The Impact of Prenatal Glucocorticoid Administration on the Development and Long Term Activity of the Hypothalamic-Pituitary-Adrenal and Metabolic Axes

By

Deborah M. Sloboda

A thesis submitted in conformity with the requirements For the degree of Doctor of Philosophy Graduate Department of Physiology University of Toronto

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The Impact of Prenatal Glucocorticoid Administration on the Development and Long Term Activity of the Hypothalamic-Pituitary-Adrenal (HPA) and Metabolic Axes

Deborah Sloboda, Doctor of Philosophy, 2001
Department of Physiology, University of Toronto

Few studies have examined the effects of clinically relevant doses of glucocorticoids on HPA and metabolic function. Moreover, there is little information available regarding differential effects based on the route of administration.

Repeated administration of betamethasone in the pregnant sheep resulted in reduced fetal weight and increases in cord plasma adrenocorticotropin (ACTH) and cortisol and a rise in cord plasma corticosteroid binding capacity (CBC), which was associated with an increase in fetal hepatic corticosteroid binding globulin (CBG) mRNA levels. This increase in CBC may result in a decrease in free circulating cortisol levels, thereby reducing negative feedback at the hypothalamus or the pituitary. Local availability of glucocorticoids is regulated by the enzyme 11βHSD1, responsible for the conversion of cortisone to cortisol. Maternal betamethasone administration resulted in an increase in fetal hepatic 11βHSD1 mRNA and protein levels. Alterations in 11β HSD1 could generate increased levels of local cortisol in the fetal liver, and affect expression of glucocorticoid sensitive hepatic enzymes involved in the regulation of glucose production.

To investigate postnatal effects of prenatal glucocorticoid administration, single or multiple doses of betamethasone were administered to the mother or fetus and lambs were challenged at 6 months and one year with CRH+AVP and glucose. One dose of maternal betamethasone resulted in elevations in basal and stimulated cortisol concentrations, without associated changes in ACTH responses at one year. Fetal betamethasone administration resulted in attenuated ACTH responses to CRH+AVP at one year not associated with alterations in cortisol levels. Both single and multiple doses of maternal betamethasone resulted in elevated
insulin responses to glucose at 6 months, which may indicate the onset of insulin resistance. At one year, this effect was only present in animals that received multiple doses. Furthermore, animals that received one dose of maternal betamethasone demonstrated elevated basal glucose levels at one year. At 6 months offspring treated with maternal betamethasone are able to maintain normal glucose tolerance through an increased insulin response. Therefore, antenatal glucocorticoid exposure altered HPA and metabolic function and these changes persisted into adult life.
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I would like to thank my family for their support and understanding through many difficult times.

Finally, I am greatly indebted to the most important person in my life, without whom this degree would have been a long and lonely road. Thank you for enduring another hard battle and standing beside me through all the challenges and highlights, always supporting and guiding me.

This degree is as much yours as it is mine.
Publications


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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotrophin hormone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBC</td>
<td>corticosteroid binding capacity</td>
</tr>
<tr>
<td>CBG</td>
<td>corticosteroid binding globulin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CLIP</td>
<td>corticotrophin-like intermediate peptide</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotrophin releasing hormone</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>dGA</td>
<td>days of gestation</td>
</tr>
<tr>
<td>DHEAS</td>
<td>dehydroepiandrosterone sulphate</td>
</tr>
<tr>
<td>DTT</td>
<td>DL-dithiothreitol</td>
</tr>
<tr>
<td>FS</td>
<td>fetal saline</td>
</tr>
<tr>
<td>F1</td>
<td>fetal one dose of betamethasone</td>
</tr>
<tr>
<td>F4</td>
<td>fetal multiple doses of betamethasone</td>
</tr>
<tr>
<td>G-protein</td>
<td>guanine nucleotide binding protein</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid response element</td>
</tr>
<tr>
<td>(^{3})H</td>
<td>tritium</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary adrenal</td>
</tr>
<tr>
<td>3(^{\beta})HSD</td>
<td>3(^{\beta})-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>11(^{\beta})HSD</td>
<td>11(^{\beta})-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>1(^{25})I</td>
<td>radiolabeled iodine</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IM</td>
<td>intramuscular</td>
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<tr>
<td>ir-</td>
<td>immunoreactive</td>
</tr>
<tr>
<td>IUGR</td>
<td>intrauterine growth restriction</td>
</tr>
<tr>
<td>iv</td>
<td>intravenous</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>K(_d)</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>K(_m)</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MPA</td>
<td>medroxyprogesterone acetate</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
</tr>
<tr>
<td>αMSH</td>
<td>α-melanocyte stimulating hormone</td>
</tr>
<tr>
<td>MS</td>
<td>maternal saline</td>
</tr>
<tr>
<td>M1</td>
<td>maternal one dose of betamethasone</td>
</tr>
<tr>
<td>M4</td>
<td>maternal multiple doses of betamethasone</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>dihydronicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NIDDM</td>
<td>non-insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>NSB</td>
<td>non-specific binding</td>
</tr>
<tr>
<td>NT</td>
<td>no treatment</td>
</tr>
<tr>
<td>OT</td>
<td>oxytocin</td>
</tr>
<tr>
<td>P450&lt;sub&gt;αα&lt;/sub&gt;</td>
<td>cytochrome 450 side chain cleavage</td>
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<td>cytochrome 450 11β-hydroxylase</td>
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<td>prostaglandin E&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBSG</td>
<td>phosphate buffered saline with gelatin</td>
</tr>
<tr>
<td>PC1</td>
<td>prohormone convertase 1</td>
</tr>
<tr>
<td>PC2</td>
<td>prohormone convertase 2</td>
</tr>
<tr>
<td>PENK</td>
<td>pre-proenkephalin</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>POMC</td>
<td>proopiomelanocortin</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SON</td>
<td>supra-optic nucleus</td>
</tr>
<tr>
<td>SSC</td>
<td>saline sodium citrate</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
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1.1 THE FETAL ORIGINS OF ADULT DISEASE
Many have suggested the importance of a healthy pregnancy in determining neonatal health. Cigarette smoking and alcohol consumption during pregnancy have been shown to have severe detrimental effects on fetal respiratory tract and brain development, effects that are still visible in childhood and adolescence (Hill et al. 2000, Larroque et al. 2000, Wasserman et al. 2001). However, over the last 2 decades it has become clear that much more subtle changes in the intrauterine environment are important in determining the health and development of the fetus, effects that are seen much later in adulthood. Professor David Barker and his colleagues have described in detail the potential influence that an adverse intrauterine environment could play in the risk of developing particular diseases later in life. A key variable is the prevalence of intrauterine growth restriction (IUGR) and the association between fetal growth and the incidence of developing an array of diseases including coronary artery disease, hypertension, insulin resistance and non-insulin dependant diabetes mellitus (Type 2 diabetes) in adulthood (Barker 1994, Barker 1994, Barker 1995). More recently, low birth weight has been associated with high blood pressure, glucose intolerance and elevated hypothalamic-pituitary adrenal activity later in life (Levitt et al. 2000). It has therefore been proposed that the resetting of endocrine axes controlling growth and development could be responsible for the fetal programming of adult disease (Seckl 1997).

1.1.1 Glucose tolerance and insulin resistance
Many studies in England have described associations between low birth weight and the increased incidence of glucose intolerance and insulin resistance associated with non-insulin-dependent diabetes (NIDDM; Type 2 diabetes mellitus) (Hales et al. 1991, Robinson et al. 1992, Phipps et al. 1993, Phillips et al. 1994). Hales et al. (1991) found that the risk of developing glucose intolerance and diabetes later in life was 2 fold greater among men who had low birth weights. Similar trends were observed between weight at one year and the subsequent development of diabetes (Hales et al. 1991). Similarly, Phipps et al. (1992) showed a strong negative relationship between birth size and the incidence of impaired glucose tolerance in adult life, independent of the gestational age. Taken together, these studies provide strong epidemiological evidence that

Many studies have reported that low birth weight is associated with a higher incidence of syndrome X, (Barker et al. 1993, McCance et al. 1994, Levitt et al. 2000), a series of related variables including insulin resistance, glucose intolerance, hyperinsulinemia, hypertriglyceridemia, decreased high-density lipoprotein cholesterol and hypertension, that tends to occur in the same patients (Reaven 1988). It is the existence of this syndrome that has been proposed to have importance in the genesis of coronary artery disease. The relationship between reduced fetal growth and insulin resistance was confirmed by studies showing that men and women who were thin at birth as measured by ponderal index (weight/length$^3$), were more insulin resistant (Phillips et al. 1994, Lithell et al. 1996). Furthermore, prepubertal children (between 8-10 years of age) who were short exhibited significant insulin resistance during prepuberty and this insulin resistance was increased during pubertal development (Chiarelli et al. 1999). Although pancreatic β cell dysfunction is also a predictor of diabetes, attempts to show the presence of a β cell defect in human populations have been controversial. Studies have shown either that low birth weight is correlated with a reduction in pancreatic function (Hales et al. 1991, Robinson et al. 1992) or that there exists no relationship between fetal growth and pancreatic function (Phillips et al. 1994, Lithell et al. 1996). However, many studies have linked poor fetal nutrition with the prevalence of impaired β cell growth and function (Van Assche 1977, Snoeck et al. 1990, Hoet 1991, Berney et al. 1997), proposing that fetal adaptations to an adverse intrauterine environment have potential effects that may permanently alter metabolic function (Hales & Barker 1992).

1.1.2 The Hypothalamic-pituitary-adrenal (HPA) axis
Studies suggest that exposure of the fetus to excess glucocorticoids may play a role in the in utero predisposition to developing cardiovascular and metabolic (diabetes) diseases (Seckl 1997, Clark 1998). Fetuses exposed to excess glucocorticoids are growth restricted (Reinsch et al. 1978), and growth restricted babies have elevated levels of cord plasma corticotrophin releasing hormone (CRH), adrenocorticotrophin (ACTH) and cortisol (Economides et al. 1988, Goland et al. 1993). In addition, increases in urinary glucocorticoid metabolites in children 9 years of age were associated with reduced birth weight (Clark et al. 1996). Recent epidemiological studies have begun to establish a strong correlation between circulating cortisol levels and the incidence of hypertension and diabetes. Phillips et al. (1998) have shown that fasting plasma cortisol levels
in men aged 64 years were inversely related to birth weight, independent body mass index, and that elevated cortisol levels were significantly associated with higher blood pressure, plasma glucose levels, fasting triglyceride levels and insulin resistance. More recently, low birth weight has been associated with elevated fasting and stimulated cortisol concentrations in adults (Phillips et al. 2000, Levitt et al. 2000, Reynolds et al. 2001). In each case, cortisol levels were positively associated with high blood pressure and in some populations, associated with glucose intolerance (Levitt et al. 2000, Reynolds et al. 2001). These observations support a role for HPA axis function in the predisposition of adult disease. A recent study has reported an association between birth weight and stress susceptibility in adult males (Nilsson et al. 2001). Nilsson et al. (2001) found that men with lower birth weight and a small head circumference at birth scored poorly on psychological assessment surveys compared to their heavier counterparts. It was suggested that impaired fetal growth was predictive of suboptimal psychological functioning and increased stress susceptibility (Nilsson et al. 2001). Although the mechanisms regulating these associations in human populations are poorly understood, it is apparent that elevated HPA activity later in life and a predisposition to diabetes are linked to alterations in fetal intrauterine growth and development.

1.1.3 Cardiovascular disease and hypertension

In early studies, Barker & Osmond (1986) described associations between infant mortality and the risk of death from cardiovascular disease independent of adult lifestyle, suggesting that poor intrauterine conditions increased the susceptibility of ischemic heart disease. Barker and colleagues explored the link between growth in utero and the onset of cardiovascular disease in adulthood through a series of epidemiological studies based on the birth records of men and women in the UK. Based on death rates of men born between 1911-1930, Barker et al. (1989) found that men whose birth weights were below 5.5 pounds had the highest death rates from ischemic heart disease. This association was still evident at one year of age and it was suggested that the combination of poor prenatal and postnatal growth led to the highest death rates from heart disease in this population (Barker et al. 1989). This relationship was later confirmed in women as well (Osmond et al. 1993), although the evidence is stronger in men than in women (figure 1.1). Similarly, studies found that men who had a small head circumference and/or were thin at birth (as measured by ponderal index; weight/length³) had higher rates of cardiovascular disease than those with a large head circumference or who were fat at birth (Barker et al. 1993). Studies reporting similar associations (Stein et al. 1996, Frankel et al. 1996, Forsen et al. 1997)
Figure 1.1
Standardized mortality ratios for men and women for cardiovascular disease below the age of 65 according to weight. Rates of cardiovascular disease fell with increasing birth weight.
(From, Osmond et al. 1993, British Medical Journal).
provided more evidence that the relationship between poor fetal growth and the onset of adult cardiovascular disease might be independent of genetics, social class or adult lifestyle.

In many studies, raised blood pressure was also associated with a reduction in birthweight (Barker et al. 1990, Barker 1996). Barker et al. (1990) found that systolic and diastolic pressures in men and women aged 46-54 years were strongly related to placental weight in addition to birth weight. The highest blood pressures occurred in people who had been small babies with large placentae, independent of body mass index as adults, suggesting that altered placental and fetal size may lead to circulatory adaptations in the fetus, altered arterial structure in childhood and hypertension in the adult (Barker et al. 1990). Furthermore, a study of four-year-old children found similar associations between birth weight, placental weight and blood pressure (Law et al. 1991).

1.1.4 Fetal programming

Therefore based on the numerous observations that in utero fetal adaptations to an adverse environment are linked to the onset of disease that occurs much later in postnatal life, the fetal programming hypothesis emerged. Programming has been suggested to reflect the ability of a factor, acting during a critical developmental period, to exert organizational effects that persist throughout life (Seckl 1997). Many factors have been suggested to play roles in the fetal programming of postnatal diseases such as the ones previously described. This review of the literature will focus on the development and programming of the HPA axis and metabolic function.

1.2 DEVELOPMENT OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

Glucocorticoids are essential for life and have a wide spectrum of effects including, maintaining glucose production and supporting vascular responsiveness. In addition, glucocorticoid levels rise in response to a threat in homeostatic balance. In mammals, the primary glucocorticoids are cortisol (primates and sheep) and corticosterone (rodents). The major role of the hypothalamic-pituitary-adrenal (HPA) axis is to control the synthesis and release of glucocorticoids from the adrenal cortex and glucocorticoids in turn regulate their own release through the action of a negative feedback system.

Briefly, a stressful stimulus causes the synthesis and release of corticotrophin-releasing hormone (CRH) and/or arginine vasopressin (AVP) from neurosecretory cells of the paraventricular nucleus (PVN) of the hypothalamus into the hypophyseal portal system to target corticotroph cells within the pars distalis (anterior lobe) region of the pituitary gland. Here, CRH
and AVP stimulate the synthesis of a polypeptide precursor pro-opiomelanocortin (POMC), which is then cleaved by processing enzymes (prohormone convertase 1 and 2) to produce adrenocorticotropic hormone (ACTH) in addition to smaller molecular weight peptides (Dallman et al. 1987). ACTH in turn is secreted by the pituitary into the circulation and stimulates the synthesis and release of glucocorticoids from the zona fasciculata of the adrenal cortex (Dallman et al. 1987). Circulating glucocorticoid levels are maintained through the action of a negative feedback system present within the brain (hippocampus and hypothalamus) and pituitary via corticosteroid receptors (Keller-Wood & Dallman 1984) (figure 1.2). It is generally thought that the hippocampus exerts an inhibitory influence on basal, circadian and stress induced HPA activity (Jacobson & Sapolsky 1991).

In several species, normal fetal HPA axis function is essential for growth, development and for the onset of birth (Liggins 1994). Glucocorticoids in general promote tissue and organ maturation at the expense of proliferation, and are therefore responsible for the maturational changes of a variety of organ systems preparing the fetus for extrauterine life (Liggins 1994, Fowden et al. 1998). Most of these changes can be induced prematurely by exogenous glucocorticoid administration (Fowden 1993, Liggins 1994). In all species studied, there is an increase in circulating glucocorticoid concentrations in the fetus towards term, although the timing and the magnitude of this increase may vary (Fowden et al. 1998; figure 1.3). Negative feedback capability of the HPA axis is apparent in the sheep fetus in the last third of gestation (Wintour et al. 1985, Norman & Challis 1985). However, over the last 15 days of gestation, the negative feedback effects of glucocorticoids on HPA function are attenuated, permitting concomitant increases in fetal plasma ACTH and cortisol levels (Challis & Brooks 1989). It is this increase in circulating fetal cortisol concentrations that provides the stimulus for organ maturation and the trigger for parturition in the sheep (Liggins 1994, Challis et al. 2000). This overview will outline the development and function of the fetal HPA axis with emphasis on the fetal sheep where possible.

1.2.1 The hippocampus

The hippocampal formation is made up of four cortical regions that include the dentate gyrus, the hippocampus proper (CA1-CA4), the subicular complex and the entorhinal cortex (Amaral & Witter 1989). The hippocampus plays a major role in learning, memory and the regulation of HPA activity. In rats, removal of the dorsal hippocampus resulted in elevated basal and stress induced adrenocortical responses (Feldman & Conforti 1980), and hippocampal cholinergic
Figure 1.2
Diagram representing the regulation of the hypothalamic-pituitary adrenal axis. CRH, corticotrophin releasing hormone; AVP, arginine vasopressin; ACTH, adrenocorticotrophic hormone; GR, glucocorticoid receptor; MR, mineralocorticoid receptor. Stimulatory (+) and Inhibitory (-) actions.
Figure 1.3
Mean fetal concentrations of plasma cortisol with respect to time period from delivery in the sheep (●), pig (○), man (▲), guinea pig (□) and horse (△), Labour, B, birth.
(From, Fowden et al. 1998, Proceedings of the Nutrition Society).
blockade resulted in enhanced HPA responses to stress (Bhatnager et al. 1997). Conversely, stimulation of the hippocampus decreased basal plasma corticosteroid levels (Mandell et al. 1963, Rubin et al. 1966). The hippocampus therefore exerts an inhibitory influence on basal, circadian and stress induced HPA activity (Jacobson & Sapolsky 1991). In rats, increased hippocampal corticosteroid receptor occupancy was associated with suppressed plasma ACTH levels and a decrease in portal levels of ACTH secretagogues such as AVP and CRH (Sapolsky et al. 1990).

It has been suggested that the hippocampus exerts inhibitory control over HPA axis activity primarily by mediating glucocorticoid feedback. Central corticosteroid receptors in the hippocampus are thought to play a critical role in the regulation of HPA activity (De Kloet et al. 1990, Meijer & De Kloet 1998, De Kloet et al. 1998). Two corticosteroid receptors are present in the hippocampus; type 1, mineralocorticoid receptor (MR), identical to the kidney MR, and type 2, the classic glucocorticoid receptor (GR). MR bind cortisol/corticosterone with an affinity that is 10 fold greater ($K_d \approx 0.5\text{nM}$) than that of GR ($K_d \approx 5.0\text{nM}$) (De Kloet et al. 1998, Bamberger et al. 1996). In most species, the hippocampus exhibits the highest levels of corticosteroid receptors of any brain region (Reul & De Kloet 1985, Jacobson & Sapolsky 1991, De Kloet et al. 1998) and is one of the few regions to express both MR and GR (Reul & De Kloet 1985). The hippocampus therefore represents a potential negative feedback site over a wide range of corticosteroid concentrations (Jacobson & Sapolsky 1991). Due to their high affinity, MR have been shown to be ~ 90% occupied by endogenous corticosteroids under most circumstances and are thought to regulate basal or circadian trough levels of ACTH and cortisol (Reul & De Kloet 1985, Reul et al. 1987, De Kloet & Reul 1987). GR occupancy however varies from a minimum of 10% at basal and circadian trough corticosteroid levels, to a maximum of ~75% with stress or administration of synthetic corticosteroids (Reul & De Kloet 1985). It was therefore proposed that GR mediate the effects of circadian peak or stress induced increases in HPA activity (Reul & De Kloet 1985, Jacobson & Sapolsky 1991). Corticosteroid receptor levels can alter negative feedback at the hippocampus. High corticosteroid levels primarily regulate hippocampal GR expression, since chronic stress or corticosteroid administration reduced GR number but not MR expression (Sapolsky et al. 1984, De Kloet & Reul 1987). Based on these extensive observations, alterations in MR and GR expression could therefore potentially influence basal and stress induced increases in HPA activity.

Most developmental studies describing the ontogeny of hippocampal MR and GR expression have been focused primarily on rats. In the rat, the period of rapid brain growth
occurs after birth, unlike that of the human, sheep or guinea pig (Dobbing & Sands 1979; figure 1.4). From approximately 4 days of postnatal life, the rat HPA system is suppressed, characterized by low plasma corticosterone levels and a general lack of HPA responsiveness to stimuli (Meaney et al. 1985, Sapolsky et al. 1985, 1986). Hippocampal corticosteroid receptors increase gradually after birth, MR expression reaching adult levels by the second week of life and GR expression increasing somewhat later (Meaney et al. 1985, Rosenfeld et al. 1988). Matthews (1998) has described the presence of both GR and MR mRNA in the guinea pig hippocampus by day 40 of gestation (term 65 days). To date there is relatively little information on the development of GR or MR in the fetal sheep hippocampus. One study has reported the presence of GR mRNA and protein in the fetal ovine hippocampus at 120 days of gestation (term 150 days) (Andrews & Matthews 2000), a time in which glucocorticoid negative feedback is emerging in this species (Brooks et al. 1996). GR mRNA levels increased dramatically by 130 days and were significantly reduced in the last 15 days of gestation (Andrews & Matthews 2000). The decrease in GR expression at term may be indicative of a potential decrease in negative feedback at the level of the hippocampus. Although there are no studies to date regarding hippocampal MR expression in the fetal sheep, changes in GR expression may reflect activation or regulation of fetal HPA activity in utero, affecting HPA function in such a way as to sustain the observed increase in glucocorticoid levels near term. Alternatively, these observations could reflect the potential regulation of hippocampal GR expression by high levels of circulating glucocorticoids.

1.2.2 The hypothalamus

The hypothalamus is divided into several nuclei including the paraventricular (PVN) and supraoptic nuclei (SON). The PVN is a highly differentiated nucleus containing discrete regions of neurons that can be classified into three groups; those that project to the posterior pituitary (pars nervosa), those associated with the autonomic nervous system, and those that project to the median eminence and affect anterior pituitary (pars distalis) function (Kupfermann 1991). It is within this nucleus that CRH and AVP neurons are primarily localized in discrete areas, in addition to the supraoptic nucleus (SON) where AVP is localized (Page 1988).

CRH containing neurons are localized primarily to the parvocellular neuroendocrine cells of the PVN. AVP containing neurons are localized to both parvocellular and magnocellular neuroendocrine cells of the PVN as well as magnocellular neurons of the SON (Page 1988). The axons of the parvocellular neurons project through the external lamina of the median eminence
Figure 1.4

Brain growth spurts of 7 mammalian species expressed as a first order velocity curves of the increase in weight with age. The units of time arbitrary and do not reflect specific peak velocity relative to birth. The graph is a representation to give a visual impression of the proportion of brain growth spurt in each case which is prenatal or postnatal, determined by the size of the areas beneath the curve either side of birth.

(Adapted from, Dobbing & Sands 1979, *Early Human Development*).
where neuropeptides are secreted into the hypophyseal portal vessels to ultimately reach the pars distalis and influence the synthesis and secretion of ACTH (Levidiotis et al. 1987, Swanson & Sawchenko 1983). The axons of the magnocellular neurons of both the PVN and the SON project through the internal lamina of the median eminence and nerve terminals reside in the pars nervosa (Swanson & Sawchenko 1983, Everett 1988). Therefore the parvocellular neurons of the PVN are thought to modulate pars distalis function, and magnocellular neurons modulate pars nervosa function and systemic AVP and oxytocin (OT) levels (Swanson et al. 1989). Other important hypothalamic neuropeptides, whose neurons are localized with the hypothalamic PVN, have been shown to regulate hypothalamic control of pituitary ACTH secretion (Kemppainen et al. 1993, Link et al. 1992, Young & Lightman 1992, Brooks & Challis 1988). Met-Enkephalin as well as oxytocin containing neurons are localized within the parvocellular and magnocellular neurons of the PVN (Kupfermann 1991) and have been shown to regulate pituitary function (Brooks & Challis 1988, Kemppainen et al. 1993, Matthews 1999).

CRH and AVP are considered to be primary factors driving ACTH release from the pars distalis corticotroph of the fetal sheep in vivo (Norman & Challis 1987) and in vitro (Durand et al. 1986, Matthews & Challis 1997). Furthermore, hypothalamic PVN lesions in fetal sheep have been shown to prevent the normal gestational rise in circulating ACTH and cortisol levels and decrease the ACTH and cortisol response to hypotensive stress (McDonald et al. 1988, 1991). In the fetal sheep, CRH and AVP mRNA have been detected in the PVN by day 60 of gestation (term 150 days) (Matthews & Challis 1995). CRH mRNA levels increased gradually with gestation with a further increase at term. After birth, CRH mRNA decreased to adult levels by 30-60 days. Similarly, immunoreactive (ir) CRH was identified by 48-50 days of gestation in the fetal sheep hypothalamus, levels increased significantly with advancing gestation and remained elevated to term (145 days) (Watabe et al. 1991, Currie & Brooks 1992, Saoud & Wood 1995). Ir-CRH and CRH bioactivity have also been detected in hypothalamic extracts from human fetuses by 12-13 weeks of gestation (term 37-40 weeks) (Ackland et al. 1986). Therefore, CRH synthesis and secretion in the fetal hypothalamus increases with advancing gestation.

