequestered from circulating pools. However, Genazzani *et al.* (1974) were the first to show extra-pituitary POMC production. They showed that human placental cells could produce ir-ACTH *in vitro* (Genazzani *et al.* 1974). These studies were confirmed and extended by the finding of two high molecular weight proteins (48kDa, 36kDa) both of which were detected in βendorphin, βLPH, and ACTH radioimmunoassays (Odagiri and Sherrell, 1979). Subsequently, uptake studies with radiolabeled amino acids have conclusively established the placenta as a site of POMC biosynthesis (Liotta *et al.* 1980). Pulse-chase experiments on cultured human placental trophoblasts showed that ACTH$_{1-39}$ and β-LPH are the initial products, however, after 4 hours the predominant placental products are αMSH and βendorphin (Liotta *et al.* 1977; Liotta *et al.* 1982). POMC-

derived peptides separated from extracts of normal term human placenta by gel filtration were found to coelute with $ACTH_{1-39}$, β-endorphin, βLPH, and a putative high molecular weight precursor (Liotta *et al.* 1977; Liotta and Krieger, 1980).

Glucocorticoids stimulate placental CRH production *in vitro* (Jones and Challis, 1990) and *in vivo* (Korebrits *et al.* 1998; Marinoni *et al.* 1998), which in turn may increase placental ACTH (Petraglia *et al.* 1987) and thereby provide a potential, glucocorticoid-stimulated source of extra-pituitary ACTH. The function of POMC-derived peptides produced in the placenta is not known. ACTH may act as an immunomodulator, or as vasodilator in the placental vasculature (Clifton *et al.* 1996), or ACTH may be secreted into the circulation and exert effects on HPA axis function.

The presence of ACTH peptide in the ovine placenta was reported by Keller-Wood and Wood (1991a). Placental ir-ACTH concentrations increased from 43 to 65 days of gestation and remained elevated until term (Jacobs and Challis, 1989). However, placental POMC mRNA levels have not been examined thoroughly during pregnancy in the sheep. POMC mRNA has been detected in the rat placenta (Pintar *et al.* 1984), and there was no change in POMC mRNA abundance during pregnancy (Bardin *et al.* 1987). In the sheep fetus during late-gestation (122-142 days of gestation), differences in ACTH concentrations in the umbilical vein compared to a femoral and/or carotid artery could not be detected under basal or hypoxemic conditions (Keller-Wood and Wood 1991a). The authors concluded the ovine placenta did not secrete ACTH (Keller-Wood and Wood 1991a), however, this study does not address the possibility that the placental uptake equaled placental secretion of ACTH.

The data regarding CRH secretion from the ovine placenta are conflicting. There is an increase in umbilical vein CRH concentrations during the final week of pregnancy (Jones *et al.* 1989; Keller-Wood and Wood, 1991b). In addition, fetal plasma CRH concentrations rose in response to reduced uterine blood flow, inducing an hypoxemic insult of reduced fetal pO_2 by 10mmHg, at days 123-135, and greater increases were seen at days 139-143 of gestation (Jones *et al.* 1989). A hypoxemic insult of reduced fetal pO_2 of ~5mmHg provoked no change in plasma CRH concentrations in the umbilical vein compared to the fetal femoral artery or in fetal or maternal carotid artery plasma CRH concentrations at 128-130 days or at 138-142 days of gestation (Keller-Wood and Wood, 1991b). Plasma CRH and ACTH concentrations increased when uterine blood flow was reduced such that fetal pO_2 was decreased by ~8mmHg between 120-125 days of gestation, but there was no difference in umbilical vein compared to femoral artery plasma concentrations of either ACTH or CRH (Sue-Tang *et al.* 1992). Therefore, it appears that the placenta can secrete CRH later in gestation with more severe hypoxemic insults; this is consistent with both a more responsive HPA axis to stress and higher circulating plasma cortisol concentrations in late gestation.

1.2.2 ACTH in the fetal lung

In the fetal sheep lung POMC mRNA is present and the levels increase from 135 days of gestation to term (Deol *et al.* 1995). Similarly, ir-ACTH peptide was present in the fetal sheep lung from 64 days of gestation until term, and from day 100 until term ir-ACTH was present within the bronchiolar epithelium and the sero-

mucous glands (Deol *et al.* 1995). It has also been shown that the content of ir-ACTH in the ovine fetal lung declines from 90 days of gestation to adulthood (Cudd *et al.* 1993). Western blot analysis showed that the ir-ACTH measured in the fetal lung by Cudd *et al.* (1993) was predominantly large molecular weight ACTH, and no $ACTH_{1-39}$ was detected. This may be due to a rapid secretion of $ACTH_{1-39}$ as it is produced, or it may be indicative of the lung as a source of ACTH-precursors which may be cleaved to bioactive $ACTH_{1-39}$ in plasma or at the site of action as has been previously suggested (Roebuck *et al.* 1980). A decrease in ACTH content in the fetal lung may reflect an increase in secretion with advancing gestation. It has been reported that the fetal lung releases ACTH in sufficient amounts to contribute to circulating hormone concentrations of ACTH (Cudd *et al.* 1993). Pulmonary arterio-venous differences measured in ovine fetuses from 120-145 days of gestation, showed that at fetal plasma ir-ACTH ($ACTH_{1-39}$ and large molecular weight ACTH-related peptides) concentrations of <200pg/ml the lung secretes ir-ACTH into circulation, and at concentrations >200pg/ml the lung clears ir-ACTH from plasma (Cudd and Wood, 1995). However, ir-ACTH isolated from the fetal sheep lung did not stimulate steroidogenesis in an adrenal cell bioassay. Since the lung contains predominantly large ACTH peptides, homogenates would contain primarily large ACTH peptides (Cudd *et al.* 1993), and therefore does not preclude the steroidogenic capability of ACTH secreted from the fetal lung.

1.3 Glucocorticoid effects on ACTH

Regulation of the HPA axis relies on cortisol action at the hippocampus, hypothalamus and pituitary to inhibit ACTH output in a classical negative feedback loop. Some aspects of this mechanism hold true for the fetus, however in late-gestation when plasma ACTH and cortisol concentrations rise concomitantly, the efficacy of cortisol negative feedback appears to decrease.

1.3.1 Hippocampus and Hypothalamus

The hippocampus is sensitive to corticosteroid inhibition (Jacobson and Sapolsky, 1991). The hippocampus contains both mineralocorticoid receptors and glucocorticoid receptors. The mineralocorticoid receptor binds cortisol with greater affinity than the glucocorticoid receptor, and therefore mineralocorticoid receptors are thought to mediate basal HPA function when circulating cortisol concentrations are low, while glucocorticoid receptors regulate HPA axis activity during stress when plasma cortisol concentrations are elevated (Meijer and de Kloet 1998).

Glucocorticoid negative feedback on hypothalamic function *in vivo* is evident between 120-130 days of gestation since CRH mRNA levels in the PVN decrease with fetal glucocorticoid treatment and increase after fetal adrenalectomy (Myers *et al.* 1991). However, the sensitivity of the hypothalamus to glucocorticoid inhibition appears to vary with gestational age. *In vitro*, perifusion of the ovine fetal hypothalamus, with the addition of dexamethasone (2.5×10^{-5} M) inhibits basal CRH secretion at day 140 but not at day 100 of gestation. Furthermore, CRH release was stimulated by potassium induced depolarization of the nerve terminals, even in the

presence of dexamethasone, suggesting that dexamethasone suppression of basal CRH release does not occur at the nerve terminals (Brooks *et al.* 1989).

1.3.2 Pituitary

Glucocorticoids suppress pituitary ACTH synthesis and secretion by inhibition of hypothalamic CRH, and by direct inhibition of POMC expression via the glucocorticoid regulatory element on the POMC gene. In addition, glucocorticoids can inhibit ACTH secretion from the corticotroph (Birnberg *et al.* 1983), possibly by decreasing intra-cellular Ca^{2+} levels (Holm and Majzoub, 1995). Alternatively, in the fetus glucocorticoids also play an important role in corticotroph maturation, mediating both the switch from "fetal" to "adult" corticotrophs, and the change in the ratio of ACTH-related peptides in the pituitary and plasma from bioactive to ir-ACTH seen during late gestation (as discussed previously).

Pars intermedia cells collected from adrenalectomized fetal sheep had increased ACTH secretion in response to CRH-stimulation *in vitro* compared to pars intermedia cells collected from intact fetuses (Fora *et al.* 1996). These data suggest that cortisol may inhibit pars intermedia function however, the direct effects of cortisol on the fetal pars intermedia have not been examined. In the adult rat, POMC mRNA in the pars intermedia has been shown to increase in response to glucocorticoids, while pars distalis levels decrease (Autelitano *et al.* 1987). The adult rat pars intermedia has also been shown to lack a functional glucocorticoid receptor (GR) and is therefore resistant to glucocorticoid inhibition (Antakly and Eisen, 1984). This suggests that the glucocorticoid-stimulation of pars intermedia POMC mRNA in

the adult rat (Autelitano *et al.* 1987) was not due to the direct effects of glucocorticoids on the pars intermedia. The human adult pars intermedia has also been shown to be less sensitive than the pars distalis to the negative effects of glucocorticoids. In humans exposed to elevated glucocorticoids, the pars distalis corticotrophs undergo a morphological change called Crooke's hyaline change (Asa *et al.* 1995). The corticotrophs of these patients contain a glassy, homogenous substance in the cytoplasm, which is composed of keratin filaments and contains no ir-POMC-derived peptides (Asa *et al.* 1995). However, in the pars intermedia of patients with glucocorticoid excess there is absent or only mild filament accumulation and a complete lack of the Crooke's hyaline change that is found in the pars distalis of these patients (Asa *et al.* 1995). This is compelling evidence that in adult rats and humans, pars distalis and pars intermedia POMC mRNA levels are differentially affected by glucocorticoids. If this is also true in the fetus, the pars intermedia may provide an important site of ACTH production in the presence of high circulating cortisol levels.

The fetal sheep pars intermedia does express GR mRNA (Matthews *et al.* 1995), however, there are no studies published that examine GR function in the sheep pars intermedia. During prolonged periods of hypoxemia in the fetal sheep the pars distalis and the pars intermedia are differentially regulated. After 48 hours of hypoxemia pars distalis POMC mRNA levels were significantly increased but pars intermedia POMC mRNA levels were significantly suppressed (Braems *et al.* 1996). After 21 days of placental embolization pars distalis POMC mRNA levels were not different from control levels however, POMC mRNA levels in the pars intermedia

were significantly suppressed (Murotsuki *et al.* 1996). The decrease in pars intermedia POMC expression may be a direct effect of increased plasma cortisol concentrations or it could be due to increased dopamine suppression of the pars intermedia. Despite the ambiguity of cause, these studies provide evidence for differential regulation of POMC in the pars intermedia and pars distalis by glucocorticoids and therefore reinforce the need to explore the role of the pars intermedia in the regulation of the fetal HPA axis.

1.3.3 The fetal lung and placenta

Glucocorticoids impact upon lung and placental function in fetal life, stimulating structural maturation in the fetal lung, and inducing placental $P450_{C17}$. However, there are few studies examining the effects of cortisol on ACTH production, synthesis, release or action in the fetal lung or sheep placenta. ACTH secretion from the ovine placenta was not detected under basal or hypoxemic conditions in late gestation (Keller-Wood and Wood, 1991a; Sue-Tang *et al.* 1992). Placental POMC mRNA levels were below the level of detection by Northern blot analysis in intact or adrenalectomized fetuses (McMillen *et al.* 1990). However, placental CRH secretion did increase in response to a 10mmHg reduction in fetal pO_2 in late gestation (Jones *et al.* 1989). In the human placenta, cortisol stimulates CRH mRNA and peptide levels (Petraglia *et al.* 1987; Jones and Challis 1989, 1990). CRH in turn increases placental ACTH output (Margioris *et al.* 1988) indicating that there may be an indirect effect of glucocorticoids on placental ACTH output. However, the direct effects of glucocorticoids upon human placental ACTH

production have not been reported. The effects of sustained increases in fetal plasma cortisol concentrations on ovine placental ACTH production are similarly not known.

1.4 Regulation of Glucocorticoid Action

1.4.1 Corticosteroid-binding globulin (CBG)

1.4.1.1 Discovery and characterization of CBG

W. H. Daughaday first discovered the presence of a moiety in plasma that bound cortisol with high affinity (Daughaday, 1956). Studies conducted by Sandberg *et al.* (1957) examining cortisol binding activity in whole blood confirmed the existence of a high affinity cortisol binding protein, and using equilibrium dialysis techniques, Bush (1957) also identified a high-affinity binding protein with low capacity. This protein was called transcortin, and subsequently corticosteroid-binding globulin or CBG (Daughaday, 1958; Slaunwhite and Sandberg, 1959).

CBG functions as a monomer and has one steroid binding site per molecule (Mickelson *et al.* 1982). The association constant of CBG in the sheep is 1.2×10^{-8}M in the fetus (Fairclough and Liggins, 1975) and 0.87×10^{-8}M in the adult (Paterson and Hills, 1967). It has been reported that CBG may also bind other steroids, such as progesterone (Westphal, 1986). In humans the association affinity is approximately the same for cortisol and progesterone ($K_a=10^{-8}$M). However, CBG in the circulation predominately binds cortisol, although cortisol and progesterone will compete for CBG binding sites in progesterone rich tissues (Hammond, 1997). In the sheep fetus, a 100-fold excess of progesterone only displaced 11.4±2.2% of

1nM [^{3}H]cortisol in plasma (Ali *et al.* 1992), therefore indicating that ovine CBG has a low cross-reactivity between cortisol and progesterone (Ali *et al.* 1992; Challis *et al.* 1985).

CBG is well conserved throughout vertebrates and the molecular weight of CBG in all species examined is consistently 50-60 kDa when estimated using polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (Kato *et al.* 1988). The molecular weight of ovine CBG was 57 kDa by PAGE (Berdusco *et al.* 1993), which is consistent with the sedimentation coefficient determined value of 57.5 kDa (Kato *et al.* 1988).

The cloning of CBG from the human (Hammond *et al.* 1987), rat (Smith and Hammond, 1989), and rabbit (Seralini *et al.* 1990) revealed sequence homologies with α1-proteinase inhibitor and thyroxine binding globulin (Hammond *et al.* 1987), both of which are members of the serine proteinase inhibitor (SERPIN) superfamily (Travis and Salvasen, 1983). Furthermore, the CBG gene has been localized to chromosome 14 in the human genome (Seralini *et al.* 1990), where α1-proteinase inhibitor and thyroxine binding globulin are also localized (Seralini *et al.* 1990). Ovine CBG was cloned by Berdusco *et al.* (1993), and reported to be greater than 75% homologous within the coding regions for rat, rabbit and human CBG (Berdusco *et al.* 1993). Ovine CBG has five consensus sites for N-glycosylation and glycosylation at position 237 in the ovine is conserved in all species examined to date (Hammond *et al.* 1991). Glycosylation at this site is necessary for the formation of the steroid-binding site (Avvakumov *et al.* 1992), because it interacts with the nascent polypeptide chain and is required for the proper folding of CBG during synthesis to

form a functional steroid-binding site (Avvakumov *et al.* 1993). Human CBG contains three biantennary and two triantennary N-linked carbohydrate chains. In the sheep fetus the number of biantennary chains increases and triantennary decreases with advancing gestational age (Berdusco *et al.* 1995). These differences in glycosylation may be important in altering the metabolic clearance rate of the protein (Berdusco *et al.* 1995). Ovine CBG has 68% sequence identity with human CBG, however, ovine CBG includes an additional 25 residues at the C-terminus (Berdusco *et al.* 1993). Ovine CBG contains a GAA codon in the position of a TAA stop codon as found in the CBG cDNA's of other species (Berdusco *et al.* 1993). The function of this 3' extension is not known.

1.4.1.2 Function of CBG

CBG is primarily considered as a cortisol transport system that maintains a circulating pool of cortisol, regulates cortisol bioavailability, and increases the circulating half-life of cortisol. CBG is a specific substrate for the serine-protease elastase, which cleaves the glycoprotein close to its carboxy-terminus to release the bound glucocorticoid (Hammond *et al.* 1990). This occurs at sites of inflammation where elastase is activated, providing a mode of delivery of the anti-inflammatory, cortisol, to the required site of action (Hammond *et al.* 1990). In addition, membrane-binding sites for CBG have been reported within the adult rat pituitary (Koch *et al.* 1977) and liver (Maitra *et al.* 1990), the human placenta (Avvakumon *et al.* 1989) and mammary carcinoma cells (Nakhla *et al.* 1988). Two classes of CBG receptors have been characterized with kD values in the μM and nM range respectively. The

lower affinity receptor was isolated from decidual epithelium and its structure has been described as a complex of four identical sialoglycoproteins of 20kDa each (Strel'chyonok and Avvakumov, 1991). However, the function of this receptor is not yet known. In adult rat hepatocyes, *in vitro*, unliganded CBG binds to the CBG receptor and with the subsequent binding of cortisol, elicits a rise in intra-cellular cAMP (Maitra *et al.* 1990), which may impact on gene expression.

There has only been one reported case of total CBG deficiency in humans (Roitman *et al.* 1984); however this claim has not been confirmed. Furthermore, a study examining CBG levels in over 10,000 subjects failed to reveal any individuals with a lack of CBG (Rosner *et al.* 1973). The latter findings suggest that CBG is necessary for independent life and a null mutation is likely lethal *in utero* (Rosner *et al.* 1973).

1.4.1.3 Age and species variation

The liver is the major site of CBG mRNA abundance in all species examined including the human adult (Hammond *et al.* 1987; Smith and Hammond 1989), the baboon fetus (Pepe *et al.* 1996), the sheep fetus (Berdusco *et al.* 1995), and the fetal (Scrocchi *et al.* 1993b) and adult mouse (Scrocchi *et al.* 1993a). However, in the neonatal mouse the kidney is the predominate site of CBG mRNA (Scrocchi *et al.* 1993a). Subtotal hepatectomy in the rat results in a decrease in plasma concentrations of CBG (Gala and Westphal, 1966), and there is a reduced plasma concentration of CBG in humans with liver disease (Doe *et al.* 1964). These studies indicate that hepatic production of CBG is reflected by plasma CBG concentrations,

and furthermore, plasma CBG concentrations in the rat, rabbit and sheep correlate well with hepatic CBG mRNA abundance (Smith and Hammond 1991; Berdusco *et al.* 1995).

CBG has also been localized to extra-hepatic sites of production. Immunohistochemical studies have shown CBG in lymphocytes (Werthamer, 1974), and uterus (Gueriguian, 1974) in humans, the rat kidney, uterus, thyroid, and anterior pituitary (Kuhn *et al.* 1986), as well as the rabbit lung (Giannopoulus, 1976). CBG mRNA expression has been reported in the maternal lung, spleen and ovary, and in the fetal kidney of the rabbit (Seralini *et al.* 1990). CBG mRNA has also been localized in the fetal sheep kidney, pituitary, adrenal, lung and hypothalamus (Berdusco *et al.* 1995), the fetal mouse exocrine pancreas, (Scrocchi *et al.* 1993b) and in the neonatal mouse kidney (Scrocchi *et al.* 1993a). The expression of CBG within extra-hepatic sites is low relative to the liver, and its significance has not yet been clearly defined. CBG has also been reported in human bronchioalveolar fluid (Loric *et al.* 1989), cerebrospinal fluid (Predine *et al.* 1984), breast milk (Rosner, 1976) and amniotic fluid (Challis and Bennett, 1977). However, the function of CBG in these fluids in not clear.

Generally, CBG levels are high during fetal life and decline either just prior to or after parturition and remain low in adulthood. In the fetal sheep plasma CBG, measured as corticosteroid-binding capacity (CBC), increased progressively from day 122 (28±6 ng/ml) until the day of delivery (85±14 ng/ml) (Berdusco *et al.* 1995). The changes in CBC reported by Berdusco *et al.* (1993) are consistent with those reported in previous studies (Fairclough and Liggins, 1975; Ballard *et al.* 1982). Ali *et*

al. (1992) also examined the level of cortisol binding earlier in gestation and found very high levels at days 40 and 56 of gestation, which decreased by 50% at mid-gestation (90 days). It is interesting to note that the ontogeny of plasma CBG levels is similar to that of adrenal responsiveness and plasma cortisol concentrations in the fetal sheep, as discussed previously (Section 1.1.3.3).

In the sheep fetus, the percent of free cortisol in plasma remains relatively unchanged during the final 20 days of gestation. However, due to the increases in total plasma cortisol concentrations during this period, the concentration of free cortisol increases moderately 5-10 days before parturition (compared to 15-20 days prior to parturition). Plasma free cortisol concentrations increase 8-fold on the day of parturition compared to the free cortisol concentrations measured five days previous to parturition (Berdusco *et al.* 1995). CBG mRNA levels in fetal sheep liver were significantly elevated at day 140 compared to day 100 and day 125 of gestation, but levels were decreased in liver collected from fetuses in active labour (Berdusco *et al.* 1995). The authors postulated that the pre-partum fall in hepatic CBG mRNA might be the emergence of the adult pattern of decreased CBG in response to glucocorticoids, as a consequence of the total and free cortisol concentrations reaching their highest values (Berdusco *et al.* 1995). In the fetal sheep, CBG appears to be important in regulating free cortisol concentrations up to the day of parturition (Berdusco *et al.* 1995), and thereby may reduce cortisol inhibition of pituitary ACTH production/secretion allowing for the concomitant rise in ACTH and cortisol which precedes parturition in that species.

Maternal plasma CBG levels increase during human pregnancy and are approximately two-fold greater by the end of second-trimester compared to plasma CBG levels in non-pregnant humans (Doe *et al.* 1964; Moore *et al.* 1978). Maternal CBG levels continue to rise throughout gestation to reach maximal levels just prior to birth (Murao *et al.* 1986). This rise in plasma CBC has been attributed to elevated serum estradiol concentrations during pregnancy (Westphal, 1971; Brien, 1981). The plasma profiles of CBG in pregnant women are in contrast to those in sheep where plasma concentrations of CBG in the ewe do not change during gestation (Patterson and Hills 1967; Fairclough and Liggins, 1975; Ali *et al.* 1992). There is no change in the affinity of CBG in pregnant compared to non-pregnant sheep (Patterson and Hills, 1967). In humans the affinity constant of CBG in fetal and maternal circulation is 5-8 x 10^{-8}M at 4°C (Hadjian, 1972), and this not different from that in the non-pregnant adult. In humans, a pregnancy-associated variant of CBG has been identified in all serum samples tested from the sixth month of pregnancy to term, and at term the variant accounted for 7-14% of total CBG in the circulation (Avvakumov and Strel'chyonok, 1987). The variant has only triantennary carbohydrate moieties (Strel'chyonok, *et al.* 1984), unlike the usual combination of two triantennary and three biantennary oligosaccharide chains (Akhrem *et al.* 1982; Strel'chyonok *et al.* 1982). The source and function of this pregnancy-associated variant have not been identified.

1.4.1.4 CBG Regulation

Elevated estrogen levels in women, whether due to endogenous increases during pregnancy, or exogenous administration, stimulate an increase in plasma CBG (Doe *et al.* 1964; Sandberg *et al.* 1964). However, there is no difference in plasma CBG concentrations between men and non-pregnant women, and no variation during the menstrual cycle (Rosner, 1990). Progestin administration has been reported to increase serum CBG concentrations by more than 20% in both males and females (Gala and Westphal, 1965). Conversely, glucocorticoid excess, in Cushing's patients or patients receiving exogenous natural or synthetic glucocorticoids significantly reduces plasma CBG levels (30-40%) (Schlechte and Hamilton, 1987; Frairia *et al.* 1988).

Glucocorticoid infusion to fetal sheep (Berdusco *et al.* 1993; Berdusco *et al.* 1994), neonatal mice (Zhao *et al.* 1997a) and the baboon fetus in mid-gestation (Pepe *et al.* 1996), stimulated hepatic CBG mRNA and plasma CBG levels. However, the CBG response to glucocorticoids appears to be reversed with maturation. In baboon fetuses, betamethasone treatment near-term did not alter plasma CBG levels (Pepe *et al.* 1996), and in adult primates exogenous glucocorticoids suppressed plasma CBG (Stanczyk *et al.* 1985). In the sheep the switch occurs later in gestation than the baboon, since fetuses at day 130-135 of gestation (term =145 days) responded to exogenous glucocorticoids with increased hepatic CBG mRNA and raised plasma CBC levels (Berdusco *et al.* 1993). However, in mice the change in CBG responsiveness to glucocorticoids does not occur until post-puberty, since serum CBG and hepatic CBG mRNA levels are increased by

dexamethasone treatment up to post-natal day 14 (Zhao *et al.* 1997a). Glucocorticoid administration to adult sheep (Berdusco *et al.* 1993) and mice (Zhao *et al.* 1997a) significantly suppressed hepatic CBG mRNA and plasma CBC levels. The effects of glucocorticoids on CBG production occur despite the apparent lack of a glucocorticoid regulatory element on the characterized human (Underhill *et al.* 1989) and rat (Zhao *et al.* 1997b) CBG gene. Therefore, these effects may be mediated by the inhibition of binding of stimulatory transcription factors.

1.4.2 Glucocorticoid Receptors

Glucocorticoids exert effects at the cellular level via a nuclear glucocorticoid receptor (GR). GR belongs to a superfamily of nuclear receptors, which includes receptors for mineralocorticoids, progestins, estrogens, androgens, vitamin D, retinoic acid, and thyroid hormone, as well as a large number of orphan receptors for which no specific ligand has yet been identified (Bamberger *et al.* 1996). All members of the nuclear hormone receptor family share a common three-domain organization. The N-terminal domain contains sequences responsible for activation of target genes and interacts with components of the basal transcription machinery and/or other transcription sites. The central portion of the receptor molecule contains the DNA-binding domain and is involved in receptor dimerization, nuclear translocation and transactivation (Giguere *et al.* 1986). The C-terminal region contains the ligand-binding domain, and sequences important in heat-shock protein (hsp) binding, nuclear translocation, dimerization and transactivation (Hollenberg and Evans 1988; Hutchinson *et al.* 1993). Glucocorticoids are lipophilic and diffuse

across the plasma membrane and bind to GRs in the cytosol. The action of ligand binding activates the GR, and it sheds the chaperone proteins consisting of two molecules of hsp90, and one molecule each of hsp70, hsp 56, and an immunophilin, revealing the DNA binding sites (Hutchinson *et al.* 1993). The activated glucocorticoid-GR complex travels into the nucleus where it binds to a glucocorticoid regulatory element (GRE) which may stimulate or inhibit gene expression. Alternatively the complex may interfere with the binding of other transcription factors (eg AP-1) and inhibit gene transcription in this manner (Bamberger *et al.* 1996). In the human alternative splicing of the glucocorticoid receptor primary transcript produces a second isoform of GR, called GRβ, which does not bind cortisol (Bamberger *et al.* 1995). GRβ are reportedly expressed at modest but varying levels in a range of tissues and were proposed to act as ligand-independent negative regulators of glucocorticoid action (Funder, 1997). Although GRβ does not bind glucocorticoids and it is transcriptionally inactive on a glucocorticoid response element, when both forms of GR are expressed in the same cell GRβ inhibits the hormone-induced GR-mediated stimulation of gene expression (Oakley *et al.* 1996). However, the GRβ isoform is not present in the mouse (Otto *et al.* 1997) and there are no reports of the presence of an alternative isoforms of GR in the sheep.

The role of GR-mediated glucocorticoid action is very important during fetal development as is evidenced in mice with a disrupted GR gene. The GR knockout resulted in neonatal death of 80% of homozygous mice within a few hours due to lung atelectasis. Other effects of GR knockout included impaired induction of liver gluconeogenic enzymes and perturbations in the feedback control of glucocorticoid

synthesis via the HPA axis. The alterations in cortisol feedback on the HPA axis resulted in increased plasma ACTH and cortisol concentrations, and extensive hypertrophy and hyperplasia of the adrenal cortex, which was accompanied by medullary disorganization, and the absence of adrenaline synthesizing cells (Berger *et al.* 1996).

1.4.2.1 Hypothalamus

Glucocorticoid receptor binding in the fetal sheep hypothalamus is highest at day 100 and decreases progressively until term, when binding was 12% of day 100 levels (Yang *et al.* 1990). GR binding was not reflected by changes in hypothalamic GR mRNA levels. GR mRNA levels, assessed by northern blot analysis utilizing a cDNA specific for ovine GR, in the fetal sheep hypothalamus remained unaltered from day 60 of gestation until term (Yang *et al.* 1992). Subsequently, Saoud and Wood (1996b) have examined the abundance of ir-GR by western blot and chemiluminescence. The GR antibody used identified two molecular weight species, a 97kDa protein similar to the previously reported size (94kDa) of GR (Bamberger *et al.* 1996), and a 45kDa protein (Saoud and Wood, 1996b). The authors suggest that the half-length ir-GR protein is a proteolytic fragment of the native receptor, and that its abundance reflects the rate of intracellular receptor clearance or turnover (Saoud and Wood, 1996b). Although there was no apparent change in the abundance of the 97kDa ir-GR protein from day 70 to day 141 of gestation, there was a significant increase in the abundance of the half-length ir-GR protein (Saoud and Wood,

1996b). The authors postulate that this reflects an increase in GR turnover as gestation approaches term (Saoud and Wood, 1996b).

1.4.2.2 Pituitary

In the pars distalis of the fetal sheep pituitary, GR binding is highest at d110, and decreases approximately 70% by day 125-130 of gestation; but binding doubled between day 130 and term (Yang *et al.* 1992). Immunoreactive GR protein levels were also measured by western blot analysis (as described above in Section 1.4.2.1). Similar to findings in the hypothalamus, there were no changes in the full-length 97kDa GR protein. The half-length, or proteolytic fragment, of GR protein however, had increased, which may reflect increased intracellular receptor clearance or turnover late in gestation (Saoud and Wood, 1996b). GR mRNA levels measured by northern blot analysis of the fetal sheep pars distalis, were not significantly altered from day 60 of gestation until term (Yang *et al.* 1992). Matthews *et al.* (1995) eliminated the possibility of pars intermedia contamination in the pars distalis by using *in situ* hybridization on full pituitary sections to quantify GR mRNA. This technique also allowed the investigators to assess GR mRNA levels in both pars distalis and pars intermedia. GR mRNA levels in the pars distalis were low throughout gestation, until term when levels increased 4-fold (Matthews *et al.* 1995). Pars intermedia levels of GR mRNA were undetectable in fetuses 60-80 days of gestation, were low at day 100-120, and increased 3-4 fold in fetuses at 130-135 days of gestation and remained at this elevated level until term (Matthews *et al.* 1995). Therefore, GR mRNA levels in the pars distalis appear to remain low during

development but increase dramatically at term when circulating cortisol levels are at their highest. Conversely, pars intermedia GR mRNA levels are present at relatively high levels throughout the late gestational rise in plasma cortisol concentrations. Infusion of cortisol to the ovine fetus for 12 hours did not alter GR mRNA abundance in the pars distalis or the pars intermedia (Matthews *et al.* 1995) however, the effects of longer duration cortisol treatment is unknown. GR binding in the pars distalis (Yang *et al.* 1990) reflected the pattern of GR mRNA levels as assessed by *in situ* hybridization (Matthews *et al.* 1995), and may be accompanied by an increase in the rate of GR turnover (Saoud and Wood, 1996b).

Despite the elevated GR mRNA levels during periods of elevated circulating glucocorticoids, POMC mRNA levels in the pars distalis and the pars intermedia remain elevated. This indicates that either the GR is not functional or that glucocorticoids are not available to bind to the GR, perhaps due to the activity of CBG or 11β-HSD.

1.4.2.3 The fetal lung and placenta

Studies in the fetal rabbit lung showed that cortisone is rapidly converted to cortisol and that the concentration of cortisol binding sites per cell increases during fetal development in the rabbit (Ballard *et al.* 1984). These data indicate that the lung is a target of high glucocorticoid activity during fetal life. As mentioned previously, disruption of the mouse GR gene causes death within the first few hours of life due to severe lung atelectasis (Berger *et al.* 1996), underscoring the importance of glucocorticoids for lung development and fetal outcome. During rat embryogenesis

GR expression appears to be highest just prior to final differentiation, indicating the importance of glucocorticoids in organ maturation (Kitraki *et al.* 1997). In the ovine fetal lung and placenta GR binding was highest at mid-gestation (91-130 days), and decreased at term. Therefore, when plasma cortisol levels are high the receptor binding is low (Flint and Burton, 1984). Hypophysectomy of the fetal sheep significantly decreased plasma cortisol concentrations and resulted in an increase in placental GR binding, but did not alter GR binding in the lung (Flint and Burton, 1984). This indicates that GR levels in the lung and placenta are differentially influenced by cortisol. However, the direct effects of cortisol on GR expression in the lung or placenta have not been examined.

Chapter Two

Rationale

The concomitant rise in plasma ACTH and cortisol concentrations in the fetal sheep during late-gestation indicates that there may be a decrease in cortisol negative feedback at this time. Plasma CBG levels also rise during late gestation and it has been previously hypothesized that in the sheep fetus CBG may be important in maintaining low levels of free and bioactive cortisol, thereby decreasing negative feedback (Liggins *et al.* 1973; Challis and Brooks, 1989; Berdusco *et al.* 1995). Previous studies indicate that the rise in CBG may be stimulated by glucocorticoids. Low dose cortisol infusion (100h) at 100 days of gestation (Berdusco *et al.*1993), and dexamethasone infusion (96h) at 130 days of gestation both resulted in increased hepatic CBG mRNA levels and a rise in plasma CBC (Berdusco *et al.*1994). Conversely, in the adult sheep exogenous glucocorticoids significantly reduce hepatic CBG mRNA and plasma CBG levels (Berdusco *et al.* 1993). The differential effects of glucocorticoids in fetal and adult life have been documented in several mammalian species (Section 1.4.1.4). Therefore, in fetal life, cortisol may stimulate an increase in plasma CBG levels thereby binding free cortisol and reducing the glucocorticoid inhibition of pituitary ACTH output. Furthermore, cortisol effects on POMC mRNA levels may also be regulated by the abundance of corticotroph glucocorticoid receptors. GR binding and mRNA levels were low at 120-130 days of gestation but increased significantly at term (Matthews *et al.* 1995; Yang *et al.* 1992). Therefore alterations in both CBG and GR may regulate glucocorticoid effects on POMC mRNA levels.

Cortisol has been shown previously to have important maturational effects on corticotroph morphology and function (Brieu and Durand, 1987; Antolovich *et al.*

1989; Brieu and Durand, 1989). In fetal sheep, pituitary corticotrophs undergo a change in morphology from predominately "fetal"-type corticotrophs at day 90 of pregnancy, to exclusively "adult"-type cells at term (Perry *et al.* 1985). Corticotrophs in fetuses adrenalectomized at day 120 did not undergo maturation from "fetal" to "adult"-type cells, however maturation of the corticotrophs could be induced by cortisol infusion to the ovine fetus at 109 to 115 days of gestation before the endogenous rise in cortisol (Antolovich *et al.* 1989). The change in corticotroph morphology is paralleled by changes in the abundance of POMC-derived peptides in fetal plasma and pituitary. In fetal sheep plasma at 110 days of gestation large molecular weight POMC-derived peptides are most abundant but the levels of these peptides decreased at term (139-147 days), whereas $ACTH_{1-39}$ concentrations increased 2-fold during this time (Saphier *et al.* 1993). In the fetal sheep pituitary the ratio of large molecular weight POMC-derived products:$ACTH_{1-39}$ decreases from 120 days to 145 days of gestation (Silman *et al.* 1979). Brieu and Durand have shown that corticotrophs treated with cortisol (4 days) *in vitro* have a reduced total output of POMC-derived peptides however there is a greater proportion of $ACTH_{1-39}$ secreted into the culture media (Brieu and Durand, 1989), and the ratio of bioactive:immunoreactive ACTH increases (Brieu and Durand, 1987). These data implicate cortisol in the morphological and functional changes observed in fetal pituitary corticotrophs during late gestation. However, the pituitary may not be the only important site of ACTH production during fetal life.

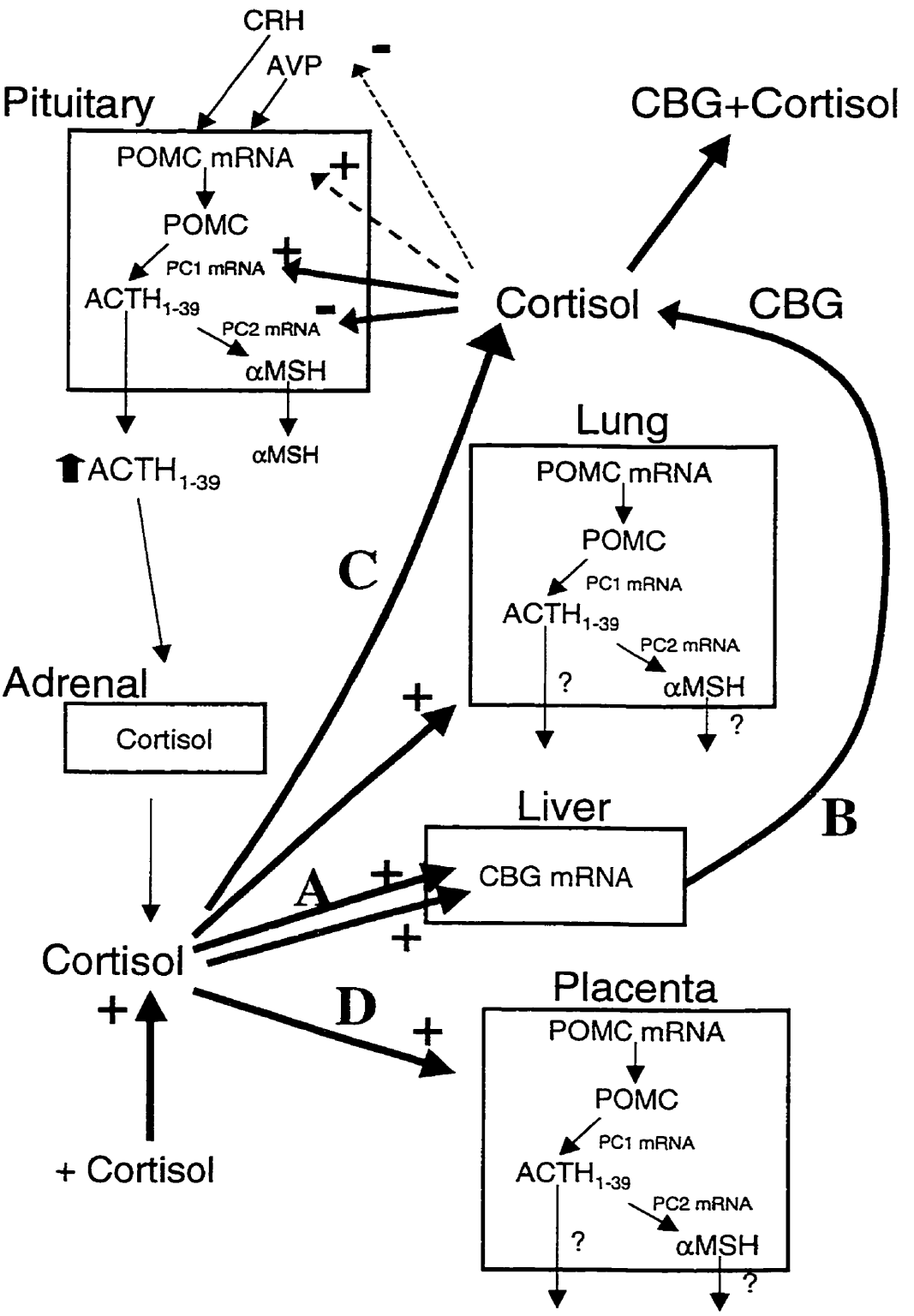
Hypophysectomized fetuses were infused with a low-dose of $ACTH_{1-24}$ to maintain adrenal function, but during a 4-hour cessation of the infusion circulating

ACTH levels were sustained at 50% of infusion concentrations (Poore *et al.* 1997). It was postulated that this indicates an extra-pituitary site of ACTH production, which contributed to circulating ACTH levels (Poore *et al.* 1997), and appeared to be dependent upon adrenal function and therefore perhaps cortisol. Previous studies have shown that the fetal sheep lung contains POMC mRNA and ir-ACTH (Deol *et al.* 1995), and secretes ir-ACTH *in vivo* (Cudd & Wood, 1993). There are several studies reporting ACTH production and secretion in the human placenta (Genazzi *et al.*1974; Margioris *et al.* 1988; Waddell and Burton, 1993). However, there is less evidence that the sheep placenta contributes to circulating ACTH concentrations. Although ir-ACTH has been localized to trophoblast and maternal stromal cells in the ovine placenta (Jacobs and Challis, 1989), ACTH secretion has not been detected in normoxemic or hypoxemic fetuses (Keller-Wood and Wood, 1991a). Further studies are required to elucidate the contribution of these alternative sites of ACTH production to circulating levels.