AVP mRNA is present at day 60 of gestation in the fetal sheep PVN but does not change dramatically over the course of gestation. However, AVP mRNA levels in the hypothalamus increased significantly in the postnatal lamb (Matthews & Challis 1995). A similar pattern of expression was observed in the fetal SON. At day 60 of gestation, AVP mRNA was differentially localized within both the parvocellular and magnocellular neurons and this pattern changed to a predominantly magnocellular localization with advancing gestation (Matthews &
Challis 1995). The developmental pattern of AVP mRNA was consistent with increases in levels of ir-AVP found in hypothalamic homogenates (Currie & Brooks 1992, Saoud & Wood 1995). These observations, together with the observation that AVP and CRH containing neurons are present in the median eminence by 42 and 105 days of gestation respectively (Levidiotis et al. 1987) suggest that both CRH and AVP are present and could act on the pars distalis early in gestation.

Oxytocin (OT) has been implicated in the regulation of pars distalis function in adult sheep (Kemppainen et al. 1993) and rats (Link et al. 1992). In fetal sheep, OT mRNA was present in the parvocellular and magnocellular neurons of the PVN and the magnocellular neurons of the SON at day 60 of gestation. In both the PVN and the SON, OT mRNA levels remained low until term. Postnatally, OT mRNA levels increased with age, reaching adult levels by days 30-60 (Matthews 1999). Matthews (1999) reported that OT significantly increased pituitary ACTH output in vitro. Furthermore, OT axons were found to project to the adenohypophysis in term fetal sheep, supporting the possibility that fetal hypothalamic OT might play a role in fetal HPA function and possibly in corticotroph maturation (Hoffman et al. 1989). The developmental pattern of OT mRNA is therefore consistent with its role as an additional hypothalamic releasing factor.

Neuromodulators may also act centrally to influence the secretion of hypothalamic releasing factors and thereby regulate pituitary function (Brooks et al. 1992). In sheep fetuses at 110 days of gestation, infusion of a potent met-enkephalin analogue increased plasma ACTH and cortisol concentrations suggesting that opioid peptides were capable of regulating the fetal HPA axis at this time in gestation (Brooks & Challis 1988). Within the adult sheep hypothalamus, pre-pro-enkephalin (PENK) mRNA was present in the parvocellular neurons of the PVN and was colocalized with CRH mRNA (Matthews et al. 1992). In the sheep fetus, PENK mRNA was present in the parvocellular neurons of the PVN at day 60 of gestation and increased to a peak at 130-140 days, followed by a dramatic decrease at term (Matthews & Challis 1995). Levels then increased in the newborn lamb. The developmental increase in PENK mRNA levels paralleled that of CRH (Matthews & Challis 1995, 1995). These observations suggest that met-enkephalin (the primary opioid in the fetal sheep, Jones et al. 1992), might regulate release of CRH from neurons in a paracrine fashion (Matthews & Challis 1995). Studies in rats have shown that met-enkephalin inhibits the release of CRH at the median eminence (Yajima et al. 1986), therefore it is possible that a decrease in met-enkephalin synthesis might facilitate CRH secretion at term (Matthews & Challis 1995).
1.2.3 Hypothalamic-hypophyseal portal system
The hypothalamic-hypophyseal portal system represents a series of capillary networks that link the basal aspect of the hypothalamus to the *pars distalis* of the pituitary. The carotid arteries branch into the superior and inferior hypophyseal arteries and supply the portal plexus (Page 1988) (figure 1.5). An intact portal system from the hypothalamus to the pituitary is established as early as 45 days of gestation in the fetal sheep (Levidiotis et al. 1989) and 11.5-16.9 weeks of gestation (term 37-40 weeks) in the human (Thliveris & Currie 1980). The early appearance of this hypothalamic-pituitary connection suggests that trophic drive to the pituitary could potentially begin early in gestation.

1.2.4 The pituitary gland
The pituitary gland (hypophysis) is a small endocrine gland situated within the sella turcica at the base of the brain. It is comprised of two morphologically different regions. The neurohypophysis (posterior lobe or *pars nervosa*) is of neural origin, resulting from the downward outpouching of the ectoderm from the brain in the floor of the third ventricle, and represents a collection of axons whose cell bodies lie in the hypothalamus. Peptide hormones that are synthesized in these neurons travel down their axons and are stored in the nerve terminals within the *pars nervosa*. The adenohypophysis can be divided into the anterior lobe (*pars distalis*), the intermediate lobe (*pars intermedia*) and the stalk (*pars tuberalis*). The *pars distalis* develops from an upward outpouching of the ectoderm from the roof of the oral cavity (Rathke’s pouch) that pinches off and becomes separated from the oral cavity by the sphenoid bone of the skull. The lumen of the pouch is reduced to a small cleft, which in some animals such as sheep, but not humans, demarcates the interwoven *pars distalis* with the *pars nervosa* and cells adjacent to the *pars nervosa* form the *pars intermedia* (Berne & Levy 1993) (figure 1.6).

1.2.4.1 Pars Distalis
The *pars distalis* of the pituitary contains at least 5 different types of secretory cells, 3-10% of which are estimated to represent corticotroph cells (Page 1988, Ganong 1999). It is these cells that synthesize and process the polypeptide precursor POMC into ACTH (Ganong 1999).

The developmental maturation of fetal pituitary corticotrophs has been well documented in the fetal sheep. Corticotroph cells that stain positively for ACTH have been described in the fetal sheep as early as 38 days of gestation and by 90 days of gestation three distinct types of
Figure 1.5
The median eminence and is perfused with capillaries that transport hypothalamic releasing hormones to the pars distalis vasculature. CRH and AVP containing neurons are localized in the PVN. The axons of the parvocellular neurons project through the external lamina of the median eminence where neuropeptides are secreted into the hypophyseal portal vessels to ultimately reach the pars distalis and influence the synthesis and secretion of ACTH as well as other important peptides. The axons of the magnocellular neurons of both the PVN and the SON project through the internal lamina of the median eminence, where nerve terminals reside in the pars nervosa.
(Adapted from, Kupfermann 1991, Principles of Neural Science).
Figure 1.6
Diagram of the pituitary gland showing its division into the *pars distalis* (anterior lobe), *pars intermedia* (intermediate lobe) and *pars nervosa* (posterior lobe).
(Adapted from, Berne & Levy 1993, *Physiology*).
corticotrophs have been identified; "fetal", "intermediate" and "adult" types (Perry et al. 1985, Mulvogue et al. 1986). Fetal corticotrophs have been described as large columnar cells that stain weakly for ACTH and are present only during fetal life. At 90 days of gestation, fetal corticotrophs are many in number but then gradually decline to 130 days of gestation. By term, these cells are almost non-existent (Perry et al. 1985, Mulvogue et al. 1986). The intermediate corticotroph cells stain positively for ACTH, fall into neither the fetal nor the adult category, and are thought to represent a transitional cell type (Perry et al. 1985). Adult corticotrophs are small stellate cells that stain strongly for ACTH and resemble the corticotrophs found in adult pituitaries. The number of adult corticotrophs increases with advancing gestation, suggesting that a cellular maturation from fetal to adult cell type exists in the fetal sheep pituitary pars distalis (Perry et al. 1985). In the human fetus, ACTH containing cells have been identified as early as 7 weeks of gestation and increased with advancing gestation (Mulchabey et al. 1987). Therefore, in both the sheep and the human, the components for HPA function are present in the pars distalis early in gestation.

This anatomical maturation of corticotrophs within the pars distalis of fetal sheep parallels a change in corticotroph function. Ir-ACTH levels increase with advancing gestation in both fetal plasma and in the pars distalis (McMillen et al. 1995, Perry et al. 1985, Norman et al. 1985). Furthermore, corticotroph maturation appears to be regulated by the fetal hypothalamus and adrenal (McDonald et al. 1992, Brieu & Durand 1987). Hypothalamic PVN lesions in fetal sheep delayed fetal corticotroph maturation into adult type cells (McDonald et al. 1992). Fetal adrenalectomy at 120 days of gestation resulted in a delay in the maturation of corticotrophs but this effect was reversed with cortisol infusion (Antolovich et al. 1989). Taken together, these observations suggest that both hypothalamic releasing factors and cortisol are necessary for the maturation of fetal corticotrophs.

1.2.4.2 Pars Intermedia

The pars intermedia is a compact component of the adenohypophysis located between the pars distalis and the neurohypophysis and is separated from the pars distalis by the occluded Rathke’s pouch (see 1.2.4). This separation from the pars distalis is visible by 40 days of gestation in the fetal sheep (Perry et al. 1982). The pars intermedia contains secretory cells typically named melanotroph cells and by 100 days of gestation it is cytologically different from the pars distalis (Perry et al. 1982). Secretory cells of the pars intermedia synthesize and secrete primarily α-melanocyte stimulating hormone (αMSH), made up of amino acids 1-13 of the ACTH peptide.
(Lundblad & Roberts 1988). Consequently, in the adult sheep the *pars intermedia* stains positively for ir-αMSH and only weakly for ACTH (Perry et al. 1985). In the fetus however, the *pars intermedia* stains positively for both ACTH and αMSH throughout gestation (Perry et al. 1985). The *pars intermedia* is well developed in the fetus, but diminishes in humans with gestation and is absent in the human adult (Mulchahey et al. 1987). In lower mammals the *pars intermedia* remains distinctive (Mulchahey et al. 1987). The major secretory products of the *pars intermedia* are αMSH, corticotrophin like intermediate peptide (CLIP), and β endorphin (Smith & Funder 1988).

Cells staining for ir-ACTH are present in the *pars intermedia* of fetal sheep by 60 days of gestation (Mulvogue et al. 1986), and have been shown to maintain a basal level of ACTH secretion throughout gestation (Fora et al. 1996). Furthermore, it has been shown that cultured *pars intermedia* cells respond to CRH and AVP stimulation *in vitro* with increased ir-ACTH output (Fora et al. 1996). Evidence that the *pars intermedia* is under tonic dopaminergic inhibitory control comes from studies in fetal sheep showing marked hypertrophy in the *pars intermedia* after hypophysectomy (Antolovich et al. 1991). In addition, infusion of the dopamine receptor 2 (D2) agonist, bromocriptine, significantly reduced POMC mRNA levels in the *pars intermedia* and decrease fetal plasma αMSH concentrations (Matthews et al. 1996, Hagan & Brooks 1996). Intravenous infusion of the dopamine antagonist sulpiride significantly increased fetal plasma αMSH levels, which was not associated with elevations in cortisol concentrations (Hagan & Brooks 1996).

1.2.4.3 Pro-opiomelanocortin and prohormone convertase enzymes

Pro-opiomelanocortin (POMC) is a large polypeptide precursor molecule cleaved by processing enzymes to yield a variety of smaller peptide hormones (Dallman et al. 1987). Although localized to many tissues (Jacobson & Drouin 1994), POMC is primarily localized to the *pars distalis* and *pars intermedia* of the pituitary (Lundbald & Roberts 1988) and cleaved to produce ACTH and αMSH respectively.

The POMC gene is comprised of approximately 1100 base pairs including 2 introns and 3 exons (figure 1.7). The regions encoding the N-terminal peptide, ACTH₁₋₃₉ and βendorphin are >95% homologous in mammals (Drouin et al. 1994, Jacobson & Drouin 1994). The POMC promoter has at least 10 regulatory elements that have been identified, including cAMP and glucocorticoid regulatory elements (GRE) that mediate transcription (increased POMC
Figure 1.7

Representation of the structure of the POMC gene and the posttranslational processing of POMC by prohormone convertase enzymes. POMC is cleaved by PC1 (primarily in the pars distalis) to yield N-terminal peptide, joining peptide (JP) adrenocorticotropic hormone (ACTH) and β lipotrophin (β LPH). These may then be cleaved further by PC2 (localized in the pars intermedia) to yield large molecular weight POMC (POMC 1-49), γ melanocyte stimulating hormone (γ MSH), α melanocyte stimulating hormone (α MSH), corticotropin-like intermediate peptide (CLIP), γ lipotrophin (γ LPH) and β endorphin. Lys-arg, arg-arg, arg-lys are dibasic amino acids that are the sites of prohormone cleavage.

transcription through cAMP and decreased transcription through glucocorticoids) (Therrien & Drouin 1995, Jacobson & Drouin 1994).

POMC is processed by 2 members of a family of prohormone convertase enzymes that cleave peptides at basic amino acid residues (Loh et al. 1985, Beinfeld 1998). Prohormone convertase 1 and 2 (PC1 and PC2) are found almost exclusively in endocrine and neuroendocrine cells, including pituitary corticotrophs and melanotrophs (Seidah et al. 1991, Beinfeld et al. 1998). In adult mice and rats, PC1 mRNA is expressed in all melanotrophs of the pars intermedia and ~20% of corticotrophs in the pars distalis. PC2 mRNA is also expressed in melanotrophs but is not expressed in corticotrophs (Seidah et al. 1991, Bloomquist et al. 1991). This pattern of localization corresponds to the posttranslational processing of POMC to 16 KDa fragment N-terminal peptide, ACTH1-39 and ßendorphin in the pars distalis by PC1 and into smaller peptides (aMSH, CLIP) in the pars intermedia by PC2 (Loh et al. 1985, Seidah et al. 1992) (figure 1.7).

In the fetal sheep, POMC mRNA levels have been shown to be present by day 60 of gestation in both the pars distalis and the pars intermedia; with levels in the pars intermedia some 5 fold higher than in the pars distalis (Matthews & Challis 1994). POMC mRNA levels increase in the pars intermedia from day 60 to day 100 of gestation, remain elevated to term and are maintained through neonatal life (Matthews & Challis 1994). In the pars distalis POMC mRNA levels increased with advancing gestation to peak at term (140-145 days) and levels remained high in neonatal and adult life (Yang et al. 1991, Myers et al. 1993, Matthews & Challis 1994, Holloway et al. 2000). Furthermore, POMC mRNA becomes differentially distributed within the pars distalis late in gestation, such that higher levels of expression were observed in the inferior region (basal aspect) of the gland and much lower levels were observed in the superior region immediately adjacent to the pars intermedia (Matthews & Challis 1994). Matthews & Challis (1994) reported that POMC mRNA levels remained unchanged in the superior region of the gland but increased significantly in the inferior region with advancing gestation. This increased pattern of expression in POMC mRNA corresponds to an increase in the proportion of cells stained for ir-ACTH in the pars distalis and increases in circulating plasma ACTH levels with advancing gestation in the fetal sheep (Norman et al. 1985, Matthews & Challis 1994).

Recent studies have shown that levels of expression for the POMC processing enzymes also change with advancing gestation. PC1 mRNA was distributed heterogeneous in the pars distalis of fetal sheep, and PC2 mRNA restricted primarily to the pars intermedia. Furthermore,
PC1 was colocalized with cells staining for POMC in the pars distalis, supporting the role of PC1 in POMC processing in the fetal sheep (Bell et al. 1998). The proportion of POMC cells containing PC1 mRNA in the pars distalis was significantly higher in 100-107 and 144-147 day fetuses than in 117-121 and 126-130 days fetuses. Levels of PC1 and PC2 mRNA in the pars intermedia were not changed over the course of gestation (Bell et al. 1998). Holloway et al. (2000) reported that in term fetal sheep, levels of PC1 and PC2 mRNA were higher in the pars intermedia than the pars distalis and that PC1 mRNA levels increased with gestational age. Therefore, a rise in both the expression of POMC and its processing enzymes may contribute to the increase in pars distalis ir-ACTH content and plasma ACTH seen later in gestation in fetal sheep.

1.2.4.4 Development of pituitary responsiveness and activation of pituitary function

Fetal plasma ir-ACTH has been detected in the sheep by 59 days of gestation and concentrations gradually increase over the last 25 days of gestation to peak levels at term (Norman et al. 1985, McMillen et al. 1995). Furthermore, Brieu & Durand (1987) have shown that the ratio of bioactive to ir-ACTH secretion increased with gestational age and was enhanced in cultured pars distalis corticotrophs at days 120-126 of gestation by either AVP or CRH treatment.

It is well established that CRH and AVP are potent stimulators of ACTH synthesis and secretion. CRH stimulates ACTH secretion from fetal corticotrophs in vivo (Pradier et al. 1985, Norman et al. 1985, Norman & Challis 1987, Brooks & Challis 1989) and in vitro (Durand et al. 1986, Lu & Challis 1994, Matthews & Challis 1997). In fetal sheep an intravenous (iv) bolus of 1μg of exogenous CRH given to fetuses at days 110-115 of gestation resulted in an increase in ACTH secretion that was not associated with any change in cortisol concentrations. By 125-130 days, the same amount of CRH produced a significantly higher ACTH response and a modest increase in cortisol. At 135-140 days, the ACTH response was reduced and the cortisol response was greater than that observed at 125-130 days (Norman et al. 1985, Norman & Challis 1987). These data suggest that maturation of the fetal pituitary preceded that of the adrenal. Similarly, CRH treatment in cultured fetal pars distalis corticotrophs resulted in significant dose dependant increases in ACTH output (Durand et al. 1986, Fora et al. 1996, Matthews & Challis 1997). CRH stimulation of ACTH output has also been shown in the adult sheep, resulting in significant increases in ACTH and cortisol output both in vivo (Donald et al. 1983) and in vitro (Kemppaninen et al. 1993).
Previous studies have shown that AVP can also stimulate ACTH secretion from the fetal sheep pituitary in vivo and vitro and may synergize with CRH to regulate ACTH output (Norman & Challis 1987, Durand et al. 1986, Brooks & White 1990, Fora et al. 1996, Matthews & Challis 1997). Infusion of AVP to fetal sheep resulted in significant increases in ACTH at 110-115 and 125-130 days of gestation but not at days 135-140, suggesting a decrease in the effect of AVP (Norman & Challis 1987). On days 110-115, a bolus of AVP+CRH produced a synergistic response in ACTH output but the role of AVP in stimulating ACTH release decreased with progressive gestational age (Norman & Challis 1987). This synergistic effect on pituitary ACTH secretion has also been shown in vitro (Durand et al. 1986, Matthews & Challis 1997). Simultaneous treatment of cultured fetal sheep corticotrophs with CRH and AVP resulted in greater elevations in ACTH output than when either peptide was given alone at 63-64, 115-116 and 123-125 days of gestation, but not at 133 and 144 days of gestation. This synergistic response in ACTH output then increased in young lambs (Durand et al. 1986). It has been shown that fetal pituitary corticotrophs respond to CRH and AVP by increasing POMC mRNA and that CRH is more potent than AVP in stimulating POMC transcription in cultured fetal sheep corticotrophs (Matthews & Challis 1997). Together these observations suggest that the fetal pituitary is sensitive to AVP and CRH stimulation early in gestation, however, this responsiveness eventually declines as gestation progresses. This synergistic effect has also been reported in adult sheep (Kemppainen et al. 1993).

The mechanism for the altered sensitivity for fetal corticotrophs to CRH and AVP has not yet been determined. However, it is possible that a change in responsiveness may be due to receptor levels. CRH and AVP act via independent receptors that are associated with different second messenger pathways. CRH acts through the CRH receptor type 1 (CRH-R1), and mediates ACTH synthesis and release through the activation of adenylyl cyclase to increase cyclic AMP (cAMP) dependant protein kinases. AVP acts through the V3 receptor stimulating phosphatidylinositol metabolism and increased intracellular calcium concentrations. The number of CRH-R binding sites in the pars distalis corticotrophs of fetal sheep has been shown to increase from 65-70 days, reaching peak values at 125-130 days and decline towards term (Lu et al. 1991, Green et al. 2000). It has been suggested that alterations in CRH receptor number might contribute to changes in responsiveness of the fetal sheep pars distalis to CRH during gestation (Lu et al. 1991, Green et al. 2000). Furthermore, CRH-R mRNA expression appears to be decreased by cortisol in fetal sheep (Green et al. 2000) and adult rats (Zhou et al. 1996). Although limited data is available concerning AVP binding in fetal sheep pituitaries, adult sheep
show much higher levels of AVP receptors than CRH receptors in the pars distalis and these receptors are reduced in the hypophysectomized sheep (Shen et al. 1990). It is possible that a change in pituitary responsiveness may be regulated by alterations in CRH and AVP binding sites and that this in turn may be regulated by the normal prepartum rise in endogenous glucocorticoid concentrations.

Another possible mechanism for altered sensitivity to CRH and AVP is a change in the functional heterogeneity of corticotroph cell types in the fetal sheep pars distalis (Perez et al. 1997, Butler et al. 1999). In vitro studies suggest the presence of corticotroph cells that respond to CRH only, AVP only or to either CRH or AVP (Schwartz 1990, Butler et al. 1999). Moreover, Perez et al. (1997) found that the number of cells that secrete ACTH and the amount of ACTH released by individual cells changes during fetal development and into adulthood. Therefore, maturation of the response of corticotrophs to CRH or AVP may depend, in part, on changes among the population of ACTH secreting cells that occurs as a function of gestational age (Perez et al. 1997). Taken together these observations suggest that developmental changes in corticotroph responses to CRH and AVP may be mediated by a change in the functional heterogeneity of corticotrophic cell types that are primarily either CRH or AVP responsive, in addition to alterations in the expression of CRH and AVP receptors with gestation.

Placental derived prostaglandin E₂ (PGE₂) has also been shown to play a role in the activation of fetal HPA function. Fetal plasma PGE₂ concentrations rise progressively late in gestation with a time course that is similar to that seen in fetal plasma cortisol (Challis et al. 1976). Infusion of PGE₂ into catheterized fetal sheep resulted in a significant elevation in circulating ACTH and cortisol concentrations (Louis et al. 1976, Young et al. 1996), furthermore, it has been shown that PGE₂ infusion into hypophysectomized fetuses was not associated in any change in either ACTH or cortisol concentrations suggesting that prostaglandins may act via the hypothalamus to stimulate ACTH secretion (Young et al. 1996).

In the fetal sheep, the melanotrophs of the pars intermedia are ACTH immunopositive early in gestation (Mulvogue et al. 1986) and contain some 5 fold higher levels of POMC mRNA than the pars distalis (Matthews & Challis 1994). Fetal melanotrophs secrete ACTH under basal conditions in vitro and respond to CRH and AVP stimulation (Fora et al. 1996). Furthermore, there appears to be a similar pattern of development in melanotroph ACTH secretion as observed in the pars distalis corticotroph. Cultured cells from sheep fetuses at 138-145 days of gestation respond to CRH and CRH+AVP with greater ACTH output than fetuses at 100-115 days of gestation (Fora et al. 1996), suggesting that the pars intermedia may play a role in the activation
of the fetal HPA axis. In addition, αMSH has been implicated as a trophic factor for the fetal adrenal gland and has been shown to stimulate cortisol secretion in catheterized lambs as early as 122-130 days of gestation (Llanos et al. 1979). High basal plasma levels of αMSH have been observed in fetuses at 116-138 days of gestation (Newman et al. 1987). It has been suggested that the pars intermedia may be a major source of large molecular weight POMC-derived peptides (Saphier et al. 1993) and that these peptides may inhibit ACTH induced synthesis and release of cortisol from the adrenal cortex (Schwartz et al. 1995). Therefore, although the fetal sheep pars intermedia may be source of ACTH and αMSH throughout gestation, its role in HPA activation and function requires further investigation.

1.2.5 The adrenal cortex

The primate adult adrenal cortex is divided into three functional zones; the zona glomerulosa that primarily synthesizes and secretes mineralocorticoids; the zona fasciculata that primarily synthesizes and secretes glucocorticoids; and the zona reticularis that synthesizes and secretes androgens. For most of gestation the primate fetal adrenal cortex is divided into three morphologically distinct zones from outer most to innermost; the definitive or adult zone, the transitional zone and the fetal zone. Rapid growth of the adrenal begins at ~10 weeks of gestation and continues to term. Growth is almost entirely due to the development of the fetal zone of the gland. The primate adrenal, unlike the fetal sheep, primarily secretes androgens, specifically dehydroepiandrostendione (DHEA) due to the low expression of 3βHSD (3β-hydroxysteroid dehydrogenase) in the fetal zone (figure 1.8). Therefore, steroid conversion occurs via the Δ5 steroidogenic pathway. In primates, the placenta lacks the enzyme P450c17 (17-hydroxylase, 17,20 desmolase) and therefore is dependant upon the production of DHEA from the fetal adrenal as the substrate for the synthesis of estrogens. (Mesiano & Jaffe 1997).

In the fetal sheep, the adrenal gland is present by 28 days of gestation (Wintour et al. 1975) and two distinct zones within the cortex are observed by day 60 (Webb 1980). Maturation of these zones begins later in gestation and although the outer zone resembles a mature zona glomerulosa and the inner zone resembles the zona fasciculata, the zona reticularis does not develop until postnatal life (Webb 1980, Robinson et al. 1979). The size and weight of the fetal sheep adrenal increases throughout gestation beginning at day 53-130 and then increases dramatically over the last 15-20 days of gestation (Boshier et al. 1989). This increase in growth late in gestation has been shown to be mainly due to an increase in the zona fasciculata (Boshier & Holloway 1991). By determining the relative changes in total steroidogenic cell volume and
Figure 1.8
Outline of hormone synthesis in the zona fasciculata and zona reticularis of the adrenal cortex. The enzymes for the reactions are shown on the left and at the top of the chart. Cholesterol desmolase, P450SCC; 17α-Hydroxylase, P450C17; 3β-Hydroxysteroid dehydrogenase, 3βHSD; 21β-Hydroxylase, P450C21; 11β-Hydroxylase, P450C11.
(From, Ganong 1999, Review of Medical Physiology).
number, Boshier et al. (1989) reported that the zona fasciculata grows in 3 phases; the first phase at 53-100 days; the second phase from day 100-130; and a third phase between 130 days of gestation and 2 days postpartum (Boshier et al. 1989). Furthermore, it has been shown in vivo that ACTH is a potent stimulus for the growth of the adrenal cortex (Liggins 1969, Robinson et al. 1983, Boshier & Holloway 1991), and fetal pituitary ablation results in adrenal cortical hypoplasia (Liggins & Kennedy 1968). These observations therefore highlight the important role of ACTH in adrenal growth and maturation. Moreover, ACTH treatment results in the precocious activation of fetal adrenocortical function (Challis et al. 1982, 1985, Manchester et al. 1983). Dispersed fetal cortical cells collected from animals that were treated with ACTH, secreted significantly more cortisol than saline infused animals (Challis et al. 1982).

Fetal adrenal responsiveness to ACTH changes over the course of gestation. Glickman & Challis (1980) showed that basal cortisol output by cultured fetal sheep adrenal cells was significantly greater at day 50 than at day 100 or 130 days, but not different from term adrenal tissue. In addition, adrenal responsiveness to ACTH stimulation followed a similar profile, in that adrenal cells responded to exogenous ACTH with elevated cortisol output early in gestation (50-60 days) followed by a loss in responsiveness at midgestation (90-125 days) and a re-emergence of responsiveness at term (Wintour et al. 1975, Glickman & Challis 1980). Altered adrenal responsiveness has been attributed to an increase in ACTH receptor number (Durand et al. 1980), enhanced sensitivity to ACTH via increased adenylyl cyclase activity, increased cAMP levels (Durand et al. 1981), or enhanced steroidogenic enzyme expression and activity (Durand et al. 1982, Challis et al. 1986).

1.2.5.1 ACTH receptors

Among the five melanocortin receptors (MC1-R to MC5-R), ACTH binds to only two of them with high affinity (MC2-R, MC5-R). MC2-R is known as the classical ACTH receptor (ACTH-R) of the adrenal cortex (Liakos et al. 1998). Once bound to its receptor, ACTH stimulates adenylate cyclase activity via Gs proteins resulting in an accumulation in cAMP, stimulation of cAMP dependant kinases and enhanced steroidogenesis (Reperant & Durand 1997). In fetal sheep, both ACTH-R mRNA levels and receptor number increase significantly between 123 days of gestation and term (Durand 1979, Fraser et al. 2001). This increase in receptor number is correlated directly with an increase in circulating cortisol levels (Durand et al. 1979). The prepartum increase in plasma ACTH and cortisol in the fetal sheep over the last 20 days of gestation (Norman et al. 1985) is consistent with a role for either ACTH or cortisol in the
regulation of adrenal ACTH receptor expression. *In vivo* (Durand et al. 1981) and *in vitro* (Picard-Hagan et al. 1997) studies have shown that ACTH upregulates ACTH receptor mRNA expression and receptor number and these changes have been correlated to an increase in cAMP production by cultured adrenal cells (Durand et al. 1981, Lye & Challis 1984). Furthermore, fetal sheep infused with ACTH for 5 days at 115 days of gestation, showed increased adrenal P450C17 and 3βHSD activity (Durand et al. 1982), supporting the role that ACTH plays in the stimulation of adrenal cortex steroidogenic enzyme expression. It has also been shown that treatment of cultured adrenal cells with glucocorticoids increases ACTH receptor mRNA levels and treatment with the antiglucocorticoid RU38486 blocked this effect (Picard-Hagan et al. 1997). The existence of glucocorticoid receptors in the fetal ovine adrenal cortex suggests a potential autocrine/paracrine role for cortisol to influence ACTH induced activation of adrenal function (Yang et al. 1989). However, *in vivo* studies have shown that exogenous glucocorticoids decrease ACTH receptor expression in the primate, with associated reductions in adrenal expression of fetal adrenal steroidogenic enzymes (Leavitt et al. 1997, Aberdeen et al. 1998). Although some data are conflicting, most evidence suggests that the upregulation of ACTH receptor number and activity, as well as increases in steroidogenic enzyme activity, by ACTH, or cortisol, in the fetal sheep may be an important event late in gestation, increasing adrenal responsiveness at the time of labour.

1.2.5.2 Steroidogenic enzymes

Tangalakis et al. (1989) have shown that high levels of expression of cholesterol desmolase (P450scс) and P450C17 are present in immature adrenal cortical tissue as early as 40-60 days of gestation and at 120 days to term, but to a very minor degree at 90-120 days. Once complete zonation occurred in the cortex, P450C17 expression was confined to the zona fasciculata. Expression of 21β-hydroxylase (P450C21) showed a gradual increase throughout gestation (Tangalakis et al. 1989). Positive 3βHSD immunostaining was observed in the zona fasciculata at 78-120 days of gestation, a period of development that is characterized by a decrease in adrenal cortisol output (Wintour et al. 1975, Glickman & Challis 1980) and by low expression levels of P450scс and P450C17 (Tangalakis et al. 1989). Therefore, the previously demonstrated triphasic responsiveness of the fetal adrenal appears to be dependant upon the expression levels of P450scс and P450C17 enzymes, rather than 3βHSD. After 125 days of gestation, 3βHSD expression was distributed principally in the zona fasciculata. The expression of the enzyme catalyzing the final step in glucocorticoid synthesis, 11β-hydroxylase (P450C11) increased
between 130-135 and 136-140 days of gestation and remained high after 141 days of gestation (Phillips et al. 1996).

Therefore late in gestation in the fetal sheep, the expression of a number of steroidogenic enzymes increases in a manner that parallels the increase in fetal plasma ACTH and cortisol concentrations. Tangalakis et al. (1994) found that infusion of the synthetic glucocorticoid, dexamethasone, at 60-70 days of gestation led to a decrease in both maternal and fetal ACTH levels and significantly lower levels of mRNA encoding P450SCC and P450C17 enzymes in the fetal adrenal. These observations support the role of ACTH as a major regulator of steroidogenic enzyme expression at that stage in gestation. Furthermore, hypophysectomy of fetal sheep at 107 and 119 days resulted in significant reductions in levels of mRNA encoding P450SCC, 3βHSD and P450C17 enzymes at term. This effect was overcome with infusion of ACTH (Simmonds et al. 2001). In addition, bilateral lesion of the hypothalamic PVN in the fetal sheep resulted in significant reductions in mRNA levels of P450SCC and P450C17 in the adrenal cortex, although had no effect on the expression of 3βHSD, P450C21 or P450C11 (Myers et al. 1992). These observations clearly demonstrate that pituitary ACTH positively regulates the expression of adrenal steroidogenic enzymes, although this regulation is enzyme specific.

1.2.6 Development of glucocorticoid negative feedback

Adrenalectomy in fetal sheep at 116-122 days of gestation resulted in elevations in fetal plasma ACTH levels at 122 days (Wintour et al. 1980) and significantly elevated POMC (McMillen et al. 1990) and CRH mRNA levels at 134 days of gestation (Myers et al. 1991). These observations suggest that negative feedback in the fetal sheep is developed by 116-122 days of gestation. A concomitant rise in fetal plasma ACTH and cortisol concentrations at term however, (Norman & Chailis 1985) suggests that the efficacy of negative feedback in this species may be attenuated. This attenuation in negative feedback capacity may therefore facilitate cortisol maturation of organ systems necessary for extrauterine survival and has been shown to be the trigger in events at the onset of labour (Liggins 1994).

The presence of glucocorticoid receptors (GR) at all levels of the HPA axis provides one of the mechanisms to facilitate negative feedback function. Glucocorticoids, because of their lipophilic nature, diffuse through the plasma membrane and bind to inactive cytoplasmic GR that are bound to chaperone proteins (heat shock protein 90 (hsp90), 70 (hsp70) and 56 (hsp56)) (Pratt 1993). Ligand binding triggers the release of hsp9 from the complex thereby activating the glucocorticoid-GR complex. Translocation of the complex to the nucleus and homo-dimerization
results in the binding of the complex to glucocorticoid regulatory elements (GRE) in the promoter region of glucocorticoid responsive genes, affecting transcription. Alternatively, the activated GR complex can interact with other transcription factors (AP-1, NFκB) to influence transcription (Bamberger et al. 1996). In some promoters, binding of the activated complex to negative GRE (nGRE) causes inhibition rather than enhancement of transcription (Bamberger et al. 1996). This type of regulatory element was first identified in the POMC promoter (Drouin et al. 1993). In the human, alternative splicing of the GR primary transcript has been shown to result in a second isoform, GRβ, which does not bind cortisol (Oakley et al. 1997). GRβ has been shown to be present in a variety of tissues at varying levels of expression and is proposed to act as a ligand independent negative regulator of glucocorticoid action (Oakley et al. 1997). This isoform however, has not been identified in the sheep.

The relative importance of GR in fetal growth and development has been examined using transgenic mice. GR knockout in the mouse results in neonatal death due to abnormal lung development in addition to impaired liver gluconeogenic enzyme activity, alterations in cortisol feedback in the form of elevated ACTH and cortisol concentrations and hypertrophy and hyperplasia of the adrenal cortex (Berger et al. 1996).

1.2.6.1 Hippocampus and Hypothalamus
As discussed in 1.2.1, hippocampal inhibitory regulation of the HPA axis function is through the action of glucocorticoid negative feedback. GR, are thought to mediate glucocorticoid negative feedback due to their low affinity for cortisol compared with that of MR (Jacobson & Sapolsky 1991). Adrenalectomized rats were infused with different concentrations of glucocorticoids to evaluate the relationship between extent of hippocampal receptor occupancy and CRH and AVP levels in the hypophyseal portal system (Sapolsky et al. 1990). Increased portal levels of CRH and AVP were associated with suppressed hippocampal GR. Furthermore, human males treated with RU38486 (non-specific GR antagonist) had elevated levels of morning basal ACTH and cortisol levels (Cooney & Dinan 1996), supporting the hypothesis that glucocorticoid binding at the level of the hippocampus results in a decrease in HPA activity. Few studies have evaluated the ontogeny of glucocorticoid negative feedback in the fetal sheep hippocampus. Andrews & Matthews (2000) demonstrated that GR mRNA levels in fetal sheep hippocampus, hypothalamus and cortex reached high levels at mid gestation (110-130 days) and decreased at term. These observations are consistent with the emergence of negative feedback at this time in gestation.
(Wintour et al. 1980). Furthermore, a decrease in GR mRNA levels at term would be consistent with a decrease in negative feedback sensitivity at term in this species.

In the hypothalamus, GR number and mRNA levels were highest at ~100 days of gestation in the fetal sheep and decreased progressively until term (Yang et al. 1990, Andrews & Matthews 2000). The decrease in hypothalamic GR at 125 days of gestation may reflect an emerging response to the beginnings of the prepartum cortisol rise in fetal plasma (Yang et al. 1990). Indeed, since ACTH infusion at 125 days of gestation resulted in decreased hypothalamic GR number, it is likely that the effect of ACTH was indirect and mediated via enhanced cortisol secretion (Yang et al. 1990). Glucocorticoid negative feedback at the level of the hypothalamus is evident between 118-120 days of gestation. Adrenalectomized fetal sheep demonstrate significantly greater CRH mRNA levels in the PVN compared with controls, suggesting that adrenalectomized animals exhibit reduced negative feedback (Myers et al. 1991). However, Matthews & Challis (1995) demonstrated that intrafetal cortisol infusion in the fetal sheep did not alter basal levels of CRH mRNA at 125-129 days of gestation, but did significantly attenuate hypoxic stress induced increases in CRH mRNA. These observations suggest that cortisol infusion in these animals had differential effects on basal and stimulated levels of CRH mRNA (Matthews & Challis 1995). This is similar to effects seen on basal and stress induced ACTH secretion (see 1.2.7.2) (Matthews & Challis 1997).

1.2.6.2 Pituitary

In the fetal sheep, GR mRNA and protein are present in the pars distalis by 60 days of gestation and levels increased dramatically at term (Matthews & Challis 1995). Although GR mRNA was uniformly distributed throughout the pars distalis early in gestation, late in gestation, GR mRNA levels were significantly higher in the inferior region of the pars distalis compared with the superior region (Matthews & Challis 1995). This pattern was consistent with the differential distribution of POMC mRNA levels within the pars distalis of fetal sheep late in gestation (Matthews & Challis 1994). These observations are similar to those of others demonstrating that GR binding was highest at 110 days of gestation, decreased by day 125-130 and then dramatically increased between 130 days and term (Yang et al. 1990, Holloway et al. 2000). Intrafetal cortisol infusion for 12 hours at 135 days of gestation did not affect GR mRNA levels (Matthews & Challis 1995). In contrast, intrafetal cortisol infusion to the fetal sheep at 125-126 days of gestation for 80-96 hours resulted in significant increases in pituitary GR mRNA expression (Holloway et al. 2000), suggesting that cortisol may alter expression of GR in the
fetal pituitary but the effect appears to be gestational age dependent. The rise in GR at term may be a mechanism allowing efficient cortisol regulation of fetal HPA activity during the stress of birth and the first few hours of postnatal life (Matthews & Challis 1995). Holloway et al. (2000) found that GR mRNA levels significantly decreased with the onset of spontaneous labour itself, suggesting that during the process of parturition, a decrease in the negative feedback efficiency of the axis may facilitate high levels of circulating cortisol.

Basal and stimulated ACTH release from fetal sheep corticotrophs is reduced by glucocorticoid treatment in vitro and these effects were observed as early as 63 days of gestation (Durand et al. 1986). Norman et al. (1987) demonstrated that intrafetal infusion of synthetic glucocorticoid (dexamethasone) at 113-116 days of gestation had no effect on basal ACTH and cortisol levels but significantly inhibited the ACTH and cortisol responses to CRH, AVP and CRH+AVP. Dexamethasone treatment at 126-130 days of gestation significantly reduced basal and stimulated in vivo ACTH levels and at 136-140 days treatment resulted in only a small reduction in basal ACTH and a greater reduction in cortisol. At this time in gestation, dexamethasone treatment did not significantly alter stimulated ACTH and cortisol responses (Norman et al. 1987). These observations support the pituitary as a site of negative feedback effect by glucocorticoids, an effect that is present from 115 days of gestation and appears to be attenuated at 136-140 days. Furthermore, it appears that basal and stimulated ACTH release is differentially regulated. These observations are consistent with those of Matthews & Challis (1997). Cortisol treatment did not affect basal ACTH output or POMC mRNA levels in fetal sheep corticotrophs cultured at 138 days of gestation, but significantly attenuated CRH- and AVP- stimulated ACTH output and POMC mRNA levels. Furthermore, this inhibition was more pronounced in CRH-stimulated ACTH output (Matthews & Challis 1997). These observations suggest that glucocorticoids are more effective in inhibiting CRH, versus AVP, induced pituitary responsiveness. These results may be reflective a change in pituitary subpopulations with increasing gestation, that are either CRH or AVP responsive (Schwartz 1990, Perez et al. 1997, Butler et al. 1999) and could provide a mechanism by which ACTH secretion increases late in gestation in the fetal sheep in the presence of elevated glucocorticoids (Matthews & Challis 1997).

In spite of the capacity for negative feedback control in the fetal sheep, ACTH and cortisol levels have been shown to rise concurrently in the final 15-20 days of gestation (Norman & Challis 1985, Wood 1988). It has therefore been proposed that in the term fetal sheep there emerges an alteration in the feedback sensitivity of the HPA axis, permitting a significant rise in
circulating fetal cortisol concentrations and contributing to both organ maturation and parturition (Brooks et al. 1996). In general, glucocorticoid treatment both in vivo and in vitro, results in significant decreases in ACTH output and ACTH responses to CRH/AVP. However, after 130 days of gestation, there is a reduction in the ability of cortisol to exert the same negative feedback effects (Wood 1988, Brooks et al. 1996). Intrafetal cortisol infusion late in gestation did not alter basal fetal plasma ACTH concentrations; instead, fetal plasma ACTH levels increased in these animals over time (Wood 1988, Jeffray et al. 1998). Furthermore, POMC mRNA levels increase consistently throughout gestation, reaching maximal levels at term (145-147 days) (Matthews & Challis 1994). This may be the result of alterations in the number of GR present in the pituitary at this time (Holloway et al. 2000), and therefore reflect the diminished ability of cortisol to exert negative feedback effects at the pituitary.

Other modulators of negative feedback have been shown to be present late in gestation in the fetal sheep. Levels of bioactive glucocorticoids can be influenced by the presence of the enzyme 11β hydroxysteroid dehydrogenase type 1 (11βHSD1). This enzyme is highly localized in the liver and primarily acts as a reductase catalyzing the conversion of inactive cortisone to active cortisol (Low et al. 1994). It has been shown that 11βHSD1 mRNA is present in fetal corticotrophs at low levels throughout gestation and then increases dramatically at term and in newborn lambs (Yang et al. 1994). However, dehydrogenase activity of the enzyme predominates in the pituitary, resulting in a rapid conversion of bioactive cortisol to inactive cortisone. Therefore, an increase in 11βHSD1 expression at term could diminish the effect of cortisol on negative feedback regulation at the pituitary by reducing local tissue cortisol levels within the pituitary (Yang et al. 1994).

Negative feedback effects of glucocorticoids may also be influenced by elevated concentrations of the high affinity binding protein corticosteroid binding globulin (CBG) present in the fetal circulation at term (see 1.3.2) (Ballard et al. 1982, Challis et al. 1985). This protein binds circulating cortisol and as a consequence maintains relatively low free cortisol levels immediately prior to birth (Ballard et al. 1982). Cortisol increases both fetal hepatic CBG mRNA levels and plasma corticosteroid binding capacity (CBC) in the fetal circulation (Berdusco et al. 1993, 1994), potentially resulting in a relative feed-forward loop where cortisol levels rise and stimulate increase production of fetal hepatic CBG. CBG therefore could play a major role in regulating the negative feedback activity of cortisol at the level of the pituitary. Furthermore, Berdusco et al. (1995) have shown that the fetal sheep pituitary expresses CBG mRNA late in gestation, supporting CBG as a regulatory protein in pituitary activity late in gestation.
Finally, placental prostaglandin (PGE₂) concentrations increase at term in the fetal sheep (Challis et al. 1976), and have been shown to stimulate ACTH secretion in vivo (Louis et al. 1976, Young et al. 1996). Placental PGE₂ may represent a unique mechanism of positive feed-forward mechanism whereby an increase in fetal glucocorticoids stimulate placental prostaglandin (PG) production and PG further stimulate and increase in fetal HPA activity (1.2.4.2) (Brooks et al. 1996).

Therefore, although the mechanisms of the alteration in negative feedback sensitivity in the fetal sheep are multifactorial (figure 1.9), it is clear that a change in fetal HPA regulation is imperative for the normal growth and maturation of the fetus in preparation for extraterine survival. Glucocorticoids late in gestation provide maturational signals to many fetal organ systems and are critical for the onset of parturition in some species. Alterations in the level of glucocorticoids could potentially disrupt this balance of HPA development and function. It is therefore critical for the fetus to strictly control the levels of and timing of this prepartum increase in glucocorticoids.

1.3 DEVELOPMENT OF THE ENDOCRINE PANCREAS AND THE LIVER

1.3.1 The endocrine pancreas

The endocrine cells of the pancreas reside in the islets of Langerhans and account for approximately 2% of the volume of the pancreas. Four islet cell types have been identified, α, β, γ and PP cells. The α cells primarily secrete glucagon, β cells secrete insulin, δ cells secrete somatostatin and PP cells secrete pancreatic polypeptide. The β cells are the most common and account for 60-75% of the cells in the islets, and are generally located in the center of each islet surrounded by α cells. Insulin and glucagon are powerful regulators of metabolism, coordinating levels of endogenous glucose, free fatty acids and amino acids.

The fetal pancreas appears at approximately 5 weeks of gestation in the human as 2 buds, dorsal and ventral, in the duodenal area (Dubois 1989). As the duodenum rotates and becomes c-shaped, the ventral bud migrates and ventral and dorsal buds fuse to form the final pancreas (Blackburn & Loper et al. 1992). In general, endocrine cells in many species, including primates and rodents, develop from duct-like cells in the embryo, fetus and neonate leading to the formation of primitive islets in the mesenchyme adjacent to the ducts (Hill et al. 1998). Initially during development endocrine cells make up ~10% of the pancreas and are seen as individual cells close to the pancreatic ducts and form into mature islets late in gestation (Hill & Duvillie 33
Cortisol cascade in the fetal sheep where cortisol occupies a central role in augmenting feed forward processes at the level of the fetal pituitary and adrenal and on the fetal liver to produce CBG. This alteration in negative feedback late in gestation is essential for organ maturation and the process of parturition where fetal cortisol can also act on the placenta and fetal membranes to influence patterns of prostaglandin output.

(Adapted from, Challis & Brooks 1989, Endocrine Reviews).
Neogenesis of mature islets, with the characteristic distribution of β cells in the center and non-insulin producing cells in the periphery, continues throughout neonatal life and ceases at about the time of weaning in rodents (Finegood et al. 1995, Sander & German 1997).

In the rodent, the endocrine cells are derived from a common precursor stem cell (Sanchez et al. 1998, De Krijger et al. 1992). This concept is supported by the coexistence of pancreatic hormone immunoreactivities in the islet cells of human, mice and guinea pig pancreata (De Krijger et al. 1992, Reddy et al. 1992). The development of endocrine cells from ductal epithelium is regulated by a sequence of transcription factors and locally acting growth factors (Sander & German 1997, Hill & Duvillie 2000). One of the most important transcription factors appears to be Pdx-1 (Sander & German 1997). In animals with targeted deletion of this factor endocrine differentiation from pancreatic buds does not occur (Offield et al. 1996). Furthermore, Pdx-1 has been associated with the maturation of developing β cells and the control of insulin secretion (Hill 1999, Hill & Duvillie 2000). The differentiation of separate endocrine cell types is controlled by the Pax family of transcription factors as well as Pdx-1 (Sander et al. 1997). In human fetuses, differentiation of islet cells begins at 10 weeks of gestation (Hay & Sparks 1985, Sanchez et al. 1998). In general, the α cells appear first followed by β, δ and PP cells (Dubois 1989, Reddy et al. 1992). In the fetal sheep, insulin immunoreactivity was observed at 65-70 days of gestation and pancreatic insulin concentrations increased from 100 days to term, then decreased in postnatal lambs (Willes et al. 1969).

Proliferation and differentiation of β cells is dependant upon a number of growth factors including insulin-like growth factors (IGFs) (Hill et al. 1998, Hill & Duvillie 2000). IGFs have been identified as cellular mitogens and differentiation factors during development in many fetal tissues (D’Ercole 1987). Two isomers of IGFs exist, IGF1, predominant in postnatal life and IGFII, predominant in fetal life (Hogg et al. 1994). IGFII mRNA and protein are highly expressed in the islet cells and some ductal epithelial cells in late fetal and early neonatal life in rats and humans (Han et al. 1988, Hogg et al. 1994, Hill et al. 1999). Furthermore, overexpression of IGFII in transgenic mice caused significant pancreatic islet cell hyperplasia although the total number of mature islets was not altered (Petrik et al. 1999). These observations suggest that in vivo, IGFII acts as a mitogen on existing islet cells, in an autocrine/paracrine manner during islet development (Hill et al. 1998, Petrik et al. 1999). Therefore, IGFII is critical for the increase in β cell mass in the development of the pancreas.
The ability to increase β cell mass is critical for the increasing insulin demands of the growing fetus late in gestation (Hay & Sparks 1985, Fowden 1989, Dornhorst et al. 1994). It has been suggested that the ontogeny of islet cells in early life involves a balance between β cell replication and neogenesis and programmed cell death (apoptosis) (Finegood et al. 1995, Scaglia et al. 1997, Hill 1999). A transient wave of apoptosis occurs in neonatal rat islets between 9-16 days of postnatal age (Finegood et al. 1995, Scaglia et al. 1997, Hill et al. 2000) that has been localized to the β cells (Hill et al. 2000). A new population of β cells compensates for this loss (Hill et al. 2000). This remodeling event also occurs in the human fetal pancreas in the perinatal period (Kassem et al. 2000). Interestingly, this apoptotic event coincides with a significant decrease in IGFII expression in rat pancreatic tissue 2 weeks after birth (Petrik et al. 1998), and overexpression of IGFII in transgenic mice resulted in a significantly lower number of apoptotic cells in the β cells of mice pancreata and greater mean islet area (Hill et al. 2000), supporting the role of IGFII as a growth factor that protects β cells from apoptosis.