These data indicate that both the regulation of cortisol and the direct effects of cortisol are critical in the concurrent rise in plasma ACTH and cortisol concentrations during late gestation. This thesis will explore the following hypotheses, which are outlined in the schematic in Figure 2.1.

1. Plasma CBG levels in the late gestation ovine fetus are regulated by plasma cortisol concentrations such that fetal plasma CBC levels will rise in parallel to an incremental infusion of cortisol and fetal adrenalectomy will attenuate the endogenous late gestation rise in CBG (Figure 2.1.A (Blue); Chapter Three).

Figure 2.1 Schematic of the hypotheses examined within this thesis. Stimulatory (+) or inhibitory (-) effects of cortisol on CBG and ACTH production.


CRH
AVP
Pituitary
POMC mRNA
POMC
PC1 mRNA
ACTH1-39
PC2 mRNA
αMSH
ACTH1-39
αMSH
CBG+Cortisol
Cortisol
CBG
Lung
POMC mRNA
POMC
PC1 mRNA
ACTH1-39
PC2 mRNA
αMSH
?
?
C
Adrenal
Cortisol
Liver
CBG mRNA
B
A
Cortisol
D
Placenta
POMC mRNA
POMC
PC1 mRNA
ACTH1-39
PC2 mRNA
αMSH
?
?
+ Cortisol

2. Intra-fetal cortisol infusion (+cortisol) will induce increases in plasma CBG levels and subsequently decrease free cortisol concentrations thereby eliciting an increase in pituitary POMC mRNA levels and $ACTH_{1-39}$ output. (Fig 2.1B (Red); Chapter Four)

3. Intra-fetal cortisol administration (+cortisol) will alter POMC processing in the fetal pituitary such that $ACTH_{1-39}$ is preferentially produced. The change in processing will be reflected by increases in PC1 mRNA levels and decreases in the level of PC2 mRNA in the fetal pituitary (Fig 2.1C (Purple); Chapter Five)

4. $ACTH_{1-39}$ production in the fetal lung and placenta will increase in response to elevated fetal plasma cortisol concentrations due to increases in POMC mRNA and PC1 mRNA levels, and a reduction in PC2 mRNA. (Fig 2.1D (Green); Chapter Six).

5. Exogenous glucocorticoid administration during pregnancy in humans will elicit a differential response, with increased plasma CBG levels in the fetus and reduced plasma CBG levels in the mother (Chapter Seven).

Chapter Three

Effects of incremental cortisol and adrenalectomy on plasma corticosteroid-binding capacity in fetal sheep

Dr. Megan Wallace (incremental infusion protocol) and Dr. Abigail Fowden (adrenalectomy protocol) conducted the animal experiments contained within this chapter. The CBG antibody was developed and characterized by Dr. Edward Berdusco. The contents of this chapter have been previously published in the Canadian Journal of Physiology and Pharmacology (1995) 73:1568-1573, and appears here with the permission of the National Research Council of Canada (see attached authorization).

3.1 Introduction

Parturition in species such as sheep results from activation of the hypothalamic-pituitary-adrenal (HPA) axis of the fetus, which is reflected in a progressive rise in ACTH and cortisol concentrations in fetal plasma (Alexander *et al.* 1968; Basset and Thorburn, 1969; Magyar *et al.* 1980). It has been previously hypothesized that this paradoxical late-gestation rise in ACTH and cortisol is due to the simultaneous increase in plasma corticosteroid-binding globulin (CBG) levels. CBG binds cortisol with high affinity therefore increases in plasma CBG decrease free and bioactive cortisol, and thereby reduce the inhibitory effects of cortisol on ACTH output (Challis *et al.* 1985; Berdusco *et al.* 1995). The late-gestational rise in cortisol occurs concomitantly with an increase in the fetal plasma concentration of CBG (Fairclough and Liggins, 1975; Ballard *et al.* 1982; Berdusco *et al.* 1995). It has been suggested previously that the rise in CBG occurs in response to fetal glucocorticoids (Challis *et al.* 1985; Berdusco *et al.* 1993). Dexamethasone, infused into fetal sheep at day 125 of gestation, increased plasma CBG levels (Berdusco *et al* 1993), and infusing physiological levels of cortisol beginning at day 100, also stimulated an increase in plasma CBG (Berdusco *et al.* 1994). CBG glycosylation was also altered with advancing gestational age and in response to glucocorticoid treatment, such that the amount of CBG that eluted from Concanavalin A columns was increased. This alteration in glycosylation may increase the half-life and thereby contribute to the observed increase in circulating CBG (Berdusco *et al.* 1995; Berdusco *et al.* 1994; Berdusco *et al.* 1993).

In the earlier studies (Berdusco *et al.* 1993), the glucocorticoid was infused at a constant rate. However, the prepartum increase in fetal plasma corticosteroid in the sheep (Norman *et al.* 1985) occurs, in part, as a result of a progressive increase in the kinetically determined secretion rate of cortisol from the fetal adrenal gland (Wintour *et al.* 1975). In the present study, we have therefore, infused the natural corticosteroid, cortisol, incrementally over 10 days (120-130d) to imitate the prepartum cortisol rise in fetal plasma which normally occurs at days 135-145 of gestation. The period of time from day 120-130 corresponds to the initial period in the prepartum activation of fetal adrenal function (Brieu *et al.* 1988). We have examined whether incremental rates of cortisol infusion continued to affect the CBG of fetal plasma, and we determined whether changes in plasma CBG correlated with the prevailing fetal plasma cortisol concentration. To obtain evidence for the endogenous prepartum cortisol rise (Alexander *et al.* 1968; Basset and Thorburn, 1968; Magyar *et al.* 1980) in stimulating the endogenous concomitant rise in CBG, we also examined the effects of bilateral fetal adrenalectomy on CBG biosynthesis and plasma CBG concentrations at term.

3.2 Materials and Methods

3.2.1 Animals and experimental protocols

3.2.1.1 *Effects of incremental cortisol on CBG*

Dr. Megan Wallace conducted these animal studies with prior approval of the Animal Care Committee, Monash University, Melbourne, Australia. Twelve pregnant merino ewes carrying singleton fetuses underwent aseptic surgery under halothane

anaesthesia between 110-116 days of gestation as described previously (Wallace *et al.* 1995). Catheters were implanted into the carotid artery, both jugular veins, the trachea and amniotic cavity of each fetus, and into a carotid artery and jugular vein of each ewe. Animals were allowed to recover for at least five days postoperatively before experiments commenced.

The animals were divided randomly into two groups of six ewes. Age-matched control animals received a saline infusion (4.32ml/day) from 120.5±0.2 (mean±SEM) days of gestation until 130.8±0.3 days, which was the day of sacrifice. Treatment animals received infusions of cortisol, starting on day 120.7±0.2 and continuing until 130.8±0.3 days, the day of postmortem. Cortisol (hydrocortisone, Solu-Cortef, 50mg/ml, Upjohn Pty. Ltd., Australia) was prepared fresh every two days, and the infusate was replaced each day. For the first three days, cortisol was infused at 1.5mg/day (4.32ml/day). For the next five days cortisol was infused at 2.5mg/day, and at a dose of 3.5mg/day for the final two days. The same volume of infusate (4.32ml/day) was given each day. Separate experiments, reported elsewhere (Wallace *et al.* 1995), were carried out on day 120.7, 125.7 and 129.3 to determine lung liquid secretion rates, and lung liquid volumes using dye dilution techniques. A single sample of fetal carotid blood (2.5ml) was collected from each fetus every day if possible. All samples were generally collected about two hours (maximum range 1-3h) after feeding the ewe. The samples were collected into chilled heparinized syringes, and centrifuged at 1000xg for 15min at 4°C. The plasma was divided into aliquots and stored at –80°C.

3.2.1.2 Effect of adrenalectomy on CBG biosynthesis

These studies were conducted by Dr. Abigail Fowden with the approval of the British Home Office Regulations (Project License No. 80/000736). In five Welsh Mountain ewes of mixed breed, carrying twin fetuses, bilateral adrenalectomy (ADX) was performed on one of the twins at 115±1.3 days of gestation using the anaesthetic regime and surgical techniques described previously (Barnes *et al.* 1977). The second twin was left intact. At 143±0.5 days, both fetuses were delivered by Cesarean section under general anesthesia (30mg/kg I.V. Sodium pentobarbitone) and blood samples were taken from the umbilical artery of each fetus by direct venipuncture. After administration of a lethal dose of anesthetic, a section of fetal liver was obtained from the right lobe, and frozen immediately at -80°C. The blood samples were collected into heparinized syringes, centrifuged, and the plasma was stored at –20°C until analysis.

3.2.2 Assays

3.2.2.1 Plasma cortisol concentrations

Plasma cortisol concentrations were quantified by radioimmunoassay as described previously (Challis *et al.* 1981). Plasma samples (100µl) were pipetted into 16 x 125 mm test tubes and extracted with 5ml diethyl ether. The tubes were mixed on a vortex mixer (20sec) and then shaken in a mechanical shaker (1h). The aqueous phase was separated from the organic phase by freezing (acetone and dry ice). The organic phase was decanted into a second set of test tubes (12 x 75mm), dried under air, and then reconstituted with 1ml phosphate buffered saline and

gelatin (PBSG: 0.4M $NaH_2PO_4 \cdot 2H_2O$, 0.6M $Na_2HPO_4 \cdot 7H_2O$, 0.15M NaCl, 0.015M NaN_3 and 0.1% w/v gelatin; pH 7.1).

Aliquots (400µl and 100µl) of the reconstituted samples were transferred into assay tubes (12 x 75mm, borosilicate), and incubated with rabbit anti-cortisol antiserum (100µl; final dilution 1:30 000, titrated to give a B/B_o of approximately 40%) and [^{3}H]cortisol (100µl; 10 000cpm; purified as described below) overnight at 4°C. The bound and 'free' cortisol were separated using dextran coated charcoal (DCC: 0.625% w/v dextran, 6.25% w/v charcoal). The antibody used was generated by this laboratory and has been previously characterized (Challis *et al.* 1981). The cross-reactivities reported are as follows: cortisol sulphate, 76%; 11-deoxycortisol (4-pregnen-17,21-diol-3,20-dione), 35%; 21-deoxycortisone, 33%; cortisone (3-pregnen-17,21-diol-3,11,20-trione), 6%; corticosterone (4-pregen-11β,21-diol-3,20-dione), 0.84%; progesterone (4-pregnen-3,20-dione), 0.31%; pregnenolone (5-pregnen-3β-ol-20-one), 17α-hydroxypregnenolone (5-pregnen-3β,17-diol-20-one), and 11α-hydroxyprogesterone (4-pregnen-11α-ol-3,20-dione), less than 0.1%. The combined intra and interassay coefficients of variation was 12%.

^{3}H-cortisol purification

Thin layer chromatography (TLC) was used to separate [1,2,6,7-^{3}H]Cortisol (40µl: specific activity 80-100 Ci/mmol) from free [^{3}H] and breakdown products of labeled steroid. A 20 x 20 cm TLC plate (Fisherbrand, Fisher Scientific, Pittsburgh, PA) was set into a glass incubation chamber containing 2-5cm of chloroform:ethanol (95:5) and the solvent front was allowed to advance to the top of the plate. The plate

was then air-dried and [1,2,6,7-^{3}H]Cortisol and non-labeled cortisol (20μl; 1mg/ml) spotted at the same level (>5cm from the bottom) on opposite halves of the plate. The TLC plate was returned to the incubation chamber and the solvent front allowed to rise to the top of the plate once more. After the plate had air-dried the unlabeled steroid was visualized using UV light. The powder from the corresponding area on the [1,2,6,7-^{3}H]cortisol side of the plate was scraped off and placed into a 12x75mm test tube and mixed with 4ml of ethyl acetate (ACP Chemicals Inc., Montreal, Que.). The contents of the test tube were mixed on a vortex (20sec), centrifuged (10 min x 1500 *g*), and the supernatant collected. The pellet was resuspended in an additional volume of ethyl acetate (4ml), vortex-mixed, centrifuged and the supernatant collected and added to the first volume. An aliquot (1μl) of the pooled supernatant was added to scintillation fluid (4ml) and counted in a beta counter (Tri-Carb 2100TR Liquid Scintillation Analyzer, Packard Instrument Co., Meriden, CT).

3.2.2.2 Corticosteroid-binding capacity

Plasma CBG levels were measured as corticosteroid-binding capacity (CBC) using the saturation binding assay of Ballard *et al.* (Ballard *et al.* 1982), with modifications described previously (Challis *et al.* 1985). Duplicate aliquots (50μl) of plasma were added to borosilicate tubes (10 x 75mm) containing [^{3}H]cortisol (10 000cpm) and non-radioactive cortisol (16ng), which had previously been added dissolved in ethanol and dried under air. Additional duplicates were set up for each sample, with [^{3}H]cortisol (10 000cpm, purified within 24 hours of use) in the presence of an excess of non-radioactive cortisol (1μg, dried under air as above), to

determine the non-specific binding of [^{3}H]cortisol in each sample. Tubes were vortex mixed, incubated at 37°C for 30min, followed by an additional 12-18h incubation at 4°C. Bound and free cortisol were separated using dextran-coated charcoal (90μl: 0.625% w/v dextran (dextran T70, Pharmacia Fine Chemicals, Baie d'Urfe, Que.), 6.25% charcoal (Norit A, Fisher Scientific, Toronto, Ont.), in tricine buffer (0.15M tricine, pH 7.4)). Tubes were centrifuged at 1500 x *g* for 10 min at 4°C, and an aliquot (100μl) of the supernatant was added to 4ml of scintillation fluid and counted. The CBC was calculated from the percent of bound [^{3}H]cortisol, minus the percent of non-specific binding, multiplied by the total concentration of cortisol (endogenous cortisol in 50μl of plasma, measured previously, plus the unlabeled and [^{3}H]cortisol added) and corrected to concentration in 1 ml of plasma. The assay did not measure cortisol binding to albumin since this complex dissociates rapidly after the addition of charcoal, and unbound cortisol is adsorbed and precipitated with the charcoal upon centrifugation.

3.2.2.3 $ACTH_{1-39}$

Plasma ACTH concentrations were measured using a commercial radioimmunoassay (RIA) kit (Incstar, Stillwater, MN) that was validated previously for use in the fetal sheep (Norman *et al.* 1985). The intra- and inter-assay coefficients of variation were 9 and 13%, respectively, and the mean assay sensitivity was 6.5pg/ml. This ACTH antibody cross-reacts <0.01% with α-MSH, β-MSH, β-endorphin, and β-LPH, and does not recognize pro-ACTH or POMC (Matthews *et al.* 1996). The antibody recognized >95% immunoreactivity corresponding to

$ACTH_{1-39}$ when samples of fetal sheep plasma obtained during normoxemia or during hypoxemia were assayed after high-performance liquid chromatography separation of ACTH-related peptides (Challis *et al.* 1989).

3.2.3 Western blot analysis

A specific antibody was raised against purified ovine CBG in rabbits, by Dr. Edward Berdusco. The CBG was purified by steroid affinity chromatography from the serum of sheep at days 120-140 of gestation as described elsewhere (Berdusco *et al.* 1993). The purity of the CBG was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 4% and 7.5% acrylamide in the stacking and separating gels respectively. The protein was visualized with Coomassie brilliant blue. This material was used to generate polyclonal antibodies against CBG in rabbits. For immunization, purified CBG was added to Freund's complete adjuvant. Three booster injections were made at monthly intervals. The generation of antibodies was determined by their ability to recognize purified CBG after SDS-PAGE using 4% and 10% acrylamide in the stacking and separating gels respectively.

Plasma was diluted 1:50 and electrophoresed in 10% SDS-PAGE at 100V for approximately 1.5 hours. Proteins were transferred to a nylon membrane (Zetaprobe, Bio-Rad, Hercules, CA), washed, and incubated in 4% fetal calf serum in TTBS (150nM NaCl, 50mM Tris-HCl, pH 7.4 containing 0.05% Tween 20) for 30 min at 24°C. The membranes were incubated with the primary antibody (1:500) for 18h at 4°C, washed, incubated with biotinylated secondary antibody (1:1000, Sigma

Chemical Company, St Louis, MO), then with extra-avidin (1:1000, Sigma Chemical Company, St Louis, MO). The brown reaction product was visualized after addition of 3,3'-diaminobenzidine substrate (SIGMA*FAST*, Sigma Chemical Company, St Louis, MO) in 0.1% hydrogen peroxide. The blot was then photographed and the negative was subjected to image analysis (Imaging Research Inc., St. Catherines, Ont.) to determine the relative optical densities of the resultant bands.

3.2.4 Northern blot analysis

3.2.4.1 Total RNA extraction

Total RNA was extracted using the LiCl/Urea method. Fractions of tissues, frozen in liquid nitrogen at the time of collection, were weighed (approximately 0.5-0.75g of liver) and placed in chilled sterile falcon tubes (50ml: Falcon Labware, Becton Dickinson and Co., Lincoln Park, NJ) containing 3M LiCl / 6M Urea (20ml) and heparin (10 000 U/ml). The tissues were homogenized (PT2000 Homogenizer, Polytron, Kinematica AG, Switzerland) on ice, and allowed to stand overnight (4°C). The homogenates were decanted into a Nalgene centrifuge tube (30ml: Nalge Co., Rochester, N.Y.) and centrifuged at 31 000 x *g* for 30 minutes (Sorvall RC26 Plus, DuPont Company, Newton, CT). The supernatants were poured off and the pellets resuspended in heparinized LiCl as above (20ml). The samples were centrifuged a second time (31 000 x *g*, 30min), and the supernatants discarded. The resultant pellets were dissolved in Tris-SDS-heparin (TSH: 3ml, 10mM Tris-HCl, 0.5% w/v SDS, and 100U/ml heparin) and transferred to sterile round-bottom polypropylene Falcon tubes (14ml, Becton Dickinson Labware, Lincoln Park, NJ). An equal volume

(3ml) of chloroform:isoamyl alcohol (24:1) was added and the solution was mixed well using a vortex mixer. The samples were centrifuged at 2700 x *g* for 10 minutes at room temperature. The aqueous layer on top was transferred to a second set of round-bottom Falcon tubes. An additional 2ml of TSH was added to the first set of round-bottom tubes, and the samples were mixed and centrifuged as before. The aqueous phase was collected and added to the second set of round-bottom tubes. The second set of tubes were then re-extracted with an equal volume of chloroform:isoamyl alcohol (24:1), as before, with the aqueous phase being transferred to a glass centrifuge tube (30ml, Corex Brand, Fisher Scientific Ltd., Nepean, Ont.). The RNA, collected in the Corex tube, was precipitated from the TSH by the addition of 3M sodium acetate (40µl: 1:10 v/v) and 100% ethanol (1ml: 2.5:1 v/v), and allowed to sit overnight at –20°C. The following day the tubes were centrifuged for 30 min (2700 x *g* at 4°C), the supernatants decanted and the pellets dried under vacuum. The RNA pellets were resuspended in 400µl of DEPC water (autoclaved 0.02% v/v diethyl-pyrocarbonate in distilled water). The dissolved RNA was transferred to screw-top microcentrifuge tubes (1.5ml: Fisherbrand, Fisher Scientific Ltd., Nepean, Ont.), 100% ethanol (1ml) was added and the samples stored at –70°C.

The purity and concentration of total RNA was determined using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Piscataway, NJ). The absorbance of an aliquot of the total RNA (2µl RNA in 498µl DEPC water) was determined at 260nm (for nucleic acid concentration), and at 280nm (for protein content). Ratios of the readings at 260:280 greater than 1.5 were deemed

acceptable for further analysis. The integrity of the isolated RNA was also assessed. Samples of total RNA (10μg) were electrophoresed on a 1% (w/v) agarose formaldehyde gel, and stained with ethidium bromide (0.001% v/v 10mg/ml ethidium bromide, 0.13% v/v 2β-mercaptoethanol), destained (0.1% 2β-mercaptoethanol), and visualized with UV light. Intact RNA was viewed as two distinct bands corresponding to 18S and 28S ribosomal RNA.

3.2.4.2 Electrophoresis and hybridization

Total RNA (20μg) was electrophoresed through a 1% agarose formaldehyde gel (Rave *et al.* 1979) with northern running buffer (0.2M MOPS, 50mM sodium acetate, 5mM EDTA). The eletrophoretically separated RNA was transferred to a nylon membrane (Zetaprobe, Bio-Rad, Hercules, CA.) by capillary blotting (Thomas, 1980). The resultant blots were exposed to UV light for 1min and then baked in an 80°C vacuum oven for one hour. The northern blots were then prehybridized with buffer (50% deionized formamide, 4X SSPE (0.6M NaCl, 0.05M NaH_2PO_4, $4.0x10^{-3}$M EDTA), 1% sheared salmon sperm DNA) for 12-24h at 42°C. The blots were hybridized using the same buffer and conditions, in the presence of ^{32}P-labeled probe (see below). The blots were washed to high stringency with 0.1% SSC (1x SSC = 0.15M NaCl, 0.015M sodium citrate) and 0.1% (w/v) SDS at 42°C for 30min. The labeled blots were exposed to x-ray film (XAR-5, Kodak Eastman Co., Rochester, N.Y.) at –70°C. After autoradiographic exposure, the blots were stripped (5x 200ml: 0.5% w/v SDS, 0.1% w/v SSC, at 95°C) and reprobed with a ^{32}P-labeled cDNA to mouse 18S rRNA to allow correction of variations in gel loading and

transfer. The relative optical densities were determined using image analysis within the linear range of the x-ray film (Imaging Research Inc., St Catherines, Ont.). Results are expressed as the ratio of mRNA : 18S rRNA.

3.2.4.3 ^{32}P-labeling of cDNA probes

An oligolabeling kit (Pharmacia Biotech Inc., Baie d'Urfe, Que.) was used to label cDNA probes utilizing the random primer technique (Feinberg Vogelstein 1983). The cDNA was denatured by heating in boiling water for 10 minutes and then cooled on ice for 5 minutes. The cDNA (~100ng) was then added to the reagent mix (10μl: supplied with kit), [^{32}P]dCTP (5μl: 3000 Ci/mmol), Klenow fragment (1μl, from kit) and DEPC-water (0.02% v/v DEPC, autoclaved) to a final volume of 50μl. This reaction mixture was incubated at 37°C for one hour. The labeled probe was eluted through a Sephadex G-50 minicolumn (Nick column: Pharmacia Biotech Inc., Baie d'Urfe, Que.) with TE buffer (10mM Tris/HCl pH7.4, 1mM EDTA pH 7.4). An aliquot of the labeled probe (1μl) was taken before and after elution through the Nick column, and the percent of incorporation was calculated. The ^{32}P-labeled cDNA probe was added to hybridization buffer at a concentration of approximately 1-1.5x10^6 cpm/ml.

3.2.5 Data Analysis

All values are reported as mean ± SEM for the number of animals stated. Statistical significance was determined as $p \leq 0.05$. Changes in plasma CBC and cortisol in response to exogenous cortisol administration were analyzed by a two-

way analysis of variance corrected for repeated measures. The effects of individual times of treatment were assessed by Student-Newman-Keuls multiple range tests. The relationship between plasma CBC and plasma cortisol concentrations in saline- and cortisol-infused fetuses was sought for individual data points and for daily mean values by linear regression analysis. Effects of adrenalectomy on plasma hormones and CBC levels were assessed by paired t-test in twin pregnancies.

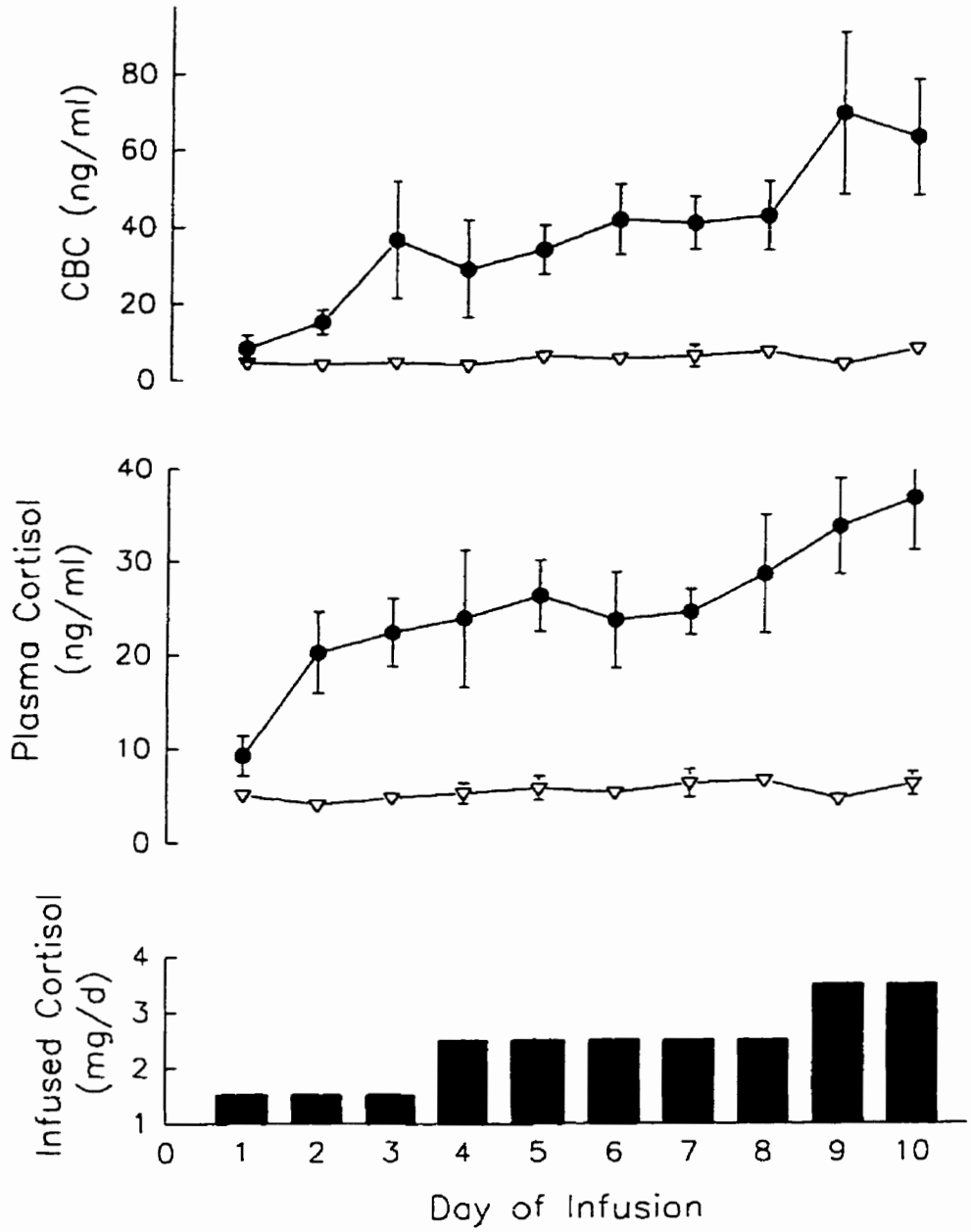
3.3 Results

3.3.1 Effects of cortisol infusion on plasma cortisol and CBC

The effects of cortisol infusion on plasma cortisol and CBC are shown in Figure 3.1. The mean plasma cortisol concentration rose during incremental cortisol infusion from 9.2 ± 2.1 ng/ml (n=5) at the start of infusion to 24.6 ± 2.5 ng/ml (n=6), on day 6, and 36.9 ± 5.7 ng/ml (n=6), on day 10. Overall there was no significant change in the mean concentration of cortisol in the plasma of the saline-treated fetuses.

The mean plasma CBC was 8.1 ± 3.4 ng/ml (n=5) and 4.4 ± 0.7 ng/ml (n=6) before the start of infusion in the cortisol- and the saline-treated fetuses, respectively. During cortisol infusion there was a significant increase in CBC to 36.8 ± 15.3 ng/ml (n=5) by day 3. The plasma CBC rose to 42.2 ± 9.14 ng/ml (n=6) by day 6, during the second increment of the cortisol infusion, and increased to 70.1 ± 21.3 ng/ml (n=6) by 9 days of cortisol infusion. There was no significant change in the plasma CBC levels in saline-infused control fetuses.

Figure 3.1 Changes in plasma corticosteroid-binding capacity (top panel) and cortisol concentrations (middle panel) in fetuses given either an incremental cortisol (•, n=5-6) or a saline (∇, n=6) infusion beginning at a gestational age of 120.5 ± 0.2 days and continuing for ten days. The incremental dose of cortisol infused (■) is depicted by the histogram (bottom panel). Values are mean ± SEM.


CBC (ng/ml)
80
60
40
20
0
Plasma Cortisol (ng/ml)
40
30
20
10
0
Infused Cortisol (mg/d)
4
3
2
1
0 1 2 3 4 5 6 7 8 9 10
Day of Infusion

In fetuses treated with cortisol there was a significant correlation between individual plasma concentrations of cortisol and individual plasma values of CBC (linear regression; $r=0.80$; $p<0.001$; Figure 3.2). There was also a significant correlation between individual values of cortisol and CBC in the saline-infused control animals during the experimental period ($r=0.53$; $p<0.001$; data not shown).

3.3.2 Plasma CBG by Western blot analysis

The CBG antibody recognized purified ovine CBG as a doublet on SDS-PAGE, with an average molecular weight of 57kDa (Figure 3.3, upper panel). The antibody recognized ir-CBG in fetal plasma; the staining intensity determined by the relative optical density was greater in plasma obtained from fetuses that had been infused with cortisol (1.71 ± 0.06) than the saline controls (1.56 ± 0.02) ($p=0.05$; Mann-Whitney U test). In a second gel, run at the same time under similar conditions, the antibody was preabsorbed with excess purified CBG (Figure 3.3; lower panel).

3.3.3 Effect of ADX on plasma hormones and CBG biosynthesis

The mean concentration of cortisol in the plasma of ADX fetuses (7.6 ± 0.8 ng/ml, $n=5$) was significantly less than that in intact animals (54.0 ± 11.7 ng/ml, $n=5$), whereas ir-ACTH concentrations were higher in ADX than intact fetuses (1128 ± 242 vs. 142 ± 23 pg/ml, both $p<0.05$, Figs. 3.4A and B). The plasma CBC, however, was

Figure 3.2 Relationship between individual plasma concentrations of cortisol and CBC, in cortisol treated fetuses (d120-130). Linear regression analysis shows a significant positive correlation ($r=0.80$, $p<0.001$) between these variables.

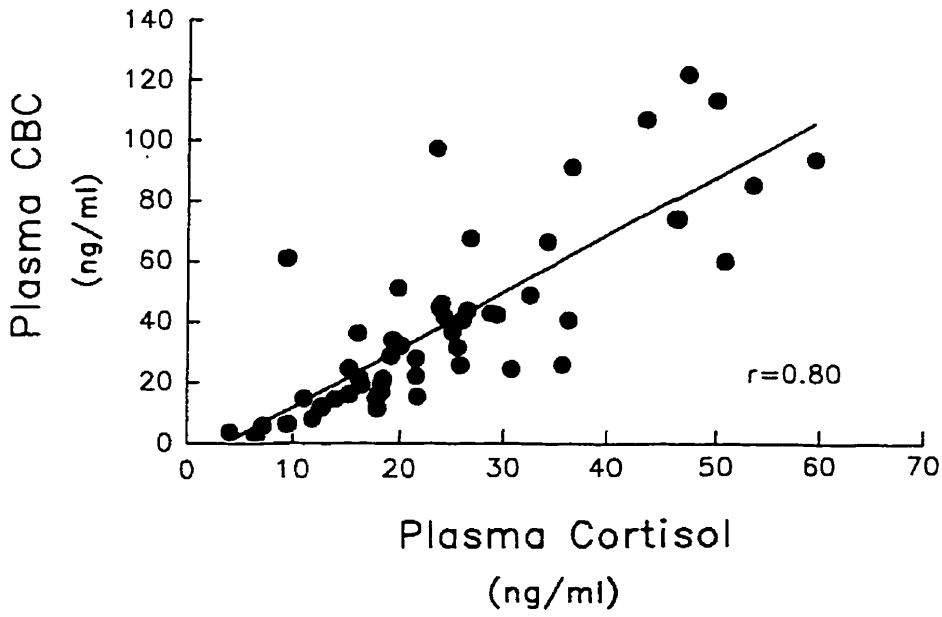
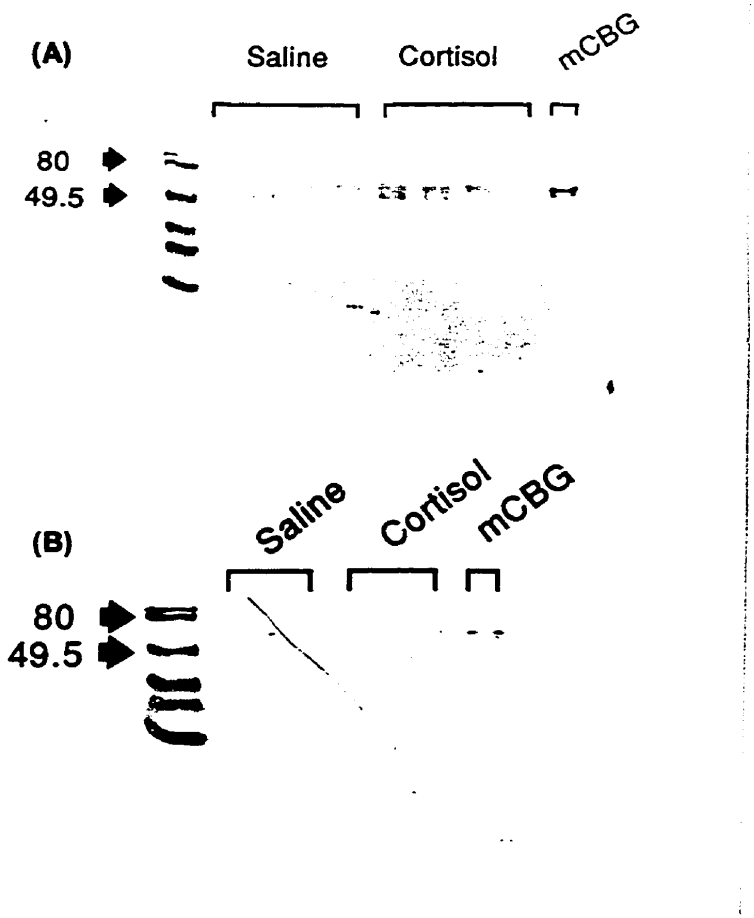

Plasma CBC
(ng/ml)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
r=0.80
Plasma Cortisol
(ng/ml)

Figure 3.3 Western blot analysis of plasma immunoreactive CBG in fetuses treated for 10 days with either a saline (left) or an incremental cortisol infusion (right). The CBG antibody recognized purified maternal ovine CBG (mCBG) as a doublet with an average molecular weight of 57kDa (top panel). The blot in the lower panel was run at the same time under similar conditions but the primary antibody was preabsorbed with excess purified ovine CBG.


(A)
Saline
Cortisol
mCBG
80
49.5
(B)
Saline
Cortisol
mCBG
80
49.5

significantly lower in the ADX fetuses (9.4 ±2.1 ng/ml) than in their intact twins (56.7 ± 6.1 ng/ml) ($p<0.05$, Figure 3.4C).

By northern blotting of liver RNA, 1.8kb CBG mRNA transcripts were detected in both ADX and control fetuses. Two autoradiogram exposures are shown (Figure 3.5). Densitometry was performed on the autoradiograms shown in the upper panel. Additional longer exposures were conducted to produce darker bands for photography (lower panel). The relative optical density of CBG mRNA was not significantly different between the intact and ADX fetuses (Figure 3.5, $p=0.74$).

Figure 3.4 Changes in fetal plasma (A) ACTH, (B) cortisol, and (C) CBC concentrations at 143±0.5 days of gestation in twin fetuses that were intact (□), or were adrenalectomized (ADX, ■) at day 115±1.3. Values are mean ± SEM, n=5; *$p<0.05$ (paired t-test).

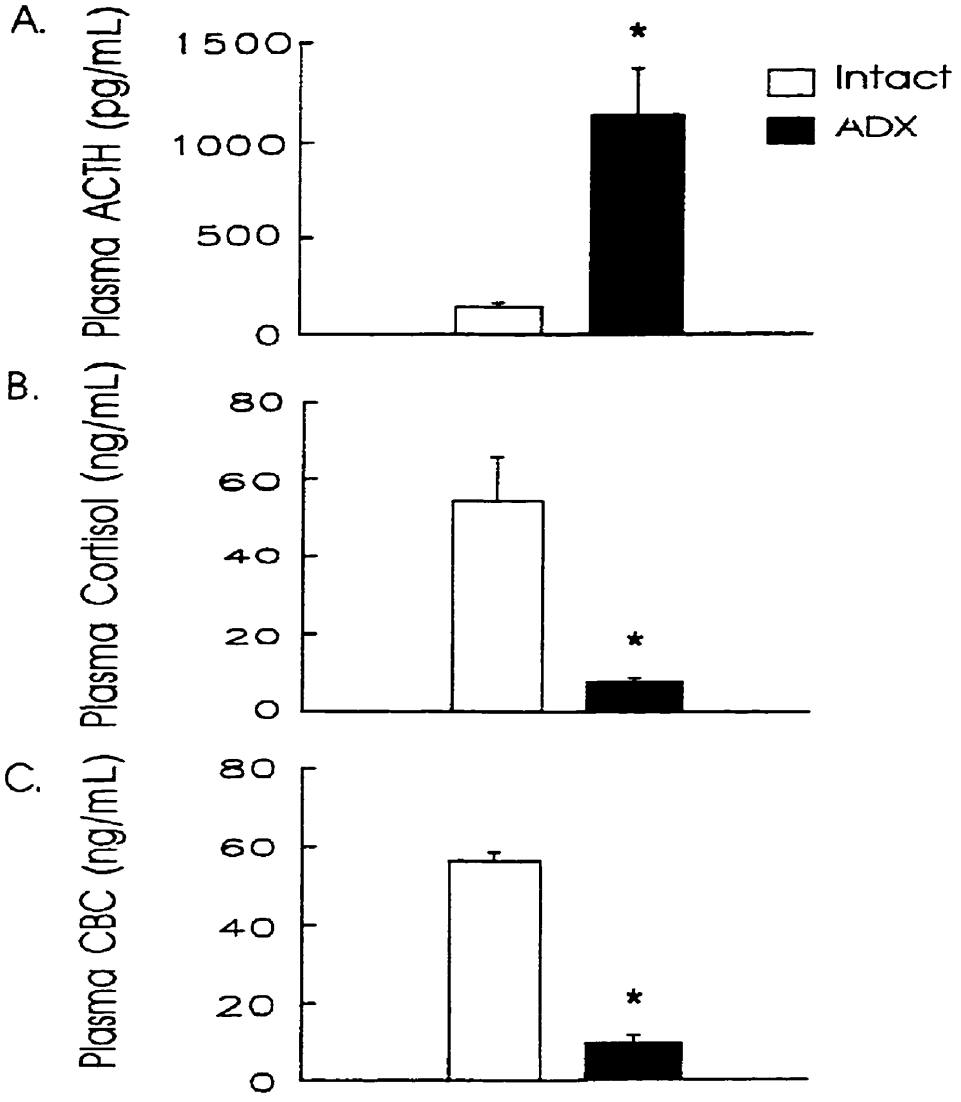
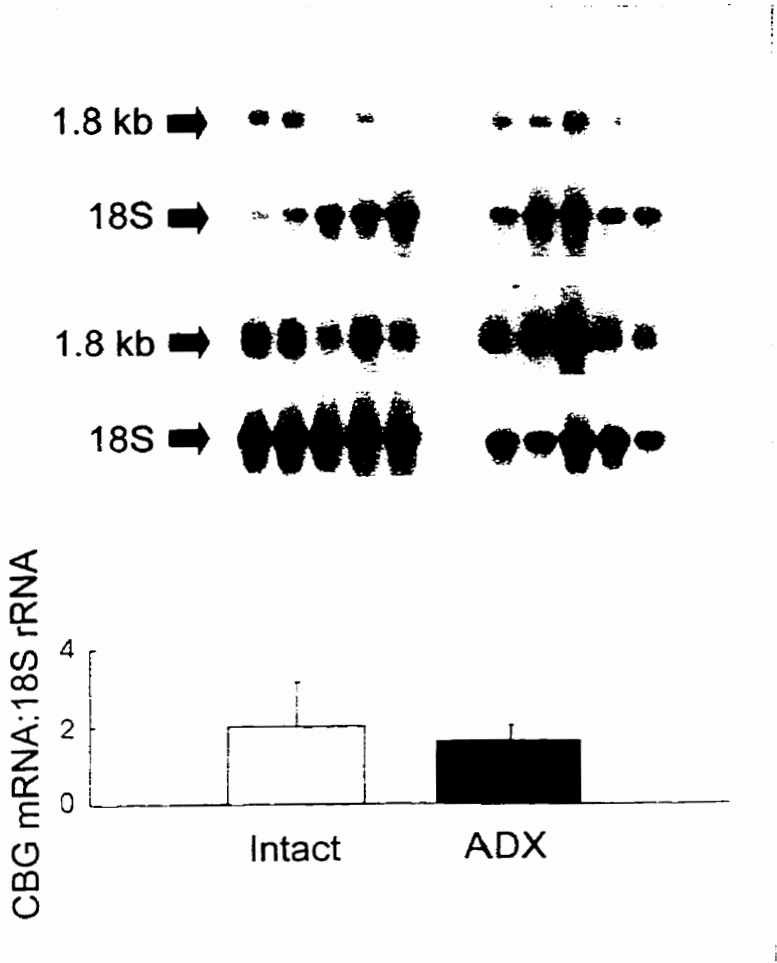

A.
B.
C.
Plasma ACTH (pg/mL)
Plasma Cortisol (ng/mL)
Plasma CBC (ng/mL)
1500
1000
500
0
80
60
40
20
0
*
Intact
ADX

Figure 3.5. Hepatic CBG mRNA in twin fetuses at term (143±0.5 days). The upper panel shows the autoradiogram exposure used for densitometry after northern blot analysis of CBG mRNA (1.8 kb) and 18S ribosomal RNA in the liver of five individual intact (left) or bilaterally adrenalectomized (right) fetuses. In the lower panel histograms show the ratio of CBG mRNA to 18S rRNA in the intact and adrenalectomized twins measured from the autoradiograms in the upper panel. Additional exposures of longer duration are represented in the lower panel. Values are the mean ± SEM, n=5 pairs, $p>0.05$. ROD, relative optical density.