Little information is available concerning pancreatic islet development and differentiation in the sheep. Titlbach et al. (1985) demonstrated the presence of two populations of islets, large and small, in sheep pancreata. Both populations were present in fetal and neonatal pancreata, whereas 2 and 15 month old lambs showed no evidence of large islets. Over 90% of the large islets showed characteristic insulin staining of β cells but were irregular in shape. Smaller islets were characteristic of classical mammalian islets of Langerhans, where β cells occupy the central portion surrounded by α and PP cells (Titlbach et al. 1985). At birth, large islets predominated although many small islets were present, developing from the ductal epithelium. By 1-4 days postpartum, large islets contained strands of connective tissue and split up the parenchyma. Small islets appeared to proliferate not only from ductal epithelium but also from the periphery of some large islets. By 2 months of age, large islets were low in concentration and in the adult no further morphological changes were evident (Titlbach et al. 1985). The existence of a dual population of islets has also been shown in the bovine pancreas (Bonner-Weir & Like 1980). There are no reported data concerning a possible wave of apoptosis and β cell neogenesis in the fetal or neonatal sheep. It is currently unknown if the postnatal development of islets observed by Titlbach et al. (1985) represents apoptosis of large islets. Further investigation into the development of the sheep pancreas is therefore warranted.

Glucocorticoids have been implicated in β cell mass and phenotype regulation. The GR is present in the β cells of the rat pancreas (Fischer et al. 1990) and glucocorticoid binding sites
increase in the neonatal rat pancreas at postnatal day 15, at a time when plasma corticosterone levels rise sharply (Lu et al. 1987). Glucocorticoids have been shown to decrease Pdx-1 expression (Sharma et al. 1997) as well as decrease IGFII expression (Price et al. 1992, Li et al. 1993). Therefore, exposure to elevated glucocorticoid concentrations similar to that seen in the neonatal rat at the time of pancreatic remodeling (Sapolsky & Meaney 1986) may precipitate a reduction in pancreatic IGFII and a wave of apoptosis in β cells within the islet (Hill 1999). The availability of glucocorticoids in the pancreas may be regulated by 11β hydroxysteroid dehydrogenase type 2 (11βHSD2), which catalyzes that conversion of bioactive glucocorticoids to biologically inactive 11-keto metabolites. 11βHSD2 is expressed in the pancreas (Albiston et al. 1994), raising the possibility that altered activity of this enzyme might permit control of local tissue levels of glucocorticoids.

1.3.1.1 Development of fetal insulin responses to glucose
Data concerning whether or not the fetus is capable of responding to glucose with increased insulin secretion appear conflicting. A number of reports suggest that in the human and rat fetus, insulin release is independent of plasma glucose concentrations. Moreover insulin release appears to be relatively unresponsive to glucose, but highly responsive to amino acids and agents that increase cAMP levels in the absence of glucose (Simpson & Tuch 1995, Dubois 1989, Hill 1999). Bassett & Thorburn (1971) reported that in the sheep the concentration of fetal plasma insulin in response to a glucose challenge, rose very slowly and only to a small extent. It was suggested that insulin secretion is sluggish in the fetus due to an immature adenylyl cyclase system (Bassett & Thorburn 1971). In fact, it has been shown that inhibition of cAMP in cultured human fetal pancreatic cells reduced insulin synthesis (Simpson & Tuch 1995).

However, other reports suggest that fetal insulin secretion is directly related to fetal glucose concentrations (Alexander et al. 1968, Hay & Sparks 1985, Philipps et al. 1978, Fowden 1989). Insulin has been detected in fetal sheep plasma very early in gestation (~40-50 days of gestation) and is present until term (Alexander et al. 1968). In vitro studies have demonstrated that insulin release can be stimulated by glucose in fetal sheep pancreatic cells at 120-147 days of gestation (Bassett et al. 1973). In vivo studies suggested that insulin sensitivity to glucose is similar in fetal and adult sheep and that a positive correlation exists between plasma glucose and insulin concentrations before, during and after maternal fasting (Philipps et al. 1978, Gresores et al. 1997). Houghton et al. (1989) reported increasing fetal basal glucose levels between 110-115 days of gestation and 140-145 days, without an increase basal insulin levels. However, the
administration of an acute glucose challenge stimulated insulin release in fetuses throughout gestation. Fetuses between 125-140 days of gestation had a significant increase in the magnitude of the peak insulin response, suggesting maturation in the insulin response to glucose (Houghton et al. 1989). Furthermore, significant insulin responses were observed in adult sheep, and peak glucose and insulin concentrations were significantly greater than those in fetuses. Therefore, in fetal sheep, insulin responses to an acute exogenous glucose load are present at 110 days of gestation.

The onset of pancreatic responsiveness therefore appears to be species specific. It has been suggested in the rodent model, that insulin release from fetal type of islets (prior to the apoptotic event) are poorly responsive to glucose. Shortly after birth in rodents, apoptosis appears to remove fetal type β cells, which are replaced with new adult type of cells following neogenesis (Hill & Duvillie 2000). This new population of cells is now sensitive to glucose with normal insulin release. This development may then prepare the pancreas for postnatal metabolism (Hill & Duvillie 2000). Whether this occurs in the sheep pancreas is unknown and warrants further investigation.

1.3.1.2 Fetal insulin and growth
The importance of fetal insulin on growth has been long since established (Fowden 1989, Phillips et al. 1991, Verhaeghe et al. 1993, Dornhorst et al. 1994). Observations that infants of diabetic mothers have increased amounts of body fat and increased birth weights (Pedersen 1971, Van Asshe et al. 1977) led to the hypothesis that increased growth in these infants was due to excessive insulin secretion. These infants were found to have larger than normal pancreata and more endocrine tissue within the pancreas (Fowden 1989). In addition, proinsulin, C-peptide, as well as IGFI and II levels are all correlated to birth weight in humans (Verhaeghe et al. 1993, Dornhorst et al. 1994). In vivo, pancreatic β cell damage (with streptozocin) in fetal sheep resulted in relative fetal hyperglycemia, hypoinsulinemia and a decrease in fetal body weight by ~ 21% (Philipps et al. 1991). Similar observations were observed in liver, heart and kidney weights as well as skeletal growth (Philipps et al. 1991). Intrauterine growth restriction in human infants has been associated with a reduction in pancreatic tissue and a loss of β cells (Van Asshe et al. 1977, Nicolini et al. 1990). Therefore, it is evident that insulin secretion plays an important role in fetal growth especially late in gestation.
1.3.2 The liver
The liver is divided into functional acini where blood flows linearly into parenchymal structures and exhibits a radial decrease in substrate and hormone concentrations around the vasculature of afferent vessels (Jungermann & Katz 1989). At least two different zones are apparent; the periportal zone, perfused with blood that is rich in oxygen, substrates and hormones, and the perivenous zone, perfused with blood that is depleted of oxygen, substrates and hormones but contains high concentrations of carbon dioxide and metabolites (figure 1.10) (Jungermann & Katz 1989). This zonation is important in the functional heterogeneity of the liver acini in that metabolic enzymes are highly localized to one zone or the other. Based on the assumption that the activities or presence of key enzymes are indicators of metabolic activities, different functions for the 2 zones (periportal and perivenous) were proposed. This proposal is known as "metabolic zonation" (Jungermann & Katz 1989) and suggests that hepatocytes from the periportal and perivenous zones of the liver differ in their enzyme content. The periportal zone has a higher capacity for glucose synthesis from gluconeogenesis and therefore contains high activities of glucose-6-phosphate and phosphoenolpyruvate carboxykinase (PEPCK). The perivenous zone possesses a higher capacity for the breakdown of glucose to pyruvate and therefore contains high activities of glucokinase (Jungermann & Katz 1989).

The fetal liver appears in the human at ~3 weeks of gestation and receives the highest net flux of maternal glucose as it receives blood returning from the placenta. Therefore, in the fetus, hepatic enzymes for glycogen synthesis are elevated and enzymes for glycolysis and gluconeogenesis (PEPCK) are decreased compared to that of the adult (Blackburn & Loper 1992). These relationships are maintained until birth when a decrease in glucose availability and the onset of high fat feeding decreases the glycogenolytic enzymes (Blackburn & Loper 1992). Glycogen synthase can be found in the human fetal liver at ~8 weeks of gestation and like many hepatic metabolic enzymes is regulated by glucocorticoids (Blackburn & Loper 1992). Similarly, hepatic glucose-6-phosphatase, PEPCK, and pyruvate carboxylase activities all increase from 120 days of gestation to term in the ovine fetus (Fowden et al. 1990, 1993). Furthermore, the activities of these enzymes were positively correlated to plasma cortisol concentrations substantiating the role of fetal cortisol in stimulating fetal hepatic enzymes late in gestation (Fowden et al. 1990, 1993).

1.3.2.1 Corticosteroid binding globulin
The liver produces plasma proteins, including albumin, clotting factors and steroid binding proteins, including corticosteroid binding globulin (CBG). CBG is primarily produced in the
Figure 1.10 Model of the functional heterogeneity of hepatic acini

1: periportal zone in which concentrations of oxygen, substrates and hormones are high
2: intermediate zone
3: perivenous zone in which concentrations of oxygen, substrates and hormones are low but concentrations of carbon dioxide and metabolites are high
CV: central vein, P: portal field, $\triangle$: represents the triad of hepatic artery, portal vein and bile ducts
(Adapted from Jungermann & Katz 1995, Physiological Reviews).
liver of all species examined to date including the primate, sheep and guinea pig (Perrot-Applanat et al. 1981, Berdusco et al 1993, Pepe et al. 1996). CBG is well conserved throughout vertebrates; it binds glucocorticoids with high affinity and over 90% of circulating glucocorticoids are bound to CBG in human plasma (Hammond et al. 1991, Seralini 1996). CBG is considered a cortisol transport protein that maintains a constant readily accessible pool of protein bound cortisol, in addition to regulating cortisol tissue availability and increasing the half-life of cortisol (Hammond et al. 1991). Ovine CBG has been cloned (Berdusco et al. 1993) and appears to be >75% homologous with rat, rabbit and human CBG. The CBG gene contains 5 consensus sites of N-glycosylation, necessary for the formation of the steroid-binding site (Berdusco et al. 1993). CBG has also been localized to extra-hepatic sites of production, including the fetal kidney, pancreas, adrenal and the fetal pituitary (Berdusco et al. 1995, Seralini 1996).

In general, CBG levels are high during fetal life, decline perinatally, and remain low in adulthood. In plasma, CBG is measured as the cortisol binding capacity (CBC) in the circulation (Fairclough & Liggins 1975, Ballard et al. 1982). Plasma CBC is demonstrable in the fetal sheep as early as 80 days of gestation and increases dramatically from day 120 to term (Fairclough & Liggins 1975, Ballard et al. 1982, Berdusco et al. 1995). By 10 days postpartum, plasma CBC values fall to those seen in the adult (Ballard et al. 1982). The rise in fetal CBG levels has been associated with the prepartum rise in fetal cortisol concentrations (1.3.2.3). In the fetal sheep, the percentage of free cortisol remains relatively unchanged during the last 20 days of gestation, due to an increase in total cortisol levels and a concomitant increase in CBC (Berdusco et al. 1995). Furthermore, Berdusco et al. (1995) demonstrated a significant increase in fetal hepatic CBG mRNA levels from 125 to 140 days of gestation followed by a decrease during active labour. It appears that CBG is critical for regulating free cortisol concentrations up to the day of parturition. Therefore, CBG may reduce the negative feedback capacity of cortisol on the fetal pituitary, allowing the concurrent rise in ACTH and cortisol that occurs late in gestation in this species (Berdusco et al. 1995, Challis et al. 1995).

1.3.2.2 11β Hydroxysteroid dehydrogenase type 1
The major site of glucocorticoid metabolism is the liver. In mammals, at least 2 isozymes of the enzyme 11β hydroxysteroid dehydrogenase (11βHSD) exist to catalyze the interconversion of biologically active 11-hydroxylated corticosteroids (cortisol/corticosterone) and inactive 11-keto metabolites (cortisone/11-dehydrocorticosterone) (Yang 1995, Stewart & Krozowski 1999,
Krozowski 1999). Type 1 and type 2 11βHSD isozymes share 14% homology and have distinctly different physiological roles and tissue distribution. 11βHSD2 is a high affinity (nM range) NAD-dependent enzyme that primarily functions as a dehydrogenase, acting unidirectionally converting biologically active glucocorticoids to inactive metabolites (Yang 1995, Stewart & Krozowski 1999, Krozowski 1999). High levels of 11βHSD2 are found in the placenta (Stewart et al. 1995, Krozowski 1999) protecting the fetus from maternally derived glucocorticoids.

In contrast, 11βHSD1 is a low affinity (μM range), NADP (H) dependant isozyme that is bi-directional, although it acts predominantly as a reductase enzyme in vivo converting inactive cortisone to biologically active cortisol (Yang 1995, Jamieson et al. 1995, 2000, Stewart & Krozowski 1999, Krozowski 1999). 11βHSD1 is primarily localized in the liver but studies have demonstrated the presence of 11βHSD1 in the brain, pituitary, adrenal, lung, ovary and adipose tissue (Yang et al. 1992, 1995, Ricketts et al. 1998). 11βHSD1 has also been identified in human decidua and fetal membranes where it may act locally to increase bioactive glucocorticoid concentrations to facilitate the process of parturition (Patel et al. 1999). 11βHSD1 mRNA has been localized to both the periportal and perivenous zones of rat hepatic acini, although expression levels were somewhat higher in the periportal zone (Nyirenda et al. 1998), supporting its role in facilitating increases in local glucocorticoid concentrations in an area where many glucocorticoid sensitive metabolic enzymes have been localized.

Due to its role in the regulation of local cortisol levels, alterations in 11βHSD1 expression and activity could potentially influence the activity of other glucocorticoid sensitive enzymes in the liver. Studies in rats and mice have shown that alterations in hepatic 11βHSD1 result in alterations in metabolic enzyme expression and activity (Kotelevtsev et al. 1997, Jamieson et al. 1999). 11βHSD1 knockout mice demonstrate significantly lower fasting glucose levels and fail to show increases in glucose-6-phosphatase and PEPCK upon starvation stress (Kotelevtsev et al. 1997). These studies support the hypothesis that 11βHSD1 can indirectly regulate metabolic processes in the liver by altering intrahepatic glucocorticoid levels.

During fetal development, the bioactive level of glucocorticoids to which the fetus is exposed is critical for organ maturation (Liggins 1994, Yang 1995). 11βHSD1 mRNA has been demonstrated in fetal sheep liver by 60 days of gestation and has shown to increase over 4 fold towards term (Yang et al. 1992, Langlois et al. 1995) accompanied by an increase in reductase activity (Langlois et al. 1995). Dehydrogenase activity was also demonstrated in the fetal liver at
85, 100-120, and 140-143 days of gestation although was always found to be less than reductase activity (Langlois et al. 1995). This progressive increase in fetal hepatic 11βHSD1 reductase activity and subsequent increase in bioactive glucocorticoids, may play a critical role in the control of glycogen deposition and gluconeogenesis late in gestation, preparing the fetus for extrauterine survival (Langlois et al. 1995, Yang 1995). Therefore, the fetal liver may produce glucocorticoids locally that act on sensitive metabolic enzymes localized in the liver.

### 1.3.2.3 Glucocorticoid regulation of liver CBG and 11βHSD1

GR have been localized in the hepatocytes (Berdusco et al. 1993, Raddatz et al. 1996), and the role of glucocorticoids in regulating many hepatic metabolic enzymes has been well established (Fowden et al. 1990, 1993, Pilkas & Granner 1992, Friedman et al. 1997). The rate-limiting enzyme for gluconeogenesis, PEPCK, is glucocorticoid sensitive. In rats glucocorticoids increase PEPCK mRNA and activity levels both in vivo and in vitro (Benvenisty et al. 1983, Pilkas & Granner 1992, Friedman et al. 1997).

Many glucocorticoid sensitive hepatic enzymes have been localized in the fetal sheep as early as 100-119 days of gestation, including glucose-6-phosphatase, pyruvate carboxylase, and PEPCK (Fowden et al. 1990, 1993). Activities of these enzymes increase with gestational age in parallel with the normal prepartum rise in fetal cortisol levels towards term and are significantly decreased in adrenalectomized fetuses (Fowden et al. 1993). Furthermore, enzyme activities were stimulated prematurely in fetuses at 122-125 days of gestation by intrafetal cortisol infusion (Fowden et al. 1990, 1993), confirming the role of cortisol as a regulator of gluconeogenic enzyme activity in the sheep fetus late in gestation.

Glucocorticoids have also been shown to stimulate the production of fetal hepatic CBG (Challis et al. 1985, Berdusco et al. 1993, 1994, Jeffray et al. 1995). Studies in fetal sheep demonstrated that pulsatile administration of ACTH at 127 days of gestation significantly increased plasma CBC and that this effect was attenuated with the simultaneous administration of the cortisol synthesis inhibitor metopirone (Challis et al. 1985). Subsequent studies reported that the synthetic glucocorticoid dexamethasone, increased plasma CBC levels and hepatic CBG mRNA levels at 123-128 days of gestation in the fetal sheep (Berdusco et al. 1993). These observations are in contrast to those seen in the adult, since in many species, glucocorticoids have been shown to decrease hepatic CBG mRNA levels (Smith & Hammond 1989, Berdusco et al. 1993, Cole et al. 1999). Therefore, the influence of glucocorticoids on CBG expression may be developmentally regulated (Berdusco et al. 1993).
Similarly, hepatic 11βHSD1 mRNA expression and activity are regulated by glucocorticoids. Adrenalectomy in adult rats attenuated the expression and activity of hepatic 11βHSD1 an effect that was attenuated by glucocorticoid replacement (Jamieson et al. 1999). In primary cultures of rat hepatocytes, dexamethasone treatment increased in 11βHSD1 reductase activity and mRNA expression (Jamieson et al. 1995). These observations have also been shown in a cultured rat liver cell line (Voice et al. 1996). In the sheep intrafetal dexamethasone infusion at 130 days of gestation resulted in significant elevations in 11βHSD1 mRNA and activity levels (Yang et al. 1994). These observations therefore support the hypothesis that glucocorticoid regulation of fetal hepatic 11βHSD1 expression towards term is the result of the prepartum increase in fetal cortisol concentrations (Yang et al. 1995).

Therefore, activation of the fetal HPA axis, leading to the prepartum increase in circulating cortisol plays a critical role in stimulating and maintaining important hepatic enzymes and proteins. Indeed, fetal hypophysectomy in the fetal sheep at 102-108 days of gestation resulted in reduced plasma cortisol concentrations and significantly lower hepatic glucose-6-phosphatase activity (Fowden et al. 1990) and plasma corticosteroid binding capacity (Ballard et al. 1982). It is therefore evident that the normal development and maturation of the fetal HPA axis is crucial for extrauterine survival.

1.4 ANIMAL MODELS OF THE FETAL ORIGINS HYPOTHESIS

1.4.1 Fetal programming of adult disease

In light of the number of epidemiological studies linking low birth weight and the increased risk of developing adult disease, many animal studies have attempted to find a mechanism behind this relationship. Some of the most compelling data are from reports concerning fetal nutrition and the effects of maternal diet on fetal growth and the subsequent development of adult disease. Maternal undernutrition has been linked to decreased glucose tolerance (Ravelli et al. 1998) and elevated blood pressure (Law et al. 1993, Clark et al. 1998) in adults. Furthermore, it has also been shown that undernutrition may alter placental glucocorticoid metabolism and there is increasing evidence suggesting that this altered fetal exposure to glucocorticoids may be involved in the programming of adult disease.

1.4.2 Prenatal undernutrition

Animal studies have demonstrated that maternal undernutrition and nutritional state before pregnancy may alter placental growth and result in growth restricted offspring that exhibit
similar characteristics that are seen in human populations, such as glucose intolerance and elevated systolic blood pressure (Langley-Evans 1994, Langley-Evans et al. 1996). Current concepts suggest that alterations in the fetal insulin and IGF axes are central to the regulation of fetal growth (Fowden 1989, Gluckman 1997). Maternal nutrient restriction in the rat resulted in significant reductions in placental weight, birth weight and postnatal weight in offspring up to 90 days postpartum. Plasma IGFl and insulin levels were significantly reduced in these animals and plasma IGF binding proteins were significantly elevated (Woodall et al. 1996). Similarly, nutrient restriction early in pregnancy in the sheep (28-80 days or 100-20 days of gestation) resulted in significant reductions in fetal plasma insulin and IGFl concentrations (Bauer et al. 1995, Brameld et al. 2000). These observations therefore suggest that alterations in fetal insulin – IGF axis due to maternal nutrient restriction could provide one mechanism underlying long-term effects of fetal growth restriction on postnatal diseases such as glucose intolerance and diabetes.

One of the most well studied models of animal nutrient restriction is maternal protein restriction. In the rat, maternal protein restriction throughout pregnancy has been reported to cause fetal growth restriction, altered glucose tolerance and hypertension in adult offspring (Langley-Evans 1994, Holness 1996, Langley-Evans & Nwagwa 1998). Prenatal protein restriction in rats results in adult offspring with abnormal pancreas development including reduced insulin content and islet size, reduced islet vascularization and blood flow, β cell number and insulin content and impaired secretory responses to glucose and arginine (Snoeck et al. 1990, Dahri et al. 1991, 1994, Iglesias-Barreirã et al. 1996, Berney et al. 1997). Therefore, prenatal protein restriction can impair the development of the fetal β cell. Offspring of rats given a low protein diet had significantly greater levels of pancreatic islet apoptosis and diminished levels of IGFl mRNA levels (Petrik et al. 1999). Therefore, protein restriction may result in changes in the balance of β cell replication and apoptosis in fetal and neonatal life and thus contribute to impaired insulin release observed later in life (Petrik et al. 1999). Prenatal protein restriction has also been shown to reduce peripheral glucose utilization by oxidative muscles in adult rats (Holness 1996), implicating alterations in insulin action as well as release in the development of glucose intolerance. Alterations in hepatic enzymes associated with glucose homeostasis have also been demonstrated in offspring fed a prenatal low protein diet. Hepatic glucokinase activity decreased by ~50% whereas PEPCK activity increased ~100% in the adult rat following prenatal protein restriction (Desai et al. 1997), results which may lead to an increased hepatic production of glucose. Taken together, these observations suggest that fetal
glucose homeostasis can be programmed by maternal nutrient restriction in utero. Therefore, due to the role that fetal insulin plays in intrauterine growth, it appears that alterations in pancreatic development and postnatal glucose tolerance may well be directly associated with fetal growth restriction.

In addition to effects on fetal insulin and glucose regulation, undernutrition may also alter HPA function. In human infants and adults, low birth weight is linked to alterations in HPA activity (Goland et al. 1993, Clark et al. 1996, Phillips et al. 1998, Reynolds et al. 2001). Maternal nutrient restriction in the sheep resulted in reductions in pituitary and adrenal responsiveness to exogenous CRH+AVP as well as endogenous hypoxic stress (Hawkins et al. 1999, Hawkins et al. 2000). Previously, maternal nutrient restriction in the sheep at 105-115 days of gestation resulted in significant reductions in fetal adrenal P450c17 and 3βHSD steroidogenic enzymes, suggesting a down regulation in fetal adrenal function (Fraser et al. 1999).

In addition to its effect on pituitary and adrenal responsiveness, maternal protein restriction in the rat significantly blunted the diurnal pattern of ACTH but not corticosterone in offspring at 4 weeks of postnatal age. Furthermore, hippocampal GR, but not MR, binding capacity and receptor number were significantly elevated in male offspring (Langley-Evans 1994). These alterations were associated with significant elevations in systolic blood pressure in both males and females at 4 weeks of postnatal age. Previously, complete protein deficiency for 5 days during pregnancy resulted in a significant elevation in basal plasma corticosterone levels in adult rats (Jacobson et al. 1997). However, in adrenalectomized adult rats, protein deficiency in utero resulted in significant increases in basal plasma ACTH and POMC mRNA levels in the pars distalis suggesting that protein malnutrition result in an increased drive to the pituitary-adrenocortical system that is normally suppressed by elevated levels of corticosterone in these rats (Jacobson et al. 1997). Taken together, these observations suggest that alterations in brain and/or pituitary HPA regulation can be programmed by nutrient restriction. This is supported by studies that demonstrate alterations in hippocampal GR and MR mRNA levels in offspring of protein restricted mothers (Langley-Evans 1996, Lingas et al. 1999). Lingas et al. (1999) found that 48 hours of maternal nutrient deprivation in the guinea pig at 50-51 days of gestation resulted in a significant reduction in fetal weight and hypothalamic PVN and hippocampal CA1-2 GR mRNA levels in females and hippocampal CA1-2 GR mRNA levels in male fetuses at 52 days of gestation, suggesting that maternal nutrient restriction can program fetal HPA function through alterations in the brain. Furthermore, Lingas et al. (1999) found that maternal and fetal cortisol levels were significantly increased after maternal nutrient restriction, and that increased
cortisol concentrations were only associated with elevated ACTH levels in the maternal circulation. This suggests that nutrient deprivation increased maternal HPA activity, and also resulted in an increase in placental transfer of maternal glucocorticoids to the fetal circulation (Lingas et al. 1999). Recently, maternal protein restriction in rats during the last week of pregnancy decreased placental 11βHSD2 expression and reduced newborn body weight, plasma ACTH and GR and MR mRNA levels in the hippocampus (Lesage et al. 2001). However, maternal adrenalectomy with corticosterone supplementation still resulted in altered 11βHSD2 expression in the placenta and growth restriction in offspring, but eliminated alterations in newborn HPA function (Lesage et al. 2001). These observations support current concepts that fetal exposure to elevated levels of glucocorticoids may be a key mechanism underlying the fetal and postnatal alterations that occur following maternal nutrient deprivation.

1.4.3 Placental 11β hydroxysteroid dehydrogenase and fetal programming

It is clear that maternal nutritional factors can exert permanent influences on the developing fetus; this may be the result of fetal exposure to elevated levels of glucocorticoids. The placental enzyme 11β hydroxysteroid dehydrogenase type 2 (11βHSD2) plays an important role in inhibiting transplacental transfer of maternal glucocorticoids from entering the fetal circulation (Brown et al. 1993, Edwards et al. 1993). Placental 11βHSD2 acts as a dehydrogenase enzyme, rapidly converting active glucocorticoids to inactive metabolites (Edwards et al. 1993, Krozowski 1999). Because maternal glucocorticoid levels are much higher than fetal levels, a relative deficiency in placental 11βHSD2 would put the fetus at great risk of glucocorticoid exposure (Edwards et al. 1993, Seckl et al. 1995, Seckl 1997). This may therefore represent a mechanism by which the fetus is exposed to elevated levels of glucocorticoids during gestation. Studies in rats have demonstrated a strong positive correlation exists between placental 11βHSD2 activity and fetal weight at term and an inverse correlation exists between 11βHSD2 activity and placental weight (Benediktsson et al. 1993). This relationship was also found in humans, where placental 11βHSD2 activity correlated with fetal weight (Stewart et al. 1995). Furthermore, maternal protein restriction, associated with elevations in systolic blood pressure in adult offspring, attenuated placental 11βHSD2 activity in rats (Langley-Evans et al. 1996). These results suggest that fetal exposure to elevated levels of glucocorticoids may be the underlying mechanisms programming fetal development and the risk of developing adult disease.

Treatment of pregnant rats with carbenoxolone, a potent inhibitor of placental 11βHSD2, resulted in significant reductions in birth weight, and significantly higher fasting basal glucose
levels, elevated insulin responses to a glucose challenge, elevated basal corticosterone and hypothalamic CRH mRNA levels and decreased hypothalamic GR mRNA levels in offspring (Lindsay et al. 1996, Saegusa et al. 1999, Welberg et al. 2000). Since these results were abolished by maternal adrenalectomy (Lindsay et al. 1996), these effects must have been mediated via fetal exposure to maternally derived glucocorticoids.

Therefore, strong evidence exists to suggest that placental 11βHSD2 deficiency results in increased fetal exposure to glucocorticoids and therefore plays a role in fetal programming.

1.4.4 Prenatal exposure to glucocorticoids
There are several features of fetal exposure to elevated levels of glucocorticoids that support its role in the programming of adult disease (Seckl 1997). Fetal exposure to excess glucocorticoids results in growth restriction in humans (Reinisch et al. 1978, Seckl 1994) and in animals (Lindsay et al. 1996, Levitt et al. 1996, Newnharn et al. 1999, Sloboda et al. 2000; Chapter 3). For example, human studies have shown that fetal levels of ACTH and cortisol are increased in intrauterine growth restricted (IUGR) babies (Goland et al. 1993). Furthermore, glucocorticoids increase blood pressure in adults (Tonolo et al. 1988) and cortisol infusion into the fetal sheep results in elevated fetal blood pressure (Dodic & Wintour 1994). Prenatal stress or glucocorticoid administration has been shown by numerous studies to alter growth and HPA activity as well as glucose tolerance (Uno et al. 1990, Weinstock et al. 1992, Lindsay et al. 1996, Liu et al. 2001, Welberg & Seckl 2001). Furthermore, low birth weight in humans correlates with increased adult cortisol levels as well as insulin resistance and elevated blood pressure (Phillips et al. 1998, Levitt et al. 2000, Reynolds et al. 2001). Specific mechanisms underlying these associations are still unclear and require further investigation.

1.4.4.1 Prenatal Stress
Prenatal stress has been shown to permanently program the pattern of HPA and metabolic responses, although these relationships are complex and subtle differences in stimuli exert different effects (Seckl 1997). Most human data comes from retrospective studies on children whose mothers experienced psychological stress during pregnancy (Austin & Leader 2000, Niederhoter & Reiter 2000, Weinstock 1996). Several different animal models of prenatal stress have been studied including noise and light stimuli, electrical shock, and hypoxic stress (Takahashi & Kalin 1991, Maccari et al. 1995, Vallee et al. 1996, Green et al. 2000, McMillen et al. 2000). The most well described studies have been done on rats and have primarily focused on HPA axis development and function.
In general, studies have shown that maternal stress has permanent effects on the offspring, however considerable variation in the magnitude of effects and whether sex specific responses exist. This variation may be due to the types of stressors employed, the gestational age of the animal and duration of the stressor (Welberg & Seckl 2001). In general, the experimental evidence suggests that stress increases both maternal and fetal glucocorticoid levels, and that maternally derived glucocorticoids may program postnatal HPA activity (Barbazanges et al. 1996, Takahashi 1998). Stress during pregnancy has resulted in offspring with elevated basal plasma ACTH and corticosterone levels (Takahashi & Kalin 1991), increased corticosterone and ACTH responses to a stressor (Weinstock et al. 1992, Takahashi & Kalin 1991, Barbazanges et al. 1996) and altered anxiety behavior (Weinstock et al. 1992). The postnatal responses of the offspring to a prenatal stress were suppressed by maternal adrenalectomy, suggesting that maternally derived glucocorticoids program the postnatal alteration in HPA function (Barbazanges et al. 1996).

Many possible mechanisms for the programming of postnatal HPA hyperactivity have been proposed, although none have been fully defined. High glucocorticoid levels during pregnancy may alter fetal HPA axis development by downregulating fetal hippocampal/hypothalamic/pituitary corticosteroid receptors resulting in altered negative feedback, or resulting in alterations in the mechanisms regulating the stimulation of pituitary ACTH synthesis and secretion (1.4.4.3) (Maccari et al. 1995, Barbazanges et al. 1996, Lemaire et al. 2000). Furthermore, exposure to elevated glucocorticoid levels may modify the inhibitory control of hippocampal corticosteroid receptors by noradrenergic systems, thereby facilitating glucocorticoid secretion (Barbazanges et al. 1996, Welberg & Seckl 2001). In the rat, maternal restraint stress over the last week of pregnancy resulted in a reduction in neurogenesis in the dentate gyrus, a decrease in hippocampal MR and GR binding and impaired hippocampal related spatial tasks in adult male offspring (Maccari et al. 1995, Lemaire et al. 2000). These observations support a role for hippocampal alterations in the programming of increased HPA activity following prenatal stress. In addition, maternal restraint stress in the last week of pregnancy resulted in significant reductions in body weight, higher basal glucose levels and prolonged corticosterone responses to postnatal restraint stress in adult rat offspring (Vallee et al. 1996), supporting previous reports that prenatal manipulations result in alterations in both HPA and metabolic function later in life.

2001). The rodent gives birth to immature offspring, and the period of rapid brain growth associated with HPA development occurs in the first 2 weeks of postnatal life (Dobbing & Sands 1979, Rosenfeld et al. 1992). Therefore, the rat HPA axis is susceptible to programming in the early postnatal period. Early postnatal events such as maternal separation or neonatal handling, result in significant elevations in hippocampal GR binding capacity and number (Meaney et al. 1985, 1989), reduced plasma ACTH and corticosterone responses to stress and enhanced glucocorticoid feedback sensitivity (Liu et al. 1997). This occurs at time in which the HPA axis is relatively quiescent in the developing neonate (Rosenfeld et al. 1992). Therefore, neonatal handling during a critical developmental window (1-3 weeks postnatally) in the rat results in permanent alterations in HPA function as a result of alterations in hippocampal corticosteroid receptors. Several studies have shown that the prenatal effects of stress are reversible by early postnatal manipulations. Prenatally stressed rats exposed to postnatal handling exhibited significantly lower corticosterone responses to stress as adults (Vallee et al. 1996). Postnatal adoption that encourages maternal interaction with pups also reverses the effects of prenatal stress, decreasing stress induced corticosterone peak levels in adult offspring (Maccari et al. 1995). These observations raise the issue of a developmental window when elevated glucocorticoid levels may produce permanent effects. It has been suggested that in the rat prenatal stress may have to occur several days beyond birth in order to see permanent effects (Takahashi 1998). This concept is somewhat different in mammals that exhibit HPA axis development and brain growth in the prenatal or perinatal period such as in the primate or sheep (Dobbing & Sands 1979).

Prenatal hypoxic stress is also associated with elevations in fetal glucocorticoid levels. Hypoxic stress in the fetal sheep resulted in increased fetal ACTH and cortisol concentrations, increased pituitary POMC mRNA and hypothalamic CRH mRNA levels, in addition to reductions in IGFII mRNA in liver, lung and skeletal muscle (Challis et al. 1986, Matthews & Challis 1995, Green et al. 2000). In the ovine fetus, placental restriction caused a significant reduction in fetal body weight at 141-145 days of gestation (term=147-150). Furthermore, the expression of 11βHSD1 mRNA was significantly elevated in the fetal liver following placental restriction (McMillen et al. 2000). This observation suggests that there may be greater intrahepatic exposure of glucocorticoid sensitive gluconeogenic enzymes to locally produced cortisol, and may be important in the context of human evidence that has demonstrated an association between the intrauterine environment, growth restriction and the programming of adult glucose intolerance and diabetes (McMillen et al. 2000). Although these studies did not
measure postnatal HPA or metabolic function, it is possible that fetal exposure to sustained elevations in cortisol levels after hypoxic stress may result in permanent alterations in postnatal function.

In general, models of prenatal stress result in postnatal alterations that are similar to those found following prenatal undernutrition. Because an important feature of the stress response is the secretion of high level of glucocorticoids, cortisol/corticosterone have become clear candidates for the role of prenatal stress in fetal programming (Welberg & Seckl 2001). It appears likely therefore, that fetal exposure to elevated glucocorticoids may indeed be the key factor linking alterations in the intrauterine environment and the increased risk of developing adult diseases.

1.4.4.2 Antenatal clinical administration of glucocorticoids

Over 30 years ago, Liggins (1969) demonstrated that lambs delivered prematurely (118-123 days of gestation) after fetal infusions of ACTH, cortisol or dexamethasone exhibited advanced alveolar stability in their lungs, and suggested that the maturational properties of glucocorticoids caused premature pulmonary development and maturation. Subsequently, Liggins & Howie (1972) were the first to propose the administration of maternal glucocorticoids to women at risk of preterm delivery to advance fetal lung maturation and reduce neonatal morbidity and mortality from respiratory disorders. In this study, women in premature labour at 24-34 weeks of gestation were admitted into the first controlled trial of antepartum glucocorticoid treatment for the prevention of respiratory distress syndrome (RDS) in premature infants (Liggins & Howie 1972). The administration protocol consisted of an intramuscular injection of either a mixture of 6mg of betamethasone phosphate and 6mg of betamethasone acetate or a control injection, followed by a second injection 24 hours later. In all premature infants, the incidence of RDS was reduced by 50% and neonatal death in the first 7 days of life was significantly reduced, although the maximum effects were seen if delivery occurred more than 24 hours and less than 7 days after treatment (Liggins & Howie 1972). Subsequently Ballard et al. (1975) demonstrated that after betamethasone administration, peak levels of betamethasone occurred within one hour in the maternal circulation and declined within 6 hours of administration. In cord blood, betamethasone was detected within one hour and declined within 14 hours. Therefore, the half-life of betamethasone was longer in the fetal circulation, although betamethasone was not detected in infants delivered 40 or more hours after the last injection (Ballard et al. 1975). Since then, it has been shown that synthetic glucocorticoids such as betamethasone and dexamethasone are 25-30
time more potent glucocorticoids than cortisol with insignificant mineralocorticoid action (Speight 1987). Furthermore, synthetic glucocorticoids do not bind to CBG (Pugeat et al. 1981) and are poor substrates for metabolism by placental 11βHSD2 (Siebe et al. 1993) making synthetic glucocorticoids prime candidates for clinical administration to women at risk of preterm labour.

Since the first report by Liggins & Howie (1972), multiple control trials have demonstrated a decrease in the number of cases of RDS and mortality among treated infants (Kari et al. 1994, Ballard & Ballard 1996, Anyaegbunam et al. 1997, Ee et al. 1998). The National Institutes of Health Consensus Developmental Conference on the Effects of Corticosteroid for Fetal Maturation came ~25 years after the first trial by Liggins & Howie and concluded that antenatal corticosteroid therapy for fetal lung maturation reduced mortality, RDS and intraventricular hemorrhage in preterm infants (NIH Consensus Conference 1995). According to the panel, corticosteroids should be administered between 24-34 weeks of gestation and in a treatment window of 24 hours to 7 days prior to delivery. However, due to the possibility of the efficacy of the drug decreasing after 7 days many clinicians repeat corticosteroid dosing every 7 days until delivery or fetal maturity (34 weeks). Recent surveys suggest that a high percentage of obstetricians (>85%) prescribe repeat doses and 50% of obstetricians prescribe weekly doses in cases of women who have a persisting risk of preterm delivery (Quinlivan et al. 1998, Brocklehurst et al. 1998). The mechanisms regulating the onset of preterm labour are poorly understood and preterm labour is therefore difficult to diagnose accurately. Since there is increasing evidence suggesting that excessive fetal glucocorticoid exposure may have long term consequences, it is worrying that women who are not in preterm labour may be receiving unnecessary corticosteroid administration. Many animal models have highlighted the potential adverse effects of fetal exposure to elevated glucocorticoids. Recent human evidence suggests that repeated fetal exposure to synthetic glucocorticoid has detrimental effects on birth outcome (Trautman et al. 1994, French et al. 1998). Some reports have therefore suggested that weekly doses of glucocorticoids may not be advisable (Ogueh & Johnson 2000, Smith et al. 2000, Whitelaw & Thorensen 2000). Antenatal administration of glucocorticoids on a repetitive basis however, continues, and therefore some fetuses are exposed to elevated levels of glucocorticoids during gestation. Whether this practice has long term effects on fetal growth and the development of adult disease is currently unknown.
1.4.4.3 Animals models of glucocorticoid administration

There is substantial evidence in animals demonstrating that fetal exposure to elevated levels of glucocorticoids alters fetal growth and has long term effects on cardiovascular, HPA and metabolic function. Early studies with rhesus monkeys demonstrated that maternal intramuscular betamethasone administration at 120-133 days of gestation (term=167 days) resulted in significant reductions in fetal body weight of ~23% at 133 and 167 days of gestation. In addition, brain, cerebellar, pancreatic, adrenal and pituitary weights were all significantly reduced with treatment (Johnson et al. 1981). Growth restriction has also been shown in rats and rabbits treated with maternal dexamethasone in the last few days of pregnancy (Price et al. 1992, Bakker et al. 1995, Levitt et al. 1996, Nyirenda et al. 1998, Thakur et al. 2000).

Both fetal cortisol infusion and maternal intramuscular administration of synthetic glucocorticoids impairs fetal growth (Fowden et al. 1996, Johnson et al. 1981, Bakker et al. 1995, Levitt et al. 1996). One limitation in many animal studies however, is that the amounts of glucocorticoids administered are much higher than that seen in a clinical setting and in some cases over long periods of time. More recently, studies in the sheep have demonstrated that clinically relevant doses of maternal betamethasone also cause significant fetal growth restriction (Jobe et al. 1998, Newnham et al. 1999, Sloboda et al. 2000; Chapter 3). Maternal betamethasone administration (0.5mg/kg) resulted in significant reductions in fetal weight at 125 and 146 days of gestation (term=150 days) (Jobe et al. 1998). Moreover, both a single injection at 104 days and multiple doses at 104, 111, 118 and 125 days of gestation resulted in a dose dependant decrease in fetal weight, where fetuses receiving multiple doses were the most growth restricted (Jobe et al. 1998). These alterations in whole body weight have been associated with significant reductions in whole brain and cerebellum weights (Huang et al. 1999).

Studies in fetal sheep evaluating the effects of synthetic glucocorticoid administration on fetal lung function hypothesized that direct fetal intramuscular injection of betamethasone may be the most effective mode of administration to induce pulmonary maturation (Berry et al. 1997). Not only did these studies find that both maternal and fetal betamethasone administration resulted in comparable increases in pulmonary development (Berry et al. 1997), but also found that direct fetal injection of betamethasone did not affect birthweight, placental weight or fetal organ weights (Newnham et al. 1999). However, repeated betamethasone administration to the ewe throughout midgestation, resulted in significant decreases in birthweight, placental weight, lung, brain, kidney and adrenal weights (Newnham et al. 1999) that persisted up to 3 months postnatally (Moss et al. 2000). These observations suggest that the route of administration of
synthetic glucocorticoids in the fetal sheep has differential effects on growth. However, the difference between fetal and maternal glucocorticoid administration on fetal endocrine development has not been examined.

Few studies have evaluated the mechanisms regulating fetal growth restriction following glucocorticoid administration, but those that have focus primarily on the role of the fetal IGF axis (Price et al. 1992, Li et al. 1993, 1998, Thakur et al. 2000). IGFs, their receptors and binding proteins are regulated by glucocorticoids in fetal tissues (Price et al. 1992, Li et al. 1993), and therefore provide a possible pathway for the glucocorticoid effects on fetal growth. Cortisol infusion in the fetal sheep significantly reduced IGFII mRNA levels in the fetal liver and skeletal muscle by 20-55% at 145 days of gestation (Li et al. 1993). In the rat, maternal dexamethasone administration resulted in a 32% decrease in fetal body weight in addition to significant reductions in fetal liver and lung weights (Price et al. 1992).

The mechanisms linking intrauterine growth restriction and metabolic function are poorly understood, although recent animal studies suggest that prenatal glucocorticoids may target specific organs such as the liver and the pancreas (Lindsay et al. 1996, Nyirenda et al. 1998, Hill 1999). Maternal treatment with carbenoxolone, a placental 11βHSD inhibitor, allows increased passage of maternal glucocorticoids to the fetus (Whorwood et al. 1993), and resulted in reduced birthweight (Lindsay et al. 1996), an effect similar to that observed with dexamethasone treatment (Price et al. 1992, Levitt et al. 1996, Nyirenda et al. 1998). Furthermore, adult male offspring demonstrated altered glucose tolerance indicated by higher fasting glucose levels and elevated glucose and insulin responses to a glucose challenge. Maternal adrenalectomy prevented this effect, supporting the role of fetal exposure to maternally derived glucocorticoids in the programming of metabolic function (Lindsay et al. 1996). Similarly, in the sheep maternal administration of dexamethasone early in pregnancy (40-14 days of gestation), resulted in elevated fetal basal and stimulated insulin levels at 135 days of gestation (Cox et al. 1999). Taken together, these data suggest that prenatal exposure to glucocorticoids can alter pancreatic function postnataally. Whether these effects persist throughout the life of the animal is unknown.

Glucocorticoid exposure in utero could also be a significant factor in the programming of the fetal pancreas and β cell development (Hill 1999). In addition to acting directly on ductal epithelial cells and β cell proliferation (Hill 1999, Hill & Duvillie 2000), glucocorticoids have been shown to regulate important transcription factors regulating pancreatic growth and remodeling, such as Pdx-1 (Sander et al. 1997, Hill et al. 1998). Moreover, glucocorticoids have been shown to regulate fetal IGFII (Li et al. 1993), an important growth factor regulating
pancreatic apoptosis and remodeling (Hill et al. 1998, Petrik et al. 1998, Hill 1999). It is presently unknown if fetal glucocorticoid exposure permanently alters pancreatic apoptosis and neogenesis in such a way as to produce postnatal metabolic alterations.

Another key target for glucocorticoid programming may be the fetal liver, where glucocorticoids regulate several enzymes involved in carbohydrate and fat metabolism, including PEPCK (Pilkis & Granner 1992). Dexamethasone administration to pregnant rats increased PEPCK mRNA and activity levels in the periportal zone of offspring, an effect that persisted up to 8 months of postnatal age (Nyirenda et al. 1998). These rats demonstrated a significant reduction in birthweight as well as fasting hyperglycemia and elevated glucose and insulin responses to glucose loading. Recent data suggest that significant elevations in GR mRNA expression in the periportal zone may underlie the programming of PEPCK activity and hyperglycemia in this animal model (Nyirenda et al. 1998). Furthermore, the actions of glucocorticoids in the liver are modulated by 11βHSD1, which can amplify intrahepatic glucocorticoid action by catalyzing the conversion of inactive cortisone to active cortisol (Jamieson et al. 1995). Transgenic mice deficient of 11βHSD1, demonstrate decreased levels of gluconeogenic enzymes (Kotelevtsev et al. 1997) supporting the role of hepatic 11βHSD1 in regulating local glucocorticoid concentration levels. Although maternal dexamethasone administration did not alter 11βHSD1 mRNA in offspring of rats (Nyirenda et al. 1998), fetal growth restriction in sheep as a result of placental restriction, results in significant elevations in hepatic 11βHSD1 mRNA levels (McMillen et al. 2000), potentially increasing local glucocorticoid action in the fetal liver.

The potential impact of fetal glucocorticoid exposure on the developing HPA axis may occur via the GR, which are expressed at every level of the axis. Therefore, synthetic glucocorticoids can potentially impact at the level of the brain, hypothalamus, pituitary and/or the adrenal. In the fetal sheep, maternal administration of dexamethasone early in gestation (40-41 days) resulted in significant elevations in basal ACTH and cortisol levels in fetuses at 131 days of gestation, in addition to elevated ACTH responses to an exogenous CRH challenge (Cox et al. 1999). Whether these effects persist postnatally in these animals is unknown. Remarkably little is known regarding the mechanisms that regulate alterations in HPA function following maternal glucocorticoid administration and their relationship to postnatal disease. In most models, programming of the HPA axis has been associated with alterations in hippocampal corticosteroid receptor populations (Levitt et al. 1996, Dean & Matthews 1999, Uno et al. 1994, McCabe et al. 2001, Liu et al. 2001). Negative feedback at the level of the hippocampus results

In the rat, maternal dexamethasone administration (100µg/kg/day) over the last few days of pregnancy resulted in a reduction in birthweight of ~11% and male offspring demonstrated significantly elevated levels of basal corticosterone and elevated blood pressure (Levitt et al. 1996). Furthermore, prenatal dexamethasone treatment resulted in a significant decrease in GR and MR mRNA levels in specific hippocampal subfields; supporting the hypothesis that negative feedback is attenuated in these animals (Levitt et al. 1996). This is supported by observations made by Benediktsson et al. (1993) in dexamethasone treated rats whose male and female offspring demonstrated a significant reduction in birthweight and significant elevations in systolic blood pressure. These effects were associated with decreased placental 11βHSD2 activity (Benediktsson et al. 1993). Administration of lower doses of maternal dexamethasone on day 17 and 19 of pregnancy in the rat significantly decreased birthweight, did not alter basal HPA function but significantly decreased the ratio of CRH to AVP in the median eminence in offspring at 20 days postnatal age (Bakker et al. 1995). This may reflect subtle long term effects on HPA regulation at the level of the hypothalamus.