1.8 kb
18S
1.8 kb
18S
CBG mRNA:18S rRNA
4
2
0
Intact
ADX

3.3 Discussion

This study has shown that a 10-day infusion of cortisol to fetal sheep at days 120-130 of gestation results in a significant increase in the CBC of fetal plasma. Since we did not compare this result directly with that from a continuous infusion of cortisol given at the same rate over 10 days, we cannot conclude whether the progressive increase in CBC reflected the duration, or the incremental nature of the cortisol infusion. In earlier studies we found that plasma CBG continued to rise during 96h of dexamethasone infused at a constant rate into fetuses from about day 130 of gestation (Berdusco *et al.* 1993). However, the strong correlation between individual plasma cortisol concentrations and CBC values in the present experiment is consistent with the suggestion that the increase in endogenous fetal plasma cortisol concentrations beginning about day 125-130 of ovine pregnancy is at least partly responsible for the prepartum increase in plasma CBC.

This suggestion is supported by the apparent lack of rise in plasma CBC in term fetuses adrenalectomized 30 days previously. Their intact twins had CBC values that were similar to those of intact catheterized fetuses at full term (Berdusco *et al.* 1994). The low levels of cortisol in the plasma of adrenalectomized fetuses has been reported previously (Silver and Fowden, 1991; Wintour *et al.* 1980), and presumably is derived from the mother after transplacental transfer (Beitins *et al.* 1970). These results indicate that this level of cortisol in the fetal circulation is sufficient to maintain basal levels of CBG production. Since the glycoprotein does not cross the placenta from mother to fetus, CBG levels in fetal plasma probably reflect fetal biosynthesis (Berdusco *et al.* 1993).

Western blot analysis showed an increase in the amount of ir-CBG protein in fetal plasma after 10 days of cortisol treatment, which accounted for the rise in CBC. The sheep ir-CBG had an average molecular weight of about 57kDa, in agreement with published estimates determined by physical methods (Kato *et al.* 1988). Purified ovine CBG runs as a doublet during SDS-PAGE, as does purified CBG from other species (Scrocchi *et al.* 1993; Ali and Bassett, 1991). Ovine CBG has five consensus sites for N-linked glycosylation. It is possible that the additional bands in the plasma samples recognized by this antibody are different CBG glycoforms, but this will require further investigation. Importantly, this is the first report of the association between cortisol-induced increases in plasma binding capacity, and levels of immunoreactive protein.

Hepatic CBG mRNA levels were not different between the intact and adrenalectomized twin fetuses at 143 days of gestation. Previous studies have shown that the liver is the major site of CBG biosynthesis in the ovine fetus (Berdusco *et al.* 1995). However, CBG mRNA levels reached maximum values at day 140 and then fell, so that by term hepatic CBG mRNA levels were similar to those of fetuses at day 110-120 (Berdusco *et al.* 1995). It has been previously reported that there was a significant increase in the levels of CBG mRNA in the liver of fetal sheep treated with dexamethasone for 96h from d130 of gestation (Berdusco *et al.* 1993). A similar stimulatory effect of glucocorticoids on CBG gene expression has been reported in the mid-gestation baboon (Pepe *et al.* 1996) and in the newborn mouse (Zhao *et al.* 1997). Results in the fetus and newborn differ from those in adults of various species (Smith and Hammond, 1991; Scrocchi et al. 1993),

where glucocorticoids down regulate hepatic CBG expression, and decrease plasma CBG. The reasons for the different responses are not known, but presumably reflect alterations in transcriptional regulation of the CBG gene between fetus and adult. The previous demonstration of decreased hepatic CBG mRNA levels at term (Berdusco *et al.* 1994) together with the lack of difference in CBG mRNA in intact and adrenalectomized twins may reflect emergence of the adult pattern of down regulation of CBG by cortisol in late-gestation intact fetuses (Berdusco *et al.* 1995). In term animals, plasma CBC is maximal close to parturition in most (Ballard *et al.* 1982; Berdusco *et al.* 1995), although not all studies (Ali *et al.* 1992). It has been suggested previously that the difference in patterns of plasma CBC and hepatic CBG mRNA may also reflect the prolonged half-life of CBG in the fetal circulation (Berdusco *et al.* 1995), and the present findings are consistent with that possibility.

In untreated sheep fetuses there is a progressive increase in plasma CBC (Ballard *et al.* 1982; Berdusco *et al.* 1995), and in high-affinity plasma binding of cortisol (Fairclough and Liggins, 1975) during the last 10-15 days of gestation. It has been suggested that this is important in regulating the negative feedback effect of cortisol on the fetal hypothalamus and pituitary (Norman *et al.* 1985; Brooks and Challis, 1988). This is supported by the finding that purified CBG decreased the negative feedback effect of cortisol, but not of dexamethasone (which does not bind to CBG), on basal and CRH-stimulated ACTH output by ovine fetal pituitary cells in culture (Berdusco *et al.* 1995). Thus an elevation in plasma CBG may be a critical event contributing to the progressive rise in ir-ACTH concentrations in fetal sheep plasma during late pregnancy (Norman *et al.* 1985), despite a progressive rise in the

total plasma cortisol concentration (Bassett and Thorburn, 1969; Norman *et al.* 1985). The present studies show that exogenous cortisol given incrementally during late gestation stimulates increases in fetal plasma CBG. The failure of plasma CBC to rise in fetuses that had been adrenalectomized provides very strong support for a physiological role of endogenous fetal cortisol in mediating this response.

Chapter Four

Divergent changes in plasma ACTH and pituitary POMC mRNA after cortisol administration to the ovine fetus in late gestation

The data contained within Chapter Four has been previously published in the American Journal of Physiology (1998) 274:E417-E425, and appears here with the permission of the American Physiological Society (authorization is appended).

4.1 Introduction

In fetal sheep, basal plasma concentrations of ACTH and cortisol (Norman *et al.* 1985), and pituitary POMC expression (Matthews *et al.* 1994) rise concomitantly in late gestation. This occurs despite demonstrable negative feedback effects of glucocorticoids on stress-induced (Akagi *et al.* 1990) or CRH-stimulated pituitary-adrenal function (Rose *et al.* 1985), suggesting that there may be a decrease in cortisol negative feedback upon basal hypothalamic and pituitary function at this time. Previously Apostolakis *et al.* (1994) showed that administration of cortisol to the ovine fetus at 134 days of gestation altered ACTH pulsatility, increasing ACTH pulse peak and nadir values. However, the mechanisms whereby intrafetal cortisol has a positive effect on plasma ACTH concentrations during late gestation is not clear. Therefore, we infused cortisol to fetal sheep, beginning prior to the prepartum rise in endogenous cortisol, in amounts that would reproduce plasma cortisol concentrations similar to those near term, to determine the effects on plasma ACTH and to examine the underlying mechanisms of any changes.

The plasma concentrations of CBG also rise during the last third of gestation (Ballard *et al.* 1982; Berdusco *et al.* 1995), reflecting an increase in its synthesis in the fetal liver (Berdusco *et al.* 1995). Previously it has been shown that CBG biosynthesis was stimulated by exogenous cortisol in mid- and late-gestation (Chapter Three; Berdusco *et al.* 1993), and reduced after bilateral adrenalectomy of the ovine fetus (Chapter Three). Therefore, it was suggested that the rise in cortisol stimulated CBG, and would in turn help to maintain a low free cortisol concentration in plasma despite elevations in the total (free + bound) cortisol concentration

(Ballard *et al.* 1982; Challis and Brooks 1989). Therefore a CBG-mediated decrease in free cortisol (Liggins *et al.* 1973; Challis and Brooks, 1989) may in turn diminish the negative feedback effects of cortisol on the pituitary, resulting in an increase in pituitary POMC mRNA levels, and ACTH output. To examine the underlying mechanisms of the ACTH response to exogenous cortisol, we measured levels of POMC mRNA in different regions of the pituitary, and CBG mRNA in the liver at various times during an intrafetal cortisol infusion.

Therefore, the overall hypothesis of the present study was that intrafetal cortisol administration during late gestation would increase hepatic CBG synthesis and circulating CBG levels, thereby reducing free cortisol concentrations in plasma. In turn, this would reduce the negative feedback effects of cortisol on pituitary POMC expression and ir-ACTH output, resulting in an increase in circulating ir-ACTH concentrations, despite elevated total cortisol concentrations in plasma. In this manner, we would approximate the plasma profiles of ACTH and cortisol observed in the fetal sheep, near term.

4.2 Materials and Methods

4.2.1 Experimental Protocols

This protocol was approved by the Animal Care Committees of St. Joseph's Health Centre, the University of Western Ontario, and the University of Toronto, in accordance with the guidelines of the Canadian Council on Animal Care. Animals were allowed a minimum of 5 days recovery after surgery, before experimentation began. Starting on days 124-129, fetuses received an intravenous infusion of either

cortisol (11β,17,21-trihydroxy-4-pregnene-3,20-dione, Steraloids, Inc., Wilton, NH; 5μg/min, n=7) or an equal volume of saline (3ml/hour, with 2% v/v ethanol, n=6) for 96 hours. Fetal arterial blood samples were collected and stored as described below (Section 4.2.2.3) every eight hours beginning 24 hours prior to the start of infusion, and continuing throughout the experiment. Additional animals were infused for 12 hours (saline n=4, cortisol n=4) or 24 hours (saline n=4, cortisol n=4) to examine changes in POMC and CBG mRNA levels associated with the initial phase of the rise in plasma cortisol concentrations.

4.2.2 Animals

Ewes of mixed breed and known insemination dates were transported to the research facility a minimum of three days prior to surgery on days 119-122 of gestation (term~145 days). The animals were fasted for a period of 24-36 hours, and water was withdrawn approximately 2 hours before surgery.

4.2.2.1 Surgery

The animals were transported to the surgical preparation room and general anaesthesia induced with 40ml of 2.5% sodium pentothal (Abbot Laboratories, Montreal, Que.), injected into the ewe via jugular venipuncture. Animals were quickly intubated and anaesthesia maintained with 1-2% halothane (Wyeth-Aerst, Montreal, Que.). The ewe's abdominal area was sheared and scrubbed three times using iodine soap (Proviodine, Rougier Inc., Chambly, Que.). The animal was transferred

to the operating room and the surgical field was treated with an iodine and alcohol solution.

Surgery was performed using aseptic techniques. A mid-line incision was made in the lower abdomen, and a second incision was made in the ewe's flank using a trochar. The packaged catheters and myographic leads were advanced through the flank incision and along the inside of the abdominal cavity to the site of the midline incision. The uterus was exposed, the fetal head was located and an incision made in the uterus through which the fetal head was delivered. Babcock clamps were used to secure the edges of the uterine incision to the fetus to prevent loss of amniotic fluid and to maintain the integrity of the fetal membranes. An incision through the fetal skin was made to expose the fetal carotid artery and jugular vein. Each of these vessels were isolated, a small incision made in the vessel wall and a heparinized-saline filled ($1x10^6$ IU heparin / 250ml sterile saline, 0.9% Sodium Chloride), polyvinyl catheter was introduced (6cm) and secured to the vessels using 2-0 silk (Ethicon, Johnson & Johnson Medical Products, Peterborough, Ont.). The catheters were attached to either three-way (venous catheters) or four-way stopcocks (for pressure recording from arterial and amniotic cavity catheters). The incision was closed and the vascular catheters anchored to the fetal skin using 2-0 silk. A catheter with a several slit openings was inserted into the amniotic fluid cavity, and also secured to the anchoring sutures of the vascular catheters. All catheters were tested repeatedly to ensure patency. The fetus was returned to the uterine cavity and the fetal membranes and uterus were closed using 2-0 silk. Uterine electromyographic (EMG) leads (Cooner Wire Co., Chatsworth, CA)

were attached to the myometrium to monitor uterine electrical activity. The abdominal incision was closed in two layers to prevent herniation. The visceral layer was closed with a line of interrupted figure 8 sutures in conjunction with a running set of simple sutures in 2-silk. The skin was subsequently closed with 0-vicryl (Ethicon, Johnson & Johnson Medical Products, Peterborough, Ont.) and the trochar perforation was sewn shut with a purse-string suture (0-vicryl).

The maternal femoral artery and vein were also catheterized. These vascular catheters were advanced 20cm to lie in the maternal abdominal aorta and inferior vena cava and secured using 0-silk (Ethicon, Johnson & Johnson Medical Products, Peterborough, Ont.). The incision was closed and catheters secured into place with anchors attached to the maternal skin using 0-silk.

4.2.2.2 Post-operative care

Ewes were administered 4ml Pen-di-strep IM (200,000 IU penicillin G and 250mg dihydrostreptomycin/ml, Rogar, London, Ont.) at the time of surgery and for 3 days post operatively. Crystapen (1ml: $1x10^6$ IU Penicillin G; Wyeth-Ayerst, Montreal, Que.) was injected into the fetal vein and into the amniotic cavity during surgery and every day for three days after surgery. The animals were allowed a minimum of five days recovery before experimentation began. Catheters were flushed with sterile heparinized saline daily during the recovery period and experimental protocols to maintain patency.

The animals were housed in individual metabolic cages from surgery to the time that the animals were euthanasized. The metabolic cage allows backward and

forward movement, and the animal may lay or stand, while the catheters at the ewe's side remain protected. The animals were housed in a 14-hour light cycle (0600 to 2000 hours) and a 10-hour dark cycle. Ewes were fed a 14% protein diet of grain pellets (500mls, B&W Feed) and hay pellets (1000mls, B&W Feed) daily, supplemented with hay and water *ad libitum*.

4.2.2.3 Blood sampling

Blood samples were collected daily for determination of maternal and fetal blood gas status. In addition, fetal blood samples (4ml) were collected as outlined in the experimental protocol (Section 4.2.1) for hormone analysis. Two sterile syringes, that previously contained heparinized saline, were aseptically attached to the stopcock, and a volume equal to twice the catheter dead space volume (approximately 2ml for fetal, and 8ml for maternal catheters) drawn into one syringe. The sample volume was then collected into the second syringe and placed onto ice immediately. The dead space volume was replaced and the catheter was flushed with fresh heparinized saline.

A small aliquot of whole blood was used to determine pH, pCO_2, and pO_2 with an ABL-5 blood gas analyzer (Radiometer, Copenhagen, Denmark). The collected blood samples were transferred to chilled polystyrene tubes and centrifuged at 1500 x *g* for 10min at 4°C. The plasma was collected and aliquots stored in eppendorf tubes at –20°C until analysis.

4.2.2.4 Uterine activity and blood pressure recording

Fetal blood pressure and amniotic fluid pressure were measured continuously using Statham pressure transducers (P23XL, Spectramed, Oxnard, CA) and displayed on a chart recorder (model 78D, Grass Instrument). Mean fetal blood pressure was calculated as 0.4 x (systolic pressure – diastolic pressure) + diastolic pressure – amniotic fluid pressure. Blood pressure was measured at five different time points every hour and the mean was calculated. The daily mean was then calculated from these hourly values.

Uterine EMG activity was recorded continuously using a Grass wide-band AC pre-amplifier (Grass model 78D) and was assessed as the number of episodes of low-amplitude activity lasting >5min, referred to as 'contractures" (Nathanielsz *et al.*1976), and the number of contractions (activity lasting 0.5-1min) per two hour period (Harding *et al.* 1982). Since intrafetal administration of cortisol can induce premature parturition in sheep (Liggins, 1968), uterine activity was monitored to ensure that any changes in plasma ir-ACTH concentrations were not attributable to the process of labour.

4.2.2.5 Euthanasia and tissue collection

At the conclusion of the experimental protocol, the ewes were killed with an overdose of Euthanyl (24% pentobarbital: MTC Pharmaceuticals, Cambridge, Ont.). The fetus(es) were delivered by cesarean section, and killed by administration of 5-10ml of Euthanyl via cardiac puncture. Tissues were subsequently dissected and collected quickly. Fetal liver was cut into ~1cm^2 pieces, flash frozen in liquid nitrogen

and stored in PolyQ scintillation vials (20ml, Beckman Instruments (Canada) Inc, Toronto, Ont.) at –80°C. The fetal pituitaries were frozen intact on dry ice oriented with the pituitary stalk extended upwards.

4.2.3 Measurement of plasma ACTH, cortisol, free cortisol and CBG

Plasma CBG, cortisol and $ACTH_{1-39}$ levels were measured as described in Section 3.2.2.

4.2.3.1 Measurement of free cortisol in fetal plasma

The percentage of free cortisol in fetal plasma (duplicate samples) was measured by the centrifugal ultrafiltration-dialysis technique developed and described by Hammond *et al.* (1980). [^{3}H]cortisol was purified on the day of the assay, using a Lipidex-5000 (hydroxyalkoxypropyl Sephadex, Packard Instrument Co., Inc. Mississauga, Ont.) chromatography column (5ml), and eluted with chloroform:hexane (50:50). Plasma samples (450μl) were pipetted into glass tubes (12 x 75mm) containing [^{3}H]cortisol (approximately 120 000cpm in choloroform: hexane (50:50), previously dried under nitrogen) and [^{14}C]glucose (30 000cpm; 1μl of distilled H_2O). The tubes were incubated for 1 hour at 37°C. Aliquots of these samples (200μl) were transferred to ultrafiltration vials (glass tubes, 10mm diameter, with dialysis membrane (24Å) covering one end). The ultrafiltration vials were inserted into a flat-bottom borosilicate shell vial (VWR Canlab, Mississauga, Ont.) containing 3 discs of filter paper (Whatmann 1: Fisher Scientific, Toronto, Ont.).

Both tubes were centrifuged at 1500 x g for 1hour at 37°C. A sample (20μl) of the plasma from the inner tube was added to a second set of flat-bottom shell vials containing filter paper (as above). Distilled water (350μl) was added to all of the vials, samples were mixed well using a vortex mixer, and incubated at room temperature for 30min. Scintillation fluid (4ml) was added and the radioactivity within each vial measured using a beta counter adjusted for the simultaneous measurement of ^{3}H and ^{14}C. Since [^{14}C]glucose does not bind to plasma proteins or the dialysis membrane (Hammond *et al.* 1990), the ratios between [^{3}H]cortisol and [^{14}C]glucose may be used to calculate the relative concentrations of [^{3}H]cortisol. After correction for background counts and overlap of ^{14}C-beta emission into the ^{3}H window the percentage of free cortisol was calculated as follows:

$$\% \text{ free cortisol} = \frac{\text{ultrafiltrate}}{\frac{[^{3}H]\text{cortisol}}{[^{14}C]\text{glucose}}} \div \frac{\text{plasma}}{\frac{[^{3}H]\text{cortisol}}{[^{14}C]\text{glucose}}} \times 100.$$

4.2.4 Northern blot analysis of CBG mRNA

It has been previously shown that the liver is the major site of CBG biosynthesis in fetal sheep (Berdusco *et al.* 1995). Total cellular RNA was extracted from the fetal liver, and subjected to northern blot analysis as outlined in Section 3.2.4.

4.2.5 *In situ* hybridization of pituitary POMC

Frozen pituitaries were sectioned (15μm) in a coronal plane using a cryostat (Tissue-Tek, Miles Canada, Etobicoke, Ont.) and mounted onto poly-L-lysine (Sigma Chemical Company Chemical, St. Louis, MO) coated slides. The mounted sections were fixed in 4% paraformaldehyde, and stored under 90% ethanol until analysis (Matthews *et al.* 1994). An oligonucleotide (45mer) complementary to bases 711-756 of the porcine POMC gene (Gossard *et al.* 1986) was used in this study. The oligonucleotide probe (1μl of 5ng/L), tailing buffer (1.5μl: Gibco-BRL), and DEPC-water (8.25μl) were combined in an eppendorf tube. ^{35}S-deoxyadenosine 5'-(α-thio) triphosphate (dATP: 1μl, 1300 Ci/mmol; Du Pont) and terminal deoxynucleotidyl transferase (TdT: 1.5μl: Pharmacia, LKB) were added, the solution was gently mixed and incubated at 32.5°C for 1 hour. At the conclusion of the incubation period the reaction was quickly terminated by the addition of DEPC-water (40μl, 1% v/v DEPC in distilled water). Separation of the labeled probe from the free fraction was achieved by eluting the mixture through a Sephadex G50 spin column. The spin column was comprised of a sterile 1ml syringe, containing a siliconized glass wool plug and filled with Sephadex (5% w/v in TENS buffer (0.1M NaCl, 0.1M Tris/HCl, 0.001M EDTA), housed in a sterile 15ml Falcon tube (Fisher Scientific Ltd., Nepean, Ont.). The column was spun at 1500 x *g* for 2 minutes, the labeled-probe was loaded onto the column and spun as before. The eluant was collected and 1M DL-Dithiothreitol (2μl: DTT, Sigma Chemical Company, St Louis, MO) was added immediately. An aliquot (1μl) was counted to determine the specific activity of the

labeled probe which was then diluted to a concentration of 1-1.5 x 10^5 cpm/100µl of hybridization buffer (50% deionized formamide, 4X saline sodium citrate (SSC), 0.01M sodium pyrophosphate, 5X Denhardts solution, 0.02% w/v salmon sperm DNA (Sigma Chemical Company, St Louis, MO), 0.01% w/v polyadenylic acid, 0.01% w/v heparin, 10% w/v dextran sulphate).

Slides were air dried and placed in plastic incubation chambers. The probe in hybridization buffer was spotted onto the tissue sections and a Parafilm (Parafilm "M" Laboratory Film, American National Can, Chicago, IL) coverslip was applied. The moisture levels in the incubation chambers were maintained by the addition of a moistened tissue paper (50% formamide, 4X SSC, and DEPC-water), and incubated overnight at 42°C. The slides were washed (1X SCC, 0.5% β-mercaptoethanol) for 30 minutes at room temperature, followed by a 1-hour wash at 55°C, and approximately 10 seconds each of 1X SSC, 0.1X SSC, 70% and 90% ethanol. The slides were air-dried and placed against x-ray film (Kodak, BioMax) at room temperature, for two hours and then re-exposed with a second film for four days to measure the levels of POMC mRNA in the pars intermedia and pars distalis respectively. The two exposure times were necessary for the hybridization signal to be within the linear range of the film for the two regions of the pituitary. Linearity was established by the simultaneous exposure of the film to ^{14}C-standards. The autoradiograms were then analyzed using computerized image analysis (Imaging Research Inc., St. Catherines, Ont.). Results are expressed as relative optical density (ROD) for a minimum of fifteen pituitary sections per animal. A control

45mer sense oligonucleotide was also synthesized. No signal was observed when it was hybridized with fetal sheep pituitary sections (Broad *et al.* 1993). The fetal pituitary sections were then coated with Ilford K5 liquid emulsion (Ilford Limited, Cheshire, England), developed and counter-stained with Carazzi's hematoxylin to identify nuclei. The resultant silver grain deposits were visualized using light microscopy.

4.2.6 Immunohistochemistry

Immunohistochemical detection of ir-ACTH was performed on adjacent 15μm frozen pituitary sections prepared as described previously for *in situ* hybridization. Sections were rehydrated in series of progressively weaker alcohols (100%, 90%, 70%, 50%), and washed in 0.1M phosphate buffered saline (PBS). Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol (30min). Immunostaining was accomplished in conjunction with the Vectastain ABC kit (Vector Laboratories Burlingame, CA) as described previously (Riley *et al.* 1991). The non-specific background binding was blocked using normal goat serum (NGS: 0.15% v/v, 20 min). Excess goat serum was blotted off and sections were incubated with a polyclonal antibody to human $ACTH_{1-24}$ (DAKO Corporation, Carpinteria, CA) for 18-24h at 4°C in a moist chamber. Sections were washed with 0.1M PBS, and incubated with biotinylated second antibody (0.05% v/v in 0.1M PBS with 0.15% NGS) for 2 hours at room temperature. After an additional wash with 0.1M PBS, slides were incubated with Vectastain ABC (Vector Laboratories Burlingame, CA) for 2 hours at room temperature. After a PBS (0.1M) wash, specific immunostaining

was visualized by the addition of the chromagen 3,3'-diamobenzidine (SIGMA*FAST* Sigma Chemical Company, St Louis, MO), and the reaction stopped with a water wash. The slides were counter-stained with Carazzi's hemotoxylin, placed under a running water wash (5min), dehydrated in ethanol, and mounted using Permount (Fisher Scientific Ltd, Nepean, Ont.).

The ACTH antibody used here has been characterized extensively, and the antigenic site shown to be between amino acids 18 and 24 (Jacobs *et al.* 1991). This ACTH antibody cross reacts <1% with α-MSH, β-MSH, βLPH (Jacobs *et al.* 1991), The number of immunopositive cells within ten fields (475μm X 350μm = 1.7 X $10^5 \mu m^2$), was counted for each animal. Adjacent sections were incubated with the primary antibody in the presence of an excess of antigen (human $ACTH_{1-24}$), to provide negative controls.

4.2.7 Data Analysis

Blood pressure and plasma hormone concentrations were measured in samples collected every eight hours, and the daily mean value was calculated for each animal. These values are reported as mean ± SEM for the number of animals stated. The maximum and minimum changes in ir-ACTH concentrations were calculated, from the three plasma samples collected each day, relative to the mean plasma ir-ACTH value during the control period, for individual animals, and reported as mean ± SEM. Changes in plasma cortisol, CBG, ir-ACTH, maximum and minimum change in ir-ACTH, mean arterial pressure, and uterine activity were

analyzed by two-way analysis of variance corrected for repeated measures (two-way RM-ANOVA). Statistical significance was determined as $p \leq 0.05$. The effects of individual times of treatment were assessed by Student-Newman-Keuls multiple range tests. Values for hepatic CBG mRNA levels, free cortisol concentrations, and the number of corticotrophs staining positively for ir-ACTH were not distributed normally, and were therefore assessed by the Kruskal-Wallis analysis of variance followed by Dunn's Test, or the Mann-Whitney rank sum test.

4.3 Results

4.3.1 Plasma cortisol concentrations

The daily mean concentration of cortisol in fetal plasma rose during cortisol infusion from basal values of 4.4±0.6ng/ml (n=6) to19.3±3.1ng/ml within 24 hours ($p<0.05$, Figure 4.1). The mean plasma cortisol concentration rose progressively to a maximum concentration of 38.6±2.7ng/ml, which is similar to that seen in the ovine fetus near term (Norman *et al.* 1985). Plasma cortisol concentrations in the control animals did not change significantly throughout the infusion period.

4.3.2 Fetal blood gases and uterine activity

Fetal arterial pH, pO_2, pCO_2, and O_2 saturation were unchanged throughout the study in both the cortisol and saline-treated animals (Table 4.1). Mean arterial pressure increased significantly within the first 24 hours of cortisol administration

Figure 4.1 Plasma cortisol concentrations before and during a 96h cortisol (■, n=7), or saline (□, n=6) infusion, starting at time zero, to fetal sheep in late gestation. Values are mean ± SEM, *$p<0.05$, two-way RM-ANOVA, followed by Student-Newman-Keuls.

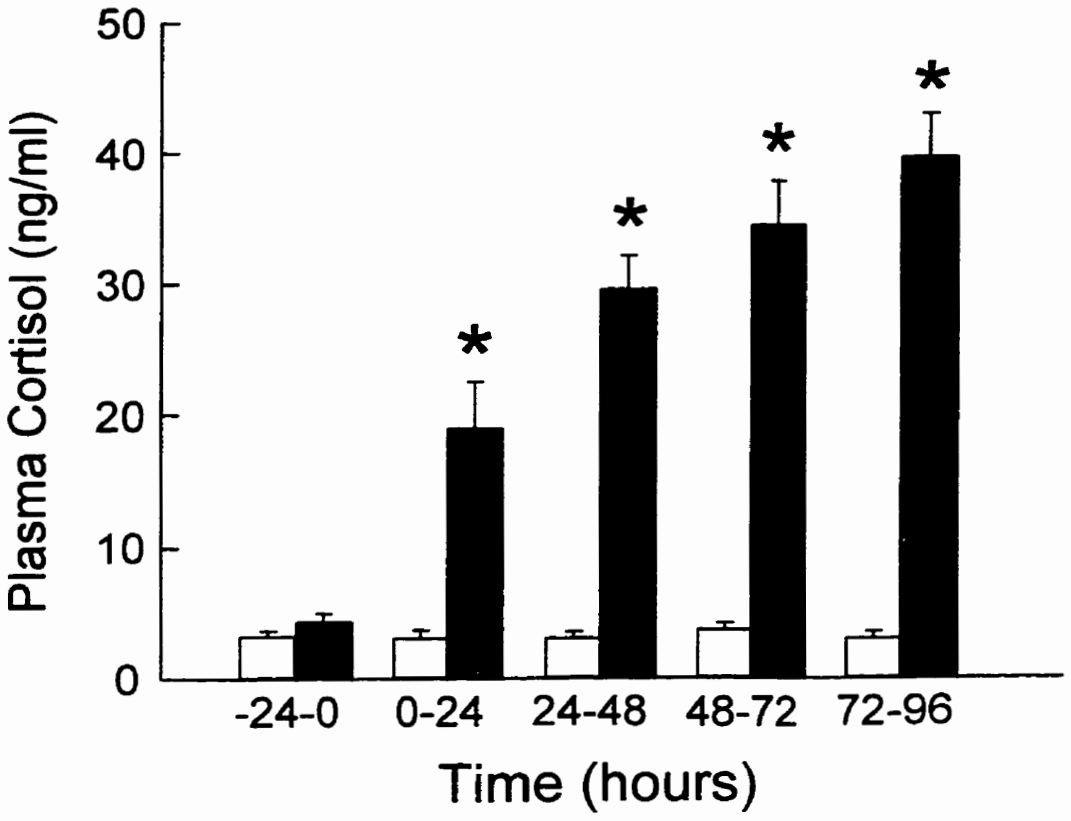

Plasma Cortisol (ng/ml)
50
40
30
20
10
0
*
*
*
*
-24-0
0-24
24-48
48-72
72-96
Time (hours)

Table 4.1 Fetal arterial blood gases during the first 24 hours prior to, and the first and last 24 hours of the infusion of saline (n=5) or cortisol (n=7). Values are mean ± SEM.

	Saline			Cortisol		
	-24-0h	0-24h	72-96h	-24-0h	0-24h	72-96h
pH	7.36 ± 0.01	7.34 ± 0.01	7.35 ± 0.01	7.38 ± 0.01	7.37 ± 0.01	7.36 ± 0.01
pO_2 (mmHg)	22.2 ± 0.7	20.65 ± 0.9	21.41 ± 0.6	21.8 ± 1.0	22.1 ± 1.3	24.4 ± 1.7
pCO_2 (mmHg)	53.5 ± 0.8	55.6 ± 0.9	54.9 ± 1.4	60.2 ± 2.5	56.6 ± 4.0	56.0 ± 2.3
O_2 Sat (%)	60.2 ± 2.5	56.6 ± 4.0	56.0 ± 2.3	60.8 ± 2.5	61.4 ± 3.5	65.5 ± 3.5

from 42.6±1.0 to 48.7±0.7mmHg, and remained at this level throughout the infusion period (Figure 4.2). There was no significant change in mean arterial pressure in the saline-infused fetuses (Figure 4.2). The number of contractures or contractions per two-hour interval were not significantly altered throughout the 96-hour infusion of either saline or cortisol (Figure 4.3).

4.3.3 Effects of cortisol on plasma ir-ACTH

Plasma ir-ACTH concentrations rose significantly in the cortisol treated fetuses from values of 25.4±2.7pg/ml during the control period, to a significantly elevated level of 37.8±3.9pg/ml, at 24-48 hours (Figure 4.4). Fetal ACTH values remained elevated throughout the cortisol infusion period ($n=7$, $p<0.05$; Figure 4.4). In the saline-infused animals plasma ACTH concentrations did not change significantly from control values of 27.0±1.7pg/ml ($n=6$, Figure 4.4). ACTH is secreted in pulses, therefore plasma ir-ACTH concentrations were variable between samples within individual fetuses. The maximum changes in plasma concentrations of ir-ACTH for the three samples collected each day were calculated relative to the initial 24-hour control period, for individual animals. The maximum change in ir-ACTH was significantly higher at 24-48 hours of cortisol infusion and remained significantly elevated throughout the infusion compared to saline-treated animals (Table 4.2). There was no difference in the minimum change in ir-ACTH for either of the infusion groups throughout the experiment (data not shown).

Figure 4.2 Mean arterial pressure in fetuses infused with cortisol (●, n=3) or saline (○, n=3), measured during the control period (24-hours prior to the start of infusion), and throughout the 96-hour infusion. Values are mean ± SEM, *$p<0.05$.

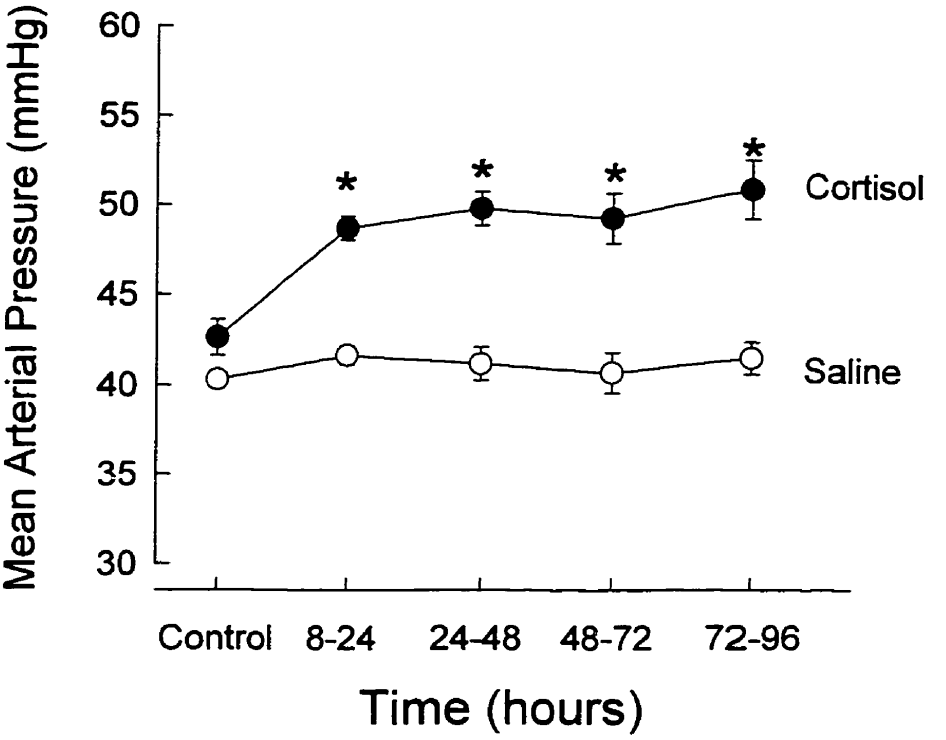
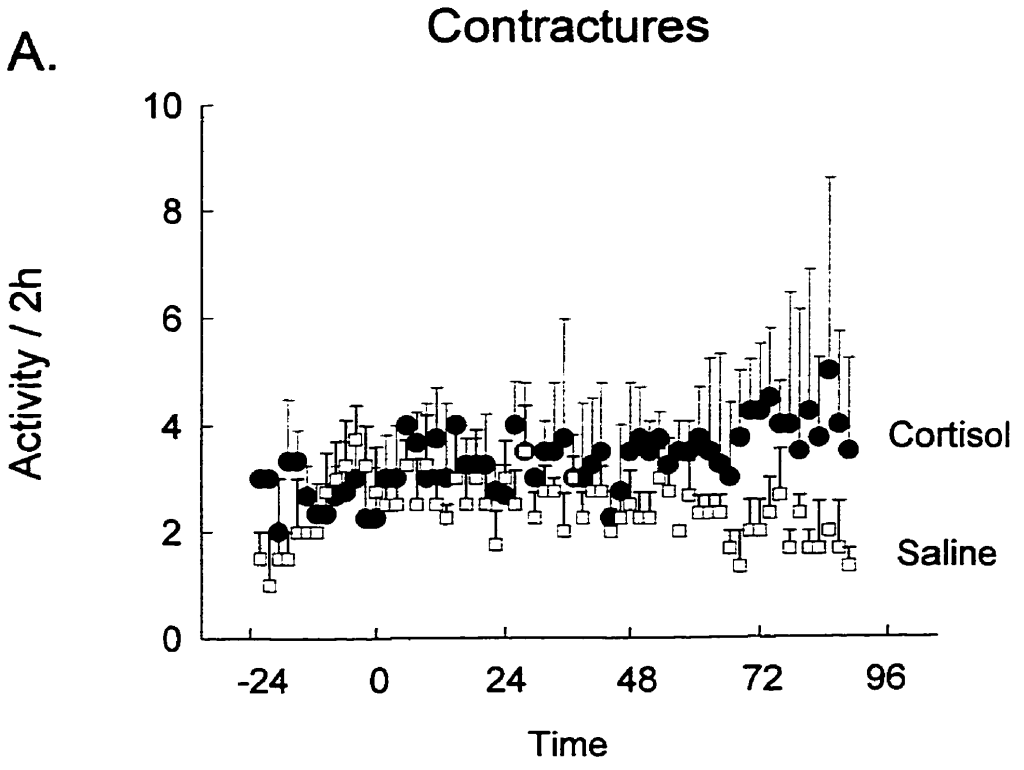

Mean Arterial Pressure (mmHg)
60
55
50
45
40
35
30
*
*
*
*
Cortisol
Saline
Control
8-24
24-48
48-72
72-96
Time (hours)

Figure 4.3 Uterine activity assessed before and during a 96 hour infusion of either cortisol (n=4) or saline (n=4). Contractures (A), are periods of uterine activity lasting >5 minutes. Contractions (B), are bursts of uterine activity lasting <1 minute and > 30 seconds. Values are mean ± SEM.

A.


Contractures
Activity / 2h
10
8
6
4
2
0
-24
0
24
48
72
96
Time
Cortisol
Saline

B.