Maternal administration of dexamethasone in the rhesus monkey at 132 and 133 days of gestation (term=165) resulted in significant alterations in the cytoarchitectural development of hippocampal neurons at 135 days of gestation (Uno et al. 1990). Degeneration of neurons and a significant reduction in the size of the whole hippocampal formation were observed in dexamethasone treated fetuses at 135 and 162 days of gestation. Those fetuses that received multiple injections showed more severe damage, suggesting that these effects were dose dependant (Uno et al. 1990). Furthermore, at 10 months of postnatal age, dexamethasone treated offspring demonstrated higher basal cortisol levels and higher plasma cortisol levels following stress (Uno et al. 1994). Moreover, maternal dexamethasone treatment in the guinea pig on days 50-51 days of gestation (term=70 days) resulted in significant increases in basal cortisol in female fetuses but not male fetuses. Furthermore, dexamethasone exposure resulted in significant increases in MR and GR mRNA in the hippocampus of female fetuses but not in males (Dean & Matthews 1999). The discrepancy between this study and others that report hippocampal receptor downregulation may be due to species differences and/or timing and dose of
glucocorticoid administration. Very recent studies have shown that repetitive synthetic glucocorticoid administration to the pregnant guinea pig results in a decrease in fetal cortisol concentrations 24 hours following the final glucocorticoid treatment (McCabe et al. 2001). This was associated with a significant reduction in CRH mRNA in the PVN of both male and female fetuses. Glucocorticoid treatment significantly increased hippocampal MR mRNA expression in the female fetuses with little effect on GR mRNA expression. It was suggested that a reduction in endogenous cortisol in these animals resulted in alterations in MR expression (McCabe et al. 2001). Lower basal and stimulated cortisol levels have been shown in male offspring treated prenatally with repeated glucocorticoids (Liu et al. 2001), although female offspring demonstrated significantly higher basal and stimulated cortisol levels. These observations suggest that synthetic glucocorticoid exposure may result in modifications in GR/MR populations in the hippocampus of developing fetuses that are sex specific and could lead to alterations in HPA development in a way that persists into postnatal life.
CHAPTER 2

2 Rationale and Hypotheses

2.1 RATIONALE

It has become increasingly evident that fetal exposure to elevated levels of glucocorticoids may be an important link between fetal growth restriction and an increased risk of developing adult disease. In humans, low birthweight has been correlated with an increased risk of developing coronary artery disease, hypertension, glucose intolerance and insulin resistance later in life (Barker 1995, 1998, Seckl 1997). Prenatal exposure to glucocorticoids restricts fetal growth in humans (Reinisch et al. 1978) and growth restricted infants demonstrate elevated levels of ACTH and cortisol in cord blood (Goland et al. 1993). Furthermore, elevated fasting cortisol levels in adult men were related to increases in systolic blood pressure, glucose intolerance and insulin resistance (Phillips et al. 1998). More recently, low birth weight was associated with higher blood pressure, glucose levels, and elevated basal and ACTH stimulated cortisol levels in a young nonobese population that failed to demonstrate catch up growth (Levitt et al. 2000). It has therefore been proposed that the resetting of hormonal axes controlling growth and development could be responsible for the intrauterine programming of adult disease (Barker et al. 1993, Seckl 1997).

Animal studies have shown that prenatal exposure to endogenous and exogenous glucocorticoids produce permanent effects on cardiovascular, HPA and metabolic function in offspring (Levitt et al. 1996, Lindsay et al. 1996, Langley-Evans 1997, Seckl 2000). Alterations in HPA and metabolic function have been associated with changes in hippocampal corticosteroid receptor populations and alterations in glucocorticoid sensitive gluconeogenic enzymes in the liver (Barbazanges et al. 1996, Desai et al. 1997, Nyirenda et al. 1998, Lingas et al. 1999). Many studies however have employed high doses of glucocorticoids that may not have physiological relevance or correspond to current clinical practice. Given the importance of prenatal administration of synthetic glucocorticoids in obstetrical practice, further investigation into both the effects of clinical doses and the effects of single versus multiple doses is essential. Studies have suggested the existence of critical windows of development where glucocorticoid exposure may have permanent effects (Uno et al. 1994, Dodic et al. 1997, Seckl 1997). Furthermore, given the importance of glucocorticoids in the stress response, the fetus may be exposed to elevated levels of endogenous glucocorticoids under a number of circumstances (maternal stress, hypoxia, undernutrition). There is relatively little information regarding the effects of low dose...
glucocorticoids in utero and whether permanent long term effects are evident. Presently, data from human studies are conflicting and do not address the mechanisms surrounding alterations in growth and long term endocrine function. Since fetal growth restriction is associated with the programming of adult disease, further investigation is required to understand the relationship between growth restriction and endocrine function.

Maternal synthetic glucocorticoid administration alters fetal growth in a manner that persists postnatally, but fetal administration does not (Jobe et al. 1998, Newnham et al. 1999, Moss et al. 2000). However, fetal glucocorticoid administration results in fetal pulmonary maturation (Ikegami et al. 1996, Berry et al. 1997), therefore fetal administration of glucocorticoids can affect intrauterine development. The effect of fetal glucocorticoid administration on fetal and/or postnatal HPA and metabolic function has not been examined. Since maternal and fetal glucocorticoid administration has differential effects on fetal growth, it is possible that endocrine programming may be differentially regulated. Clearly, this method of glucocorticoid administration warrants further investigation.

2.2 HYPOTHESES AND AIMS

This thesis will explore the following hypotheses;

1. Multiple doses of betamethasone administered to the pregnant sheep will result in fetal growth restriction and an increase in fetal HPA activity that is reflective of alterations in hypothalamic and pituitary neuropeptides and glucocorticoid receptor populations. Specifically, the aims of this chapter were to examine fetal ACTH, cortisol and CBC concentrations following maternal administration of betamethasone, and to examine potential alterations in fetal pituitary POMC, GR and processing enzymes in addition to hypothalamic releasing peptides (CRH, AVP, OT, PENK) and GR levels (Chapter 3).

2. Multiple doses of betamethasone administered to the pregnant sheep will result in alterations in fetal glucose and insulin levels that may be associated with alterations in fetal pancreatic remodeling; more specifically alterations in pancreatic β cell apoptosis and insulin content. In addition, maternal betamethasone administration will result in elevations in hepatic 11βHSD1, GR and CBG expression thereby contributing to elevated levels of local hepatic and circulating cortisol. The specific aims of this chapter were to examine fetal glucose and insulin concentrations as well as fetal pancreatic ir-insulin staining and content following maternal betamethasone administration. In addition, fetal
apoptotic staining in the fetal pancreas was examined. An additional aim was to examine fetal hepatic 11βHSD, CBG and GR expression levels (Chapter 4).

3. Based on previous studies that demonstrated a differential effect on growth, maternal and fetal betamethasone administration may elicit a differential effect on postnatal ACTH and cortisol responses to exogenous CRH+AVP administration. It was hypothesized that maternal glucocorticoid administration will result in elevations in plasma ACTH and cortisol levels in postnatal lambs. This effect would be greater in those animals that receive multiple prenatal doses. Fetal betamethasone administration however, may have different effects on postnatal HPA function as measured by CRH+AVP challenge that may be dependant upon dose. The specific aims of this chapter were to examine postnatal basal and stimulated ACTH and cortisol levels following CRH+AVP infusion in lambs treated prenatally with betamethasone comparing the effects of fetal and maternal glucocorticoid administration (Chapter 5).

4. Maternal and fetal betamethasone administration may elicit a differential effect on postnatal glucose and insulin responses to exogenous glucose administration. Maternal glucocorticoid administration will result in elevations in plasma glucose and insulin levels in postnatal lambs and this effect will be enhanced in those animals treated with multiple prenatal doses. Fetal betamethasone administration may result in different responses in postnatal glucose and insulin responses to an exogenous glucose load. The specific aims of this chapter were to examine postnatal basal and stimulated glucose and insulin levels following glucose infusion in lambs treated prenatally with betamethasone comparing the effects of fetal and maternal glucocorticoid administration (Chapter 6).
3 Effects of Repeated Maternal Betamethasone Administration on Growth and Hypothalamic-Pituitary-Adrenal Function of the Ovine Fetus

3.1 INTRODUCTION

Liggins and Howie (1972) first suggested the potential of administering synthetic glucocorticoids to women at risk of early preterm birth to promote fetal lung maturity and prevent the development of neonatal respiratory disease. Recent practice has included repeated administration of synthetic glucocorticoids at weekly intervals to women at risk of early preterm labour in whom delivery has not occurred. However, diagnosis of preterm birth is difficult and women not at risk may be receiving antenatal glucocorticoids unnecessarily (Ballard et al. 1995).

Exposure of the fetus to elevated levels of corticosteroids has shown to be detrimental to fetal development. Various studies in animals have shown reductions in weight at birth (Jobe et al. 1998), alterations in organ (Johnson et al. 1981) and neuronal development (Uno et al. 1990, 1994, Dunlop et al. 1997) and increases in postnatal basal and stress-induced plasma cortisol levels (Uno et al. 1974) following prenatal glucocorticoid administration. These latter observations suggest that chronic fetal glucocorticoid exposure may alter the development of the hypothalamic-pituitary adrenal (HPA) axis in utero. In the human, a reduction in birth weight and neonatal head circumference was related to an increasing number of maternal corticosteroid courses (French et al. 1999). Furthermore, an increased incidence of death from cardiovascular disease has been associated with a reduced head circumference, ponderal index and birth weight in man (Barker et al. 1993), potentially linking fetal exposure to elevated glucocorticoids with increased risk of adult disease later in life.

Cortisol is essential for normal growth and development of the fetus. A late gestational rise in endogenous plasma cortisol occurs in fetuses of many species and is responsible for prenatal enzyme activation within target tissues, necessary for tissue and organ maturation (Liggins 1994). The regulation of fetal HPA function and adrenal cortisol output is multifactorial. Corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) are the primary stimulators of corticotrophs within the pars distalis (anterior lobe) of the pituitary that synthesize the polypeptide precursor, proopiomelanocortin (POMC) and secrete adrenocorticotropin (ACTH) (Antolovich et al. 1991). Two prohormone convertase enzymes, prohormone convertase 1 (PC1) and prohormone convertase 2 (PC2), cleave POMC to generate
ACTH and related peptides (Eipper & Mains 1980). In the fetal pituitary both the pars distalis and the pars intermedia (intermediate lobe) express PC1 mRNA whereas PC2 mRNA is highly localized to the pars intermedia (Bell et al. 1998). The pars distalis is therefore the main site of synthesis and secretion of ACTH, whereas the pars intermedia preferentially secretes smaller molecular weight peptides such as α-melanocyte stimulating hormone (α-MSH). Other hypothalamic neuropeptides such as oxytocin (OT) (Matthews 1999, Kemppainen et al. 1993) and Met-enkephalin (Brooks & Challis 1988, Matthews & Challis 1995) can also influence corticotroph activity. Oxytocin has been shown to stimulate ACTH output in cultured ovine fetal pituitary cells, and to synergize with CRH to stimulate ACTH output (Matthews et al. 1999). Furthermore, it has been suggested that in the fetal sheep, pre-proenkephalin (PENK) mRNA is negatively regulated by endogenous glucocorticoids, and that hypothalamic Met-enkephalin may control CRH secretion at the median eminence (Matthews & Challis 1995), potentially regulating HPA axis activity.

Cortisol regulates its own synthesis and secretion through binding to type II corticosteroid receptors (glucocorticoid receptor, GR) initiating negative feedback at the level of the pituitary, hypothalamus and hippocampus (Matthews & Challis 1995, Yang et al. 1990, Meijer et al. 1998). Cortisol has been shown to decrease POMC mRNA levels and pituitary CRH receptors both in vivo and in vitro (Lu et al. 1994, Matthews & Challis 1995, Zhou et al. 1996), and decrease basal CRH release in perifused hypothalamic neurons (Brooks et al. 1989). The hippocampus tonically inhibits HPA axis activity and is regulated by glucocorticoids, which bind to both type 1 (mineralocorticoid receptors, MR) and type 2 (GR) corticosteroid receptors (Feldman & Conforti 1980, Jacobson & Sapolsky 1991).

Despite the potential for negative feedback late in gestation in the fetal sheep, plasma ACTH and cortisol levels rise concurrently until birth occurs (Norman et al. 1985). This apparent paradox may be explained in part, by corresponding increases in circulating plasma corticosteroid binding globulin (CBG). CBG, primarily synthesized in the fetal liver (Berdusco et al. 1995), is a high affinity binding protein for cortisol and maintains a relatively low free cortisol concentration potentially reducing the impact of cortisol negative feedback on pituitary ACTH output (Ballard et al. 1982, Berdusco et al. 1995).

Since cortisol plays a pivotal role in fetal growth and development, it is therefore essential to regulate strictly circulating glucocorticoid levels in the fetus. Although the effects of glucocorticoid exposure on the fetus have been well documented, very few studies have evaluated either the effects of clinically relevant glucocorticoid doses or replicated the method of
glucocorticoid administration used in human obstetrical practice. The long-term effects of maternally administered synthetic glucocorticoids on fetal endocrine function have not yet been ascertained. In addition, there is little information describing the mechanisms underlying either the change in fetal growth or the reported changes in fetal and neonatal endocrine function following antenatal glucocorticoid exposure. Therefore, this study set out to determine the effects of repeated maternal glucocorticoid administration at mid gestation on fetal growth and on the development of the HPA axis in fetal sheep. It was hypothesized that repeated fetal exposure to synthetic glucocorticoids at midgestation would decrease fetal weight and alter gene expression of key neuropeptides and receptors regulating fetal HPA axis function thereby altering the fetal HPA endocrine profile.

3.2 MATERIALS AND METHODS

3.2.1 Animals
Merino ewes were mated and singleton pregnancy was confirmed using ultrasound examination at 42 days of gestation (term 150 days). The sheep were transported to a research station at 90 days of gestation and were kept in pastures, to graze in a field environment.

3.2.2 Experimental Procedures
Pregnant ewes received an intramuscular injection of 150mg medroxyprogesterone acetate (MPA, Depo Provera™, Upjohn, Rydalmere, NSW, Australia) at 100 days of gestation to reduce pregnancy losses due to glucocorticoid treatment. It has been previously shown that this treatment does not affect the fetal endocrine status (Nathanielsz et al. 1988). Pregnant sheep (n=37) were randomized into either control or treatment groups (at 125 or 146 day groups). Animals in the treatment group received intramuscular injections of 0.5mg/kg maternal weight of betamethasone (Celestone Chronodose™, Schering Plough, Baulkham Hills, NSW, Australia) at 104, 111 and 118 days of gestation. Control animals received saline injections at the same time points. The total dose of betamethasone was between 25-30mg, which correlates closely with clinical doses used for fetal lung maturation in women at risk of early preterm birth (Liggins & Howie 1972). This dose has been shown previously to improve lung function in fetal sheep (Ikegami et al. 1997). Dr. J. Newnham (University of Western Australia) was responsible for the ultrasound examinations and the injection protocol in this study.

On the evening prior to injections and later delivery, sheep were brought into a nearby indoor facility. Ewes were sedated with maternal intramuscular ketamine (15mg/kg) and xylazine (0.1mg/kg, Troy Laboratories, Smithfield, NSW, Australia) and spinal anaesthesia was
induced by injection of 3-4ml of lidocaine (2%). The fetus was delivered through a midline hystero-tomy and arterial blood samples were collected from the umbilical cord and from the maternal femoral vessels, following which the fetus was killed by a lethal dose of pentobarbital (30mg/kg). Cord blood gases (PO2, PCO2, and pH) were measured and samples were centrifuged at 2200g for 10 minutes and the plasma was stored at -20⁰C until further analysis. At 125 days of gestation, 22 animals were sacrificed and fetal weights recorded (control n=10, and betamethasone n=12). Tissues from 11 of these animals were collected for further analyses (control n=5, and betamethasone n=6). At 146 days of gestation, control (n=7) and betamethasone (n=8) treated animals were sacrificed, fetal weights recorded and tissue collected for further analyses. Fetal hypothalamic blocks and pituitaries from all groups were slow frozen on dry ice for *in situ* hybridization and immunohistochemistry (Matthews et al. 1994). The protocols were approved by the Institutional Ethics Committees of the Western Australian Department of Agriculture and the Animal Care Committee of the University of Toronto, according to the guidelines of the Canadian Council for Animal Care.

### 3.2.3 Radioimmunoassays (RIA)

#### 3.2.3.1 Plasma ACTH

Cord plasma (100µl) immunoreactive (ir)-ACTH concentrations were measured by a commercial radioimmunoassay (RIA) kit (Incstar, Stillwater, MN) previously validated for use in the fetal sheep (Norman et al. 1985, Jeffray et al. 1998). All samples were analyzed in a single assay. The intra-assay coefficient of variation was 15%. The following formula was used to calculate the intra-assay coefficient of variation (CV):

\[
\text{intra-assay CV} = \frac{\text{sd}}{\text{mean}}
\]

where \( \text{sd} \) = the standard deviation between quality control samples within one assay, \( \text{mean} \) = the mean concentration of hormone \( x \) in the quality control samples within one assay.

The mean assay sensitivity was 6.5 pg/ml. The ACTH antibody cross-reacts <0.01% with α-MSH, γ-MSH, β-endorphin, β-lipotropin (LPH) and CLIP (Incstar, Stillwater, MN).

#### 3.2.3.2 Plasma Cortisol

Plasma cortisol concentrations were quantified by RIA (as described previously (Challis et al. 1981, Jeffray et al. 1998). Plasma extraction: Plasma samples (100µl) were pipetted into
16x125mm test tubes and extracted with 4ml of diethyl ether. The tubes were vortexed and then shaken in a mechanical shaker for 1 hour. The aqueous phase was separated from the organic phase by freezing (acetone and dry ice). The organic phase was decanted into 12x75mm test tubes, dried under air and reconstituted with 1ml of phosphate buffered saline and gelatin (PBSG; 0.4M NaH2PO4-H2O monobasic, 0.6M Na2HPO4-7H2O dibasic, 0.15M NaCl, 0.015M NaN3 and 0.1% w/v gelatin; pH 7.0).

Aliquots of the extracted plasma (100μl and 400μl) were pipetted into 12x75mm borosilicate assay tubes and incubated overnight with rabbit anti-cortisol antiserum (100μl, final dilution of 1:30 000, titrated to give a B/Bo of approximately 40%) and purified [3H] cortisol (100μl; 10 000 – 15 000cpm; Amersham Life Sciences, USA). The antibody was generated in this laboratory and cross-reactivities are as follows; cortisone <0.1%; 21-deoxycortisone <0.1%; 11-deoxycortisol 1.60%; progesterone <0.1%; 17-α hydroxyprogrenenolone <0.1%; 11 β-hydroxyprogesterone 0.20% and pregnane diol <0.1%. The bound and free cortisol were separated using dextran-coated charcoal (0.625% W/V dextran; [Dextran T70, Pharmacia Fine Chemicals, Baie, D'Ufe, PQ] 6.25% w/v charcoal [Norit A, Fisher Scientific, Toronto, Canada]) (centrifuged at 1500g for 10min., 4°C), and the supernatant counted in a β scintillation counter (Tri-Carb 2100TR liquid scintillation analyzer, Packard Instrument Co., Meriden, CT). All samples were analysed in a single assay. The intra-assay coefficient of variation was 5%.

3.2.3.3 [3H]Cortisol Purification

[1,2,6,7-3H] cortisol (50μl; specific activity 1mCi/ml, Amersham Life Sciences, USA) was separated from free [3H] and breakdown products of labelled steroid by thin layer chromatography (TLC). A TLC plate was placed in a glass incubation chamber containing 100ml of chloroform:ethanol (90:10) and the solvent front was permitted to advance to the top of the plate. After air-drying, [1,2,6,7-3H] cortisol and non-labeled cortisol (20μl; 1mg/ml) were applied to the plate ~5cm from the bottom on opposite sides of the plate. The TLC plate was returned to the chamber and the solvent front permitted to advance to the top of the plate. The non-labeled cortisol was visualized using UV light and the corresponding area associated with [1,2,6,7-3H] cortisol was scraped off the plate into a 12x75mm tube, mixed with 4ml of ethyl acetate, centrifuged (1500g for 10min.), and the supernatant collected. This was repeated with the precipitate and pooled with the first supernatant. Specific activity of the purified [1,2,6,7-3H] cortisol was determined by counting 1μl in a β scintillation counter.
3.2.3.4 Corticosteroid Binding Capacity

Plasma corticosteroid binding globulin (CBG) levels were measured as corticosteroid binding capacity (CBC), determined using the saturation binding assay of Ballard *et al.* (1982) with modifications described previously (Challis *et al.* 1985). Duplicate aliquots of 50μl of plasma were added to 10x75mm tubes containing [3H]cortisol (10 000cpm) and non-labelled cortisol (16ng) (binding tubes; B). Additional duplicates were set up containing [3H]cortisol (10 000 – 15 000cpm) in the presence of an excess of non labelled cortisol (1μg) (non-specific binding tubes; NSB). Tubes were incubated at 37°C for 30 min. then overnight at 4°C. Bound and free cortisol were separated using dextran coated charcoal (Norit A) (90μl 0.625% w/v dextran, 6.25% charcoal) in tricine buffer (0.15M tricine, pH 7.4). Tubes were centrifuged at 1700g for 10min. at 4°C, and 100μl of the supernatant was added to 4ml of scintillation fluid and counted in a β scintillation counter. CBC was calculated as:

\[
\text{Binding (B)} = \%B - \%\text{NSB}
\]

\[
\text{Mass in 50 μl (M)} = 16\text{ng} + F + ^3\text{H} F
\]

where \( F \) = endogenous cortisol measured in the cord plasma with cortisol RIA

\[
\text{Capacity ng/ml (CBC)} = M \times B/100
\]

The assay does not measure cortisol binding to albumin, since this complex dissociates rapidly after the addition of charcoal (Ballard *et al.* 1982). All samples were analyzed in a single assay. The intra-assay coefficient of variation was 3%.

3.2.3.5 Plasma α-MSH

Cord plasma α-MSH (100μl) was quantified using a commercial RIA kit (Euro-Diagnostics, Malmo, Sweden), previously validated for use in fetal sheep (Jeffray 1999). The α-MSH antibody cross reacts 100% with α-MSH and des acetyl-α-MSH, and <0.002% with des-amido-α-MSH, ACTH1-13, ACTH1-24, ACTH1-39, β-MSH, and γ-MSH. All samples were analysed in a single assay. The sensitivity of the assay is 7.6pg/ml and intra-assay coefficient of variation was 9%.

3.2.3.6 Plasma estradiol

Plasma samples (500μl) were extracted as described previously. Duplicate aliquots of 100μl were pipetted into 12x75mm borosilicate assay tubes and incubated overnight with rabbit anti-estradiol antisemur (100μl, final dilution of 1:5000, titrated to give a B/B₀ of approximately
40%) and [2, 4, 6, 7-^3H]estradiol (100µl; 10 000 - 15 000 cpm; NEN Life Sciences). The antibody was generated in this laboratory and the characteristics and assay validation for measurements in the sheep have been described previously (Challis et al. 1985). Bound and free estradiol was separated using dextran-coated charcoal, centrifuged at 1500g for 10 min. at 4°C. The supernatant was removed and counted in a β scintillation counter. All samples were analyzed in a single assay. The intra-assay coefficient of variation was 15%.

3.2.3.7 Plasma progesterone

Plasma samples (100µl) were extracted as described previously. Aliquots of 100µl were pipetted into 12x75mm borosilicate assay tubes and incubated overnight with rabbit anti-progesterone antiserum (100µl, final dilution of 1: 8000; Endocrine Sciences, RIA Reagents, CA, titrated to give a B/Bo of approximately 40%) and [1,2,6,7-^3H] progesterone (100µl; 10 000 - 15 000cpm; NEN Life Science Products). The cross reactivities of this antibody are as follows; <0.1% pregnenolone; <0.1% 17-hydroxypregnenolone; <0.1% estradiol, <0.1% estriol; <0.1% cortisol, <0.1% cortisone; <0.1% dehydroepiandrosterone, <0.1% androstenedione. This antibody has been validated previously (Gyomorey et al. 2000). All samples were analyzed in a single assay. The intra-assay coefficient of variation was 15%.

3.2.4 146-day pituitary ACTH protein content

3.2.4.1 Tissue Preparation

The pars distalis was separated from the pars intermedia in those frozen pituitaries that remained after cryosectioning for in situ hybridisation. Each pars distalis was individually weighed and homogenized (PT200 Homogeniser, 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. 1981). Samples were centrifuged at 1775g for 10 minutes at 4°C and the supernatant stored at -80°C until further analysis.

3.2.4.2 Protein Assay

Protein concentration of each sample was quantified prior to ir-ACTH determination using the Bradford assay (Bradford 1976). A protein standard curve (range 2.5 µg/ml – 25 µg/ml standard) was set up using a 1mg/ml bovine serum albumin (BSA) protein standard stock diluted in Bio-Rad assay dye reagent (Bio-Rad, Richmond, CA, USA). Tissue samples were also diluted in Bio-Rad protein assay dye (1µl sample/ml of dye). Standards and samples were prepared in duplicate and absorbance was measured at 595nm wavelength using a spectrophotometer (Model
DU-64, Beckman Instruments, Inc. CA, USA). Samples were quantified by linear regression analysis using a standard curve derived from the absorbance and concentration values of the standards.

3.2.4.3 Measurement of ir-ACTH
Ir-ACTH levels were analysed in pars distalis homogenates (10µl; titrated to give B/Bo of approximately 40%) using a commercial RIA (Incstar, Stillwater, MN) and ACTH1-39 levels were expressed per mg of protein. Intra-assay coefficient of variation for ACTH assay was 8% and all samples were analysed in a single assay.

3.2.5 In situ hybridization of fetal pituitaries and hypothalami.

3.2.5.1 Tissue Preparation
Frozen pituitaries and hypothalami were sectioned (12µm coronal sections) using a cryostat (Tissue-Tek, Miles Canada, Etobicoke, Canada) and mounted onto poly-L-lysine (Sigma Chemical, St. Louis, USA)-coated slides, fixed in 4% paraformaldehyde for 5 min, rinsed in phosphate buffered saline (PBS; NaCl, KCl, Na2HPO4, KH2PO4; 2 x 1 min), dehydrated in an alcohol series and stored in 95% ethanol at 4°C until hybridization.

3.2.5.2 Antisense Oligonucleotide Probes
Pituitary sections were incubated with α-35S-labeled, 45 mer oligonucleotide antisense probes. In the pituitary, antisense probes were complementary to bases 711-756 of the porcine POMC gene (Gossard et al. 1986); bases 231-275 of the porcine PC1 gene (Dai et al. 1995); 153-197 of the porcine PC2 gene (Seidah et al. 1992); and bases 146-191 of the ovine GR gene (Yang et al. 1992). Hypothalamic sections were incubated with α-35S-labeled, 45 mer oligonucleotide antisense probes complementary to bases 503-547 of the ovine CRH gene (Matthews et al. 1991); bases 146-191 of the GR gene (Yang et al. 1992); bases 1-45 of the bovine pre-proenkephalin (PENK) gene (Noda et al. 1982) and to bases 771-816 of the ovine oxytocin-neurophysin gene (oxytocin; OT) (Ivell et al. 1990). All probes have been characterized and used previously (Matthews et al. 1991, 1993, Broad et al. 1993, Matthews & Challis 1995, Jeffray et al. 1998).

3.2.5.3 Probe labelling
Each oligonucleotide probe (1µl of 10ng/ml), tailing buffer (2µl) and DEPC-treated water (1%w/v diethyl-pyrocarbonate (DEPC) in distilled water and autoclaved) (7.5µl) were combined
and mixed in an eppendorf tube. \(^{35}\)S-deoxyadenosine 5'-\((\alpha\text{-thio})\) triphosphate (dTTP; 1\(\mu\)l, 1300 Ci/mmol; Comp) and terminal deoxynucleotidyl transferase (TdT; 1.5\(\mu\)l, Pharmacia, LKB) were added, mixed and incubated at 37°C for 50 minutes. The reaction was terminated with the addition of DEPC-treated water (40\(\mu\)l). The labelled probe and unincorporated fraction were separated using micro-columns that had been centrifuged (ProbeQuant G-50 Micro Columns, AmershamPharmacia, Biotech Inc., NJ) for 2min. at 1700g. The reaction mixture was placed into the columns and centrifuged again for 2min. at 1700g. The labelled probe was collected in an eppendorf tube and mixed with 1M DL-dithiothreitol (DTT; 2\(\mu\)l; Sigma Chemical Co., St. Louis, MO). An aliquot (1\(\mu\)l) was counted in a \(\beta\) scintillation counter to determine specific activity (minimum of 100 000 cpm/\(\mu\)l).

### 3.2.5.4 Hybridization

Slides were removed from 95% ethanol, air dried, placed in incubation chambers and incubated overnight (42°C) with hybridization buffer containing the labeled probe (1-1.5 \(	imes\) 10\(^5\) cpm/100\(\mu\)l). Hybridization buffer: 50% deionized formamide, 4X saline sodium citrate (SSC), 0.01M sodium pyrophosphate, 5X Denhardt's solution, 0.02% w/v salmon sperm DNA, 0.01% w/v polyadenylic acid, 0.01% w/v heparin, 10% w/v dextran sulphate. Parafilm covering of each section was used to prevent drying of slides. Moisture levels in the chambers were maintained by the addition of moistened kimwipes (50% formimide, 4X SSC, and DEPC-water). Incubation allowed for the hybridization of the radiolabeled probe to specific complementary RNA sequences contained in the tissue sections.

### 3.2.5.5 Wash

Slides were washed (1X SSC, 0.5% \(\beta\)-mercaptoethanol) for 30 minutes at room temperature, followed by a 1hour wash at 55°C, and a final rinse in each of 1X SSC, 0.1X SSC, and 70% and 90% ethanol.

### 3.2.5.6 Image Analysis

All slides were exposed together with \(^{14}\)C standards (American Radiochemical) against x-ray film (Biomax, Eastman Kodak Co., Rochester, NY) to ensure analysis within the linear range of the autoradiographic film. Cassettes were stored at room temperature undisturbed for the appropriate length of time dependant on each probe. The relative optical density (ROD) of the signal on the film of 9-14 sections per tissue was quantified using a computerized image analysis system (Imaging Research, St. Catharines, Canada). Values represent an average density over the
area measured, after background values were subtracted. Control slides were incubated with α-
$^{35}$S-labeled, 45 base pair oligonucleotide random sequences, which did not correspond to the
antisense probes. No signal was observed in these slides. All control and experimental sections
were processed together to allow direct comparisons between groups. At 146 days of gestation
pituitary POMC mRNA was distributed regionally, therefore analysis of the superior region
(region around the pars intermedia) and the inferior region (region at the base of the pars
distalis) was performed separately, in addition to analysis of the entire pars distalis. For
hypothalamic sections, values represent total paraventricular signal.

3.2.6 Immunohistochemistry
Immunohistochemical detection of ir-ACTH was performed on adjacent 12 μm frozen pituitary
sections at 146 days of gestation prepared as above for in situ hybridization. Sections were
rehydrated in series (100%, 90%, 70%, 50% ethanol), 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 using a commercial kit (Vectastain ABC
Kit, Vector Laboratories, California, USA) as described previously (Jacobs et al. 1991, Jeffray et
al. 1998). Non-specific staining was blocked using normal goat serum (NGS; 0.15% v/v, 20min).
Sections were incubated overnight at 4°C in a moist chamber with a polyclonal antibody to
human ACTH$_{1-24}$ (Dako, Carpinteria, USA). Adjacent sections were incubated with the primary
antibody in the presence of excess antigen (human ACTH$_{1-24}$) to provide negative controls.
Sections were washed with 0.1M PBS and incubated with a biotinylated second antibody (0.05%
v/v in 0.1M PBS with 0.15% NGS) for 2 hours at room temperature. Following a wash in 0.1M
PBS, slides were then incubated with Vectastain ABC (Vectastain ABC Kit, Vector
Laboratories, California, USA) for 2 hours at room temperature. Following a wash in 0.1M PBS,
immunostaining was visualized with the addition of the chromagen 3,3'-diamobenzidiné
(SIGMAFAST, Sigma Chemical Co. St. Louis, MO), followed by a final tap water wash. Slides
were counter stained with Carazzi's hemotoxylin (30sec), placed under a running water wash,
dehydrated in an ethanol series (50%, 70%, 90%, 100%) and cover slips mounted using Permunt
(Fisher Scientific Ltd., Nepean, Canada). This antibody has been previously characterized for use
in fetal sheep (Jacobs et al. 1991, Jeffray et al. 1998), where the antigenic site was shown to be
between amino acids 18-24. The antibody crossreacts <1% with α-MSH, β-MSH, βLPH (Jacobs
3.2.7 Statistical Analysis
Changes in fetal weight were analyzed using the Student’s t-test for each group (125 days and 146 days) separately. Changes in cord plasma pH, PO$_2$, PCO$_2$, ir-ACTH and CBG, and maternal plasma estradiol at both 125 and 146 days of gestation were analyzed using the Student’s t-test. Pituitary POMC, GR, PC1 and PC2 mRNA, and hypothalamic CRH, AVP, GR, OT and PENK mRNA were expressed as relative optical density (ROD) and analyzed using the Student’s t-test. The content of ir-ACTH in the *pars distalis* tissue at 146 days, cord plasma cortisol, cord plasma α-MSH and maternal progesterone at 125 and 146 days of gestation were not normally distributed and were analyzed using the Mann Whitney Rank Sum test. Statistical significance was determined as *P*<0.05. All values are presented as mean ± standard error (SEM) (SigmaStat, Jandel Scientific, California, USA).

3.3 RESULTS

3.3.1 Maternal plasma estradiol, progesterone, cortisol and CBC concentrations
Maternal plasma estradiol and progesterone were measured in order to evaluate whether maternal betamethasone exposure induced the pattern of increase in circulating estradiol and decrease in progesterone seen with onset of spontaneous and induced parturition in sheep (Liggins et al. 1973). Maternal plasma estradiol levels were significantly higher at 125 days of gestation one week following the final betamethasone injection (Table 3.1; *P*<0.05, but were not significantly different from controls at 146 days of gestation. Maternal plasma progesterone concentrations were similar between groups (Table 3.1). Neither maternal mean plasma cortisol nor CBC concentrations were altered significantly following maternal betamethasone exposure (Table 3.1).

3.3.2 Cord blood gases and pH
Fetal status at the time of delivery was assessed through the measurement of cord blood pH and blood gas measurements. Mean cord arterial PO$_2$, PCO$_2$ and pH were similar in all groups (125 and 146 days of gestation) (Table 3.2).

3.3.3 Fetal weight
Fetal weight at 125 days of gestation following repeated exposure to betamethasone *in utero* was significantly reduced by 23% (*P*<0.05; Table 3.2). At 146 days of gestation, 30 days following the last course of betamethasone, fetal weight was reduced significantly by 19% compared to controls (*P*<0.05; Table 3.2).
### Table 3.1 Maternal arterial estradiol, progesterone, cortisol and CBC concentrations

Values are expressed as mean ± SEM. *P < 0.05, betamethasone versus control. CBC, corticosteroid binding capacity.

<table>
<thead>
<tr>
<th>Variables</th>
<th>125 Days of Gestation</th>
<th>146 Days of Gestation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=5)</td>
<td>Betamethasone (n=6)</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>11.1 ± 7.3</td>
<td>58.0 ± 10.2*</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>9.8 ± 2.4</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>Cortisol (ng/ml)</td>
<td>16.7 ± 4.2</td>
<td>7.3 ± 2.6</td>
</tr>
<tr>
<td>CBC (ng/ml)</td>
<td>10.9 ± 0.0</td>
<td>10.4 ± 0.2</td>
</tr>
</tbody>
</table>
### Table 3.2 Fetal weight, cord plasma pH, PO$_2$ and PCO$_2$

For fetal weight measurements at day 125: control; $n=10$ and betamethasone treated; $n=12$ (see Methods). Values are expressed as mean ± SEM. *P < 0.05 betamethasone versus control.

<table>
<thead>
<tr>
<th>Variables</th>
<th>125 Days of Gestation</th>
<th>146 Days of Gestation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=5)</td>
<td>Betamethasone (n=6)</td>
</tr>
<tr>
<td>Fetal wt (kg)</td>
<td>2.7 ± 0.5</td>
<td>2.1 ± 0.3*</td>
</tr>
<tr>
<td>PO$_2$ (mmHg)</td>
<td>9.1 ± 1.0</td>
<td>10.4 ± 1.1</td>
</tr>
<tr>
<td>PCO$_2$ (mmHg)</td>
<td>54.3 ± 2.0</td>
<td>51.4 ± 1.9</td>
</tr>
<tr>
<td>pH</td>
<td>7.32 ± 0.03</td>
<td>7.41 ± 0.02</td>
</tr>
</tbody>
</table>
3.3.4 Basal cord plasma ir-ACTH, cortisol, α-MSH and CBC concentrations
At 125 days of gestation mean cord plasma ir-ACTH and α-MSH levels were not significantly different from control following repeated glucocorticoid exposure (Table 3.3). At 146 days however, cord plasma ir-ACTH levels were significantly higher in betamethasone treated fetuses (P< 0.05; Table 3.3). Mean cord plasma cortisol concentrations at 125 days of gestation were not altered after glucocorticoid exposure and although cord plasma cortisol levels at 146 days tended to be higher than controls, these differences did not reach statistical significance (Table 3.3). Plasma CBG, measured as CBC, was significantly higher at 125 days of gestation following prenatal betamethasone exposure (P<0.05; Table 3.3). This difference was not present however, at 146 days of gestation.

3.3.5 Pituitary ir-ACTH peptide content at 146 days of gestation
In order to evaluate the mechanisms underlying the increase in cord ACTH levels at term, the ir-ACTH<sub>1-39</sub> peptide content in 146-day pituitary tissue was determined in addition to conducting immunohistochemical localization of ir-ACTH. Positive ir-ACTH staining was observed in both the pars distalis and the pars intermedia, after betamethasone exposure, in tissue from fetuses at 146 days of gestation (figure 3.1 a-d). Tissue sections from betamethasone-treated fetuses showed less staining for ir-ACTH in the pars distalis (figure 3.1 c, d) but similar ir-ACTH in the pars intermedia (figure 3.1 a, b). Adjacent sections incubated with antibody preabsorbed with an excess of human ACTH<sub>1-24</sub> showed no positive staining for ir-ACTH (figure 3.1 e). In order to quantify changes in pituitary ACTH content, protein was extracted from the pars distalis and analyzed for ir-ACTH. Although levels of pars distalis ir-ACTH (pg/mg of protein) in the betamethasone-treated animals tended to be lower than controls, this did not reach statistical significance (control; 7.6 ± 1.2 pg/mg, n=7, betamethasone; 5.0 ± 0.8 pg/mg, n=8; P=0.09).

3.3.6 Effects on pituitary POMC, PC1, PC2, and GR mRNA levels

3.3.6.1 125 days of gestation
Figure 3.2 represents images of POMC, PC1, PC2, and GR mRNA expression patterns at 125 days of gestation. POMC mRNA expression was some 10 fold higher in the pars intermedia compared with that of the pars distalis and was distributed throughout the pars distalis as described previously (Matthews et al. 1994). PC1 mRNA was localized to the pars distalis and was uniformly distributed, whereas PC2 mRNA expression was localized to the pars intermedia.
<table>
<thead>
<tr>
<th>Variables</th>
<th>125 Days of Gestation</th>
<th>146 Days of Gestation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=5)</td>
<td>Betamethasone (n=6)</td>
</tr>
<tr>
<td>ACTH (pg/ml)</td>
<td>29.5 ± 3.0</td>
<td>36.0 ± 10.1</td>
</tr>
<tr>
<td>Cortisol (ng/ml)</td>
<td>3.2 ± 0.6</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>CBC (ng/ml)</td>
<td>17.3 ± 3.2</td>
<td>47.9 ± 10.7*</td>
</tr>
<tr>
<td>α-MSH (pmol/l)</td>
<td>34.4 ± 10.2</td>
<td>29.8 ± 5.3</td>
</tr>
</tbody>
</table>

Table 3.3 Cord plasma cortisol, ACTH, α-MSH and CBC concentrations

Values are expressed as mean ± SEM. *P < 0.05 betamethasone versus control. ACTH, adrenocorticotropic; α-MSH, α- melanocyte stimulating hormone, CBC, corticosteroid binding capacity.
Figure 3.1
Photographs of immunohistochemical staining of ACTH in the pars intermedia of saline (a) and betamethasone (b) treated animals and the pars distalis of saline (c) and betamethasone treated animals (d) at 146 days of gestation. Preabsorption control slides showed no staining (e). Magnification 400X.
Figure 3.2
Localization of proopiomelanocortin (POMC; a, b), prohormone convertase 1 (PC1; c, d), prohormone convertase 2 (PC2; e, f), and glucocorticoid receptor (GR; g, h) mRNA in the pars distalis and pars intermedia of 125-day fetal pituitaries after in situ hybridization of coronal sections with 35S-labelled oligonucleotide probes following either saline or betamethasone administration. Images are colour enhanced and the representative scale bar is an example of the relative intensity of the signals from very high (red) to very low (blue). Control sections were incubated with random nonsense sequences and showed no signal (not shown). PI, pars intermedia; PD, pars distalis; SUP, superior region; INF, inferior region. Scale bar: 500μm.
consistent with previous reports (Seidah et al. 1991, Bell et al. 1998). GR mRNA expression was found to be localized to the pars distalis, consistent with previous reports (Matthews et al. 1995) POMC, PC1, PC2 and GR mRNA levels in 125-day fetal pituitaries were unaltered by prenatal betamethasone administration (figure 3.2).

### 3.3.6.2 146 days of gestation

POMC mRNA distribution was similar to that seen previously in fetal sheep at this time in gestation (Matthews et al. 1994) but was not altered by prenatal betamethasone exposure (figure 3.3 a,b). Pituitary POMC mRNA levels were significantly higher in the inferior region of the pars distalis than in the superior region in both control and betamethasone-treated groups (P<0.05) but not significantly different between treatment groups (figure 3.4 c). Neither PC1 nor PC2 mRNA levels or distribution were altered after betamethasone exposure (figure 3.3 c-f). Maternal betamethasone administration however, increased GR mRNA levels significantly in the pars distalis of 146-day fetal pituitaries (figure 3.5; P<0.05). GR mRNA was not detected in the pars intermedia.

### 3.3.7 Effects on hypothalamic neuropeptides at 146 days gestation

The distribution of corticotrophin releasing hormone (CRH, figure 3.6a,b), arginine vasopressin (AVP, figure 3.6c,d) and glucocorticoid receptor (GR, figure 3.6a-d) mRNA in the paraventricular nucleus of 146-day fetal hypothalami was not altered by prenatal betamethasone exposure. Levels of CRH, AVP and GR mRNA, as determined by computerized image analysis were similar in both groups (figure 3.6, i,ii,iii). Levels of OT (figure 3.7a,b) and PENK (figure 3.7c,d) mRNA were also similar in both groups following maternal betamethasone administration.

### 3.4 DISCUSSION

This study has demonstrated that clinically relevant doses of betamethasone administered to pregnant sheep at 0.7 of gestation result in significant reductions in fetal weight. Repeated glucocorticoid exposure in this study resulted in significant increases in basal cord plasma CBC at 125 days of gestation and significant elevations in basal ACTH concentrations at 146 days of gestation. Plasma cortisol concentrations followed a similar trend to ACTH values, but the differences did not reach statistical significance. It appears that these changes were not associated with detectable alterations in the levels of mRNA transcripts encoding key neuropeptides of the HPA axis in either the fetal pituitary or the hypothalamus.
Figure 3.3

Localization of proopiomelanocortin (POMC: a, b), prohormone convertase 1 (PC1; c, d) and prohormone convertase 2 (PC2; e, f), in the pars distalis and pars intermedia of 146-day fetal pituitaries after in situ hybridisation of coronal sections with 35S-labelled oligonucleotide probes following either saline or betamethasone administration. Images are colour enhanced and the representative scale bar is an example of the relative intensity of the signals from very high (red) to very low (blue). Control sections were incubated with random nonsense sequences and showed no signal (not shown). PI, pars intermedia; PD, pars distalis; SUP, superior region; INF, inferior region. Scale bar: 500μm.
Figure 3.4
Bar graphs illustrating densitometric analysis of POMC mRNA levels in coronal sections of the *pars intermedia* (a), total *pars distalis* (b) and differential distribution in the superior and inferior regions of the *pars distalis* (c) of 146-day fetal pituitaries following either saline (open bar) or betamethasone (shaded bar) administration. SUP, superior region of the *pars distalis*. INF, inferior region of the *pars distalis*. Values are presented as relative optical density (ROD) and are mean ± SEM. *P<0.05.
Figure 3.5
Localization of glucocorticoid receptor mRNA in the pars distalis of 146-day fetal pituitaries after in situ hybridisation of coronal sections with 35S-labelled oligonucleotide probes following either saline (a) or betamethasone (b) administration. Images are colour enhanced and the representative scale bar is an example of the relative intensity of the signals from very high (red) to very low (blue). Control sections were incubated with random nonsense sequences and showed no signal (data not shown). Histograms represent densitometric analysis of GR mRNA levels in coronal sections of the entire pars distalis of 146-day fetal pituitaries following either saline (open bar) or betamethasone (shaded bar) administration. Values are presented as relative optical density (ROD) and are mean ± SEM. *P<0.05.
Figure 3.6
Localization of corticotrophin releasing hormone (CRH; a, b), arginine vasopressin (AVP; c, d) and glucocorticoid receptor (GR; e, f) mRNA in the paraventricular nucleus (PVN) of 146-day fetal hypothalami after in situ hybridization of coronal sections with 35S-labelled oligonucleotide probes following either saline or betamethasone administration. Images are colour enhanced and the representative scale bar is an example of the relative intensity of the signals from very high (red) to very low (blue). Control sections were incubated with random nonsense sequences and showed no signal (data not shown).

Histograms represent the results of densitometric analysis of CRH (i), AVP (ii) and GR (iii) mRNA levels in coronal sections of the entire PVN of 146-day fetal hypothalami following either saline (open bar) or betamethasone (shaded bar) administration. Values are presented as relative optical density (ROD) and are mean ± SEM.
Localization of oxytocin (OT; a, b) and pre-proenkephalin (PENK; c, d) mRNA in the PVN of 146 day fetal hypothalami following in situ hybridization of coronal sections with $^{35}$S-labelled oligonucleotide probes following either saline or betamethasone administration. Images are colour enhanced. Control sections were incubated with random nonsense sequences and showed no signal (data not shown). Histograms represent the results of densitometric analysis of OT (i) and PENK (ii) mRNA levels in coronal sections of the entire PVN of 146-day fetal hypothalami following either saline (open bar) or betamethasone (shaded bar) administration. Values are presented as relative optical density (ROD) and are mean ± SEM.

Figure 3.7
The increase in cord plasma ACTH is unlikely to be related to the onset of labour, since maternal estradiol and progesterone levels were not significantly different between groups at 146 days of gestation and neither group exhibited the pattern of maternal estradiol or progesterone change characteristic of parturition (Challis et al. 1971, Flint et al. 1975). Maternal estradiol levels were increased at day 125, closer to the time of betamethasone administration, reproducing the changes reported by Liggins et al. (1973) after fetal glucocorticoid treatment. The possibility that betamethasone stimulated prostaglandin production in the fetal placenta and that an increase in fetal circulating prostaglandins stimulated an increase in fetal ACTH secretion cannot be ignored.

The late gestation increase in circulating fetal cortisol has been linked previously to the increase in placental prostaglandin secretion that occurs at term (Challis et al. 1991, 1999). Furthermore, fetal PGE2 infusion in late gestation resulted in a rapid increase in fetal plasma ACTH and cortisol levels (Louis et al. 1976, Ratter et al. 1979, Young et al. 1996), suggesting that PGE2 may act directly on the fetal pituitary as well as the fetal adrenal to increase cortisol secretion. However, blood samples were not collected in a manner appropriate to measure cord plasma PGE2 levels.

It was therefore proposed that the elevation in cord plasma ACTH was the result of functional changes in the fetal HPA axis following exposure to elevated glucocorticoids. Glucocorticoids suppress pituitary POMC levels and ACTH secretion (Birnburg et al. 1983, Schachter et al. 1991). Day et al. (1992) found that dexamethasone treatment resulted in a 59% reduction in POMC, PC1 and PC2 mRNA levels in cultured At-20 cells (pituitary cell line), but that levels returned to control by 60 minutes of incubation. Normally PC1 mRNA expression is present in all melanotrophs of the rat pars intermedia, but in only 20% of pars distalis cells (Seidah et al. 1991). Elevated cord plasma ACTH concentrations at 146 days of gestation in this study were not accompanied by significant changes in precursor POMC mRNA levels, nor in PC1 and PC2 mRNA levels in either the pars intermedia or the pars distalis at 125 or 146 days. Therefore, the term increase in basal ACTH concentrations was not likely due to increased transcription, although mRNA stability or transcriptional activity was not evaluated. It remains possible that small changes in steady state POMC mRNA were present, but these differences could not be detected by in situ hybridization. The relative distribution of PC1 and PC2 mRNA levels within the pituitary was consistent with that reported in other studies (Marcinkiewicz et al. 1994, Bell et al. 1998). However prohormone convertase enzymatic activity was not measured and PC1 and PC2 were not localized solely to the corticotrophs in the pars distalis, so it cannot
be firmly concluded that the potential for POMC processing is unaffected by glucocorticoid exposure.

Positive ir-ACTH staining in the cells of the pars intermedia of 146-day pituitaries of fetuses treated with betamethasone was similar to that of control pituitaries. Although not measured directly, less staining appeared to be present in the pars distalis of treated pituitaries. These observations are consistent with those of Jeffray et al. (1998) who reported a decrease in the number of ir-ACTH positive corticotrophs in the fetal pars distalis after 96 hours of fetal cortisol infusion, but no change in the pars intermedia. Pituitary ACTH content in the pars distalis was similar in 146-day fetuses of both groups. It remains possible that other sites of POMC production such as the lung and/or placenta may contribute to the increase in cord plasma ir-ACTH (Jeffray et al. 1999, Cudd et al. 1995). These effects could be mediated by placental CRH. Both ACTH and CRH have been identified in the sheep placenta (Jones et al. 1989, Jeffray et al. 1999) and CRH may enter the fetal circulation and stimulate the fetal HPA axis. Although glucocorticoids increase placental CRH production in vitro in human cultured placental cells (Majzoub et al. 1999), fetal cortisol infusion did not result in any changes in ovine placental POMC, PC1 or PC2 mRNA levels (Jeffray 1999).

Negative feedback is an important regulator of cortisol synthesis and secretion in the fetal HPA axis, through glucocorticoid binding to type II receptors (GR) in the hypothalamic paraventricular nucleus (PVN) and in the pars distalis to reduce CRH and POMC production. The exact role of GR in the pituitary however is still unclear. In the fetal sheep, GR mRNA and protein levels in the pars distalis increase at term (Yang et al. 1990, Matthews et al. 1995). There are no studies that have evaluated the effect of repeated maternal betamethasone administration on fetal pituitary or hypothalamic GR mRNA levels, receptor number or activity in the fetal sheep. The increase in GR mRNA levels that were observed in the pars distalis of fetuses at day 146 after betamethasone treatment is consistent with the increase in pituitary GR seen at term as endogenous cortisol rises (Yang et al. 1990, 1992, Holloway et al. 2000). This change may contribute to the effect of cortisol in promoting an increased output of ACTH₁-₃₉ relative to large molecular weight POMC-peptides as seen previously in cultured corticotrophs (Durand et al. 1986). Although mean cord cortisol levels in betamethasone-treated animals at term were 3-fold higher than in controls, large variations between individual animals resulted in cortisol values that were not statistically different between groups. Thus, we did not find significant rises in both cord cortisol and pituitary GR mRNA after betamethasone treatment.