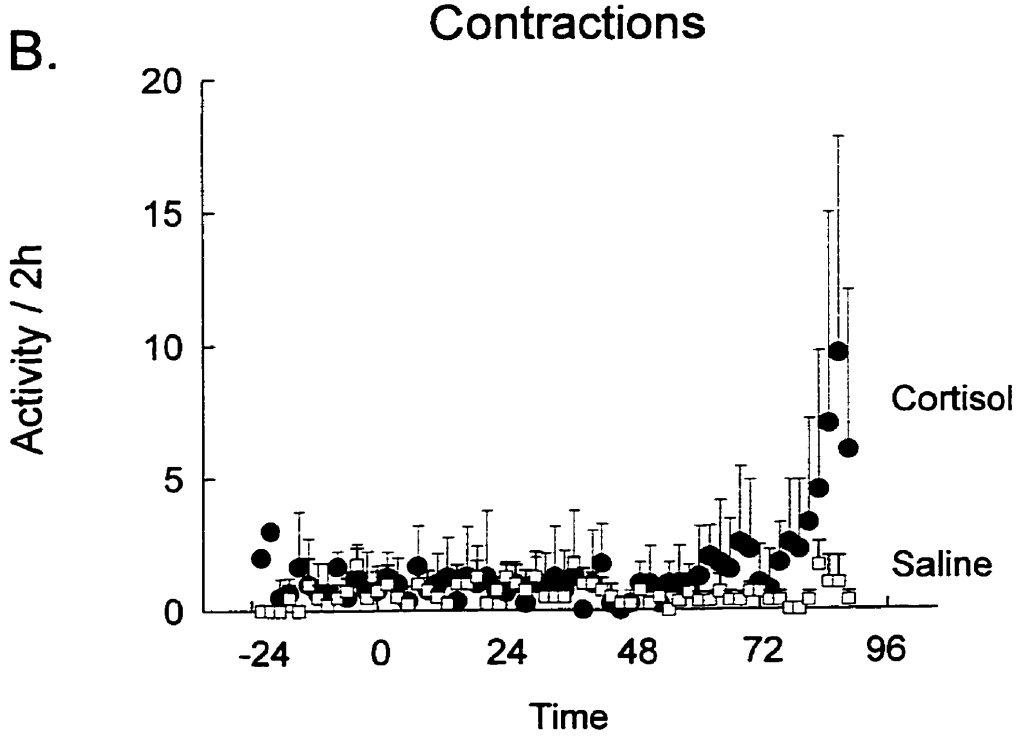

Contractions
Activity / 2h
20
15
10
5
0
-24
0
24
48
72
96
Time
Cortisol
Saline

Figure 4.4 Plasma ir-ACTH concentrations before and during a 96h infusion of cortisol (■, n=7) or saline (□, n=6), starting at time zero, to fetal sheep in late gestation. Values are mean ± SEM, *$p<0.05$ (2-way RM-ANOVA, followed by Student-Newman-Keuls).

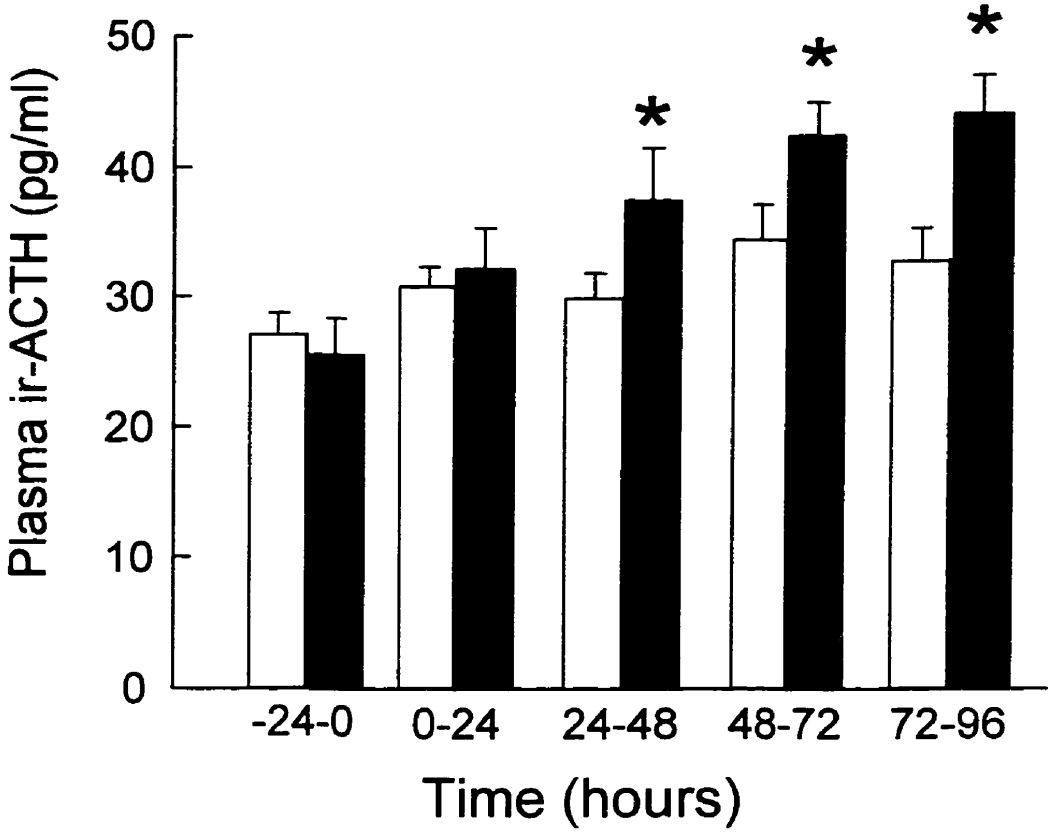

Plasma ir-ACTH (pg/ml)
50
40
30
20
10
0
*
*
*
-24-0
0-24
24-48
48-72
72-96
Time (hours)

Table 4.2 Mean maximum change in plasma ir-ACTH per 24-hours, as compared to the average plasma ir-ACTH concentrations during the 24-hour control period

	Mean plasma ir-ACTH (pg/ml)	**Maximum change in plasma ir-ACTH (pg/ml)**			
	Control	0-24h	24-48h	48-72h	72-96h
Saline (n=6)	27.0 ± 2.3	8.7 ± 2.0	7.7 ± 2.9	13.7±5.5	11.4±4.8
Cortisol (n=7)	25.8 ± 4.1	11.1±4.4	20.7±6.4*	23.7±5.4*	23.7±4.7*

Values are mean ± SEM, *p<0.05, two-way RM-ANOVA followed by Student-Newman-Keuls multiple range test.

4.3.4 Effects of cortisol on CBG biosynthesis and secretion

Plasma CBG levels were similar in both the cortisol (34.6±2.1ng/ml) and saline-treated animals (27.5±3.0ng/ml) during the control period (Figure 4.5). The plasma CBG levels of the cortisol-treated animals were elevated significantly (51.1±3.2ng/ml) by 48-72 hours of infusion and remained elevated at 72-96 hours ($p<0.05$). Northern blot analysis of RNA from the fetal liver identified a single CBG transcript of 1.8kb (Figure 4.6, top panel). Cortisol treatment elevated levels of hepatic CBG mRNA ($p<0.05$, Kruskal-Wallis analysis of variance). After 96 hours hepatic CBG mRNA levels were significantly higher ($p\leq0.05$, Dunn's Test) in the cortisol infused animals compared to the saline-infused fetuses.

4.3.5 Plasma free cortisol levels

The percent of free cortisol in plasma increased 3-fold, from 6.3±0.5 to 16.8±6.2%, at 8 hours of cortisol infusion (Figure 4.7A), but by 72 hours the percentage of free cortisol was not significantly different from saline-infused animals. However, the absolute plasma concentration of free cortisol rose within 8 hours and remained significantly elevated in cortisol-treated animals throughout the 96 hours infusion (Figure 4.7B). There was no change in the percentage of free cortisol in the plasma of saline-treated animals throughout the course of the experiment (Figure 4.7A), and the absolute free cortisol concentration did not change significantly from mean values of 0.3±0.1ng/ml (n=3; Figure 4.7B).

Figure 4.5 Plasma corticosteroid-binding capacity (CBC), before and during a 96h cortisol (■, n=7), and saline (□, n=6) infusion, starting at time zero, to fetal sheep in late gestation. Values are mean ± SEM, *$p<0.05$ (Two-way ANOVA for repeated measures, Student-Newman-Keuls).

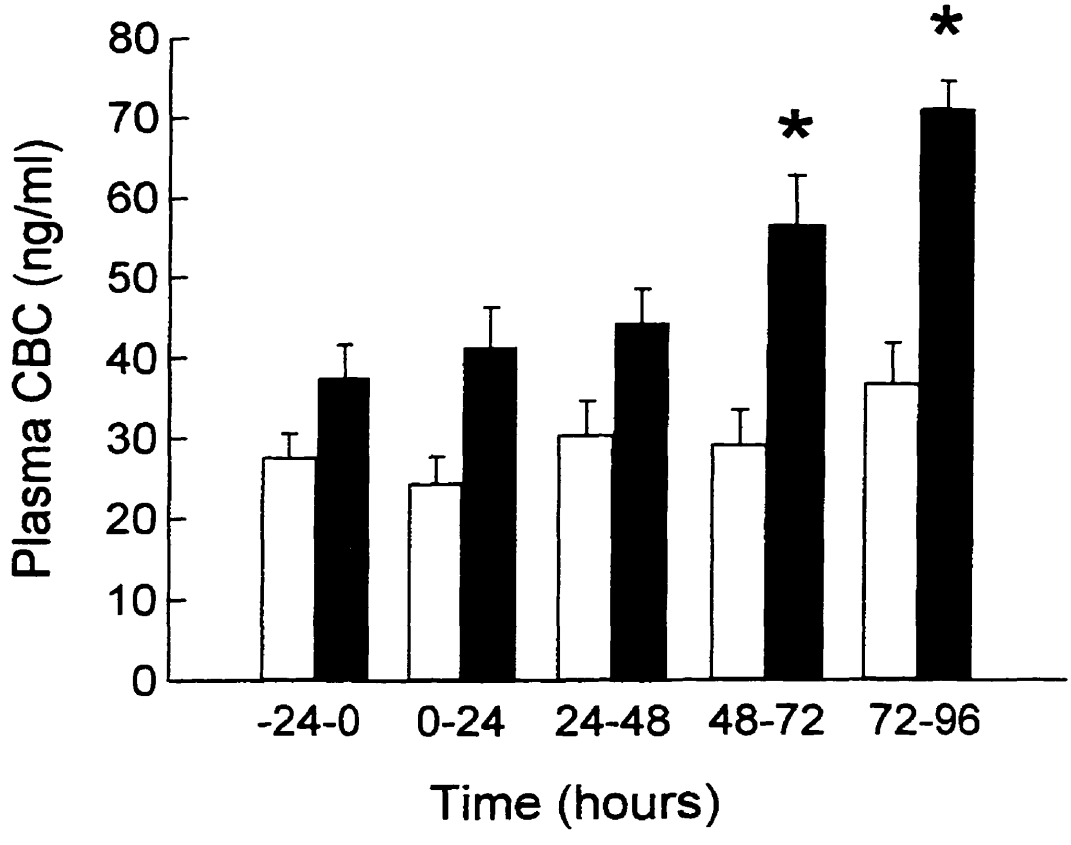

Plasma CBC (ng/ml)
80
70
60
50
40
30
20
10
0
*
*
-24-0
0-24
24-48
48-72
72-96
Time (hours)

Figure 4.6 The effect of cortisol on hepatic corticosteroid-binding globulin (CBG) mRNA levels. *Top panel,* Northern blot analysis of CBG mRNA in the livers of individual fetuses treated with cortisol or saline for 12h (n=4), 24h (n=4), or 96h (saline n=6, and cortisol n=7). A cDNA probe to 18S rRNA was used to control for the amount of RNA analyzed. The histograms show the ratio of the relative optical densities of CBG mRNA:18S rRNA for each of the cortisol (■), and the saline (□) infusion groups. Values are mean ± SEM, *$p<0.05$ (Kruskal-Wallis analysis of variance followed by Dunn's Test).

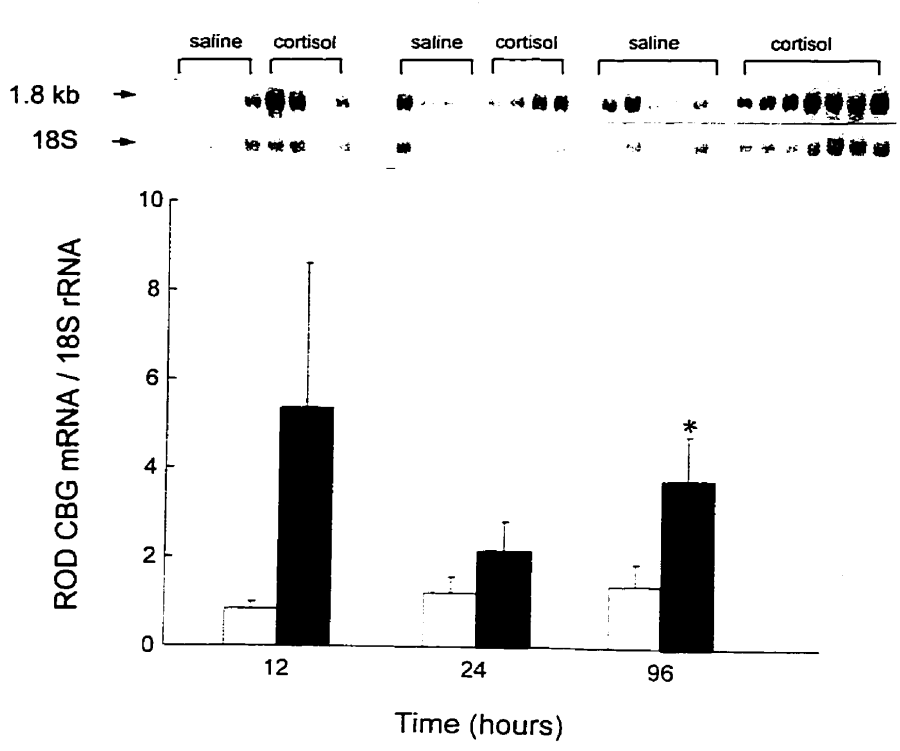
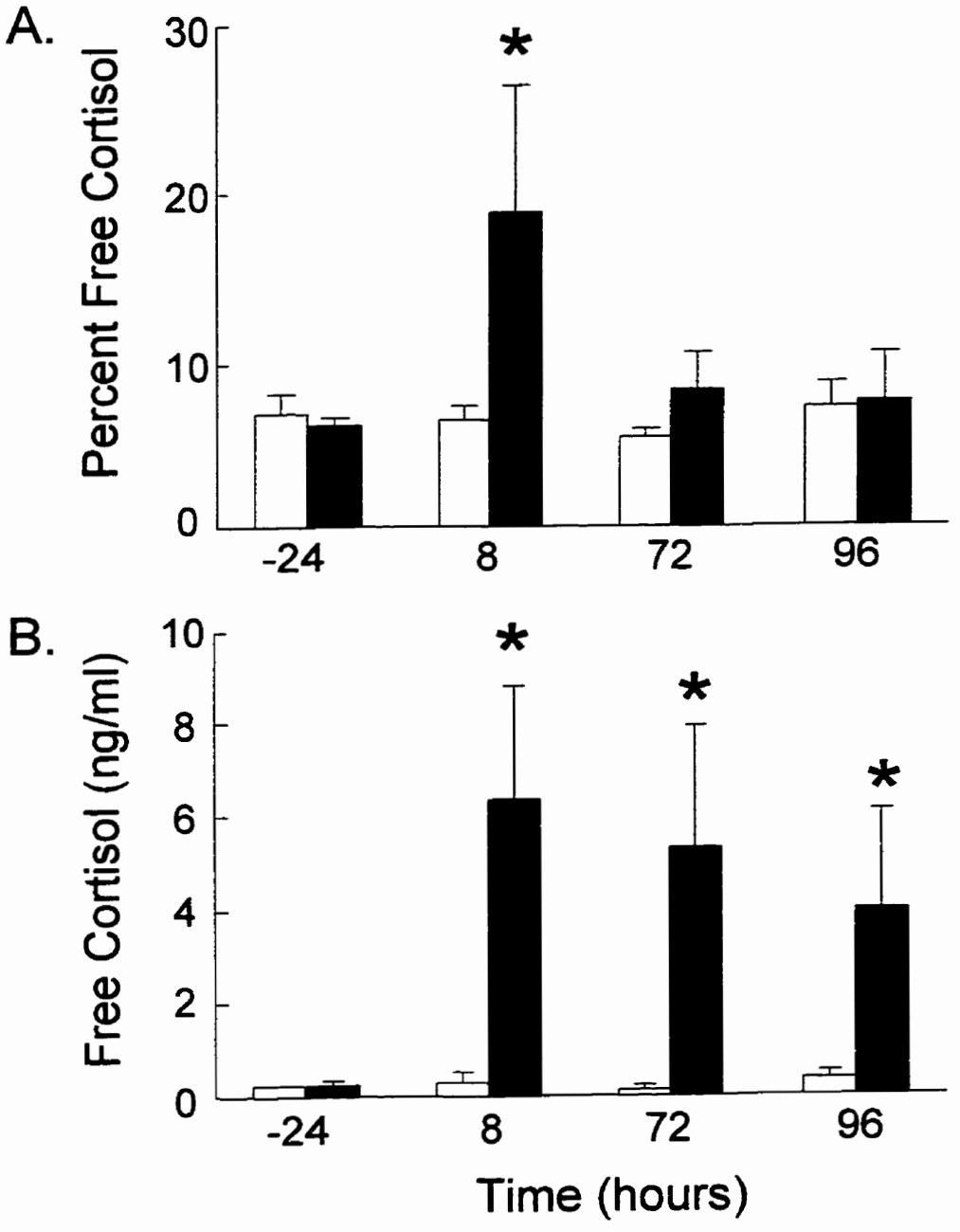

saline
cortisol
saline
cortisol
saline
cortisol
1.8 kb
18S
ROD CBG mRNA / 18S rRNA
10
8
6
4
2
0
*
12
24
96
Time (hours)

Figure 4.7 Changes in the percent of free cortisol (A), and total free cortisol (B) concentrations in fetal plasma collected during a cortisol (■) or saline (□) infusion. Values are mean ± SEM, n=6 in each group except at 96 hours, n=3 cortisol infused, and n=4 control animals, *$p<0.05$).


A.
Percent Free Cortisol
30
20
10
0
*
-24
8
72
96
B.
Free Cortisol (ng/ml)
10
8
6
4
2
0
*
*
*
-24
8
72
96
Time (hours)

4.3.6 Effect of cortisol on levels of pituitary POMC mRNA and ir-ACTH

Levels of POMC mRNA in the pars distalis of cortisol treated animals appeared markedly lower (Figure 4.8, panel B), than those in saline control animals (Figure 4.8, panel A) after 96 h of infusion. POMC mRNA levels in the pars intermedia also appeared to be decreased after 96 hours of cortisol infusion (Figure 4.8, panel D), compared to the saline-treated controls (Figure 4.8, panel C). POMC mRNA levels were quantified by computerized image analysis. There was no significant change in levels of POMC mRNA within the pars distalis or the pars intermedia after 12 hours of cortisol treatment, but the levels had fallen to 4% of controls by 96 hours (Figure 4.9). High resolution analysis using liquid silver emulsion autoradiography confirmed this finding by the near absence of silver grain deposits in the pars distalis after 96 hours of cortisol infusion (Figure 4.10, panel c), compared to the abundance of silver grain deposits seen in the saline-treated fetuses (Figure 4.10, panel a). POMC mRNA levels in the pars intermedia of the cortisol treated animals were unchanged at the end of the first 24 hours of infusion, but the levels had decreased by 38%, compared to saline controls, after 96 hours of infusion (Figure 4.9). There was no apparent change in the number of silver grains deposited between saline and cortisol infused groups (Figure 4.10), panels e and g).

The intensity of ir-ACTH peptide staining within the pars distalis did not appear different after 96 hours of cortisol treatment (Figure 4.10, panel d), compared to the saline-infused animals (Figure 4.10, panel a). However, the number of ir-ACTH positive corticotrophs in the pars distalis was significantly reduced (by 13.9%), from 218.6 ± 12.5 immunopositive cells per $1.7x10^5\mu m^2$ in the saline

Figure 4.8 Autoradiograms of coronal pituitary sections following in situ hybridization with an ^{35}S-labelled POMC oligonucleotide. Fetuses treated for 96h with saline (panels A and C) or cortisol (panels B and D). Panels A and B were exposed for four days to allow analysis of POMC mRNA in the pars distalis. Panels C and D were exposed for 2 hours to allow analysis of POMC mRNA in the pars intermedia (signal in the pars distalis is barely evident at this time). Scale: A and B = 470μm, C and D = 222μm.

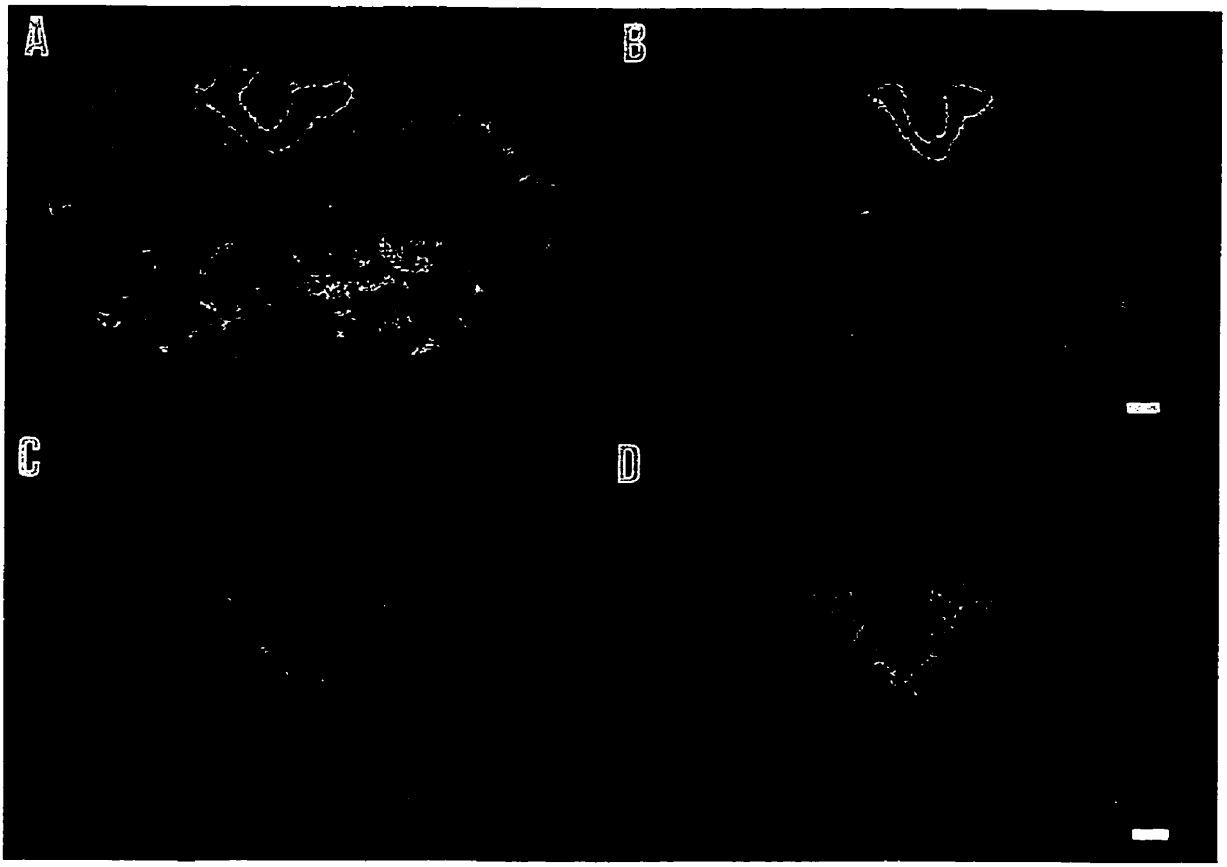

A
B
C
D

Figure 4.9 Histogram illustrating the changes in POMC mRNA levels in the pars distalis and pars intermedia following 96h of cortisol (■) or saline (□) treatment. Values are expressed as relative optical density (ROD) of the autoradiograms, and expressed as mean ± SEM, *$p<0.05$, two-way ANOVA followed by Student-Newman Keuls.

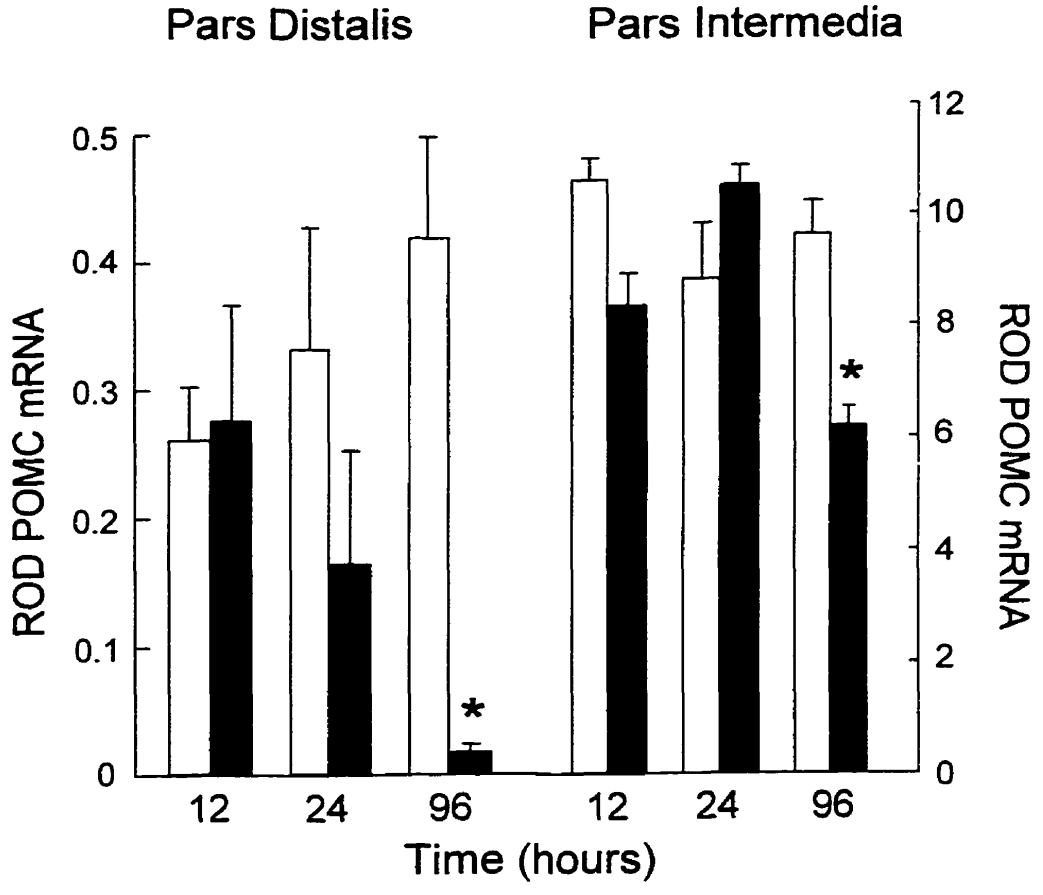
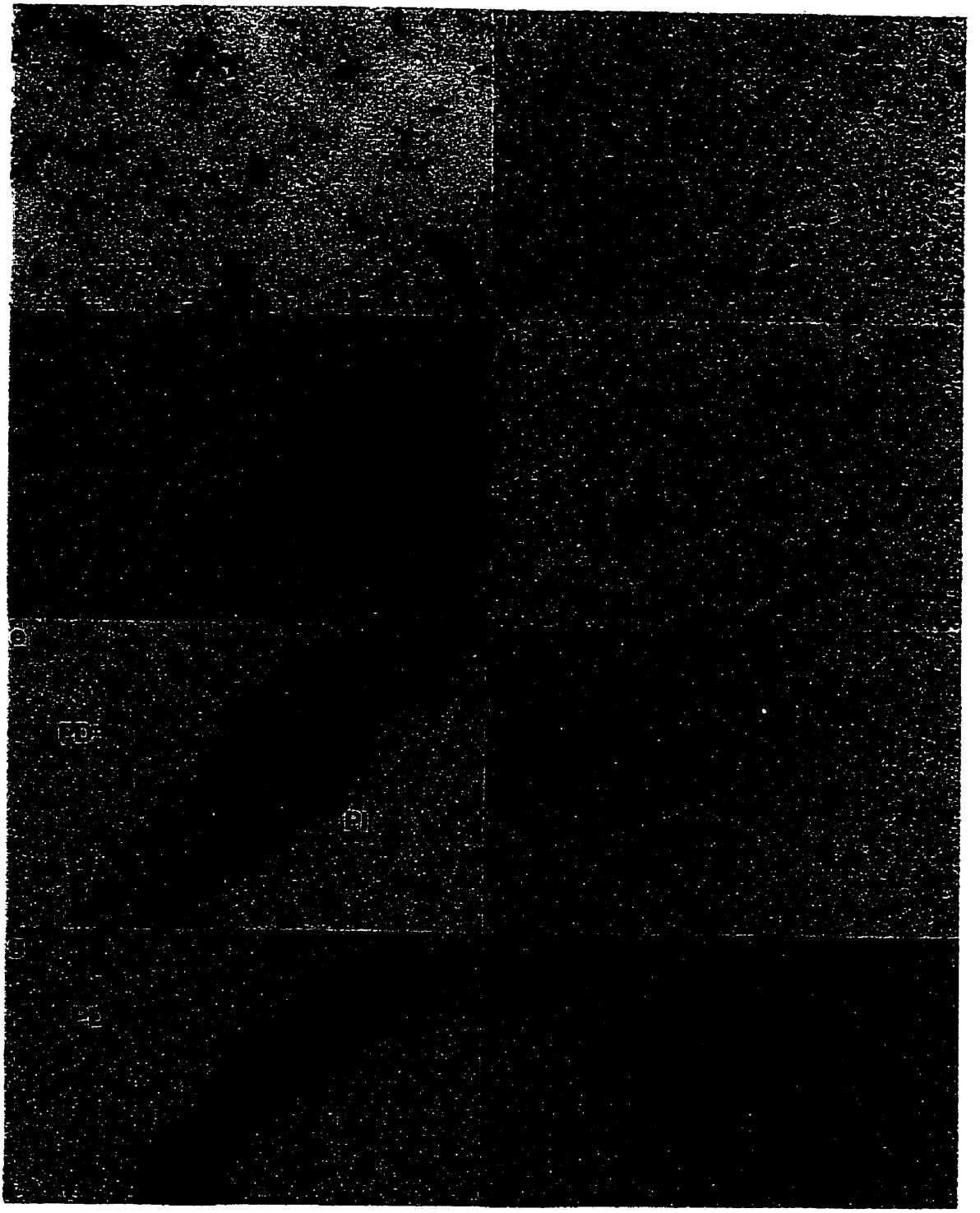

Pars Distalis
Pars Intermedia
ROD POMC mRNA
ROD POMC mRNA
0.5
0.4
0.3
0.2
0.1
0
12
10
8
6
4
2
0
*
*
12
24
96
12
24
96
Time (hours)

Figure 4.10 Photographs of the emulsion autoradiograms localizing POMC mRNA within the pars distalis (a, c) and the pars intermedia (e, g), and ir-ACTH peptide levels in the pars distalis (b,d) and pars intermedia (f, h) of the ovine fetus after a 96 hour infusion of cortisol (c, d, g, h) or saline (a, b, e, f). PI, pars intermedia; PD, pars distalis. Scale bar = 50μm.



controls, to 188.2 ± 15.1 immunopositive cells per $1.7x10^5\mu m^2$ after 96 hours of cortisol infusion. There was no diminution in the number of immunopositive cells in the pars intermedia after 96 hours of cortisol infusion (Figure 4.10, panel h), compared to control (Figure 4.10, panel f). Adjacent sections were incubated with antibody preabsorbed with an excess of human $ACTH_{1-24}$, and there was no staining present.

4.4 Discussion

We have shown that in the late gestation ovine fetus, mean plasma ir-ACTH concentrations rise significantly in response to physiological elevations in plasma cortisol concentrations. However, this does not appear to be due to a decrease in cortisol negative feedback, since plasma free cortisol concentrations remained elevated despite an increase in plasma CBG levels. The high concentration of plasma free cortisol suppressed pituitary POMC mRNA levels in both the pars distalis and the pars intermedia, and the number of ir-ACTH immunopositive cells within the pituitary pars distalis was also reduced after 96 hours of cortisol infusion. However, the number of ACTH immunopositive cells within the pars intermedia did not appear to be appreciably altered in the presence of elevated circulating free cortisol.

We believe that the increase in plasma ir-ACTH predominantly reflects changes in $ACTH_{1-39}$. The ACTH antibody used to measure fetal ACTH concentrations has been validated extensively, and does not show cross reactivity

with the higher molecular weight ACTH-related peptides (Section 3.2.2.3). We suggest that the increase in ir-ACTH reflects an increase in ACTH output in response to the infusion of cortisol. However, we cannot exclude the possibility that the metabolic clearance rate of circulating ACTH is reduced by cortisol infusion. Although, there is no significant change in the half-life of ACTH (~1 minute) in sheep between fetal and adult life (Jones *et al.* 1975), it has been previously reported in the adult rat that the metabolic clearance rate of ACTH decreases with increasing plasma ACTH concentrations (Lalonde and Normand, 1977). Mean fetal arterial pressure increased by approximately 7mmHg, within the first 24 hours of cortisol infusion and remained at this level throughout the experiment. This is similar to the increase reported during a 48 hours-cortisol infusion to the mid-gestation ovine fetus (Dodic and Wintour, 1994). However, the hypertensive effects of cortisol do not elicit an immediate ACTH response, and seem very unlikely to be causal in the later rise in plasma ir-ACTH concentrations. Previous studies have shown increases in plasma ACTH concentrations in response to oxytocin-induced uterine contractions (Lye *et al.* 1985). In the present study although uterine activity does increase slightly within the last 24 hours of cortisol infusion, this event is preceded by the ACTH rise, therefore the initial rise in plasma ir-ACTH cannot be labour induced. The increase in plasma ir-ACTH at 72-96 hours may be associated with an increase in uterine activity, however, there was no change in fetal arterial pO_2 which indicates that the rise in ACTH is not hypoxia-mediated. In addition, there was no change in the plasma ir-ACTH concentrations of the saline-treated fetuses, verifying that the rise in ACTH is not an effect of the sampling protocol employed.

It has been reported previously that a 96-hour cortisol infusion to the ovine fetus at 134 days of gestation affects pulsatility of ir-ACTH, increasing pulse peak and nadir (Apostolakis *et al.* 1994). We examined the maximum and minimum change in ir-ACTH, and compared these to the mean ir-ACTH concentration during the control period. The maximum change in ir-ACTH from the three plasma samples collected each day was significantly elevated during the cortisol infusion, compared to control fetuses. However, we did not employ a frequent sampling protocol necessary to establish whether the cortisol infusion administered in these animals altered ACTH pulse frequency over the course of the experiment.

It has been demonstrated that plasma CBC levels rise within two to four days of cortisol infusion (Apostolakis *et al.* 1994; Berdusco *et al.* 1995). In the present study, plasma CBC levels rose in response to cortisol, becoming significantly greater than controls at 48-72 hours of infusion. This was associated with an increase in hepatic CBG mRNA levels. Low dose cortisol infusion to the ovine fetus at 100 days of gestation (for 100 hours), or administration of dexamethasone at 130 days of gestation (for 96 hours), increased CBG biosynthesis and secretion, and also altered the pattern of CBG glycosylation (Berdusco *et al.* 1994; Berdusco *et al.* 1995). Changes in CBG glycoforms may increase the half-life of CBG in the circulation, and this may account for the earlier rise in corticosteroid binding capacity in plasma than in steady-state levels of hepatic CBG mRNA determined in the present study.

The percentage of free cortisol in plasma rose within 8 hours of cortisol infusion and then returned to control levels by 72 hours of infusion. However, the

absolute concentrations of free cortisol remained elevated throughout the experiment. This suggests that CBG is effective at maintaining the percent of free cortisol in the fetal circulation, but does not control the absolute concentration of free cortisol during periods of rapidly increasing plasma cortisol concentrations. This reflects the CBG response to increasing plasma cortisol concentrations in the sheep fetus at term. Plasma CBG and cortisol concentrations rise in parallel, during late gestation, and low free cortisol concentrations are effectively maintained until the last five days of pregnancy (Ballard *et al.* 1982; Berdusco *et al.* 1995). Although CBG did not appear to be effective in decreasing the negative feedback effects of the rapidly increasing plasma cortisol concentrations, this does not preclude a role for circulating CBG in modifying feedback control of the prepartum rise in plasma ACTH levels. In addition, it has been previously shown that the fetal sheep pituitary synthesizes CBG (Berdusco *et al.* 1995), which could alter local feedback mechanisms. However, we did not determine changes in pituitary CBG biosynthesis in the current study.

POMC mRNA levels in the pars intermedia and the pars distalis were not affected by 12 hours of cortisol treatment, as has been demonstrated previously (Matthews and Challis, 1994). However, after 96 hours of cortisol infusion, POMC mRNA levels were suppressed in both the pars intermedia and the pars distalis. In addition, the number of ir-ACTH positive cells within the pars distalis was decreased after 96 hours of cortisol treatment, suggesting that the elevated plasma free cortisol concentrations are exerting negative feedback effects upon the corticotrophs of the pars distalis. Nonetheless, it is possible that cortisol affects the rate of POMC

translation, or ACTH secretion. However, within the pars intermedia there was no apparent change in the number of ir-ACTH positive cells. Pars intermedia corticotrophs from fetal sheep secrete $ACTH_{1-39}$ *in vitro* (Fora *et al.* 1996). In addition, attenuation of the endogenous cortisol rise, by bilateral fetal adrenalectomy, does not alter the basal $ACTH_{1-39}$ output from subsequently cultured pars intermedia cells (Fora *et al.* 1996). The pars intermedia may therefore provide an additional source of ACTH. In the presence of high plasma concentrations of cortisol there may also be an alteration of POMC processing within the pars intermedia resulting in an increase in circulating $ACTH_{1-39}$. Alternatively, the pars intermedia may secrete large molecular weight POMC products into the circulation (Saphier *et al.* 1993), that are processed to $ACTH_{1-39}$ under the influence of elevated cortisol at other sites.

Schwartz *et al.* (1994) have examined the regulation of pituitary ACTH secretion *in vitro* and have shown that there are sub-populations of corticotrophs within the pars distalis which are differentially regulated. There are CRH-sensitive corticotrophs which secrete ACTH in response to CRH via an increase in cAMP, and AVP-responsive corticotrophs which release ACTH through a second pathway likely involving IP_3 (Todd and Lightman, 1987). In vitro studies indicate that glucocorticoids do not inhibit basal $ACTH_{1-39}$ secretion, and significantly increase ACTH precursor release from the AVP-responsive corticotrophs (Schwartz *et al.*1994). In addition, cortisol may have a direct effect on corticotroph morphological and functional maturation (Antolovich et al. 1989; Brieu and Durand 1989), as was discussed previously (Section 1.1.2.1). The "maturational" effects of cortisol on the

fetal sheep corticotroph appears to alter the ratio of POMC-derived peptides produced, with a decrease in large molecular weight ACTH-related peptides accompanied by an increase in the proportion of $ACTH_{1-39}$ or bioactive ACTH released. The pituitaries collected for the present study were slow frozen for *in situ* hybridization and therefore the cellular morphology could not be examined effectively. However, it remains possible that the rise in plasma ir-ACTH in response to the cortisol infusion could be a result of AVP-stimulated ACTH release, and/or a change in the processing of POMC such that $ACTH_{1-39}$ is preferentially released from POMC remaining in the anterior pituitary corticotrophs. Previous studies have suggested that fetal sheep plasma ACTH concentrations may be increased by elevations in 17β-estradiol (Wood and Saoud 1997), and since cortisol increases placental estrogen production (Steele *et al.* 1976), estrogen may mediate the cortisol-induced rise in plasma ACTH. However, futher studies are required to explore the role of estrogen in the cortisol-induced increase in plasma ACTH during late gestation.

We speculate that changes in prohormone convertase activities (Zheng *et al.* 1994) in the pars distalis and pars intermedia of the fetal pituitary might lead to increased proportions of $ACTH_{1-39}$ secreted, or that circulating ACTH might be derived from alternate sources, including the lung and placenta (Jacobs *et al.* 1989; Keller-Wood and Wood 1991; Cudd and Wood, 1995, Deol *et al.* 1995). The processing of POMC in the pituitary, lung and placenta are examined further in the following chapters.

Chapter Five

The effect of intra-fetal cortisol administration on pituitary POMC processing

5.1 Introduction

The concomitant rise in plasma ACTH and cortisol concentrations in the ovine fetus during late gestation (Norman *et al.* 1985) has been attributed to a decrease in cortisol negative feedback. We have shown previously that plasma ACTH concentrations also rise in response to a cortisol infusion given during late gestation (Chapter Four) but, unlike at term (Yang *et al.* 1992; Matthews *et al.* 1994), pituitary POMC mRNA levels in the pars distalis and the pars intermedia were significantly suppressed (Chapter Four). Therefore plasma ACTH concentrations increased despite apparent glucocorticoid inhibition of pituitary POMC mRNA levels.

Silman *et al.* (1979) have previously reported a change in the relative abundance of POMC-derived peptides in the fetal sheep pituitary, such that large molecular weight peptides predominate in mid-gestation and decrease to term, accompanied by an increase in $ACTH_{1-39}$. Subsequent studies have shown that cortisol may be critical in this maturation of fetal corticotroph function. Corticotrophs collected from fetuses in early-, mid- and late-gestation and incubated with cortisol for four days, had decreased output of total POMC-derived peptides, but the relative proportion of immunoreactive (ir-) ACTH peptide which co-eluted with $ACTH_{1-39}$ was increased (Brieu and Durand, 1989). In addition, the bioactivity of the immunoreactive-ACTH secreted was also augmented by cortisol treatment (Brieu and Durand, 1987). These changes in output of POMC-derived peptides may be a reflection of glucocorticoid-induced changes in the activity of prohormone convertases (PC) which cleave POMC to produce smaller biologically active peptides. POMC is cleaved by PC1 to $ACTH_{1-39}$ and β-LPH, and these peptides are

cleaved further by PC2 to produce smaller peptides including αMSH and CLIP (Figure 1.2; Benjannet *et al.* 1991; Korner *et al.* 1991). Therefore changes in $ACTH_{1-39}$ and αMSH may reflect changes in PC1 and PC2 enzyme activity. The effects of cortisol on proconvertase enzymes have not been examined in fetal life.

Glucocorticoid action may be regulated at the level of the pituitary by the availability of glucocorticoid receptors (GR). Pars distalis GR mRNA levels and GR binding are reported to be low during late gestation and to increase significantly at term (Yang *et al.* 1990; Matthews *et al.* 1995). It is not known whether the increase in free cortisol at term influences this increase in GR binding and mRNA levels in the pars distalis. Levels of GR mRNA in the fetal sheep pars distalis and pars intermedia were not altered after 12 hours of cortisol infusion at 135 days of gestation (Matthews *et al.* 1995), however, the effects of a longer exposure to cortisol on GR mRNA levels have not been examined.

Therefore, we hypothesized that intra-fetal cortisol administration would increase PC1 mRNA levels and decrease PC2 mRNA levels in the pituitary such that POMC would be cleaved to produce predominately $ACTH_{1-39}$, and increase the ratio of $ACTH_{1-39}$ to larger ACTH-related peptides would be increased. In addition, the cortisol-mediated change in POMC processing would be accompanied by an increase in GR mRNA levels similar to that seen at term when plasma cortisol and ACTH concentrations also rise in concert.

5.2 Material and Methods

5.2.1 Tissues

Fetal sheep pituitaries were collected at the conclusion of a 96-hour infusion of cortisol (5μg/min at 3ml/h) or saline (2% v/v ethanol, 3ml/min) at 129-134 days of gestation, and frozen as described previously (Section 4.2.2.5). The pituitaries were sectioned using a cryostat for in situ hybridization identification and quantification of mRNA encoding POMC, PC1, PC2 and GR, and for immunohistochemistry. All sections analyzed were from the central portion of the pituitary and contained pars intermedia. The pars intermedia and pars nervosa were removed from the remaining pituitary, and the protein extracted from the pars distalis for radioimmunoassay of $ACTH_{1-39}$ and ACTH-related peptides.

5.2.2 Protein extraction

The pars distalis was separated from the pars intermedia of the frozen pituitary remaining after cryosectioning. Each pars distalis was individually weighed and homogenized (PT2000 Homogenizer, Polytron, Kinematica AG, Switzerland), in extraction buffer (1M HCl, 5% v/v formic acid, 1% w/v NaCl, 1% v/v TFA; Bennett *et al.* 1991). Samples were centrifuged at 1775 x *g* for 10min, pipetted into smaller aliquots and stored in microcentrifuge tubes (1.5ml, Fisherbrand, Fisher Scientific Ltd, Nepean, Ont.) at –20°C until analysis.

5.2.3 Protein Assay

A bicinchoninic acid protein assay (BCA Protein Assay, Pierce, Rockford, IL) was used to quantify protein concentrations in aliquots (25μl) of the acid extracted

tissue samples. Protein standards (bovine serum albumin, fraction V, included in the kit) and the unknown samples were mixed with the working reagent according to the manufacturer's instructions. After mixing, the tubes were allowed to sit at room temperature for 1 hour. The absorbance was determined using a spectrophotometer (584nm:Ultrospec 2000, Pharmacia Biotech, Piscataway, NJ) and the unknown concentrations were determined from a standard curve generated using the protein standards of known concentration.

5.2.4 Measurement of αMSH

αMSH concentrations were assessed in plasma (100ul) and pars distalis homogenates (25μl), by commercial radioimmunoassay (Euro-Diagnostica, Malmo, Sweden). The αMSH antibody cross-reacts 100% with αMSH, and des-acetyl-αMSH, and <0.002% with des-amido-αMSH, $ACTH_{1-13}$, $ACTH_{1-24}$. $ACTH_{1-39}$, βMSH, and γMSH. The sensitivity of the assay is 7.6pg/ml and the intra- and inter-assay coefficients of variation are 12% and 13% respectively. The validity of measuring αMSH concentrations using the αMSH radioimmunoassay in pituitary homogenates was assessed by plotting the percent binding against the concentration of αMSH in serial dilutions and comparing these values to the standard curve. Pituitary αMSH was visualized by immunohistochemistry using a commercial polyclonal rabbit anti-mouse αMSH antibody (1:1000; Incstar Corporation, Stillwater, MN). The specificity for the antibody was confirmed by preabsorption of the antibody with excess antigen (10μM).

5.2.5 Gel chromatography

Sephadex G75 (8% w/v: Pharmacia Biotech Inc. Baie d'Urfé, Que.) was expanded in degassed distilled water at room temperature for 24 hours. A 1.0 x 30cm column (Bio-Rad Laboratories, Hercules, CA) was poured continuously. Once the gel bed had settled the column was run at a rate of 3ml/hr with 1% formic acid containing 0.1% Polypep (Sigma Chemical Company, St Louis, MO Chemical Co.) for 48 hours before it was calibrated with a mixture of Blue Dextran 2000 (0.2% w/v: MW 200 000, Pharmacia Biotech Inc. Baie d'Urfé, Que.), and [^{125}I]ACTH$_{1-39}$ (~20 000cpm: MW 4500, Incstar, Stillwater, MN). A sample of extracted pars distalis (1ml) was loaded onto the column and eluted with 1% formic acid containing 0.1% Polypep at a flow rate of 3ml/h and 1ml fractions were collected (2112 REDIRAC fraction collector, LKB).

5.2.6 Measurement of ACTH

Each fraction was assayed using two radioimmunoassays; a commercial ACTH assay (Incstar Corporation, Stillwater, MN) shown to be specific for ACTH$_{1-39}$ (as described in Section 3.2.2.3), and an assay developed using the less-specific ACTH antibody (GRAb) donated by Dr. G.E. Rice (Royal Women's Hospital, Department of Perinatal Medicine, Melbourne, Australia). Thus we could compare the relative amounts of the immunoreactive ACTH peptides of different molecular weight recognized by each of the ACTH antibodies. Homogenates from the pars distalis were diluted and assayed. The resultant dilution curves were compared to the standard curves in each radioimmunoassay to determine parallelism.

5.2.6.1 GRAb radioimmunoassay

The immunoreactive ACTH peptides measured using the GRAb antibody are referred to as ACTH plus related peptides (ACTH+RP). A radioimmunoassay was established to measure the concentration of immunoreactive ACTH peptides using the GRAb antibody raised in rabbits against synthetic human $ACTH_{1-24}$. This antibody has been validated previously for use in fetal and maternal sheep plasma (McMillen *et al.* 1990). Porcine ACTH (Incstar, Stillwater, Minnesota, USA.) was used as standard. Assay buffer (0.01M phosphate buffered saline; pH 7.5, with 0.25% w/v bovine serum albumin) was added to standards and samples in borosilicate tubes (12 x 75mm), such that the final volume was 200µl. The ACTH antiserum (GRAb; 50µl; initial dilution 1:15 000) was added, the contents of the tubes were mixed well, and incubated at 4°C for 16-24h. Iodinated $ACTH_{1-39}$ (100µl; 3 µCi/15ml Incstar, Stillwater, Minnesota, USA.) was added to each tube, and the tubes were mixed and incubated for an additional 16-24h. A second antibody (100µl of donkey anti-rabbit; Sac-Cel, IDS LTD. Boldon Business Park, Tyne&Wear, England) was used to separate the bound and free fractions. After a 30-minute incubation at room temperature, 1ml of distilled water was added and the tubes centrifuged at 1000 x *g* for 20min (Sorvall RC3 Plus, Dupont Company, Newton, CT). The supernatant was aspirated and the radioactivity in the pellet was counted using a gamma counter (Cobra II auto-gamma, Packard Instrument Company, Meriden, CT). Human plasma spiked with synthetic ACTH was used as a reference standard. The intra- and interassay coefficients of variation were less than 5% and

15% respectively. The GRAb, used in this assay has been previously characterized and the cross-reactivity with ovine growth hormone, ovine lutenizing hormone, ovine follicular stimulating hormone, and ovine prolactin was reported as <0.01% (McMillen *et al.* 1990).

5.2.7 *In situ* hybridization

Specific oligonucleotide probes (45 mer) were generated for PC1, complementary to bases 231-275 of porcine PC1 (Dai *et al.* 1995), and PC2, corresponding to bases 153-197 of porcine PC2 (Seidah *et al.* 1992). Northern blots of total RNA extracted from fetal sheep hypothalamus and adult sheep pituitary were hybridized with the newly synthesized oligonucleotide probes for PC1 and PC2, to ensure signal specificity. The GR oligonucleotide probe has been previously characterized (Matthews *et al.* 1995). The GR probe is 45 bases in length and complementary to a portion of the coding sequence of ovine GR (Matthews *et al.* 1995). *In situ* hybridization was performed as described in Section 4.2.5.

5.2.8 Data analysis

The relative optical density of PC1, PC2 and GR mRNA levels were assessed using computerized image analysis, and statistical significance determined by Student's t-test. Significance was set at $p<0.05$. Differences in plasma αMSH concentrations were determined by two-way ANOVA corrected for repeated measures, and the effect of cortisol on $ACTH_{1-39}$, ACTH+RP, and the ratio of $ACTH_{1-39}$:ACTH+RP was assessed by Students t-test.

5.3 Results

5.3.1 PC1 and PC2 mRNA levels

Northern blot analysis conducted on fetal hypothalamus and adult pituitary resulted in two transcripts of approximately 3kb and 5kb for PC1 and 2.8kb and 4.8kb for PC2 (Figure 5.1), as previously reported (Seidah *et al.* 1994). PC1 mRNA was distributed evenly throughout the pars distalis and the pars intermedia in the pituitaries of saline-infused fetuses (Figure 5.2). The PC1 mRNA levels appeared to be increased in the pars intermedia of three out of four of the cortisol treated fetuses, such that the PC1 mRNA levels in the pars intermedia could be distinguished from PC1 mRNA levels in the other regions of the fetal pituitary (Figure 5.2). However, since PC1 mRNA levels in the pars intermedia were not identifiable in the control animals, PC1 mRNA levels were assessed in the pituitary as a whole. Total PC1 mRNA abundance was not altered by cortisol treatment (Figure 5.3A). The level of PC2 mRNA appeared much higher in the pars intermedia compared to the pars distalis in all fetuses (Figure 5.2). PC2 mRNA levels were significantly increased after intra-fetal cortisol administration in both the pars distalis (Figure 5.3B) and the pars intermedia (Figure 5.3C) compared to saline-infused controls.

5.3.2 αMSH concentrations

The concentration of αMSH in pars distalis (Figure 5.4A) and fetal plasma (Figure 5.4B) were not altered by cortisol treatment compared to saline-infused fetuses. The distribution of ir-αMSH within the pars distalis and the pars intermedia was similar in cortisol treated fetuses (Figure 5.5, C and D) compared to control

Figure 5.1 Northern blot analysis of total RNA prepared from fetal hypothalamus (left lane) and adult pituitary (right lane) for PC1 (left blot) and PC2 (right blot). Two transcripts approximately 3kb and 5kb for PC1 and 2.8kb and 4.8kb for PC2 were visualized

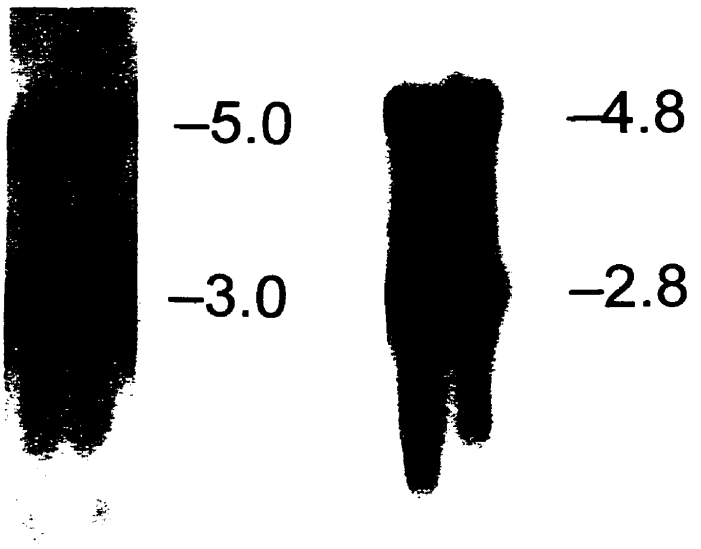

–5.0
–3.0
–4.8
–2.8

Figure 5.2 Autoradiograms from in situ hybridization showing POMC, PC1 and PC2 mRNA distribution in the pars distalis (pd) and pars intermedia (pi) of representative fetal pituitaries collected at the completion of a 96-hour infusion of either cortisol or saline. Adjacent pituitary sections were incubated with a random sequence oligonucleotide probe to determine the level of non-specific activity (random probe). PC1 and PC2 mRNA levels were determined within the same *in situ* hybridization and films were exposed for 12 days. The specific activity of the oligonucleotide labeling was PC1=2.3X10^4cpm/ng, PC2=3.7X10^4cpm/ng, and the random oligonucleotide probe=2.2X10^4cpm/ng. Scale=1mm.

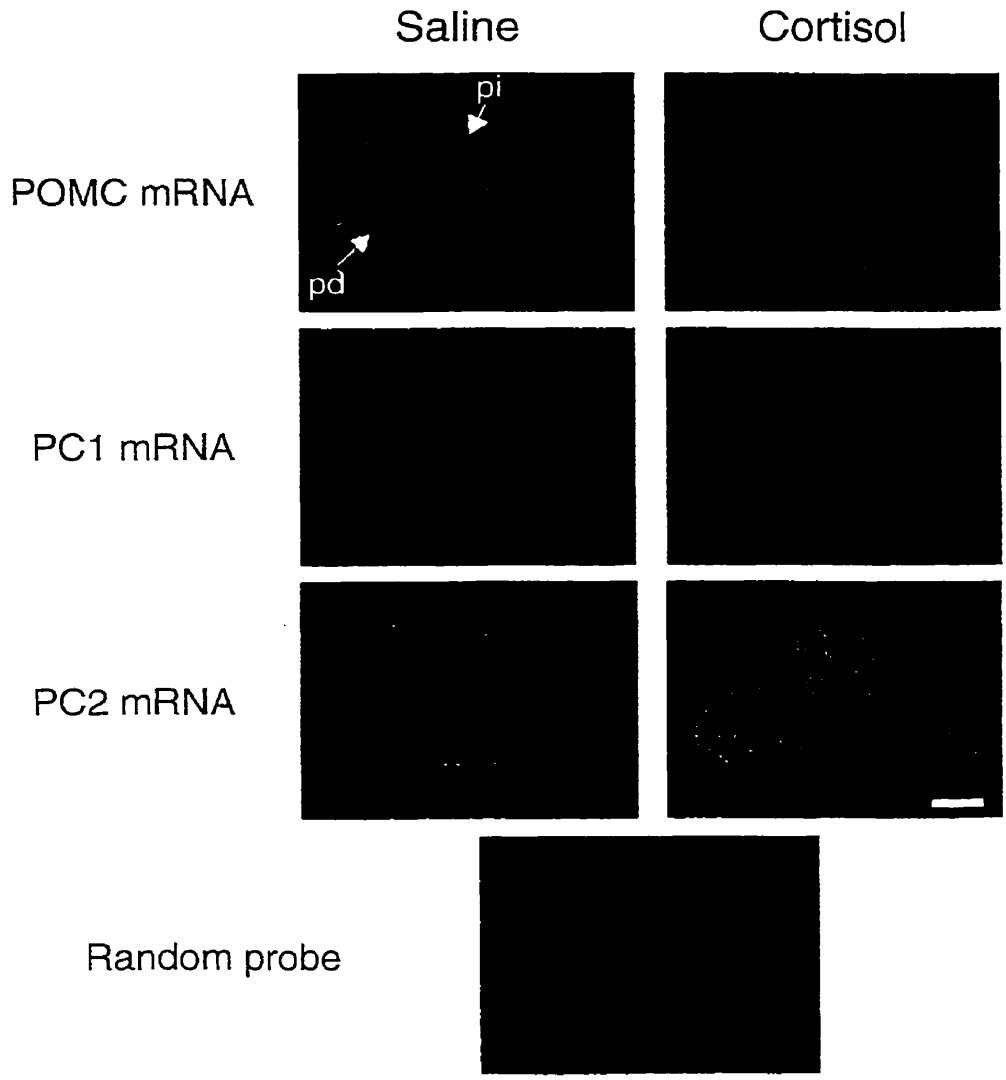
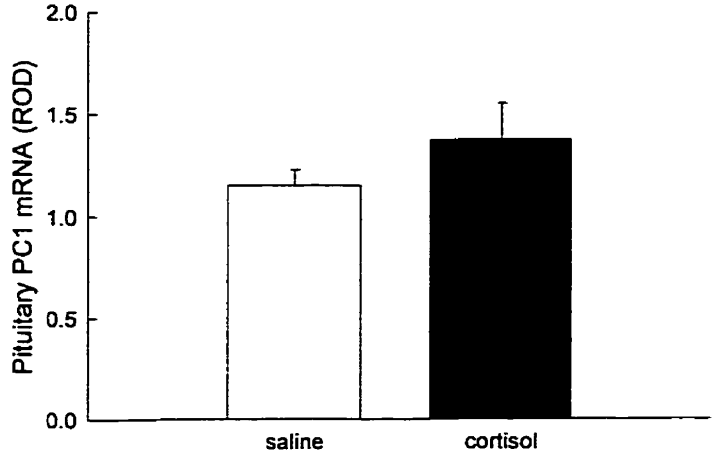
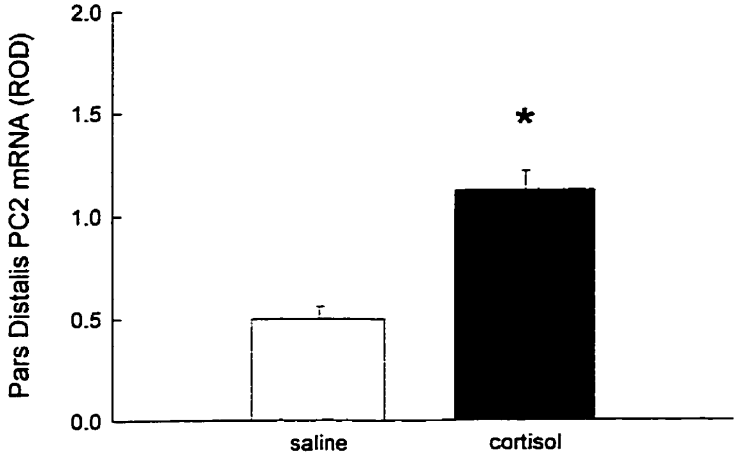

Saline
Cortisol
pi
pd
POMC mRNA
PC1 mRNA
PC2 mRNA
Random probe

Figure 5.3 Histograms illustrating the results of densitometric analysis of (A) PC1 mRNA levels in the pituitary, and PC2 mRNA levels within (B) the pars distalis, and (C) the pars intermedia, after 96 hours of intra-fetal saline (□) or cortisol (■) treatment. Results are expressed as the mean ± SEM of the relative optical density (ROD), *$p<0.05$.

A.


Pituitary PC1 mRNA (ROD)
2.0
1.5
1.0
0.5
0.0
saline
cortisol

B.


Pars Distalis PC2 mRNA (ROD)
2.0
1.5
1.0
0.5
0.0
*
saline
cortisol

C.

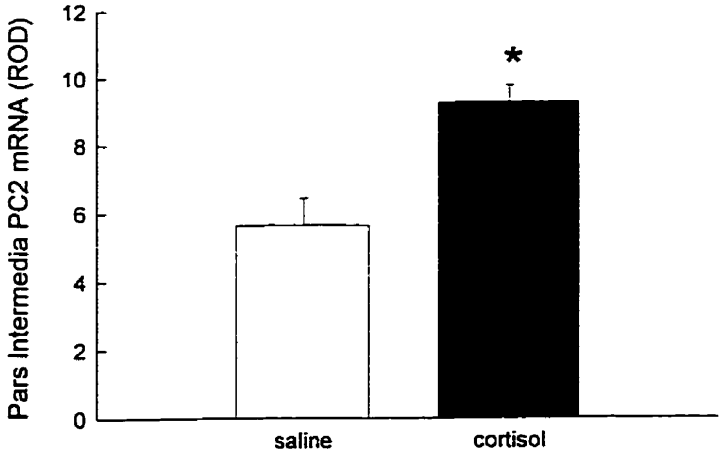
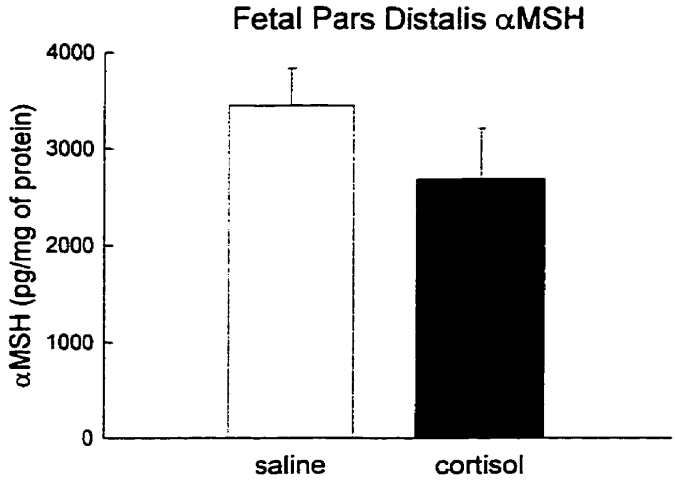

Pars Intermedia PC2 mRNA (ROD)
12
10
8
6
4
2
0
*
saline
cortisol

Figure 5.4 (A) α-Melanocyte stimulating hormone (αMSH) concentrations measured in protein extracted from the fetal pars distalis at the conclusion of 96 hour infusion of either saline (□, n=4) or cortisol (■, n=3). (B) Fetal plasma αMSH concentrations measured during the control period (control), and at 96-hours of saline (□, n=6) or cortisol (■, n=7) infusion. Values are mean ± SEM.

A.


Fetal Pars Distalis αMSH
αMSH (pg/mg of protein)
4000
3000
2000
1000
0
saline
cortisol

B.

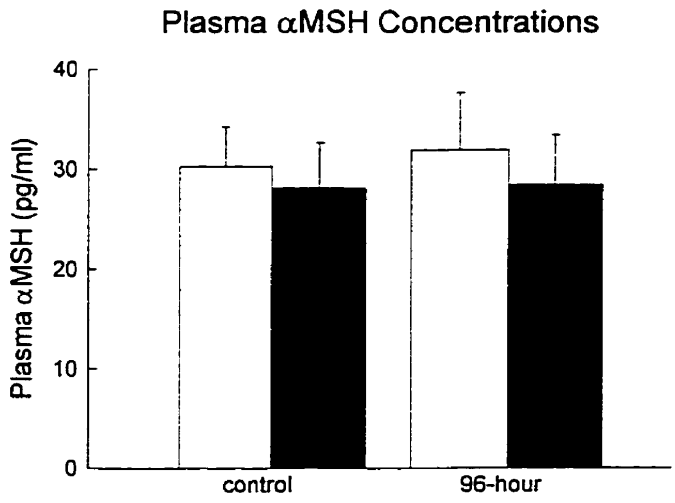
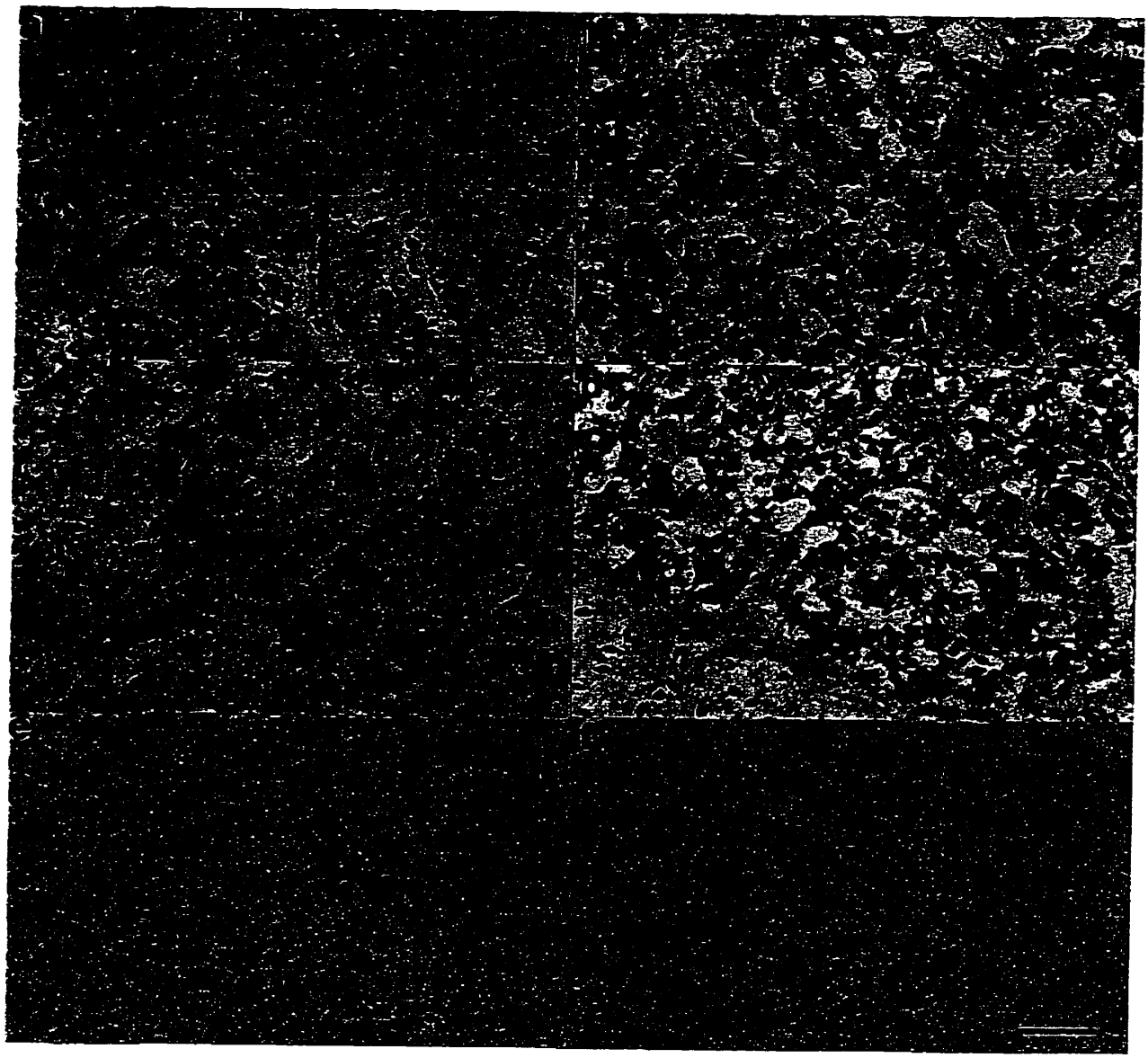

Plasma αMSH Concentrations
Plasma αMSH (pg/ml)
40
30
20
10
0
control
96-hour

Figure 5.5 Photographs of immunoreactive αMSH peptide in the pars distalis (A,C) and the pars intermedia (B,D) from fetuses after a 96-hour infusion of either saline (A, B) or cortisol (C, D). Adjacent sections (pars distalis E, and pars intermedia F) were incubated with primary antibody that had been preabsorbed with an excess of αMSH. Scale bar=50μm.



(Figure 5.5, A and B). There was negligible staining in sections of pars distalis or pars intermedia incubated with primary antibody in the presence of an excess of αMSH (Figure 5.5, panels E and F).

5.3.3 Column chromotography of pars distalis extracts

Dextran Blue 2000 (MW 200 000) eluted predominately in fraction 7, and [^{125}I]$ACTH_{1-39}$ in fraction 17 (Figure 5.6A). In fractions collected from extracted pars distalis, the Incstar ACTH antibody identified immunoreactive-ACTH predominantly in fractions 15-18 (Figure 5.6B) corresponding to [^{125}I]$ACTH_{1-39}$. However, the GRAb assay identified immunoreactive ACTH in fractions 15-18 as well as several peaks containing higher molecular weight species (Figure 5.6C).

5.3.4 ACTH in the pars distalis

The standard curves of the two assays were superimposable (Figure 5.7A). Dilution curves of fetal pars distalis homogenate were parallel to the respective standard curves in each of the Incstar ACTH (Figure 5.7B) and the GRAb assay (Figure 5.7C). Figure 5.8 shows that $ACTH_{1-39}$ and ACTH+RP concentrations measured in protein extracted from fetal pars distalis was not significantly different after 96 hours of intra-fetal cortisol administration (Figure 5.8, A and B). Similarly,

Figure 5.6 G-75 Sephadex column elution profile (A) of Blue Dextran 2000 (○); based on a colour scale of 0-10, with the most intense fraction equal to 10 (colour intensity), and [^{125}I]ACTH$_{1-39}$ (●; cpm). [^{125}I]ACTH$_{1-39}$ eluted between fractions 15 and 20. Protein from the fetal pars distalis was eluted through the column and the successive fractions were measured using both (B) the commercial (Incstar) ACTH assay, which recognizes ACTH$_{1-39}$, and (C) the GRAb assay that recognizes ACTH$_{1-39}$ and related peptides. The 30x1cm column was eluted with a flow rate of 3ml/hr.

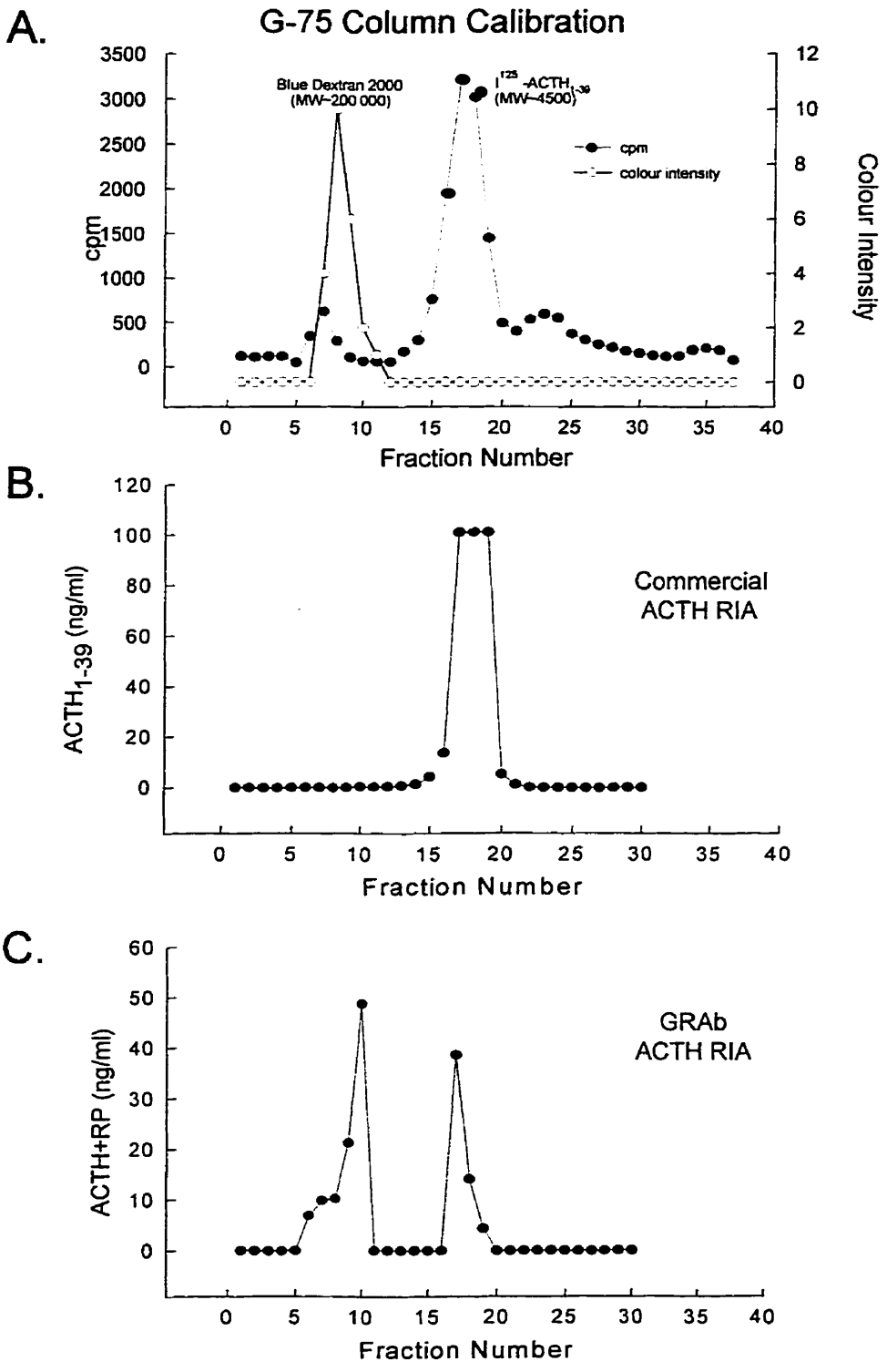
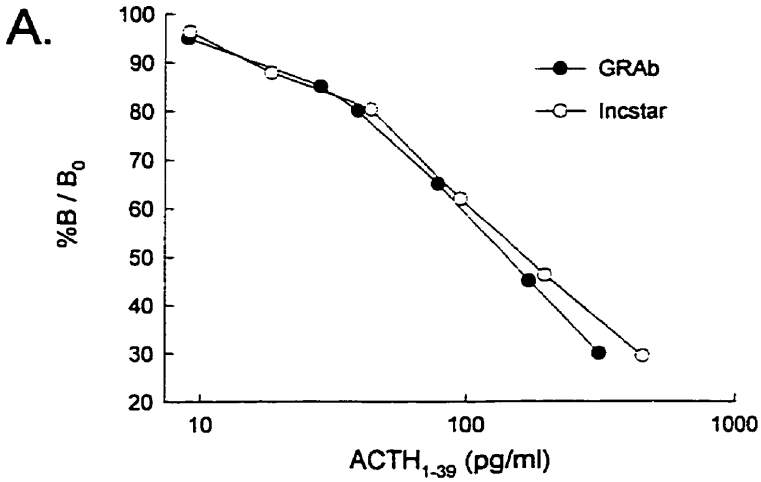
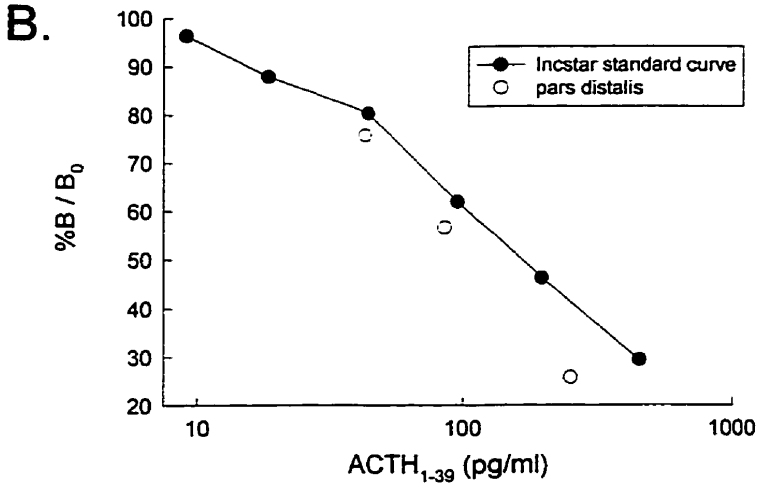

A.
G-75 Column Calibration
Blue Dextran 2000
(MW~200 000)
I125-ACTH1-39
(MW~4500)
cpm
colour intensity
cpm
Colour Intensity
Fraction Number
B.
ACTH1-39 (ng/ml)
Commercial
ACTH RIA
Fraction Number
C.
ACTH+RP (ng/ml)
GRAb
ACTH RIA
Fraction Number

Figure 5.7 (A) Comparison of the standard curves from the Incstar ACTH radioimmunoassay and the GRAb RIA. (B) Dilutions of pars distalis were assayed and compared to the standard curve the Incstar ACTH assay. (C) Homogenates of pars distalis were diluted, assayed and compared to the standard curve for the GRAb assay.


A.
100
90
80
70
60
50
40
30
20
%B / B0
GRAb
Incstar
10
100
1000
ACTH1-39 (pg/ml)


B.
100
90
80
70
60
50
40
30
20
%B / B0
Incstar standard curve
pars distalis
10
100
1000
ACTH1-39 (pg/ml)

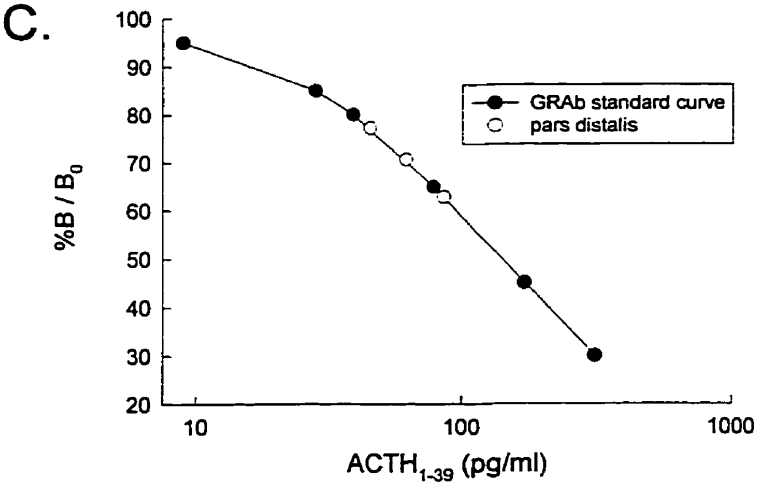
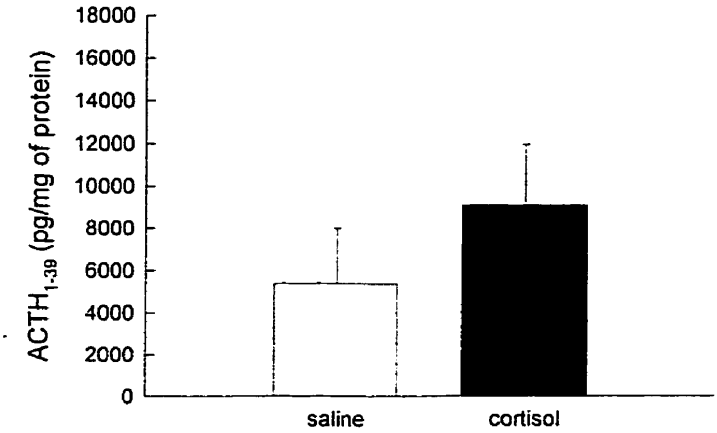
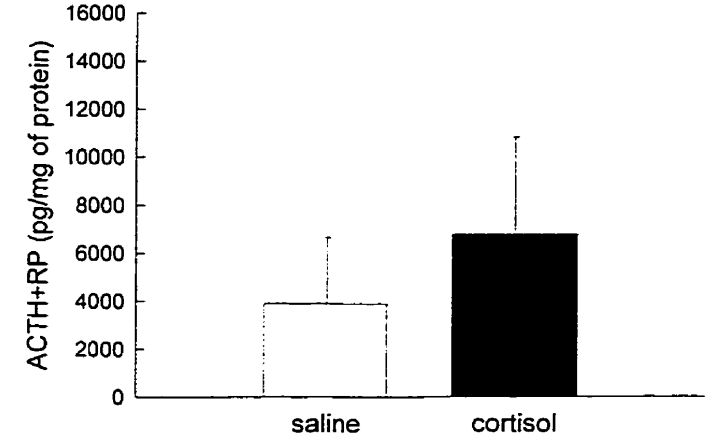

C.
100
90
80
70
60
50
40
30
20
%B / B0
GRAb standard curve
pars distalis
10
100
1000
ACTH1-39 (pg/ml)

Figure 5.8 The concentration of (A) $ACTH_{1-39}$, and (B) ACTH+RP and (C) the ratio of $ACTH_{1-39}$ to ACTH+RP in the pars distalis of saline-treated fetuses (□, n=5) compared to animals infused with cortisol (■, n=3-4). Values are mean ± SEM.

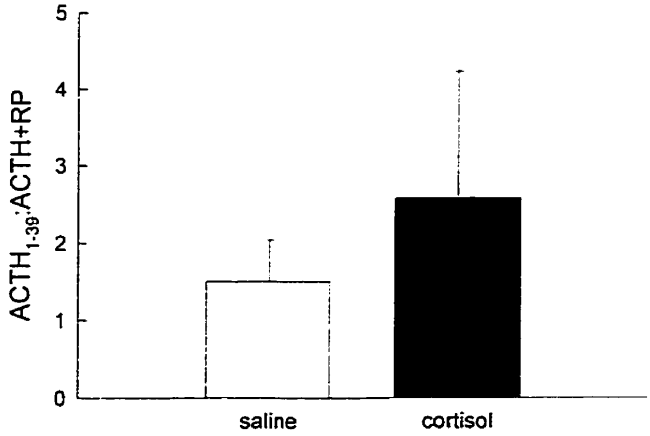
A.


18000
16000
14000
12000
10000
8000
6000
4000
2000
0
ACTH1-39 (pg/mg of protein)
saline
cortisol

B.


16000
14000
12000
10000
8000
6000
4000
2000
0
ACTH+RP (pg/mg of protein)
saline
cortisol

C.


5
4
3
2
1
0
ACTH1-39:ACTH+RP
saline
cortisol

the ratio of $ACTH_{1-39}$:ACTH+RP was not significantly altered after cortisol treatment compared to control (Figure 5.8C).

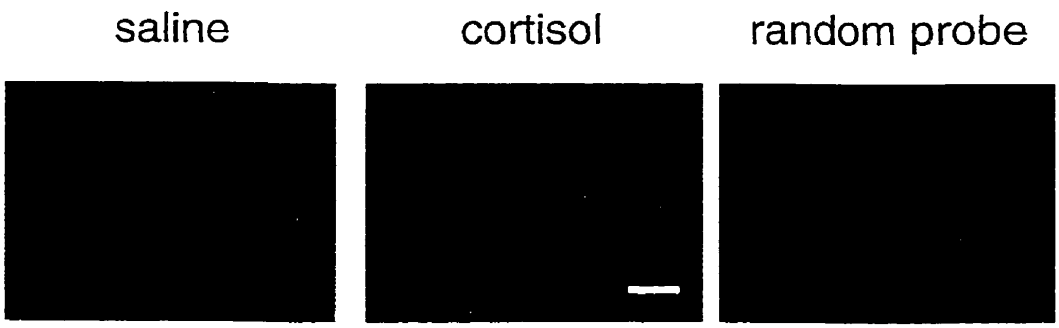
5.3.5 GR mRNA levels

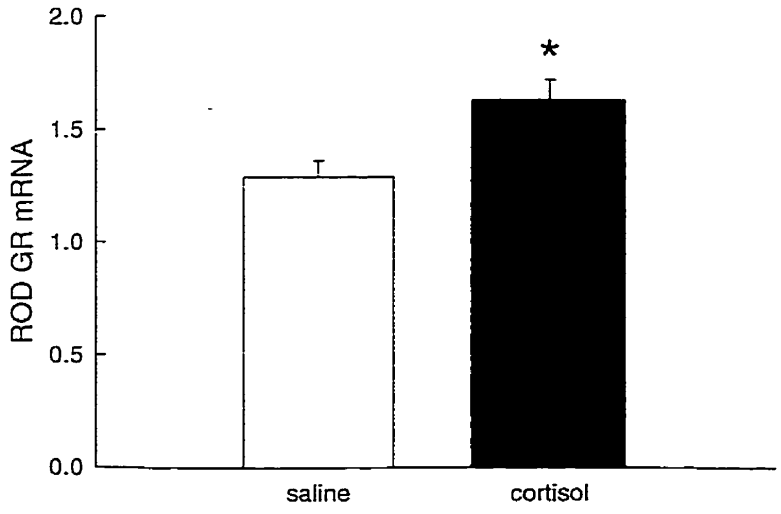
GR mRNA was expressed uniformly throughout the pars distalis and pars intermedia (Figure 5.9, upper panel) and therefore GR mRNA levels were measured in the gland as a whole and no distinction was made between expression in the pars distalis and pars intermedia. There was a significant increase in GR mRNA levels in the pituitary after 96 hours of cortisol infusion compared to saline-infused controls (Figure 5.9, lower panel).

5.4 Discussion

Intra-fetal cortisol administration during late gestation stimulated a rise in PC2 mRNA levels in both the pars distalis and the pars intermedia, however PC1 mRNA levels were not affected. Therefore PC1 and PC2 may be differentially regulated and thereby may influence POMC processing. However, the rise in pituitary PC2 mRNA levels was not reflected by an increase in pars distalis or plasma αMSH concentrations. There was no change in the concentration of $ACTH_{1-39}$ or ACTH+RP or in the ratio of $ACTH_{1-39}$:ACTH+RP within the fetal pars distalis, despite the significant decrease in pars distalis POMC mRNA levels. The pars distalis may

Figure 5.9 Fetal pituitary GR mRNA levels determined using *in situ* hybridization. The top panel shows the localization of GR mRNA in the fetal pituitary after 96 hours of either saline or cortisol treatment. Adjacent sections were incubated under the same conditions with a random sequence oligonucleotide probe (random probe). Densitometric analysis of the autoradiograms is represented by the bar graph in the lower panel, after 96 hours of cortisol infusion (■) compared to saline infused fetuses (□). Values are mean relative optical density (ROD) ± SEM, *$p<0.05$ Student's t-test. Scale=1070μm.


saline
cortisol
random probe


2.0
1.5
1.0
0.5
0.0
ROD GR mRNA
*
saline
cortisol

therefore continue to increase $ACTH_{1-39}$ secretion, as reported, during a 96-hour cortisol infusion.

The relative distribution of PC1 and PC2 mRNA levels within the pituitary was consistent with that reported in other species (Marcinkiewicz *et al.* 1993; Zheng *et al.* 1994). PC1 mRNA levels were distributed throughout the gland, which is in accordance with POMC processing to ACTH and βLPH in both the pars distalis and pars intermedia. However, PC2 mRNA expression was 10-fold greater in the pars intermedia compared to the pars distalis. This pattern of expression is consistent with the processing of POMC to predominantly smaller peptides like αMSH, CLIP and βendorphin in the pars intermedia. Intra-fetal cortisol administration did not alter total pituitary PC1 mRNA levels. In three of four pituitaries collected from cortisol-treated fetuses the presence of PC1 mRNA in the pars intermedia became distinguishable from levels of PC1 mRNA in the pars distalis. However, since levels of PC1 mRNA in the pars intermedia and pars distalis were similar in the saline controls, the change in pars intermedia PC1 mRNA levels was not assessed. However, an increase in PC1 cleavage in the pars intermedia may result in greater $ACTH_{1-39}$ production, and output thereby contributing to circulating ACTH concentrations. Previous studies have suggested a role for ACTH produced in the pars intermedia in HPA axis function (Fora *et al.* 1996; Saiardi *et al.* 1998). Dopamine type-2 receptor deficient mice, have Cushing's-like symptoms with increases in the number of melanotrophs, POMC and PC1 mRNA and plasma ACTH and cortisol concentrations (Saiardi *et al.* 1998). Fetal sheep melanotrophs

have been shown to secrete ACTH *in vitro* (Fora *et al.* 1996). However, we were not in a position to measure the concentration of $ACTH_{1-39}$ or ACTH+RP separately in the pars intermedia in the present studies. However, previously (Chapter Four) we showed by immunohistochemistry that ir-ACTH in the pars intermedia was apparently unaltered by cortisol infuson. Hence, continued secretion of $ACTH_{1-39}$ by the pars intermedia could contribute to circulating $ACTH_{1-39}$.

PC2 mRNA levels were significantly increased in both the pars distalis and the pars intermedia after 96 hours of cortisol infusion compared to fetuses infused with saline. This increase in the abundance of PC2 mRNA within the pituitaries of cortisol-treated fetuses, may be associated with an increase in PC2 processing, which would be reflected by an increase in αMSH production. However, there was no change in αMSH concentrations in either plasma or pars distalis homogenates measured by radioimmunoassay. The apparent inconsistency between PC2 mRNA levels and enzyme activity may reflect the lengthy activation process of PC2 enzyme activity (Seidah, 1993). In addition, the changes in proconvertase mRNA levels measured within the pars distalis may not reflect the changes in proconvertase expression in corticotrophs, since there are several other cell types in the pars distalis, including lactotrophs, gonadotrophs and thyrotrophs, which have all been shown to contain PC1 and PC2 (Takumi *et al.* 1998).

GR mRNA levels in the pituitary were significantly higher after cortisol treatment. Levels of GR mRNA and GR binding in the pars distalis also increased with advancing gestation (Yang *et al.* 1990; Matthews *et al.* 1995), when plasma

cortisol and ACTH concentrations increase concomitantly. In the previous and current studies GR mRNA levels or GR binding was measured in the pars distalis as a whole and there are no reports of changes in GR within the corticotroph alone. The pars distalis contains many cell types and therefore it is impossible to deduce whether there is an increase in GR within corticotrophs. Glucocorticoids appear to stimulate the morphological corticotroph maturation (Antolovich *et al.* 1989), and the switch in corticotroph production of large molecular weight ACTH peptides to $ACTH_{1-39}$ (Brieu and Durand, 1989). In the ovine fetus during late gestation the rises in fetal plasma cortisol and pituitary GR mRNA and GR binding (Yang *et al.* 1990; Matthews *et al.* 1995) are accompanied by an increase in trophic drive from the hypothalamus, which stimulates POMC mRNA levels and $ACTH_{1-39}$ secretion (Matthews and Challis, 1996). The intra-fetal infusion of cortisol in the present study suppressed POMC mRNA levels in the pars distalis and pars intermedia, unlike the rise in pituitary POMC mRNA levels seen in late-gestation (Yang *et al.* 1991; Matthews *et al.* 1994). This may be due to differences in the rate of increase in plasma cortisol concentrations or differences in hypothalamic drive to the pituitary. However, hypothalamic input to the pituitary has not been examined in this study.

The maintenance of $ACTH_{1-39}$ and ACTH+RP concentrations in the pars distalis may reflect an increase in production and secretion of peptide or a cessation of secretion such that tissue concentrations are unaltered by cortisol treatment. ACTH secretion from the pars distalis was not determined in the present study therefore the role of the pars distalis in the rise in plasma ACTH concentrations is not known. The possibility remains that other sites of POMC production, such as the

pars intermedia, the fetal lung or the placenta, may also contribute to circulating levels of ACTH.

Note: After this thesis was submitted Bell *et al.* (1998) published a study examining PC1 and PC2 mRNA levels and co-localization with POMC mRNA in the fetal sheep pituitary. The authors reported that the levels of PC1 mRNA in the pars distalis and pars intermedia, and PC2 mRNA in the pars intermedia did not change during gestation. Similarily the percentage of pars distalis cells co-expressing POMC and PC1 was greater at 100 days of gestation and near-term (144-147 days), compared with the intervening ages. PC2 mRNA expression in the pars distalis was below the level of detection in these studies. As discussed above, PC1 mRNA was detected in numerous pars distalis cell types in addition to POMC expressing cells. However at the maximum only 50% of POMC mRNA containing cells also expressed PC1 mRNA, which suggests that other processing enzymes may also be important in POMC processing (Bell *et al.* 1998).

Chapter Six

Glucocorticoid induced changes in fetal lung and placental ACTH production

6.1 Introduction

In the ovine fetus during late gestation the paradoxical rise in fetal plasma ACTH and cortisol concentrations (Norman *et al* 1985), has been previously attributed to a decrease in cortisol negative feedback. However, we have shown that plasma ACTH concentrations also rise in response to a cortisol infusion given during late-gestation (Chapter Four), but unlike at term (Yang *et al.* 1992; Matthews *et al.* 1994) pituitary POMC mRNA levels in the pars distalis and the pars intermedia were significantly suppressed (Chapter Four). Therefore plasma ACTH concentrations increased despite apparent glucocorticoid inhibition of pituitary POMC mRNA levels. The pars distalis concentrations of αMSH, $ACTH_{1-39}$, and ACTH+RP (as defined in Chapter Five), and the ratio of $ACTH_{1-39}$:ACTH+RP were not altered by cortisol infusion compared to control animals. Secretion of these peptides from the fetal pituitary was not measured and therefore we cannot exclude the pituitary as a source of the rise in plasma $ACTH_{1-39}$ concentrations previously reported (Chapter Four). Alternatively, we speculate that the reported rise in circulating ACTH concentrations might be derived from an extra-pituitary source, such as the fetal lung or placenta.

Extra-pituitary sites containing ACTH have been previously identified in human skin, brain, thyroid, pancreas, placenta, gonads, and lung (Smith and Funder 1988). In the ovine fetus POMC mRNA and ir-ACTH have also been detected in the lung (Deol *et al.* 1995). The content of ir-ACTH, including large POMC-derived peptides, in the fetal lung decreased progressively from 90 days of gestation into adulthood (Cudd and Wood 1993). From these data we cannot determine whether

the decrease in ir-ACTH is a reflection of an increase in secretion, or whether ACTH production in the lung decreases with advancing gestational age. Cudd and Wood (1995) have shown that the fetal sheep lung secretes ACTH *in vivo* when circulating ACTH levels are less than 200pg/ml. The ovine placenta has been similarly shown to contain ir-ACTH (Jacobs and Challis 1989, Keller-Wood and Wood 1991), and corticotrophic activity (Mitchell *et al.* 1986) although umbilical arteriovenous differences in ACTH concentration were not detectable under basal or hypoxemic conditions (Keller-Wood and Wood 1991a). Therefore the fetal lung and the ovine placenta are two potential sites of ACTH production in the sheep fetus, which may contribute to circulating ACTH levels. However, there are no data available regarding the impact of glucocorticoids on these extra-pituitary sites of ACTH production.

Glucocorticoid receptor binding has been previously reported in the fetal sheep lung and the ovine placenta (Flint and Burton, 1984). In both tissues, cytosolic GR concentrations were highest between 91 and 130 days of gestation when plasma cortisol concentrations are low, and GR concentrations decreased at 140 days of gestation when plasma cortisol levels have increased (Flint and Burton, 1984). The decrease in GR in the fetal lung and placenta near term is contrary to the increased GR levels seen in the fetal pituitary at term (Yang *et al.* 1990; Matthews *et al.* 1995) as plasma cortisol concentrations rise. Cortisol infusion may induce a similar reduction in placental and lung GR and thereby reduce cortisol inhibition of POMC mRNA levels, if POMC in these extra-pituitary sites is glucocorticoid regulated.

We hypothesized that the fetal lung and the placenta would be additional sources of ACTH production that are not inhibited by elevated fetal plasma cortisol concentrations due to a decrease in GR that is reflected by reduced GR mRNA levels. Therefore we examined the effects of cortisol on levels of mRNA encoding for POMC, PC1 and PC2 processing enzymes, and GR as well as the concentration of $ACTH_{1-39}$, ACTH-related peptides and αMSH in the fetal lung and the ovine placenta.

6.2 Materials and Methods

6.2.1 Tissue collection

Fetal lung and placenta tissue was collected at the end of a 96 hour infusion of either cortisol (5μg/min, n=7) or an equal volume of saline (2% v/v ethanol, 3ml/min, n=6) into chronically catheterized fetal sheep at 129-134 days of gestation as described previously (Chapter Four). Sections of fetal lung and placenta were obtained and prepared using three methods: 1) fixed in 4% paraformaldehyde and 0.2% glutaraldehyde, 2) cut into ~1cm^3 pieces, flash frozen in liquid nitrogen and stored in Poly-Q scintillation vials (20ml, Beckman Instruments (Canada) Inc, Toronto, Ont.) at –80°C, or 3) frozen flat on dry ice to maintain tissue structure and subsequently wrapped in parafilm, and stored at –80°C.

6.2.2 Protein extraction

Protein was acid-extracted from frozen samples of fetal lung and placenta and the amount of protein in the extracts was measured using a bicinchoninic acid protein assay as described in Section 5.2.3.

6.2.3 Hormone measurements

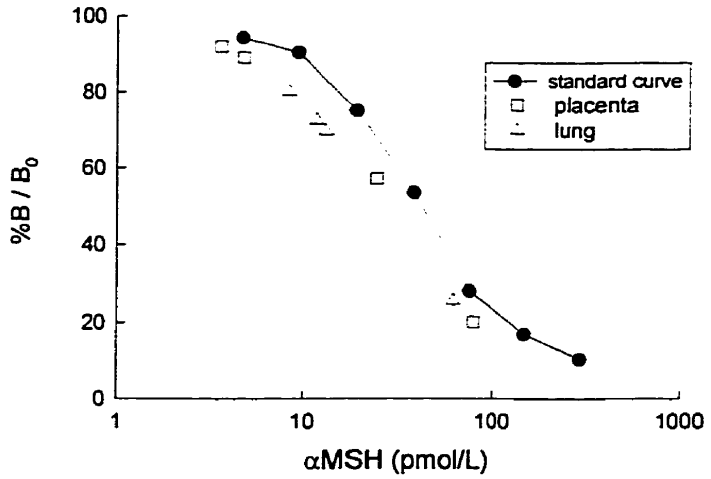
αMSH, $ACTH_{1-39}$, and ACTH-RP levels were measured in tissue extracts of fetal lung and placenta as previously described in Chapter Five (Sections 5.2.2 and 5.2.6). The dilution curves of placental and lung homogenates were parallel to the respective standard curve for the αMSH (Figure 6.1A), Incstar ACTH (Figure 6.1B) and the GRAb assay (Figure 6.1C).

6.2.4 Immunohistochemistry

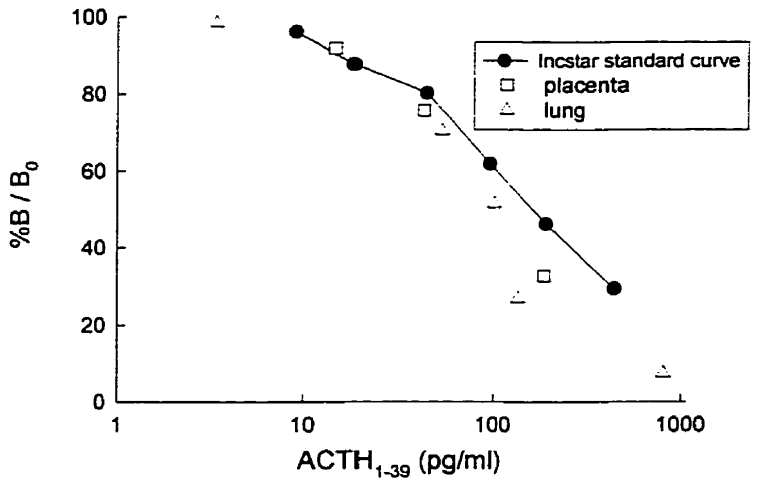
Tissue sections collected at the time of euthanasia were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde (as described above) for 18-24 hours. The tissues were washed (0.1M PBS) four times in the following two days, and then stored in 70% ethanol, until embedded in paraffin. The fixed tissues were trimmed, placed in the desired orientation within a plastic multicassette (Surgipath Medical Industries Inc., Richmond IL) and embedded in paraffin. The paraffin embedded tissues were sectioned (5μm) using a microtome (RM 2035, Leica, Heerbrugg, Switzerland), and mounted on glass slides. The mounted sections were deparaffinized in xylene substitute (BDH Chemicals, Toronto, Ontario), rehydrated in

Figure 6.1 Serial dilutions of fetal lung and placenta assayed and compared to the respective standard curve for each of the (A) αMSH, (B) Incstar ACTH, and the (C) GRAb assays.

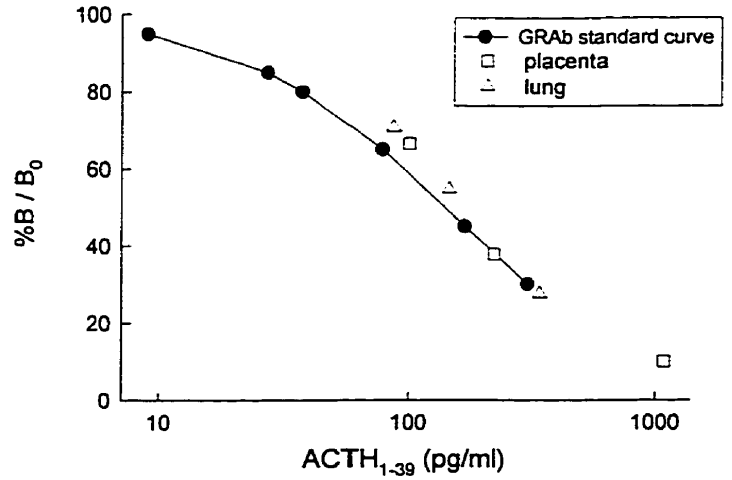
A


100
80
60
40
20
0
%B / B0
1
10
100
1000
αMSH (pmol/L)
standard curve
placenta
lung

B.


100
80
60
40
20
0
%B / B0
1
10
100
1000
ACTH1-39 (pg/ml)
Incstar standard curve
placenta
lung

C.


100
80
60
40
20
0
%B / B0
10
100
1000
ACTH1-39 (pg/ml)
GRAb standard curve
placenta
lung

alcohol and washed in 0.1M PBS as described in Section 4.2.6. The ACTH (Section 4.2.6) and αMSH (Section 5.2.4) antibodies used have been described previously.

6.2.5 mRNA analysis

In situ hybridization was performed as previously described, using 45mer oligoprobes specific for porcine POMC (Section 4.2.5), PC1 and PC2 (Section 5.2.7). All sections were hybridized at the same time and the specific activity of the radiolabeled oligonucleotide probes were as follows; POMC=5.1X10^4cpm/ng, PC1=2.3X10^4cpm/ng, PC2=3.7X10^4cpm/ng and the random oligonucleotide probe=2.2X10^4cpm/ng. GR mRNA levels in the lung and placenta were assessed by northern blot analysis (Section 3.2.4) using a 942 base pair cDNA which encodes an internal portion of the ovine GR and identifies a single 5.6kb transcript (Yang *et al.* 1992).

6.2.6 Data analysis

Computerized image analysis (Imaging Research Inc., St. Catherines, Ontario), was used to assess the relative optical density (ROD) of POMC, PC1, PC2 and GR mRNA in five areas of fixed dimension within each section of lung or placenta. A minimum of four sections per tissue was analyzed for each animal. The northern blot determination of GR mRNA was compared to the ROD of 18S rRNA for each sample to control for variations in loading and transfer. The effect of cortisol treatment on mRNA levels, and changes in tissue levels of αMSH, $ACTH_{1-39}$,

ACTH-RP and the ratio of $ACTH_{1-39}$ and ACTH-RP compared to saline treated control fetuses was determined by t-test. Significance was set at $p<0.05$.

6.3 Results

6.3.1 Placenta

POMC, PC1 and PC2 mRNA were detected within the ovine placenta after 96 hours of intra-fetal saline or cortisol infusion as shown in Figure 6.2. The measurement of relative optical density from the *in situ* hybridization autoradiograms showed that placental POMC (Figure 6.3A) and PC1 (Figure 6.3B) mRNA levels were not altered by cortisol treatment. However, placental PC2 mRNA levels were significantly increased in fetuses infused with cortisol, compared to saline-infused controls (Figure 6.3C).

The concentration of $ACTH_{1-39}$, ACTH+RP and the ratio of $ACTH_{1-39}$:ACTH+RP in placenta was not significantly different after cortisol treatment compared to control (Figure 6.4). αMSH and ACTH had the same distribution of immunostaining within the trophoblast, and the maternal stromal tissue within the placentome (Figure 6.5), and in the capsular tissue surrounding the placentome (data not shown). There was no change in the distribution of ir-αMSH or ACTH peptide in the placenta after cortisol treatment. Preabsorption of the primary antibody with an excess of antigen resulted in negligible staining for αMSH or ACTH (Figure 6.5). αMSH concentrations in the placenta were not different after cortisol treatment compared to control (Figure 6.6). GR mRNA levels in the placenta were

Figure 6.2 Autoradiograms depicting the distribution of POMC, PC1, and PC2 in sections of the ovine placenta. Placenta was collected at the conclusion of a 96-hour infusion of either saline, or cortisol, and analyzed by *in situ* hybridization using specific [^{35}S]-labeled 45mer oligonucleotide probes. An adjacent section incubated with a random oligonucleotide probe is also shown. Autoradiograms were exposed for 22 days. Scale=3mm.

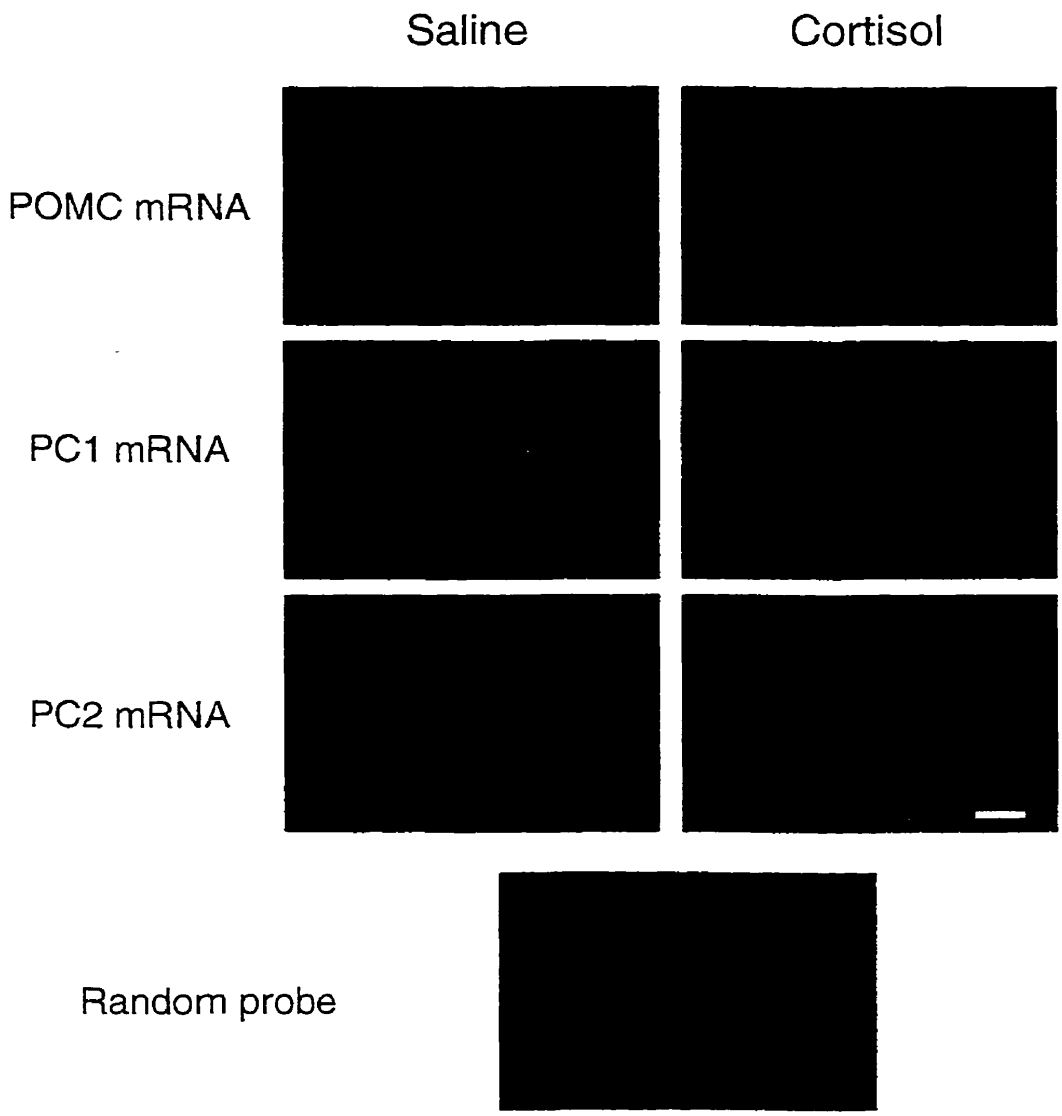
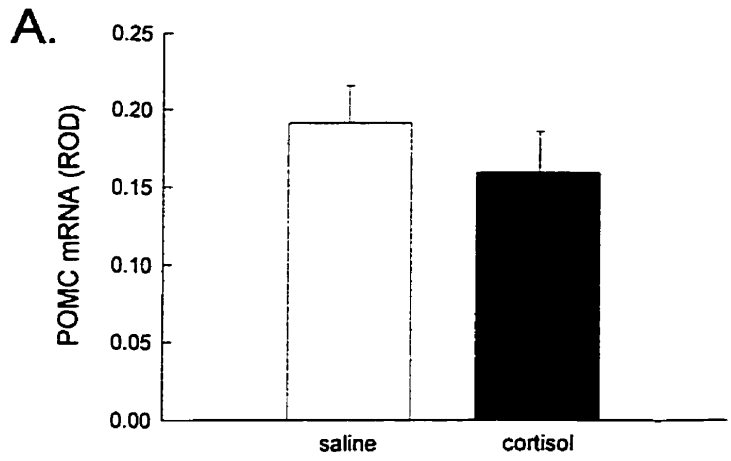
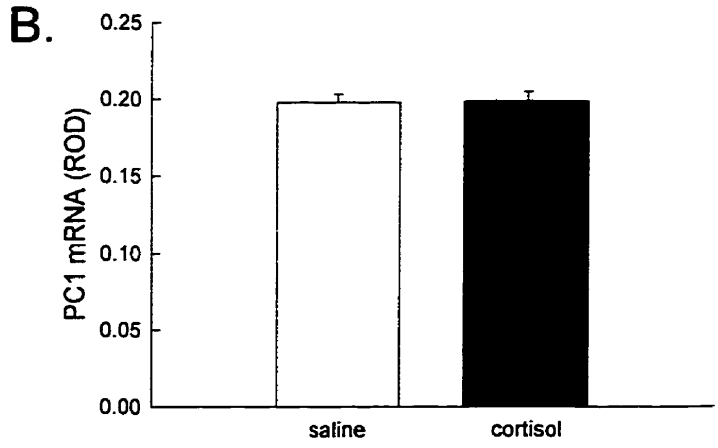

Saline
Cortisol
POMC mRNA
PC1 mRNA
PC2 mRNA
Random probe

Figure 6.3 (A) POMC, (B) PC1 and (C) PC2 mRNA levels in ovine placenta after a cortisol (■, n=7) or saline (□, n=5) infusion (96 hours) by *in situ* hybridization. The relative optical density (ROD) from the resultant autoradiograms was assessed by computerized image analysis and is expressed in the bar graphs as mean ± SEM. *$p<0.05$, Student's t-test.


A.
POMC mRNA (ROD)
0.25
0.20
0.15
0.10
0.05
0.00
saline
cortisol


B.
PC1 mRNA (ROD)
0.25
0.20
0.15
0.10
0.05
0.00
saline
cortisol

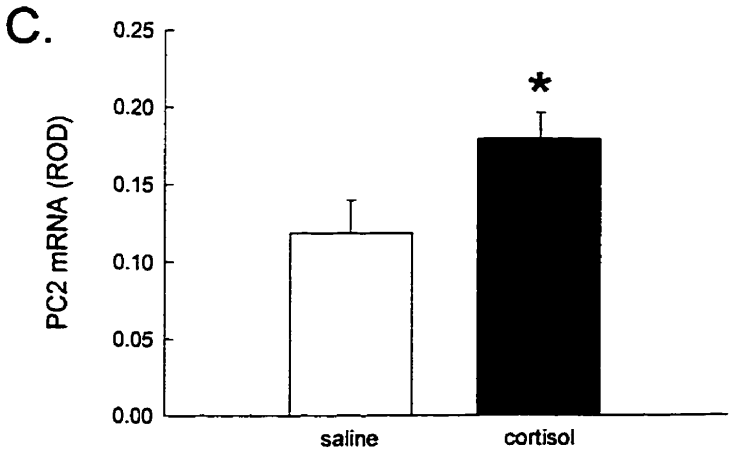
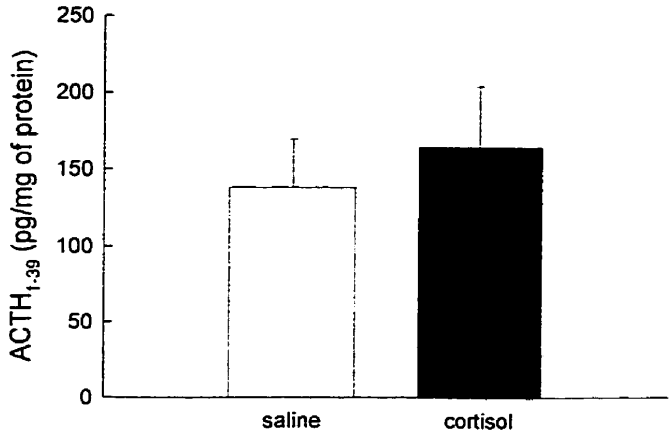
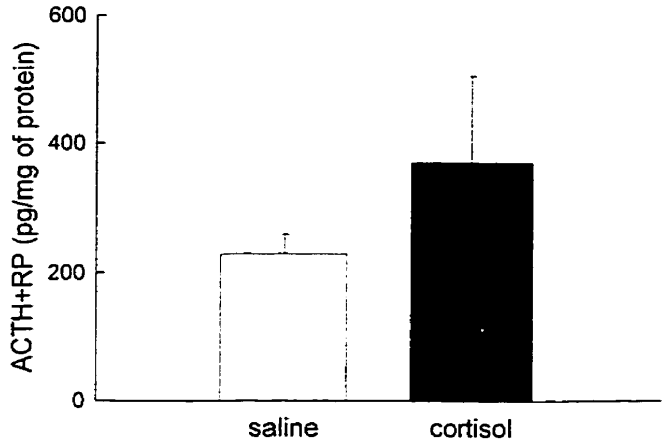

C.
PC2 mRNA (ROD)
0.25
0.20
0.15
0.10
0.05
0.00
*
saline
cortisol

Figure 6.4 The concentration of (A) $ACTH_{1-39}$, (B) ACTH+RP and the (C) ratio of $ACTH_{1-39}$:ACTH+RP in extracts from ovine placenta at the end of a 96-hour intra-fetal infusion of saline (□, n=6) or cortisol (■, n=7). Values are mean ± SEM.

A.


250
200
150
100
50
0
ACTH1-39 (pg/mg of protein)
saline
cortisol

B.


600
400
200
0
ACTH+RP (pg/mg of protein)
saline
cortisol

C.

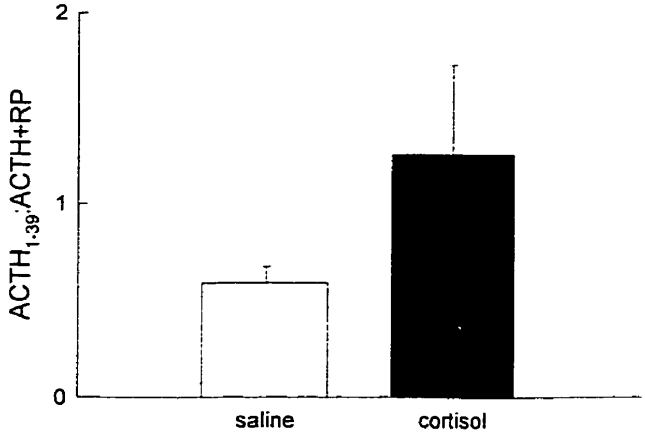

2
1
0
ACTH1-39:ACTH+RP
saline
cortisol

Figure 6.5 Immunoreactive ACTH (panels, A and B) and αMSH peptide (panels, C and D) in the ovine placenta collected after a 96-hour infusion of saline (panels A and C) or cortisol (panels B and D). Immunoreactive αMSH and ACTH were present in the trophoblast (t), and maternal stromal tissue (s). Sections of placenta were incubated with ACTH antibody (panel E) and αMSH (panel F) antibodies preabsorbed with an excess of antigen. Scale=50μm

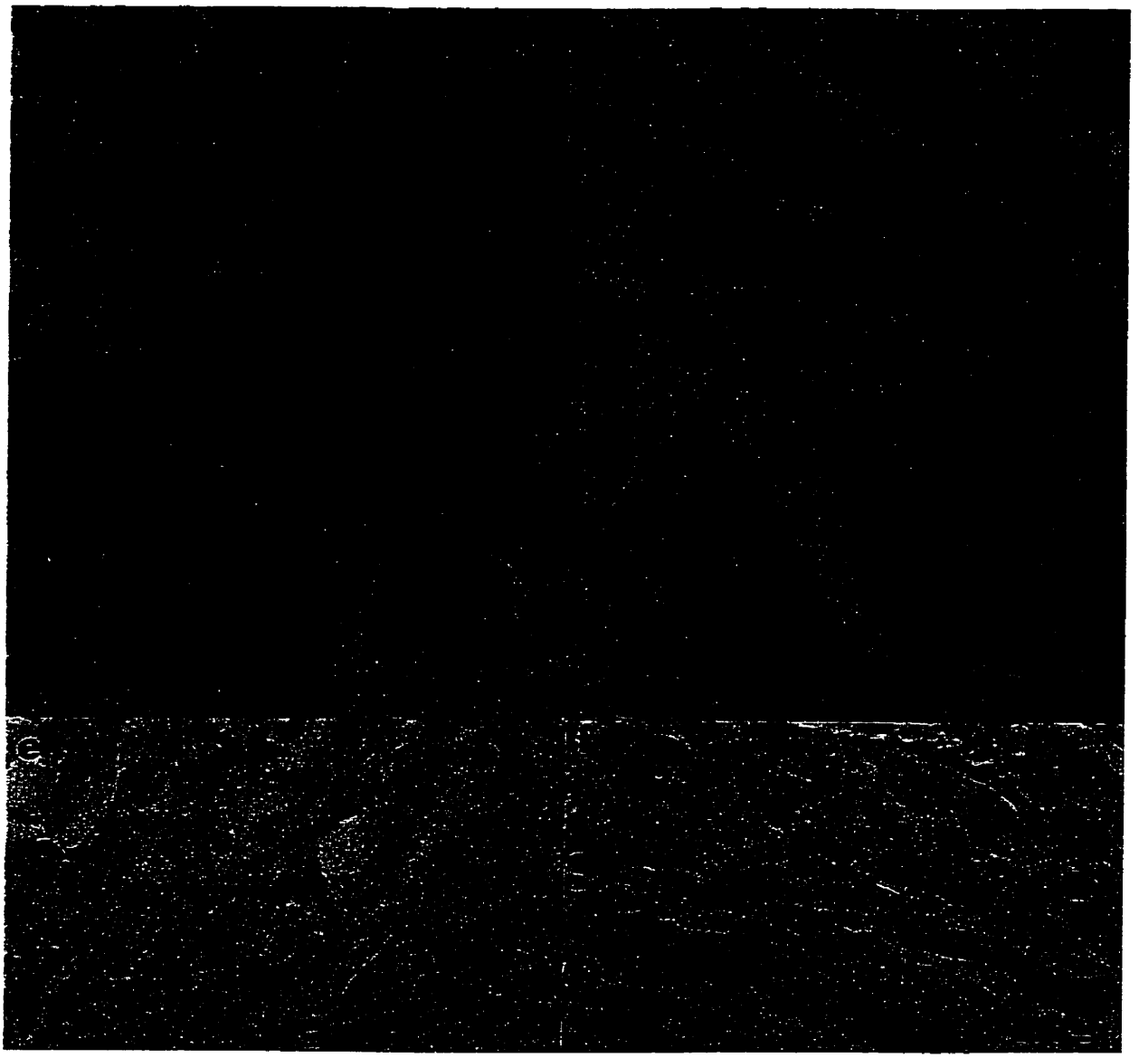
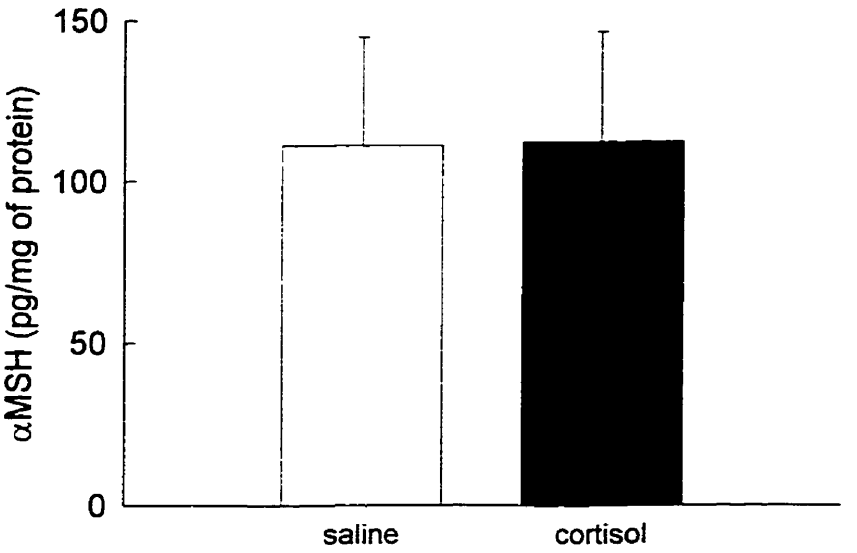


Figure 6.6 αMSH concentrations in the placenta collected at the conclusion of a 96-hour infusion of either saline (□, n=4) or cortisol (■, n=4) to the late-gestation ovine fetus. Values are mean ± SEM.


150
100
50
0
αMSH (pg/mg of protein)
saline
cortisol

significantly reduced after 96 hours of cortisol infusion to 25% of the level of GR mRNA in the saline-infused controls (Figure 6.7).

6.3.2 Fetal Lung

The mRNA encoding for POMC, PC1 and PC2 was expressed in the fetal sheep lung (Figure 6.8). Intra-fetal cortisol administration significantly reduced POMC (Figure 6.9A) and PC2 (Figure 6.9C) mRNA levels in the fetal, while PC1 mRNA levels remained unaltered compared to controls (Figure 6.9B).

There was no change in the concentration of $ACTH_{1-39}$, ACTH+RP or the ratio of $ACTH_{1-39}$ to ACTH+RP (Figure 6.10). Immunoreactive αMSH and ACTH peptide were present in the epithelium of tertiary bronchi and bronchioles, sero-mucous glands and at lower levels in the alveoli (Figure 6.11). Adjacent sections were incubated with the primary antibody preabsorbed with an excess of antigen, and the immunostaining was abolished in all areas with the exception of the sero-mucous glands which contained some residual staining (Figure 6.11, I and J). There was no marked change in the distribution of ir-ACTH peptide in the lung after cortisol treatment (Figure 6.11, B and D) compared to control (Figure 6.11, A and C). Intra-fetal cortisol infusion reduced bronchiolar epithelium and alveolar levels of αMSH (Figure 6.11, F and H) compared to saline-infused fetuses (Figure 6.11, and G).

However, αMSH concentrations, measured by radioimmunoassay, in the fetal lung were not different after cortisol treatment compared to control (Figure 6.12). GR mRNA levels were not altered by cortisol treatment (Figure 6.13) compared to control animals.

Figure 6.7 Northern blot analysis of placental glucocorticoid receptor (GR) mRNA from individual animals after intra-fetal cortisol (n=5) or saline (n=5) treatment. The autoradiograms are shown in the upper panel. The blot was subsequently probed using an 18S rRNA cDNA. The relative optical density (ROD) of GR mRNA was expressed as a ratio of 18S rRNA and shown in the histogram (lower panel). Values are mean ± SEM, *$p<0.05$.

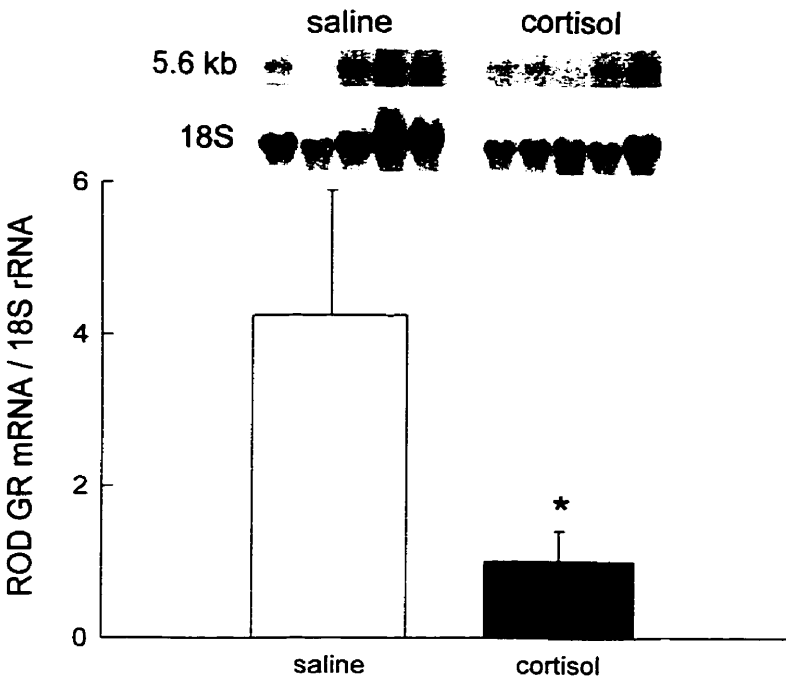

saline
cortisol
5.6 kb
18S
ROD GR mRNA / 18S rRNA
6
4
2
0
*
saline
cortisol

Figure 6.8 *in situ* hybridization localization of POMC, PC1, and PC2 mRNA in sections of lung collected from fetuses that received an infusion of either cortisol or saline, for 96 hours. An adjacent section incubated with a random oligonucleotide probe is also shown (lower panel). Films were exposed for 21 days.

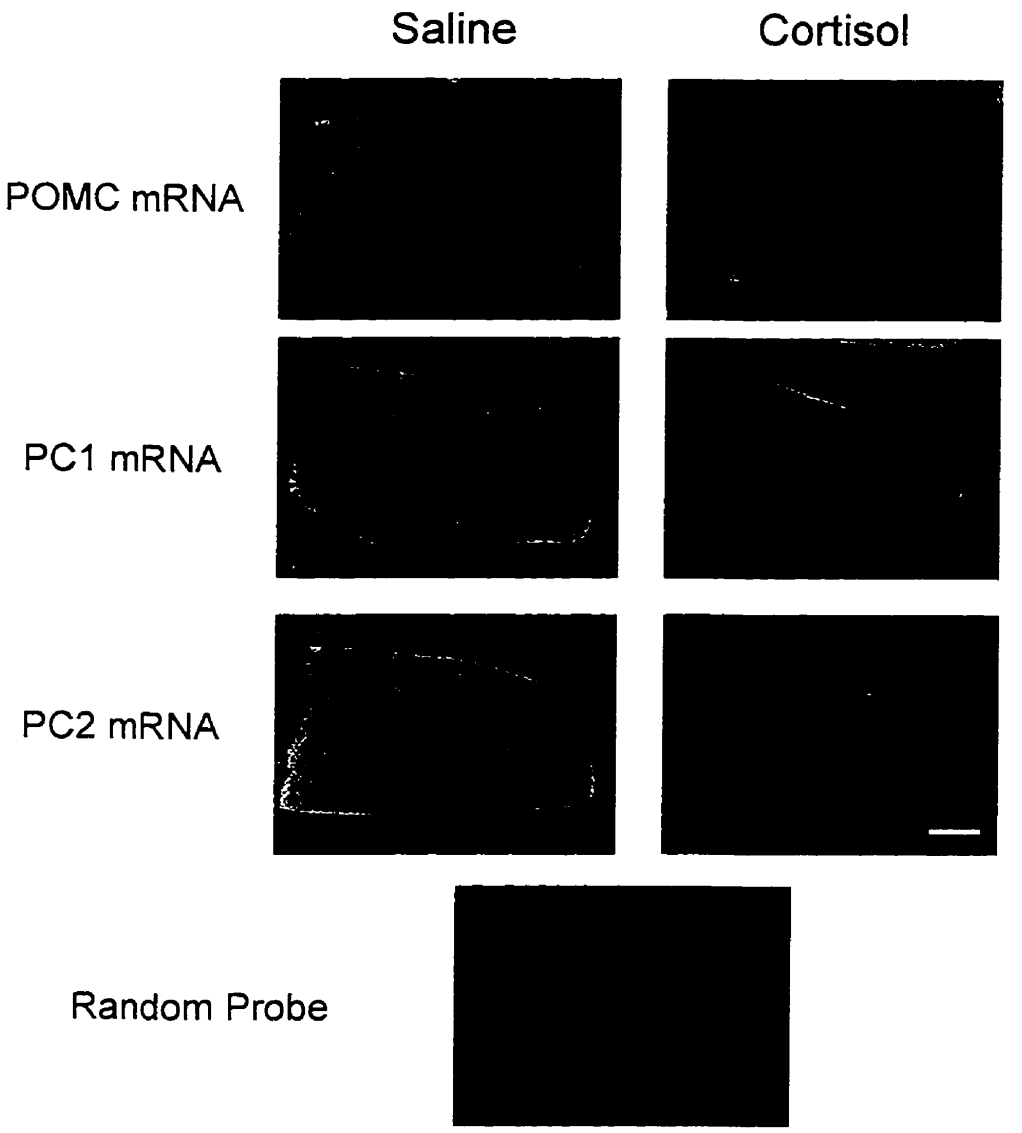
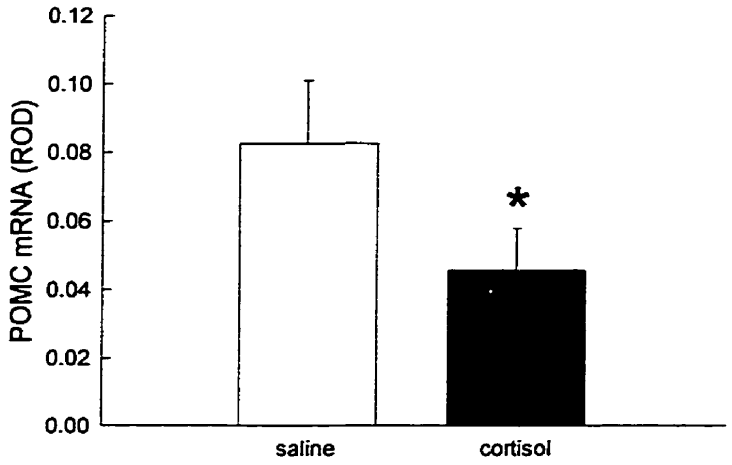
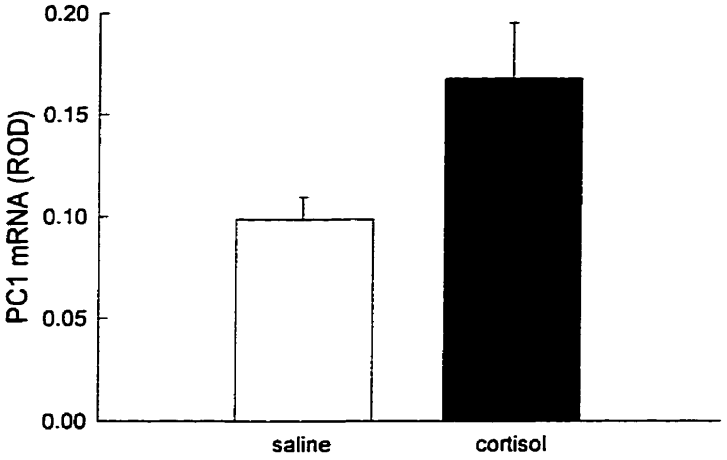

Saline
Cortisol
POMC mRNA
PC1 mRNA
PC2 mRNA
Random Probe

Figure 6.9 Densitometric analysis of autoradiograms from *in situ* hybridization analysis of (A) POMC, (B) PC1 and (C) PC2 mRNA levels in sections of lung collected from infused with either cortisol (■, n=7) or saline (□, n=5) for 96 hours. Values are mean ± SEM, *$p<0.05$. ROD = relative optical density.

A.


0.12
0.10
0.08
0.06
0.04
0.02
0.00
POMC mRNA (ROD)
*
saline
cortisol

B.


0.20
0.15
0.10
0.05
0.00
PC1 mRNA (ROD)
saline
cortisol

C.

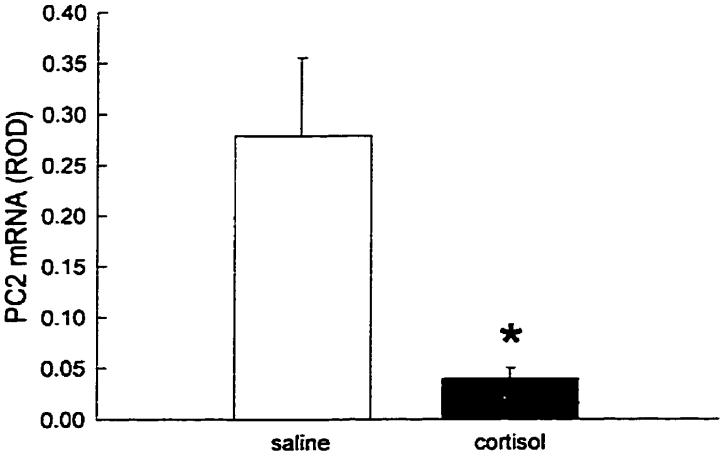
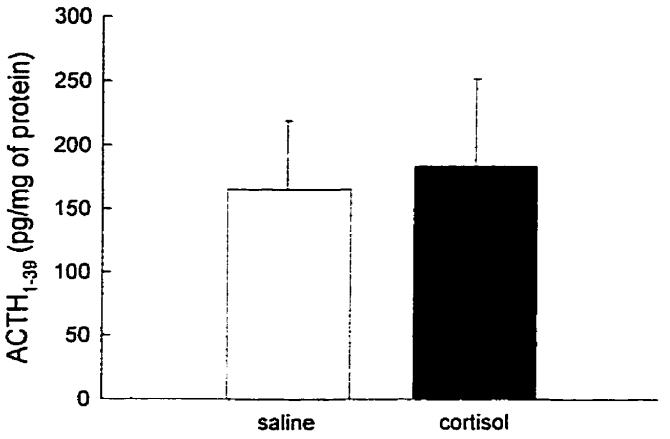
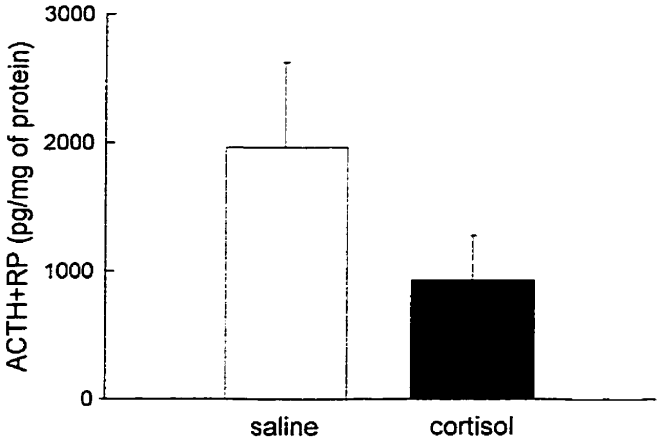

0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
PC2 mRNA (ROD)
*
saline
cortisol

Figure 6.10 Concentration of (A) $ACTH_{1-39}$, (B) ACTH+RP and (C) the ratio of $ACTH_{1-39}$:ACTH+RP in the fetal lung after intra-fetal administration (96 hours), of either saline (□, n=3) or cortisol (■, n=5). Values are mean ±SEM.

A.


300
250
200
150
100
50
0
ACTH1-39 (pg/mg of protein)
saline
cortisol

B.


3000
2000
1000
0
ACTH+RP (pg/mg of protein)
saline
cortisol

C.

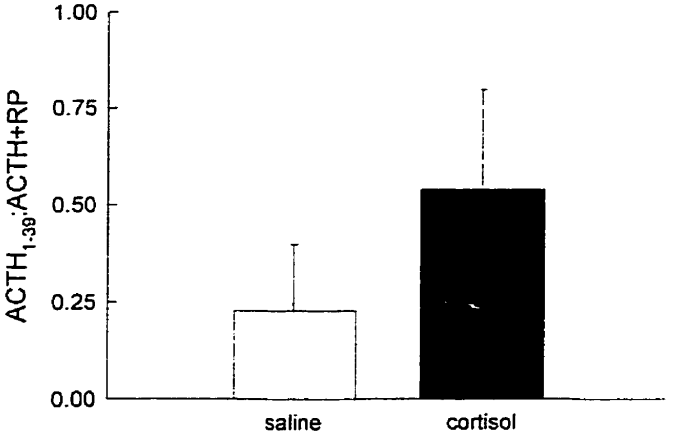

1.00
0.75
0.50
0.25
0.00
ACTH1-39:ACTH+RP
saline
cortisol

Figure 6.11 Immunoreactive ACTH (panels A, B, C, and D) and αMSH (panels E, F, G and H) peptides in the fetal sheep lung, were localized to the epithelium of tertiary bronchi (3B) and bronchioles (b), sero-mucous glands (g), smooth muscle (sm) and in the alveoli (a). Representative sections of fetal lung after 96-hours of intra-fetal saline (panels A, C, E, and G) and cortisol (panels B, D, F and H) administration are shown. Sections of lung incubated with either ACTH (panel I) or αMSH (panel J) antibody preabsorbed with an excess of antigen are also shown. Scale=50μm.

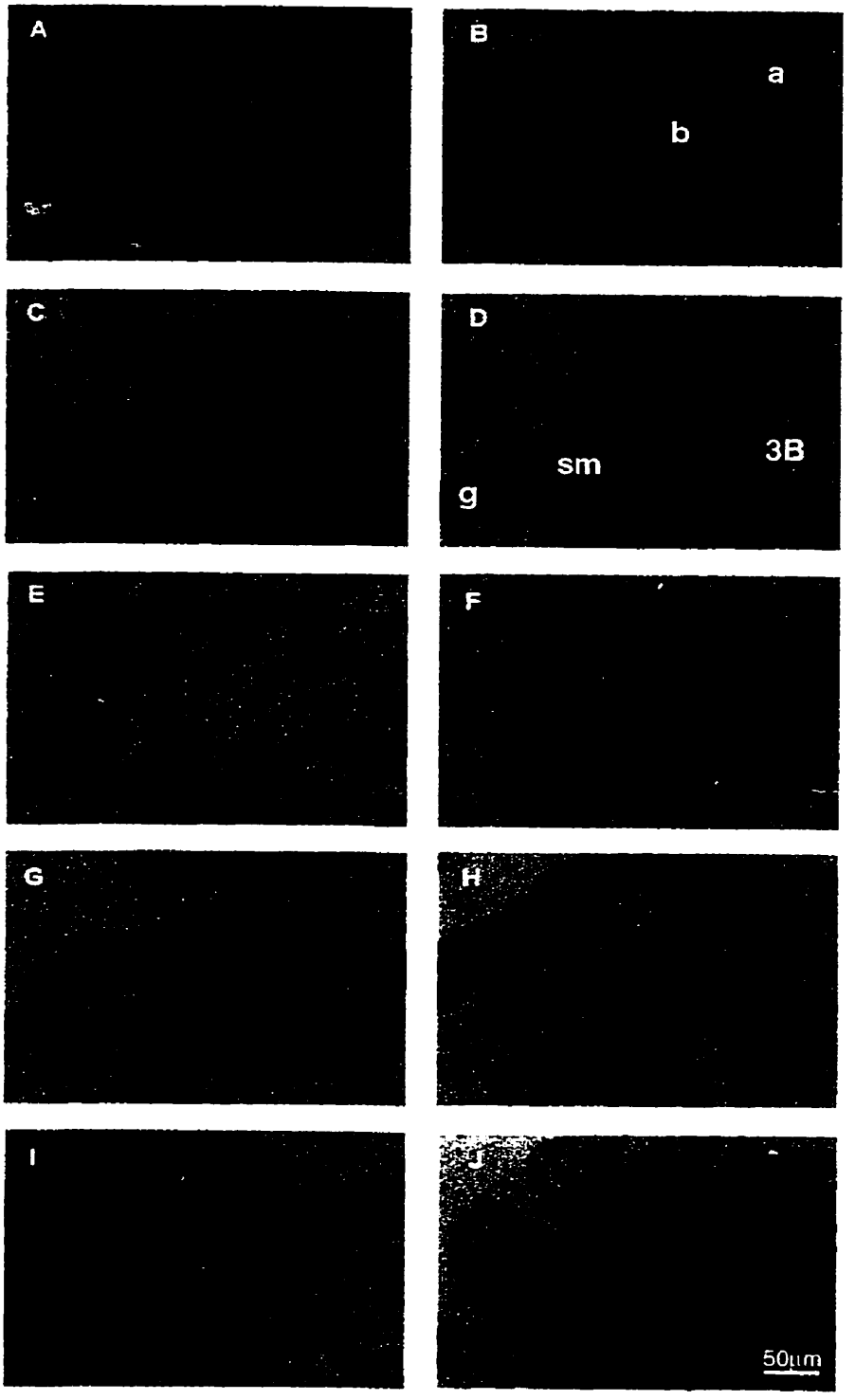

A
B
a
b
C
D
sm
3B
g
E
F
G
H
I
J
50µm

Figure 6.12 αMSH concentrations in the lung collected at the conclusion of a 96-hour infusion of either saline (□, n=4) or cortisol (■, n=4) to the late-gestation ovine fetus. Values are mean ± SEM.

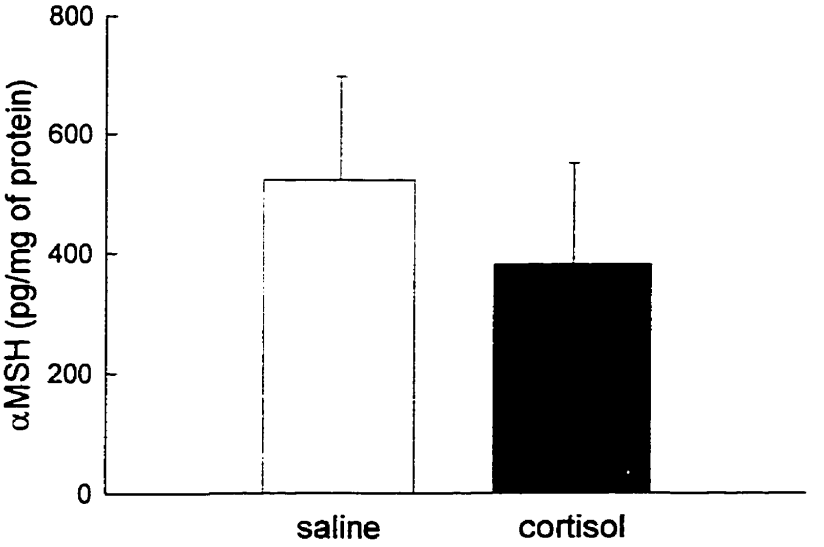
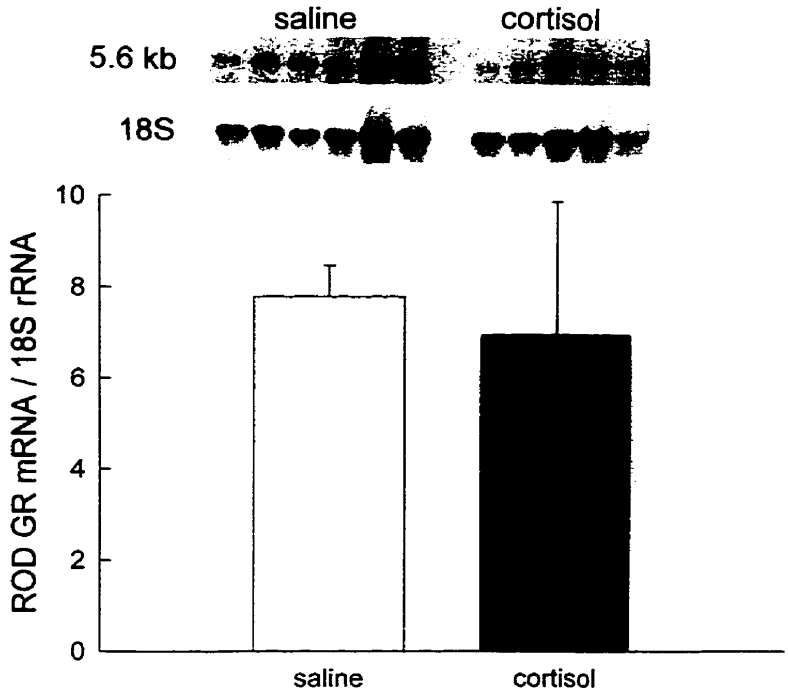

800
600
400
200
0
αMSH (pg/mg of protein)
saline
cortisol

Figure 6.13 Northern blot analysis of glucocorticoid receptor (GR) mRNA in fetal lung from individual fetuses infused with saline (n=6), or cortisol (n=5) for 96 hours. The blot was subsequently probed using an 18S rRNA cDNA to control for the amount of RNA analyzed (autoradiograms are shown in the upper panel). The relative optical density (ROD) of GR mRNA was expressed as a ratio of 18S rRNA and shown in the histogram (lower panel). Values are mean ± SEM.


saline
cortisol
5.6 kb
18S
ROD GR mRNA / 18S rRNA
10
8
6
4
2
0
saline
cortisol

6.4 Discussion

This is the first report of POMC mRNA expression in the ovine placenta, and the first time PC1 and PC2 mRNA have been identified in the fetal lung and sheep placenta. POMC mRNA and $ACTH_{1-39}$ levels in the placenta were not altered by intra-fetal cortisol administration. Despite decreases in POMC mRNA levels in the fetal lung, $ACTH_{1-39}$ levels were not suppressed during cortisol administration. This is consistent with the lung and placenta as potential sources of $ACTH_{1-39}$ in the late-gestation ovine fetus when plasma cortisol concentrations are elevated.

Studies conducted previously, using *in vitro* overexpression systems have shown that an increase in PC1 or PC2 mRNA levels is reflected by an increase in the respective enzymatic activity (Bloomquist *et al.* 1991, Zhou and Mains, 1994). In addition, PC2 mRNA levels within the pituitary are greater in the pars intermedia where PC2 cleavage of POMC is greater compared to the pars distalis (Benjannet *et al.* 1991). There are few reports directly examining the relationship between changes in PC1 and PC2 mRNA levels and enzyme activity. However, if the changes in placental PC2 mRNA levels reflect changes in prohormone convertase activity an increase in PC2 would be expected to increase cleavage of $ACTH_{1-39}$ to produce αMSH. Despite unchanged POMC mRNA, PC1 mRNA, $ACTH_{1-39}$, ACTH+RP, the ratio of $ACTH_{1-39}$:ACTH+RP levels and increased PC2 mRNA levels, there was no change in the concentration or distribution of αMSH staining in the placenta, or in the plasma αMSH concentrations (Chapter Five). These data may indicate that processing POMC by PC1 and PC2 is not altered by cortisol treatment, despite changes in PC2 mRNA levels.

The placental ACTH concentrations reported here are similar to those published previously by Jacobs *et al.* (1989), using similar extraction and assay techniques. However, these values are 3-fold greater than the values reported by Keller-Wood and Wood (1991a). This may be due to variations in dissection and extraction techniques. Keller-Wood and Wood (1991a) removed the outer capsule and boiled the tissues in acid before homogenization. In the present study and in the report of Jacobs *et al.* (1989), a section of the entire placentome including the capsule was homogenized in acid and the samples were not boiled. The distribution of POMC mRNA seen in the *in situ* autoradiograms indicates that the capsule contains POMC mRNA and therefore may be an important source of ACTH. In addition, the studies of Keller-Wood and Wood (1991a) utilized a different ACTH radioimmunoassay. In the present study, $ACTH_{1-39}$ concentrations measured in the fetal lung are similar to the concentrations of ACTH reported previously by Cudd *et al.* (1993).

Intra-fetal cortisol administration significantly reduced fetal lung POMC mRNA and PC2 mRNA levels, however PC1 mRNA abundance was not altered. This expression pattern would expected to result in a lower abundance of POMC peptide cleaved to produce predominantly $ACTH_{1-39}$, with very little αMSH produced. There was a marked decrease in the levels of ir-αMSH seen in the bronchiolar epithelium by immunohistochemistry. However, there was no detectable change in the concentration of αMSH measured in lung homogenates, perhaps indicating that there might be a shift in the distribution of αMSH within the fetal lung. In addition, different αMSH antibodies were used for immunohistochemistry and

radioimmunoassay and it is possible that differences in antibody specificity may confound comparison of the radioimmunoassay and immunohistochemistry results. Cortisol treatment did not alter the concentration of $ACTH_{1-39}$, ACTH+RP, nor the ratio of $ACTH_{1-39}$:ACTH+RP in the fetal lung. This lack of change does not reflect the alterations one might have anticipated based upon the levels of mRNA encoding the processing enzymes. It emphasizes that changes in levels of mRNA may not equate to enzyme levels or activity. Also, we have not co-localized PC1 and PC2 mRNA with POMC mRNA therefore we cannot comment on whether PC1 and PC2 are expressed in both POMC and non-POMC expressing cells in the lung or in the placenta. A change in overall PC1 or PC2 mRNA levels may not reflect the changes in levels of these enzymes specifically in POMC-expressing cells.

Intra-fetal cortisol administration did not inhibit placental POMC mRNA levels. Conversely, POMC mRNA abundance in the fetal lung was suppressed significantly by exogenous cortisol administration. These changes in POMC mRNA may relate to changes in GR mRNA. In the placenta where POMC mRNA levels did not change, GR mRNA levels were significantly reduced. However, in the lung GR mRNA levels were not altered and POMC mRNA levels decreased. These data would be consistent with the impact of glucocorticoids on extra-pituitary sites of POMC being mediated, at least in part, by alterations in levels of mRNA encoding glucocorticoid receptors.

Studies reported in this Chapter have shown that the fetal sheep lung and placenta contain mRNA transcripts encoding POMC, PC1 and PC2. Immunoreactive $ACTH_{1-39}$ and related peptides were detected by radioimmunoassay and

immunostaining. However, in the placenta levels of POMC mRNA , $ACTH_{1-39,}$ and the ratio of $ACTH_{1-39}$:ACTH+RP were unaltered by cortisol infusion *in vivo*. In the fetal lung $ACTH_{1-39}$ and ACTH+RP levels were also unaltered by the increase in plasma cortisol concentrations. The change in total PC2 mRNA levels was not reflected in changes in levels of αMSH in the lung or placenta. However, the differential effect of cortisol on POMC mRNA levels in the placenta and the lung were consistent with the cortisol effect on GR mRNA levels. Therefore, in the sheep fetus both the lung and the placenta may contribute to circulating ACTH levels in the presence of elevated plasma cortisol concentrations. However, further studies are required to establish the level of influence each has on plasma ACTH concentrations.

Chapter Seven

Prenatal betamethasone effects on fetal and maternal CBG levels during human pregnancy

This chapter is a collaborative study. The patient groups included in this chapter are sub-populations of patients from previously studies. Patients in Group 1 (London, Ontario) have been previously published by Korebrits *et al.* (1998), and Group 2 (Rome, Italy) patients were included in the publication by Marinoni *et al.* 1998. The data presented in this chapter has been submitted for publication.

7.1 Introduction

Maturation of the fetal HPA axis is a consistent feature of late pregnancy in many animal species as well as the human fetus (Challis and Brooks, 1989; Grino et al. 1991; Cudd *et al.* 1995). An increase in the fetal plasma cortisol concentration provides part of the stimulus to maturation of those organ systems that are required for extra-uterine life, and may contribute to the stimulus to parturition (Liggins, 1976; Challis et al. 1977; Challis *et al.* 1995). In the circulation, cortisol levels are regulated, in part, by the extent of binding to the high affinity glycoprotein CBG, which is synthesized predominately in the liver (Westphal, 1983; Hammond, 1990). In fetal sheep, levels of hepatic CBG mRNA rise during late pregnancy to peak at 7-10 days before parturition and then begin to decline before birth (Berdusco *et al.* 1995). Levels of CBG in fetal plasma peak some days later, but also begin to decline immediately pre-partum (Ali et al. 1992; Berdusco *et al.* 1995). Previously we reported that glucocorticoids stimulated increases in plasma CBG binding capacity and hepatic CBG mRNA levels in fetal sheep at 0.65-0.80 of gestation, in contrast to the decreases in plasma CBG and levels of CBG mRNA in the liver of adult sheep after glucocorticoid administration (This thesis, Chapter Three; Berdusco et al. 1993; Berdusco *et al.* 1994). Glucocorticoids administered to the baboon fetus in mid-gestation (day 100; term ~180 days) (Pepe *et al.* 1996), and to mice in the newborn period (Zhao *et al.* 1997) also produced rises in plasma CBG. However, exogenous glucocorticoid did not alter circulating CBG levels in the late-gestation baboon fetus (Pepe *et al.* 1996), or in the adult pregnant baboon (Pepe *et al.* 1996). Conversely, glucocorticoid treatment suppressed plasma CBG levels in the adult mouse (Zhao *et*

al., 1997), rat (Smith and Hammond 1992), and rhesus monkey (Stanczyk *et al.* 1985). These observations suggest that the pattern of the CBG response to glucocorticoids changes from a fetal response that is characterized by stimulation, to an adult response that is generally inhibitory. Alteration in the response patterns appears to occur either late in fetal life or just after birth.

Adult humans receiving glucocorticoid replacement therapy or pharmacological amounts of synthetic corticosteroids have modest (30-40%) decreases in plasma CBG binding capacity (Schlechte and Hamilton 1987, Frairia *et al.* 1988). Plasma CBG levels are elevated in human pregnancy (Doe *et al.* 1964) and this is generally considered to reflect stimulation of hepatic CBG biosynthesis by raised levels of maternal estrogens (Coe *et al.* 1986, Feldman *et al.* 1979) however, the effects of glucocorticoids on maternal and fetal levels of CBG in mid-pregnancy are not known.

Women presenting in preterm labor often receive synthetic glucocorticoids, which cross the placenta and promote fetal lung maturity. We collected both maternal and fetal blood of treated patients to examine the separate effects of glucocorticoids on CBG levels in both the fetal and maternal compartments. Moreover, CBG is present in amniotic fluid, although the source of this CBG is unclear (Challis and Bennett, 1977). Therefore we have determined CBC in maternal and umbilical cord plasma and in amniotic fluid from patients at risk of preterm delivery at 24-35 weeks of gestation, and who received prenatal betamethasone to promote fetal lung maturity.

7.2 Materials & Methods:

7.2.1 Patients

We obtained samples from two separate groups of patients; one in London, Ontario, Canada, and the second in Rome, Italy. These were subgroups from patient populations that have been reported previously (Korebrits *et al.* 1998, Marinoni *et al.* 1998).

Group 1

Forty women admitted to St. Joseph's Health Care Centre, London, Ontario, Canada in preterm labor (24-32 weeks of pregnancy), were studied (Group 1). Gestational age was determined by assessment of menstrual dates, early pelvic examination, and first or second trimester ultrasound. Women with multiple pregnancies, fetal anomalies, diabetes, preeclampsia, cervical dilatation more than 4 cm, intrauterine growth restriction and/or clinical signs of infection were excluded from the study. The study was approved by the Review Board for Research on Human Subjects of the University of Western Ontario, and all of the participants gave informed consent.

Upon admission to hospital, maternal blood samples (5ml) were collected, by venipuncture, into heparinized tubes. The women received betamethasone (Celestone, Schering, Canada; 2 x 12mg IM, q 12 or 24h) to stimulate fetal lung maturation, and blood was collected again at 24h and 1 week post-treatment, if the

patient had not delivered. Blood samples were centrifuged at 1900 x *g* for 20 minutes at 4°C. The plasma was collected and stored at -80°C until analysis.

Group 2

An additional group of patients was studied in Rome, Italy (Group 2). Women with multiple pregnancies, diabetes, preeclampsia, intrauterine growth restriction and clinical infection were excluded. Patients with prolonged premature rupture of membranes (PROM) and placenta praevia received tocolytic management with oral ritodrine at a dose of 2 x 10mg daily (Miolene, Lusofarmace S.p.a., Milan, Italy). Indications for elective cesarean section included maternal disease, pregnancy complications and fetal malformations. This protocol was approved by the ethics committee of University "La Sapienza", Rome, Italy.

Ten patients between 29 and 35 weeks of gestation with the diagnosis of threatened pre-term delivery were enrolled in the study. Betamethasone (Celestone Cronodose, Essex S.p.a., Milan, Italy) was given 2 x 12mg, IM, 24h apart at 8 a.m, on consecutive days. Patients who had not delivered 1 week later received an additional dose of 12mg.

Maternal blood samples (10ml) were obtained by venipuncture just before betamethasone administration (basal samples) and at 3h, 6h, 12h, and 24h after the first dose of betamethasone. Patients that had not delivered 1 week later received an additional dose (12mg) of betamethasone and a further blood sample was obtained 24h later. Blood was collected into heparinized tubes, and centrifuged at

650 x *g* for 15 min at 4°C. The plasma was divided into aliquots and stored at –20°C until analysis.

Mixed cord blood samples were obtained at elective cesarean section at various times after treatment with betamethasone. Patients were divided into sub-groups according to the time that had elapsed between the final dose of betamethasone and cesarean section delivery (<24h; n=9, <48h; n=6, <1week; n=6, or >1week; n=3). Umbilical cord blood samples were collected and stored as described for the maternal blood samples.

Samples of amniotic fluid were collected either by transabdominal amniocentesis (performed to test for fetal lung maturity), or by transuterine amniocentesis at the time of elective cesarean section. In some patients multiple samples of amniotic fluid were obtained. Patients were divided into the following groups: amniotic fluid collected within 24h of betamethasone (n=10), within 48h (n=12), within 1 week (n=8) or after 1 week (n=8). Amniotic fluid samples were centrifuged at 1800 x *g* for 15 minutes at 4°C, and stored at –20°C until analysis.

Eight patients (32.4 ± 0.8 weeks of gestation) presented at risk of preterm delivery, but did not receive corticosteroid therapy. Maternal blood samples were taken at the same frequency (3, 6, 12, 24 hours and 1 week after collection of the basal sample), beginning at ~10.00h using the same techniques as outlined for the betamethasone treated patients. Amniotic fluid (n=10) and fetal plasma (n=9) samples were collected at elective cesarean section.

7.2.2 Measurement of plasma CBG, cortisol and estradiol

CBG and cortisol levels in plasma and amniotic fluid were measured as described previously (Section 3.2.2).

Estradiol

Plasma 17β-estradiol in human plasma was measured using a commercial radioimmunoassay (ICN Biomedicals, Inc., Costa Mesa, CA). This assay has been extensively characterized for use in humans and exhibits the following cross-reactivities: estradiol-17β 100%, estrone 20%, estriol 1.51%, estradiol-17α 0.68%, and <0.01% for ethinyl estradiol, androstenedione, DHEA, 5α-dihydrotestosterone, 20α-dihydroprogesterone, DOC, progesterone, testosterone, pregneneolone, 17-hydroxypregnenolone, DHEA-sulfate, aldosterone, cortisol, 11-desoxycortisol, 17α-hydroxyprogesterone and cholesterol. The sensitivity of the assay was 10pg/ml, and the intra-assay coefficient of variance was 5.9%. All plasma estradiol concentrations were assessed within a single assay.

7.2.3 Western blot analysis

Maternal plasma samples were diluted 1:50 in 0.5M Tris-HCl buffer (pH 6.8) and subjected to Western blot analysis (Section 3.2.3).

7.2.4 Data Analysis

Results are expressed as mean ± SEM. Differences in maternal plasma cortisol and CBC levels were assessed using Kruskal-Wallis one-way analysis of

variance on ranks (non-parametric). CBC levels in fetal plasma and amniotic fluid were assessed using analysis of variance, followed by Student-Newman-Keuls if a significant F ratio was obtained. Statistical significance was set at $p<0.05$.

7.3 Results

7.3.1 Betamethasone effects on maternal plasma cortisol and CBC

7.3.1.1 Group 1

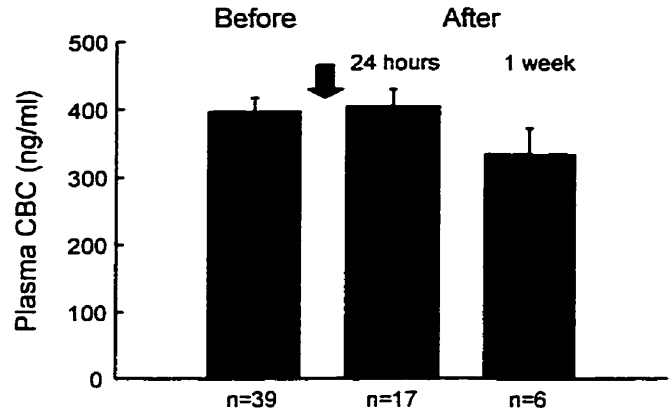
The mean maternal plasma cortisol concentration at admission was 223.1 ± 21.2ng/ml (Figure 7.1A). Twenty-four hours later, after 2 x 12mg of betamethasone, cortisol concentrations had fallen by more than 70%, and remained suppressed 1 week later (Figure 7.1B). Maternal plasma CBC levels were not altered significantly at 24h and 1 week after maternal betamethasone administration (Figure 7.1B). The ratio of cortisol:CBC in maternal plasma fell from 0.60 ± 0.05 before betamethasone, to 0.15 ± 0.05 24h later, and 0.26 ± 0.10 at one week later. Western blot analysis revealed a major 57 kDa immunoreactive (ir-) CBG isoform in maternal plasma. There was no apparent change in the size of CBG isoforms or the amount of ir-CBG present in maternal plasma after betamethasone treatment (Figure 7.2).

7.3.1.2 Group 2

Plasma CBC levels in the control patients did not change significantly throughout the study period (Figure 7.3B). Maternal plasma CBC levels were not altered by betamethasone treatment (Figure 7.3A). The ratio of cortisol:CBC in maternal plasma decreased from 0.90 ± 0.22 before betamethasone to 0.35 ± 0.05

Figure 7.1 Maternal plasma (A) CBC and (B) cortisol levels from preterm labor patients before and 24 hours after and 1 week after receiving prenatal betamethasone treatment (12.5mg IM). Values are mean ± SEM, for numbers of samples (patients) indicated in the histograms *$p<0.05$, Kruskall-Wallis one-way ANOVA on ranks. The arrow denotes the time of maternal betamethasone injection.

A.


Before
After
24 hours
1 week
Plasma CBC (ng/ml)
500
400
300
200
100
0
n=39
n=17
n=6

B.

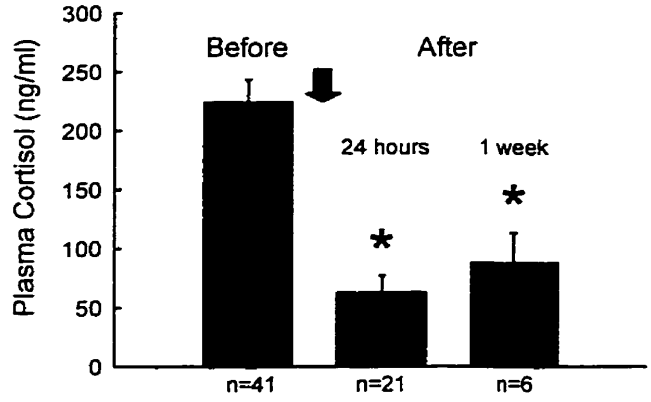

Before
After
24 hours
1 week
Plasma Cortisol (ng/ml)
300
250
200
150
100
50
0
*
*
n=41
n=21
n=6

Figure 7.2. Western blot analysis of maternal plasma before and 24 hours after betamethasone treatment. Samples are from the same 4 individual patients at the two times (same order of gel loading).

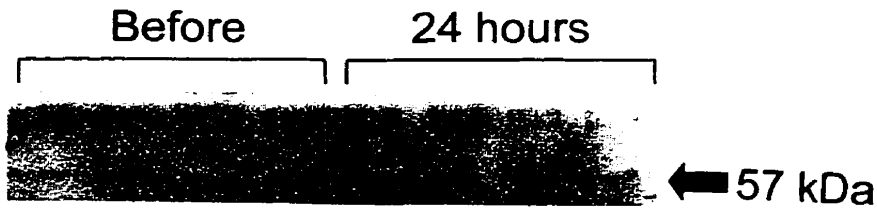
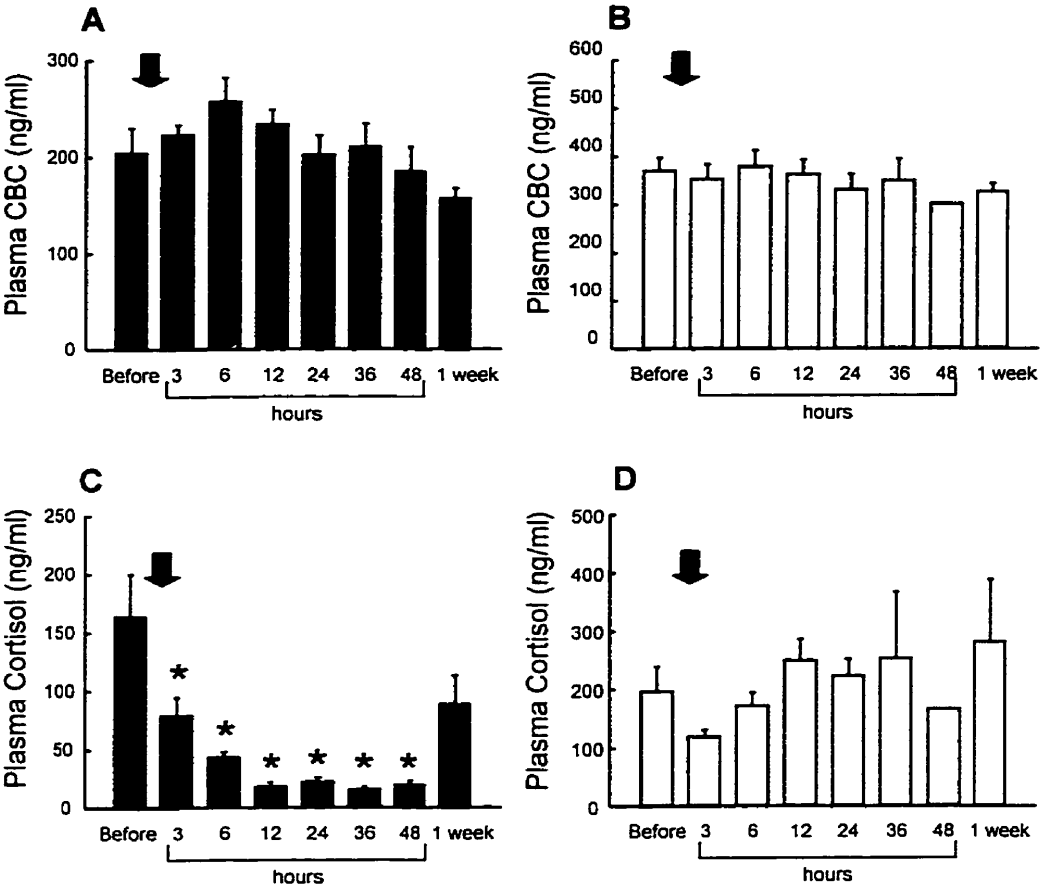

Before
24 hours
57 kDa

Figure 7.3 Maternal plasma CBC (A, B) and cortisol (C, D) levels in response to betamethasone treatment (■, n=3-10) or in control (□; non-steroid treated, n=3-8) patients at risk of preterm delivery. Values are mean ± SEM, *$p<0.05$, Kruskal–Wallis one-way ANOVA on ranks. The arrow denotes the time of maternal betamethasone injection.

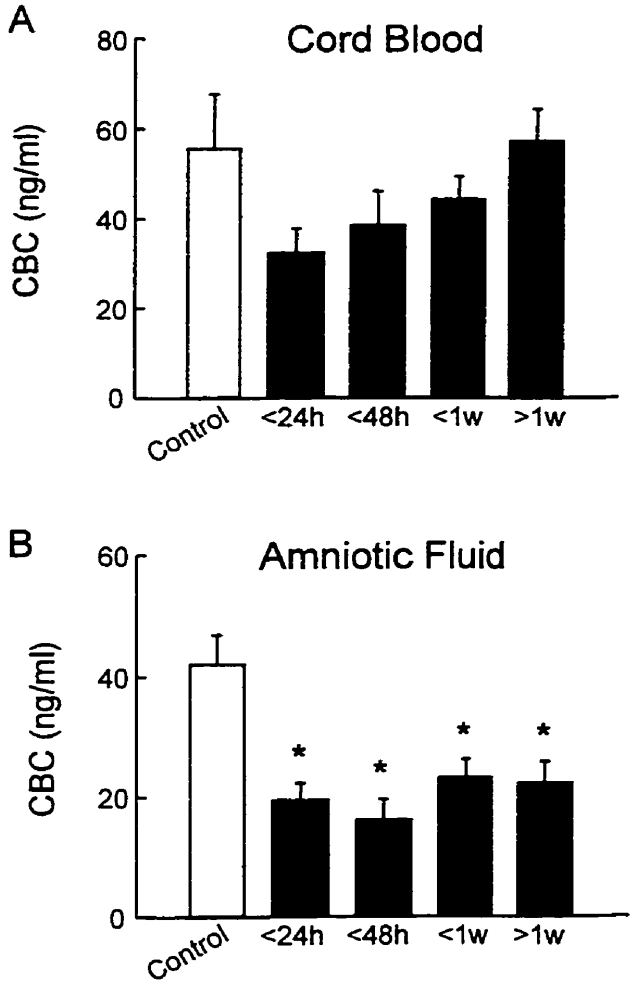

A
Plasma CBC (ng/ml)
300
200
100
0
Before
3
6
12
24
36
48
1 week
hours
B
Plasma CBC (ng/ml)
600
500
400
300
200
100
0
Before
3
6
12
24
36
48
1 week
hours
C
Plasma Cortisol (ng/ml)
250
200
150
100
50
0
*
Before
3
6
12
24
36
48
1 week
hours
D
Plasma Cortisol (ng/ml)
500
400
300
200
100
0
Before
3
6
12
24
36
48
1 week
hours

by 3h after administration. There was no change in CBG isoforms or protein levels in maternal plasma in either betamethasone, or control patients (data not shown). Maternal plasma cortisol concentrations were suppressed significantly after a single dose of betamethasone, and reached their lowest values at 12-24h after betamethasone (Figure 7.3C). Plasma cortisol concentrations in the control patients varied in a manner consistent with a circadian rhythm (Figure 7.3D).

7.3.2 Fetal CBG response to maternal betamethasone administration

There were no overall significant effects of betamethasone on cord CBC concentrations, although values at 24 h were significantly lower than in control patients when compared by a non-parametric Mann-Whitney test ($p<0.05$) for these two groups alone (Figure 7.4A). Amniotic fluid CBC levels were significantly lower than controls in patients at 24h after betamethasone administration, and these values remained significantly lower at one week after betamethasone (Figure 7.4B). Cortisol concentrations in umbilical cord plasma decreased from control values (24.9±4.7ng/ml) to 5.3±1.7ng/ml within 24 h and remained significantly depressed 48 hours after treatment, but were not significantly different from control at 1 week. However, amniotic fluid cortisol concentrations were significantly lower than control within 24h and continued to be significantly reduced (data not shown, see Marinoni *et al.* 1998).

Figure 7.4. CBC levels in (A) cord blood samples and in (B) amniotic fluid collected within 24 hours (<24h, cord blood; n=9, amniotic fluid; n=12), within 48 hours (< 48h, cord blood; n=6, amniotic fluid; n=12), within 1 week (<1w, cord blood; n=6, amniotic fluid; n=8), and greater than 1 week (>1w, cord blood; n=3, amniotic fluid; n=8) after betamethasone (■), compared to CBC levels measured in non-steroid treated control patients (□). The number of patients per group is indicated in the histograms. *$p<0.05$, one-way ANOVA, followed by Student-Newman-Keuls.


A
Cord Blood
CBC (ng/ml)
80
60
40
20
0
Control
<24h
<48h
<1w
>1w
B
Amniotic Fluid
CBC (ng/ml)
60
40
20
0
*
*
*
*
Control
<24h
<48h
<1w
>1w

7.4 Discussion

Betamethasone given to women in threatened preterm delivery in order to promote fetal lung maturity had no statistically significant effect on maternal or umbilical cord CBC. However, there was a tendency for CBC levels to be lower in cord blood obtained 24 h after betamethasone administration. CBC levels were reduced significantly and remained reduced in amniotic fluid for up to 1 week after betamethasone administration. Western blot analysis showed that the principle immunoreactive CBG isoform corresponded to a 57 kDa protein, and the relative concentration of CBG before and 24 hours after betamethasone administration was not altered with betamethasone treatment. The present study was conducted within the clinical management routines of the two hospital settings, and we were less able to control for time of day of betamethasone injection, and blood sampling in the Canadian patients. We recognize that all of the study patients had a diagnosis of threatened preterm delivery, which may have altered the maternal or fetal response to exogenous betamethasone.

We found no effect of betamethasone administration on maternal CBC levels in either group of patients. The synthetic corticosteroid was bioactive since plasma cortisol concentrations were decreased by negative feedback, and maternal peripheral plasma CRH concentrations rose, reflecting placental CRH output (Marinoni *et al.* 1998). Conversely, CBC is decreased in patients with Cushing's disease, and in males and non-pregnant females receiving physiological or pharmacological doses of glucocorticoids (Schlechte and Hamilton, 1987; Frairia *et al.* 1988). Furthermore, in adult animals exogenous glucocorticoids decrease

circulating CBG concentrations, and levels of hepatic CBG mRNA (Stanczyk et al. 1985; Smith and Hammond, 1992; Berdusco et al. 1993). The present results are consistent with studies in pregnant baboons, which reported no effect of betamethasone on CBG levels in the maternal circulation (Pepe *et al.* 1996).

It is generally regarded that the increase in maternal plasma CBG in human pregnancy reflects a stimulatory effect of estrogen on maternal hepatic CBG gene expression (Wesphal, 1971). It would appear that this effect is not overcome by exogenous betamethasone, at least at the concentrations given clinically. However, maternal estrogen concentrations should decrease after betamethasone administration since provision of fetal and maternal adrenal precursors for placental aromatization is reduced by negative feedback (Mesiano and Jaffe, 1997). Therefore one might anticipate finding a decrease in maternal CBG after betamethasone as a consequence of suppressing maternal estrogen concentrations. There was a tendency (not significant) for lowered CBC values at one week after betamethasone administration, in the Italian patients and in two of these patients who received three additional weekly doses of betamethasone there were modest decreases in maternal estrogen concentrations and a 25% decrease in maternal CBC levels. It is possible that the long half-life of CBG (Sandberg *et al.* 1964) makes it difficult to detect significant changes in maternal plasma values within the time frame of the present studies.

We have suggested recently that the ratio of cortisol to CBG in maternal plasma remains relatively constant through the course of human pregnancy, but might be elevated in patients at risk of preterm delivery (Korebrits *et al.* 1998). After

betamethasone, plasma cortisol concentrations were suppressed, but CBG was unaltered, hence the cortisol:CBC ratio fell dramatically. It follows, therefore, that this ratio would only be of clinical value in predicting the patient at risk of preterm delivery (Korebrits *et al.* 1998) prior to the administration of maternal betamethasone.

We found no significant change in the levels of CBC in umbilical cord plasma after betamethasone administration, although there was a tendency to decrease CBC levels at 24h. Clearly we found no evidence for an increase in cord CBC values in the human fetus at 26 to 32 weeks gestation, in contrast to results in the baboon fetus at mid-gestation (Pepe *et al.* 1996), the sheep fetus throughout late pregnancy (Berdusco *et al.* 1993; This thesis, Chapter Three) and in the mouse at 4 and 10 days postnatal age (Zhao *et al.* 1997). Betamethasone administration to the baboon fetus later in gestation did not stimulate CBG (Pepe *et al.* 1996), a response similar to that of the current study. It is possible that a rise in plasma CBG and hepatic CBG synthesis after glucocorticoid treatment is characteristic of a "fetal" response and fall in CBG characterizes the normal "adult" response. The switch from fetal to adult response pattern may occur prenatally in some species and in the neonatal period or postnatally in others. The present study suggests that the human fetus during the early to mid-third trimester of pregnancy does not show the increased CBG response to glucocorticoids seen in fetuses of other species.

The mechanisms by which glucocorticoids affect CBG expression and which underlie an altered response are unclear. There does not appear to be a consensus sequence corresponding to a glucocorticoid response element in the proximal

promoter region of CBG (Underhill and Hammond, 1989). Therefore the effects of glucocorticoids on CBG are likely mediated through other transcription factors. An alteration in the glucocorticoid-induced expression pattern of these elements may account for the stimulatory or inhibitory effects of glucocorticoid on CBG. It is of interest that other glucocorticoid-responsive genes in the liver, for example 11β-hydroxysteroid dehydrogenase - Type 1 are also affected differently between fetal and adult life (Yang *et al.* 1994).

It has been suggested that increased CBG synthesis in response to glucocorticoids (Chapter Three; Berdusco *et al.* 1993; Berdusco *et al.* 1994) assists in attenuating the normal negative feedback effect of cortisol in the late gestation ovine fetus (Challis and Brooks, 1989; Ballard *et al.* 1982). Thus ACTH and cortisol concentrations rise concurrently in the fetal circulation during late pregnancy (Norman *et al.* 1985). Although basal circulating corticosteroid levels are higher, it is not clear why the human and baboon fetus should have a different CBG response pattern to glucocorticoids in late pregnancy. One possibility would be that the absence of an increase in CBG would allow a more rapid increase in free cortisol in response to fetal HPA activation under circumstances of fetal stress. However, because of the relative deficiency of 3β-hydroxysteroid dehydrogenase in the primate fetal adrenal gland (Mesiano and Jaffe, 1997), the major product of acute ACTH stimulation is likely to be increased estrogen precursor, rather than cortisol (Mesiano *et al.* 1997). This may increase CBG, but through an estrogen-responsive pathway (Westphal, 1986).

The CBC concentration of amniotic fluid was reduced after betamethasone administration, and remained lower than controls for up to 1 week. Maternal plasma CBC values were significantly higher in the control patients than in the women treated with betamethasone (Group 2), but there is no evidence that CBG crosses the human placenta. Although CBG levels rise in maternal plasma, CBG levels in amniotic fluid do not change in late gestation (Challis and Bennett, 1977). It has been argued that amniotic fluid CBG levels are independent of maternal CBG (Challis and Bennett, 1997). This is supported by the findings in the present study, which suggest that betamethasone does not affect maternal CBG but suppresses amniotic fluid CBG. Although the source of CBG in amniotic fluid remains unclear, the different patterns of CBC during gestation raise the possibility that CBG in amniotic fluid may be derived from a different source than CBG in the fetal or maternal circulation. The kidney (Scrocchi *et al.* 1993) and pituitary (Berdusco *et al.* 1995) are capable of CBG gene expression. In the mouse, CBG mRNA levels in the fetal kidneys change dramatically during the course of gestation (Scrocchi *et al.* 1993). Human placenta or fetal membranes could also be sources of CBG in amniotic fluid; however, the possibility of CBG expression and regulation in these tissues has not been resolved

We conclude that CBG in maternal and fetal plasma is not suppressed significantly by exogenous synthetic glucocorticoid administration during third-trimester human gestation and may function to protect the fetus and mother from high free cortisol concentrations. Conversely, CBG levels in amniotic fluid appear to

be affected differentially by maternal betamethasone treatment and therefore may originate from a source other than the fetal or maternal liver.

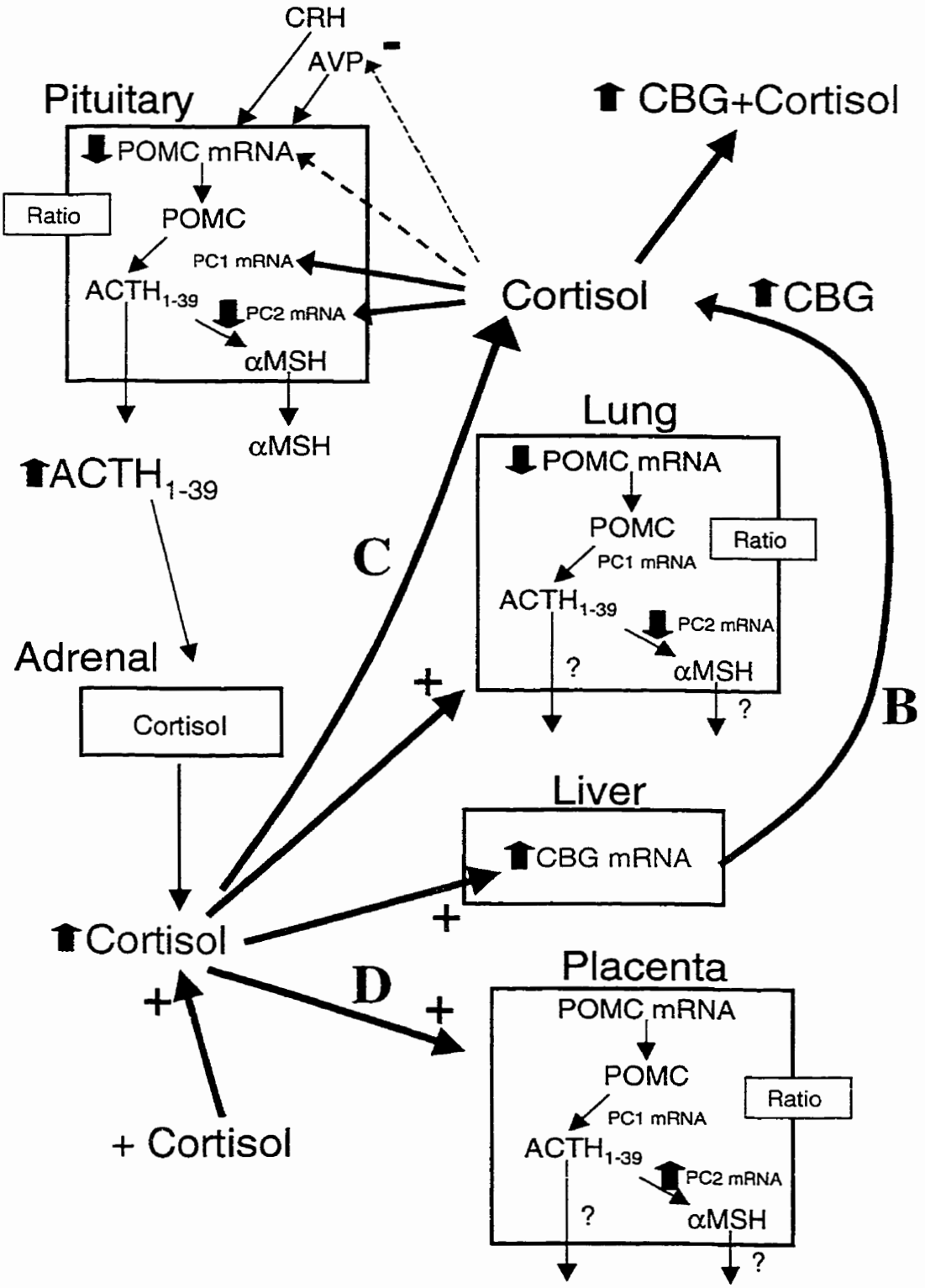
Chapter Eight

General Discussion

Activation of the hypothalamic-pituitary-adrenal (HPA) axis occurs during late-gestation in both human and sheep fetuses. There is a progressive rise in plasma cortisol and ACTH concentrations, which is consistent with a decrease in cortisol negative feedback within the fetal HPA axis. Cortisol is critical in organ maturation thereby preparing the fetus for extra-uterine life, and in sheep, cortisol is the trigger to parturition. The apparent decrease in cortisol negative feedback on ACTH output may be attributable to many factors. Previous studies have shown that there was a decrease in pituitary glucocorticoid receptor binding from 110 days of gestation until term when glucocorticoid specific binding and GR mRNA levels increased dramatically (Yang *et al.* 1992; Matthews *et al.* 1995). This is accompanied by an increase in plasma CBG (Berdusco *et al.* 1995), and an increase in pituitary 11βHSD-1 activity (Yang *et al.* 1995). Thus several mechanisms are activated which could regulate feedback of cortisol at the level of the pituitary. Cortisol appeared to be central to these mechanisms. Therefore, this thesis examined the effects of cortisol on CBG and ACTH production in late-gestation when the phenomena of reduced negative feedback occurs.

Cortisol appears to regulate CBG levels in the ovine fetus (Chapter 3; summary Figure 8.1A). An incremental cortisol infusion over 10 days (120-130 days of gestation) stimulated a progressive rise in plasma CBG levels. Conversely, fetal adrenalectomy at 115 days of gestation prevented the prepartum rise in plasma cortisol concentrations, and also attenuated the prepartum rise in plasma CBG. These results revealed the positive influence of cortisol on plasma CBG levels in the ovine fetus during late gestation.

Figure 8.1 Summary of results contained in this thesis. Text in colour represents information gathered, and whether cortisol-infusion resulted in an increase (↑), decrease (↓) or no change (no arrow) as compared to the saline-infused control animals. The colours represent the hypothesis (B-D) stated in Chapter Two (Figure 2.1). Red = Chapter Four, Purple = Chapter Five, and Green = Chapter Six. Ratio = $ACTH_{1\text{-}39}$:ACTH+RP.


CRH
AVP
-
Pituitary
POMC mRNA
Ratio
POMC
PC1 mRNA
ACTH1-39
PC2 mRNA
αMSH
αMSH
↑ CBG+Cortisol
Cortisol
↑CBG
↑ACTH1-39
Lung
POMC mRNA
POMC
Ratio
PC1 mRNA
ACTH1-39
PC2 mRNA
αMSH
?
?
C
B
Adrenal
Cortisol
+
Liver
↑CBG mRNA
+
↑Cortisol
D
+
Placenta
+
POMC mRNA
POMC
Ratio
PC1 mRNA
ACTH1-39
PC2 mRNA
αMSH
?
?
+ Cortisol

However, the effect of glucocorticoids on CBG levels was very different during early third-trimester human pregnancy. Betamethasone given to women at risk of preterm delivery, to stimulate fetal lung maturation, did not alter maternal or fetal plasma CBC levels. Based upon data from the sheep we would have anticipated an increase in fetal CBC after glucocorticoid treatment (Berdusco *et al.* 1993; Berdusco *et al.* 1994). Studies of adult humans and sheep would suggest that CBC levels in adults decrease after glucocorticoids (Schlechte and Hamilton, 1987; Frairia *et al.* 1988, Berdusco *et al.* 1993). However, pregnancy appeared to attenuate the inhibitory effects of glucocorticoids on CBG typically seen in adults. Further studies are required to explore whether the lack of glucocorticoid inhibition of CBG during pregnancy is a result of the stimulatory action of elevated circulating estrogens. These data collected during human pregnancy are consistent with studies conducted by Pepe *et al.* (1996) in the baboon. In late gestation maternal and fetal baboon plasma CBG levels were not altered by glucocorticoid treatment (Pepe *et al.* 1996). This may reflect a difference in the timing of the change in CBG responsiveness to glucocorticoids. The switch in glucocorticoid stimulation to glucocorticoid inhibition of CBG does not occur until the time of parturition in the sheep and not until post-puberty in the mouse (Zhao *et al.* 1997a). The cause of the switch in glucocorticoid regulation of CBG, from stimulatory to inhibitory, is presently unknown. It has be suggested that very high free cortisol concentrations may be the trigger to the change in regulation of CBG in the sheep fetus (Berdusco *et al.* 1995) however, further research is necessary to determine the mechanism of this change. Amniotic fluid CBC levels were significantly suppressed after maternal betamethasone

treatment. This suggests that amniotic fluid CBG (Challis and Bennett, 1977) may originate from an alternate and differentially regulated source of production than fetal and maternal plasma CBG.

It has been postulated that the rise in plasma CBG in late gestation maintains low free cortisol concentrations in fetal plasma and thereby allows the concurrent rise in plasma ACTH and cortisol (Challis and Brooks, 1989). We examined these relationships between ACTH, cortisol and CBG in the late gestation ovine fetus by infusing cortisol for 5 days prior to the endogenous rise in plasma cortisol concentrations, at a dose which produced concentrations similar to that seen in the near term fetus (Chapter Four; summary Figure 8.1B). This infusion protocol stimulated a rise in plasma ACTH concentrations within 24-48 hours. Plasma CBC levels were significantly increased, however, not until 48-72 hours, almost 24 hours after the initial rise in ACTH. The rise in CBG maintained a low percentage of free cortisol but did not suppress free cortisol concentrations. Therefore, free and presumably bioactive cortisol was available to act on the pituitary. The increase in bioactive cortisol is reflected by the significant decrease in pituitary POMC mRNA levels. Despite the apparent glucocorticoid inhibition of pituitary POMC mRNA levels, plasma ACTH concentrations increased. We hypothesized that the increase in circulating ACTH was due to a glucocorticoid-induced change in processing such that POMC was cleaved to preferentially produce $ACTH_{1-39}$ and/or that additional sites of POMC production, specifically the fetal lung and the placenta, contributed to plasma ACTH levels, under the influence of exogenous cortisol.

Pituitary levels of PC1 mRNA examined in the entire pituitary were not altered by cortisol treatment, but PC2 mRNA levels in the pars distalis and the pars intermedia were increased (Chapter Five and Figure 8.1C). If changes in enzymatic activity are reflected by changes in mRNA levels these data may suggest that POMC in the pars distalis and the pars intermedia would be cleaved predominately to αMSH. However, this was not reflected by concentrations of αMSH in the pars distalis, or in peripheral plasma. In addition, the levels of $ACTH_{1-39}$, ACTH+RP and the ratio of $ACTH_{1-39}$ to ACTH+RP in the pars distalis were not altered by cortisol. Therefore, the pars distalis of fetuses treated with cortisol appeared to contain abundant $ACTH_{1-39}$, which may result from maintained production and secretion, despite the decrease in POMC mRNA levels. This $ACTH_{1-39}$ may contribute to circulating ACTH concentrations. Alternatively, a reduction of peptide secretion may also explain the unaltered pars distalis concentration of $ACTH_{1-39}$. We examined the possibility that the pars intermedia might be important as a source of circulating ACTH. The pars intermedia contains 5-fold more POMC mRNA than the pars distalis (Matthews *et al.* 1994), and appears to be less sensitive to cortisol inhibition since levels of POMC were reduced by only 38% compared to the 96% decrease in levels of POMC mRNA in the pars distalis after a 96 hour cortisol infusion. Therefore the pars intermedia could be an important source of POMC-derived peptides when plasma cortisol concentrations are elevated. Further experiments are critical to understand the role of the pars intermedia in HPA axis function during fetal life.

The fetal sheep lung may also contribute to circulating ACTH concentrations (Chapter Six and Figure 8.1D). Although intrafetal cortisol administration

significantly decreased levels of POMC mRNA, PC1 mRNA levels were not altered and PC2 mRNA levels were significantly suppressed by intrafetal cortisol administration. This raised the possibility that there could be a decrease in cleavage of $ACTH_{1-39}$ to αMSH and therefore greater $ACTH_{1-39}$ production from the remaining POMC. However, there was no significant change in αMSH concentrations in the lung, although αMSH immunoreactivity in the bronchiolar epithelium was reduced. We suggest this could reflect a change in distribution of αMSH in pulmonary tissue, without significant changes in the pulmonary concentration of αMSH. The concentration of $ACTH_{1-39}$, and ACTH+RP in the lung were not significantly different in cortisol-infused fetuses compared to controls. Therefore the lung may continue to produce and secrete ACTH despite increases in fetal plasma cortisol concentrations. In addition, the decrease in PC2 mRNA levels in the fetal lung raise the possibility that cleavage of $ACTH_{1-39}$ may be reduced, and thus may be reflected by an increase in $ACTH_{1-39}$ output. However, we have not assessed the contribution of fetal lung $ACTH_{1-39}$ secretion to total production and circulating levels of $ACTH_{1-39}$. Therefore, further studies are required to elucidate the contributions of the fetal lung to circulating ACTH concentrations especially during periods of elevated fetal plasma cortisol concentrations.

The ovine placenta expressed POMC mRNA, PC1 mRNA and PC2 mRNA. The levels of POMC mRNA and PC1 mRNA were not altered by cortisol infusion to the fetus, but PC2 mRNA was increased after cortisol (Chapter Six and Figure 8.1D). These changes raise the possibility that the placenta would produce more αMSH and less $ACTH_{1-39}$ in response to cortisol. The placenta contained abundant

ir-αMSH. However, the concentration and distribution of αMSH in the placenta did not change after cortisol treatment. Neither $ACTH_{1-39}$, nor ACTH+RP concentrations nor the ratio of $ACTH_{1-39}$:ACTH+RP in the placenta were altered significantly by cortisol infusion. POMC mRNA levels were not suppressed and there remained abundant $ACTH_{1-39}$ in the ovine placenta after 96 hours of cortisol treatment thereby implicating the placenta as a potential source of circulating $ACTH_{1-39}$.

In all tissues examined, PC1 mRNA levels were not altered by glucocorticoids. The changes in PC2 mRNA levels did not appear to reflect the changes seen in the tissue levels of $ACTH_{1-39}$ or αMSH. This may be due to cell heterogeneity in the pars distalis, lung and placenta. In addition,, the synthesis of active PC2 is relatively slow and regulated by the specific binding protein 7B2, which may account for the increase in PC2 mRNA levels without apparent changes in PC2 activity. We have not co-localized PC1, or PC2 with POMC, therefore we cannot assume that changes in proconvertase mRNA levels reflect changes in proconvertase mRNA levels in POMC expressing cells. In fact, the pars distalis contains several cell types which express PC1 and PC2 including lactotrophs, gonadotrophs, thyrotrophs as well as corticotrophs. The pars intermedia is predominantly corticotroph cells, unfortunately we were unable to isolate and assess pars intermedia concentrations of POMC-derived peptides.

The total ACTH content of the fetal pars distalis, lung and the placenta was assessed, based on the previously published average organ weights of untreated fetuses of comparable gestational age and total body weight (Gagnon *et al.* 1995; David Cox, personal communication). The placenta and lung contain 95-fold and 40-

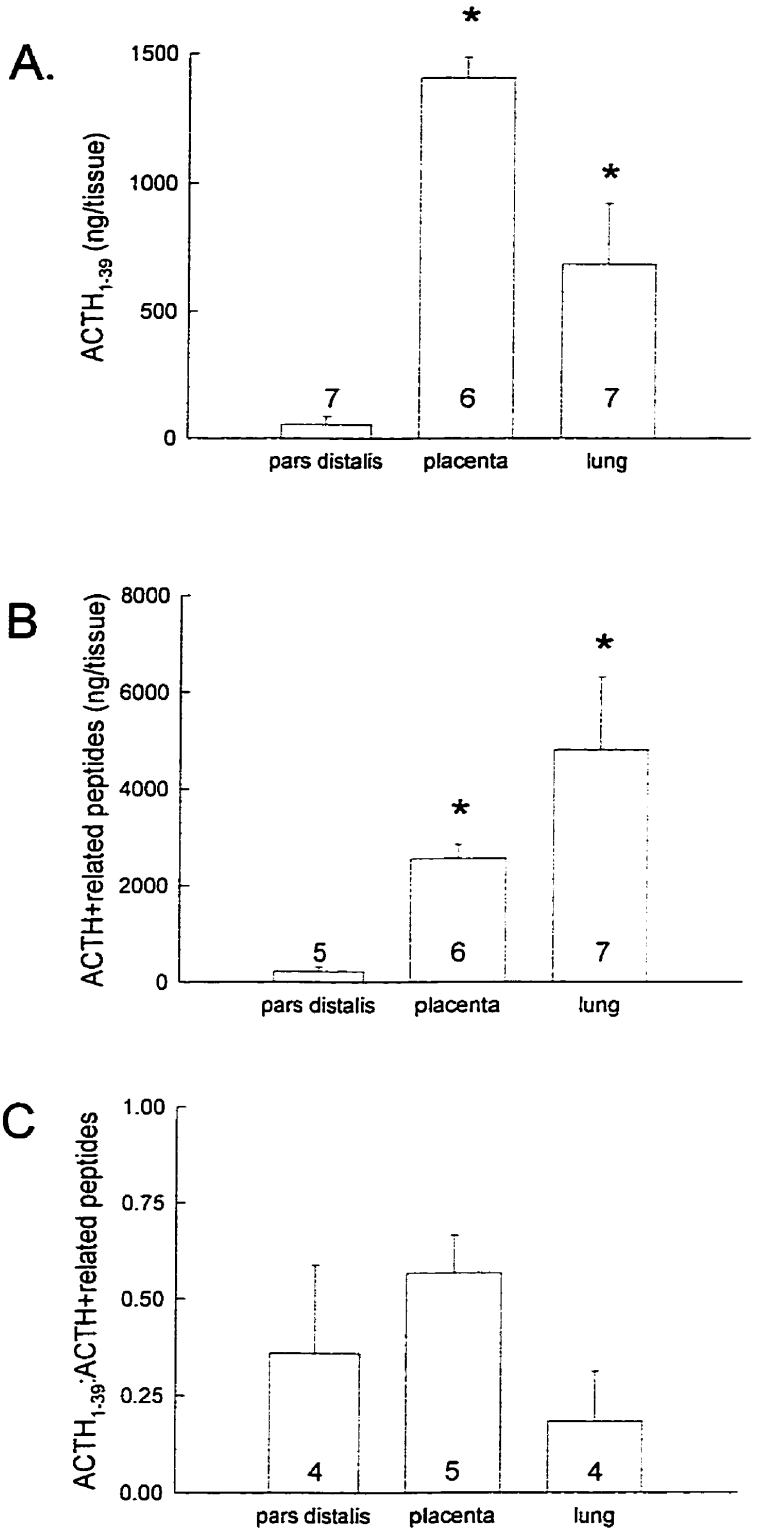
fold more total $ACTH_{1-39}$ than the pars distalis, respectively (Fig. 8.2, A and B). This suggests that the lung and placenta should produce abundant amounts of $ACTH_{1-39}$ and therefore have the potential to impact significantly on circulating $ACTH_{1-39}$ levels. The fetal lung and placenta also contain 50X more αMSH than the pars distalis (data not shown). However, the role of αMSH in fetal development is not clear.

The effect of cortisol on POMC mRNA levels varied between tissues. POMC mRNA levels were significantly suppressed in the pituitary and lung while levels in the placenta were unaltered. These alterations in tissue POMC mRNA levels correspond to changes in the abundance of GR mRNA in each of these tissues. GR mRNA expression was high in the pituitary and lung and decreased in the placenta. This is consistent with the effects of intra-fetal cortisol administration on POMC mRNA levels being GR-mediated, and reflected by changes in GR mRNA levels. PC2 mRNA levels also appeared to be differentially regulated by cortisol. However, changes in PC2 expression did not correlate with changes in GR mRNA levels. PC2 mRNA increased in the pituitary and placenta, whereas GR mRNA increased in the pituitary and decreased in the placenta. In the lung, GR mRNA did not change significantly, but PC2 mRNA levels were significantly suppressed.

The studies contained in this thesis document a strong positive correlation between fetal sheep plasma cortisol and CBG levels. However, in the human during early third trimester, both maternal and fetal plasma CBG levels were not altered by glucocorticoid treatment. This may be the emergence of the change in

cortisol regulation of CBG seen between fetal and adult life. The precise timing and cause of the switch in cortisol regulation of CBG, from stimulatory to inhibitory, remains a mystery in all species. It is remarkable that infusing cortisol to the ovine fetus late in gestation, prior to the endogenous rise in fetal plasma cortisol concentrations, closely mimics the prepartum rises in plasma CBC and ACTH. This experimental paradigm offers a unique model in which to study cortisol negative feedback on the HPA axis during this critical period of reduced cortisol inhibition on HPA axis function. However, it must be noted that this is not a model of normal term development due to the more rapid rise in plasma cortisol concentrations than the endogenous rises seen during late gestation. The rise in fetal plasma $ACTH_{1-39}$ concentrations in response to intra-fetal cortisol infusion occurs despite a significant decrease in pars distalis POMC mRNA levels. This emphasizes the importance of POMC processing and $ACTH_{1-39}$ secretion within the pars distalis as well as the possibility that other sites of POMC production may be important during fetal development. We have considered the fetal pars intermedia, lung and the placenta, as potential sources of $ACTH_{1-39}$ production. The high abundance of $ACTH_{1-39}$ within the fetal lung and ovine placenta suggests that these sites could impact significantly on circulating $ACTH_{1-39}$ concentrations. However, more research is required to elucidate the relative contributions of extra-pituitary sources of ACTH production.

Figure 8.2 The calculated content of (A) $ACTH_{1-39}$, (B) ACTH+RP and (C) the ratio of $ACTH_{1-39}$:ACTH+RP in protein extracted from fetal pars distalis, lung and the placenta. Tissues were collected from fetuses infused for 96 hours with saline. The number of fetuses in each group is noted on the histogram. Values are mean ±SEM, Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's test, *$p<0.05$ different from pars distalis levels.


A.
1500
1000
500
0
ACTH1-39 (ng/tissue)
*
*
7
6
7
pars distalis
placenta
lung
B
8000
6000
4000
2000
0
ACTH+related peptides (ng/tissue)
*
*
5
6
7
pars distalis
placenta
lung
C
1.00
0.75
0.50
0.25
0.00
ACTH1-39:ACTH+related peptides
4
5
4
pars distalis
placenta
lung

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