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These data suggest that increases in cord plasma ACTH were not associated with changes in mRNA levels of hypothalamic neuropeptides. Matthews et al. (1995) reported that fetal cortisol infusion late in gestation did not alter basal hypothalamic CRH or AVP mRNA levels but suppressed hypoxemia-induced increases in CRH mRNA levels (Matthews & Challis 1995). Therefore, glucocorticoids have differential effects on basal and stimulated levels of CRH mRNA. This study also suggests that it is unlikely that elevations in cord plasma ACTH are the result of changes in steady state expression of oxytocin or pre-proenkephalin mRNA. Furthermore, a substantial body of evidence exists to suggest that fetal glucocorticoid exposure results in changes in hippocampal GR and MR (Sapolsky et al. 1990, Uno et al. 1994, Meaney & Aitken 1985). In the present study, if such changes have occurred, they have not resulted in detectable alterations in levels of mRNA encoding key hypothalamic neuropeptides or pituitary POMC. Nevertheless, such measurements will be of interest in the context of long-term programming of HPA function by antenatal corticosteroids.

Plasma corticosteroid binding capacity (CBC) was significantly elevated at 125 days of gestation after betamethasone treatment. It has been suggested previously that a rise in fetal ovine plasma corticosteroid binding globulin (CBG) late in gestation maintains low concentrations of circulating cortisol and therefore maintains low negative feedback on the pituitary and the hypothalamus (Ballard et al. 1982, Berdusco et al. 1994, 1995). Glucocorticoids have been shown to increase plasma CBC and hepatic CBG mRNA in fetal sheep (Berdusco et al. 1994). Therefore repeated fetal betamethasone exposure between days 104 and 118 could have resulted in an increase in CBG production and an increase in plasma CBC measured at 125 days of gestation. In turn, it is possible that CBG binds circulating cortisol, resulting in a decrease in negative feedback at the level of the hypothalamus/pituitary and an increase in ACTH secretion, which persists through later gestation, even though CBG values have been restored to control. In effect, maternal betamethasone may have altered the set point(s) of the fetal HPA negative feedback.

These results are consistent with others that have shown changes in fetal or newborn circulating ACTH and cortisol levels following in utero betamethasone exposure (Uno et al. 1994). Uno et al. (1990) found that the offspring of pregnant rhesus monkeys exposed to betamethasone showed elevations in both basal and stimulated levels of ACTH and cortisol at 10 months of age compared to controls (Uno et al. 1990). Recent data in humans also link reductions in fetal growth with alterations in circulating glucocorticoids (Phillips et al. 1998). It has been reported that fasting plasma cortisol concentrations of 64 year old men were inversely
related to their birth weight and that raised plasma cortisol levels were significantly associated with an increase in blood pressure, plasma glucose and insulin resistance (Phillips et al. 1998). These data support the hypothesis that an adverse intrauterine environment can permanently reset the developing fetal HPA axis leading to an increased risk of adult diseases. It appears that precocious exposure of the fetus to glucocorticoids creates an adverse intrauterine environment within which this may occur.

Glucocorticoid exposure results in reductions in weight at birth (Reinisch et al. 1978, Johnson et al. 1981, Jobe et al. 1998), a finding that was substantiated using smaller amounts of steroid in the present study. Organ weights from a larger cohort of animals treated alongside this group have been published elsewhere and showed reductions in liver, brain and kidney weight and a significant elevation in the brain to liver weight ratio (Newnham et al. 1999). Treatment of pregnant rhesus monkeys with betamethasone resulted in reductions in fetal brain, liver, pancreas, heart and adrenal weights at term (Johnson et al. 1981). A recent report by French et al. (1999) provides clear evidence that decreased birth weight ratios of infants of mothers treated with prenatal glucocorticoids, are associated with number of glucocorticoid courses. Head circumference analyses showed significant reductions with greater than one glucocorticoid course (French et al. 1999). The mechanisms altering fetal growth however, are still unknown. Insulin-like growth factors (IGFs) are generally believed to influence fetal growth by stimulating cell proliferation (D’Ercole et al. 1987), and it has been proposed that a rise in endogenous fetal glucocorticoid regulates IGF-II mRNA expression and thereby regulates fetal growth, especially later in gestation (Li et al. 1993). It is possible that the reduction in fetal growth following repeated betamethasone exposure in this study may be mediated through a reduction in tissue specific IGF-II expression. Further studies are required to investigate fully this possibility. Maternal betamethasone administration between days 104 and 118 may have resulted in a shift in the fetal growth trajectory to a parallel but lower profile, resulting in similar differences in weight between treated animals and controls at term, as at day 125 of gestation.

This is the first study to evaluate levels of mRNA transcripts encoding key pituitary and hypothalamic neuropeptides regulating the fetal HPA axis following repeated exposure to clinically relevant doses of glucocorticoids. Repeated maternal betamethasone administration may produce long-term changes in growth and the development of the fetal HPA axis. These observations raise the possibility that corticosteroids administered during the course of pregnancy to promote maturational changes in the fetus may have adverse consequences on both short term and long term fetal development.
CHAPTER 4

4 Effects of Repeated Maternal Betamethasone Administration on Fetal Ovine Hepatic CBG and 11β HSD 1 expression and Pancreatic Morphology

4.1 INTRODUCTION
Numerous epidemiological studies have suggested that intrauterine factors are important determinants of the risk of developing a variety of adult diseases independent of lifestyle risks and social class (Barker et al. 1993, 1998, Osmond et al. 1993, McCance et al. 1994). Low birth weight has been associated with impaired glucose tolerance and insulin resistance, possibly linked to alterations in pancreatic function (Van Asshe et al. 1977, Hales et al. 1991, Phipps et al. 1993, Lithell et al. 1996, Ravelli et al. 1998). The fetal programming hypothesis has been proposed to explain these associations. It reflects the possibility of an intrauterine factor mediating cellular growth and development at a vulnerable time in gestation, subsequently resulting in permanent alterations in tissue and organ function that are apparent later in life (Dennison et al. 1997, Barker et al. 1998, Hill et al. 1999, Dodic et al. 1999, Seckl 2000).

Although the mechanisms regulating these effects are not fully understood, the hypothesis that prenatal perturbations alter fetal pancreatic development in a way that persists into adulthood is strongly supported (Hoet et al. 1993, Ozanne 1999). Maternal undernutrition has been proposed by a number of researchers to be a major factor in determining fetal programming of glucose tolerance and insulin sensitivity (Hoet 1991, Phillips 1996, Desai & Hales 1997, Barker 1998). Mothers exposed to famine during pregnancy have offspring that exhibit decreased glucose tolerance as adults in addition to elevated insulin responses to a glucose load (Ravelli et al. 1998). This effect was exaggerated in mothers exposed to famine late in gestation. In rats, protein restriction (5-8%) administered either prenatally (Snoek et al. 1990, Dahri et al. 1991) or postnatally (Swenne et al. 1987) resulted in alterations in glucose tolerance and insulin secretion and altered pancreatic morphology and β cell dysfunction in offspring when mature. Prenatal protein restriction in the pregnant rat resulted in a reduction in offspring body weight (Snoek et al. 1990, Berney et al. 1997, Petrik et al. 1999) that persisted into adulthood (Iglesias-Barreira et al. 1996). Furthermore, offspring demonstrate reductions in pancreatic islet size, number of β cells, insulin content and pancreatic islet cell blood flow and vascularization (Snoek et al. 1990, Iglesias-Barreira et al. 1996, Berney et al. 1997, Petrik et al. 1999). Furthermore, prenatal protein restriction resulted in alterations in the balance of fetal pancreatic
β cell replication and apoptosis possibly contributing to smaller islet size and impaired insulin release seen later in life (Petrik et al. 1999). In rats it has been shown that the developing neonatal pancreas undergoes remodeling that involves a wave of apoptosis and neogenesis that participates in the replacement of fetal β cells to adult β cells, capable of responding to glucose postnatally (Scaglia et al. 1997, Hill et al. 1999). It is this developmental remodeling that appears to be vulnerable to intrauterine perturbations.

Offspring subjected to maternal protein restriction show permanent growth retardation in lung, brain, spleen, muscle, and pancreas as well the liver (Desai et al. 1996). Because the liver plays a key role in metabolic regulation (Pilkis & Granner 1992), many studies have evaluated the effect of prenatal perturbations on liver development and function (Desai et al. 1997, Nyirenda et al. 1998, Saegusa et al. 1999, McMillen et al. 2000). Maternal protein restriction and fetal growth restriction have been associated with dramatic alterations in key enzymes involved in circulating glucose levels and local glucocorticoid regulation (Desai et al. 1997, McMillen et al. 2000). Although the mechanisms linking intrauterine growth restriction and metabolic function are poorly understood, recent animal studies suggest that prenatal glucocorticoids may target specific organs such as the liver and the pancreas (Lindsay et al. 1996, Nyirenda et al. 1998, Hill 1999). It has been shown that maternal protein restriction specifically attenuates placental 11 β hydroxysteroid dehydrogenase type 2 (11 βHSD2) activity, a unidirectional enzyme that catalyzes the conversion of the biologically active glucocorticoids to the inert 11-keto metabolites (Chapman et al. 1997), thereby permitting maternal derived glucocorticoids to influence fetal development (Langley-Evans et al. 1996). Maternal treatment with a placental 11βHSD inhibitor, carbenoxolone, allows increased passage of maternal glucocorticoids to the fetus (Whorwood et al. 1993). In rats, maternal carbenoxolone treatment reduced birthweight (Lindsay et al. 1996), an effect similar to that observed with dexamethasone treatment (Price et al. 1992, Levitt et al. 1996, Nyirenda et al. 1998). Furthermore, adult male offspring demonstrated altered glucose tolerance indicated by higher fasting glucose levels and elevated glucose and insulin responses to a glucose challenge. Maternal adrenalectomy prevented this effect, supporting the role of fetal exposure to maternally derived glucocorticoids in the programming of metabolic function (Lindsay et al. 1996). Glucose intolerance in adult rats exposed to maternal dexamethasone in utero has been associated with elevations in hepatic glucocorticoid receptors (GR) and glucocorticoid sensitive enzymes involved in gluconeogenesis such as phosphoenol- pyruvate carboxykinase (PEPCK) mRNA levels (Nyirenda et al. 1998, Saegusa et al. 1999).
Glucocorticoid exposure could be a significant factor in the programming of the fetal pancreas and the developing β cells (Hill 1999). Glucocorticoid receptors (GR) have been localized to pancreatic β cells in the rat (Fischer et al. 1990) and glucocorticoids have been shown to regulate insulin secretion in vitro (Lambillote et al. 1997) and in vivo (Delaunay et al. 1997). In addition, glucocorticoids have been shown to regulate many important prenatal growth factors involved in the remodeling of the developing pancreas (Price et al. 1993, Li et al. 1993, Petrik et al. 1999, Hill 1999). Furthermore, due to the importance of glucocorticoids in the regulation of carbohydrate metabolism (Pilkis & Granner 1992) it has been suggested that alterations in hypothalamic-pituitary-adrenal (HPA) activity may be associated with the development of insulin resistance and glucose intolerance in adulthood (Phillips et al. 1998). Indeed, prenatal exposure to synthetic glucocorticoids resulted in elevations in fetal and postnatal HPA activity in sheep (Sloboda et al. 2000, 2001; Chapters 3, 5) and in primates (Uno et al. 1994). Phillips et al. (1998) have recently reported that low birth weight was related to elevated levels of fasting plasma cortisol levels, elevated systolic blood pressure, plasma glucose and triglyceride levels and insulin resistance in adulthood. In addition, Levitt et al. (2000) recently suggest that the intrauterine programming of the HPA axis may be a key mechanism underlying the association between low birth weight, insulin resistance and hypertension in human adults.

The level of tissue exposure to glucocorticoids can be regulated in several ways. The binding of glucocorticoids to a high affinity binding globulin (CBG) regulates levels of free (unbound) circulating bioactive glucocorticoids (Ballard et al. 1982, Berducso et al. 1993). Intracellular exposure is regulated through the action of 11β HSD enzyme. Type 1, primarily converts inactive 11-keto metabolites to bioactive glucocorticoids (Chapman et al. 1997). Both CBG and 11 βHSD1 are present in the fetal liver and their expression has been shown to be vulnerable to glucocorticoid exposure in the fetal sheep (Berdusco et al. 1993, Yang et al. 1995). It is presently unclear whether maternal glucocorticoid administration alters fetal hepatic CBG or 11 βHSD1 expression, although studies in rats suggest that prenatal maternal dexamethasone administration either does not change (Nyirenda et al. 1998) or decreases (Saegusa et al. 1999) 11β HSD1 expression levels in adult offspring. Studies in sheep suggest however, that exogenous glucocorticoids exert opposite effects on hepatic CBG mRNA expression in fetal and adult sheep (Yang et al. 1994, Berdusco et al. 1993) where glucocorticoids act to increase fetal hepatic 11βHSD1 and CBG expression levels. Therefore, the mechanism by which excess glucocorticoids act on the developing liver and the mature liver may differ.
It is presently unknown whether synthetic glucocorticoid administration to the pregnant sheep results in alterations in fetal glucose and insulin regulation or fetal hepatic \(11\beta\text{HSD1}\) and CBG levels. Furthermore, very little is known regarding the development of the sheep pancreas and whether prenatal glucocorticoid exposure alters pancreatic development. We have previously shown that repeated maternal glucocorticoid administration resulted in alterations in fetal growth and HPA function (Sloboda et al. 2000, Chapter 3). We hypothesized that maternal betamethasone administration would result in changes in fetal regulation of intrahepatic glucocorticoid levels that may contribute to alterations in fetal metabolic and HPA function. Therefore this study set out to describe fetal ovine pancreatic development in terms of pancreatic islet morphology and determine the expression levels of CBG, \(11\beta\text{HSD1}\) and GR in the liver of fetal sheep, following maternal administration of synthetic glucocorticoids.

4.2 MATERIALS AND METHODS

4.2.1 Animals
The animals in this chapter were the same animals in Chapter 3. Detailed information on animals and experimental procedures are given in sections 3.2.1, 3.2.2.

4.2.2 Experimental Procedures
Cord blood samples were collected at the time of sacrifice, centrifuged at 2200g for 10 minutes and the plasma was stored at \(-20^\circ\text{C}\) until further analysis. At 125 (control \(n=5\), and betamethasone \(n=6\)) and 146 (control \(n=7\) and betamethasone \(n=8\)) days of gestation, fetal liver samples were collected from the quadrate lobe of the liver lateral to the gallbladder. These were slow frozen on dry ice for northern and western blot analysis. The entire fetal pancreas was removed and half was slow frozen for determination of insulin content and half was fixed in 4\% paraformaldehyde (Sigma Chemical Co., St. Louis, USA) and 0.2\% gluteraldehyde (8\% EM Grade, Polyscience Inc., Warrington PA, USA) for morphological analysis. The protocols were approved by the Institutional Ethics Committees of the Western Australian Department of Agriculture and the Animal Care Committee of the University of Toronto, according to the guidelines of the Canadian Council for Animal Care.

4.2.3 Measurement of cord plasma glucose and insulin levels
Cord plasma glucose levels were analyzed using a glucose analyzer (Beckman Glucose Analyzer 2; Beckman Instruments, Ireland) via the glucose oxidase method. Cord plasma immunoreactive
(ir) insulin concentrations (in 100μl of plasma) were measured using a commercial radioimmunoassay (RIA) kit (Linco Research, Inc., USA). The intra-assay coefficient of variation was 8% and the mean assay sensitivity was 0.1ng/ml. All samples were analyzed in a single assay. The insulin antibody cross-reacts 100% with rat, sheep and porcine insulin; cross-reactivity to rat C-peptide, glucagon, somatostatin, pancreatic polypeptide and IGF-I is undetectable (Linco).

4.2.4 Measurement of pancreatic insulin content

4.2.4.1 Protein extraction

Frozen pancreatic tissue was homogenized (PT200 Homogeniser, Polytron, Kinematica AG, Switzerland) in 5ml of extraction buffer (HCl 1M; 5% formic acid; 1% trifluoroacetic acid TFA; 1% NaCl; distilled water) in two aliquots and centrifuged at 1500g at 4°C for 10 minutes. Samples were pooled and further extracted using Sep-Pak cartridges. Sep-Pak cartridges (Classic cartridge, Waters Corp. USA) attached to 20ml syringes were flushed with 80% 2-propanol (containing 0.1% TFA), then primed with 0.1% TFA and flushed with the sample (flushed through twice). Cartridges containing the sample were then flushed with 0.1% TFA to release any unbound protein from the cartridge. Samples were then eluted from the cartridges with 80% 2-propanol (containing 0.1% TFA) and stored at -20°C for protein determination.

4.2.4.2 Protein assay

The protein concentration of each sample was quantified prior to ir-insulin determination using the Bradford assay (Bradford 1976). Detailed methodology is described in 3.2.4.2.

4.2.4.3 Measurement of ir-insulin content

Ir-insulin levels were analyzed in pancreatic homogenates (titrated to give B/B<sub>0</sub> of approximately 40%) using a commercial radioimmunoassay (RIA) kit (Linco Research, Inc., USA) as described in 4.2.3. The intra-assay coefficient of variation was 8%. All samples were analyzed in a single assay.

4.2.5 Immunohistochemistry

4.2.5.1 Detection of ir-insulin

Immunohistochemical detection of ir-insulin was performed on adjacent 12μm fixed and paraffin embedded sections of pancreas at 125 and 146 days of gestation. Detailed methodology is
described in 3.2.6. Sections were incubated overnight at 4°C in a moist chamber with a polyclonal antibody to porcine insulin (1:50 dilution, Cedarlane Laboratories Ltd, Hornby, Canada). Adjacent sections were incubated with the antibody dilution buffer in the absence of the primary antibody to provide negative controls.

4.2.5.2 Determination of pancreatic islet area
Analysis was performed using a transmitted light microscope (Carl Zeiss Canada, Ltd, North York, Canada) with Northern Eclipse version 2.0 analysis software (Empix Imaging Co., Mississauga, Canada). The total islet area and the area that stained specifically for insulin was determined in at least 15 different islets in three sections per animal from each age (125 and 146 days of gestation) as described previously (Petrik et al. 1998). Results are presented as total islet area, ir-insulin area and insulin area as a percentage of total area.

4.2.5.3 Detection of apoptotic cells in pancreatic sections
Detection of apoptotic bodies was performed on adjacent 12 m fixed and paraffin embedded sections of pancreas at 125 and 146 days of gestation using the Promega Apoptosis Detection System, Fluorescein (Promega Corporation, Madison, USA). Slides were incubated in xylene solution for 2x10 minutes followed by an alcohol dehydration series (100%-70%), then incubated in 0.85% NaCl in water for 5 minutes. Slides were washed in PBS for 5 minutes and each section was incubated with 50µl of proteinase K buffer for 20 minutes (water, 0.5M EDTA, 2.5M Tris; Promega Corporation, Madison, USA) to permeabilize the membranes to the kit reagents. Following a 5-minute PBS wash, each section was incubated with 50µl of Equilibration Buffer (Promega Corporation, Madison, USA) for 13 minutes. The following steps were performed in the dark to minimize quenching of fluorescein. Sections were then incubated with 45µl of TdT Incubation Buffer (equilibration buffer, nucleotide mix, terminal deoxytransferase (TdT) enzyme; Promega Corporation, Madison, USA) for 2 hours at 37°C in order to label fragmented DNA with fluorescein-12-UTP. Slides were washed in 2xSSC to terminate the reaction and washed in 5g bovine serum albumin + 1ml triton in PBS for 10 minutes to wash out unincorporated fluorescein, followed by a PBS wash for 5 minutes. Nuclei were then stained in propidium iodide, washed in tap water and coverslips were mounted with Permount (Fisher Scientific Ltd., Nepean, Canada). Sections of pancreas from neonatal mice were processed as controls for apoptotic staining. All slides were stored at -10°C in a closed dark container until analysis.
4.2.5.3.1 Fluorescence microscopy
Detection of localized green fluorescence of apoptotic cells was visualized using a fluorescent microscope (Incident-light fluorescence Axioskope, Carl Zeiss Canada Ltd, North York Canada). Pancreatic islets were first identified using a light filter and then using a red filter, green fluorescent staining within the islets was determined. Comparison between the two visual fields was used in order to ensure that red blood cells were not counted as apoptotic cells, since red blood cells readily take up the fluorescein dye used in the system. Within each islet, total cell number was counted and compared with the number of cells stained with fluorescein dye. Results are presented as % of apoptotic cells of total number of cells within each islet. For each animal, at least 15 islets were analyzed in various areas of the sections.

4.2.6 Northern blot analysis

4.2.6.1 Total RNA extraction
Aliquots of frozen tissues were placed in chilled polypropylene tubes containing 2ml TRIZOL reagent (Life Technologies, Inc., Maryland USA) and homogenized (PT200 Homogeniser, Polytron, Kinematica AG, Switzerland) on ice for at least 1 minute and then transferred into sterile eppendorf tubes. Chloroform (Sigma Chemical Co., St. Louis, USA; 0.2ml per 1ml of TRIZOL Reagent) was added to each sample, vortexed and centrifuged at 31 000g for 15 minutes at 4°C to allow separation of RNA, DNA and protein. The aqueous phase containing RNA was transferred to a new eppendorf tube. The RNA was precipitated out of solution by the addition of 500µl of isopropyl alcohol per 1ml of TRIZOL Reagent and the mixture was centrifuged at 31 000g for 10 minutes at 4°C. The precipitated RNA pellet was washed with 1ml of 75% ethanol, allowed to air-dry for ~20 minutes and dissolved in DEPC treated water at 60°C for 10 minutes. The purity and concentration of RNA in each sample was determined by measuring the spectrophometric absorbance (Ultrispec 2000, Pharmacia Biotech, Piscataway, USA) of isolated RNA (2µl in 498µl DEPC water) at 260nm (for nucleic acid concentration) and at 280nm (for protein concentration). Ratios of the readings at 260:280nm between 1.6 and 1.8 were considered acceptable for further analysis. The isolated RNA in each sample was also assessed for integrity. Samples of isolated RNA (10µg) were electrophoresed on a 1% agarose formaldehyde gel, stained with ethidium bromide overnight (0.001% v/v 10mg/ml ethidium bromide, 0.13% v/v 2β-mercaptoethanol), destained for 2 hours (0.1% 2β-mercaptoethanol) and
bands visualized under UV light. Intact RNA was viewed as 2 distinct bands corresponding to 18S and 28S ribosomal RNA (rRNA).

**4.2.6.2 Electrophoresis and hybridization**

Total RNA from each sample (30µg) and a standard RNA ladder (one per blot; Life Technologies, Inc.) were size fractioned by horizontal electrophoresis (Horizon 20X25, Life Technologies, Inc.) on a 1% agarose formaldehyde gel at 100 volts for approximately 2-2.5 hours. The electrophoretically separated RNA was transferred to a nylon membrane (Zeta Probe GT Blotting membrane, Bio-Rad Laboratories, Mississauga, Canada) by capillary blotting. The following day, blots were removed from the gel and RNA ladders separated from the rest of the samples. The resultant northern blots containing transferred sample RNA were exposed to UV light for 1 minute to permit crosslinking of the RNA to the membrane. The RNA ladders were exposed to methylene blue dye to mark the RNA ladder and stored at -20°C for future reference. The blots were prehybridized in 30ml glass cylinders for 45 minutes at 50°C in a rotating hybridization oven (Autoblot Hybridization Oven, Bellco Technology, Bello Glass Inc., USA). The blots were then exposed to the same buffer containing 32P-labelled complementary DNA (cDNA) antisense probe (see 4.2.4.3) overnight at 50°C in a rotating hybridization oven to allow hybridization of labeled 32P antisense probes to specific RNA sequences. Each blot was washed once in 150mM sodium phosphate (NaP)/0.1% sodium dodecyl sulphate (SDS) for 15 minutes rotating at 55°C followed by 3 washes in 30mM NaP/0.1% SDS of 15 minutes each rotating at 55°C. Each labeled blot was exposed to film (XAR-5, Kodak, Eastman Co., NY, USA) within a cassette at -80°C, with an intensifying screen (Biomax Transcreen LE, Eastman Kodak Co., NY, USA) in order to intensify the signal and shorten exposure time. Following exposure (length of time was specific for each probe), the blots were stripped of the hybridized label (0.01xSSC, 0.5% SDS in DEPC water at 95°C for 3 washes of 15 minutes each). Blots were then reprobed with 32P labeled cDNA antisense probe to mouse 18S rRNA as an internal control to allow for corrections in gel loading and transfer. The signals for both the mRNA of interest and 18S rRNA were analyzed using an image analysis system (Imaging Research Inc, St. Catharines, Canada) within the linear range of the film and represented as relative optical densities (ROD). Results are expressed as the ratio of ROD for mRNA: 18S rRNA.
4.2.6.3 $^{32}$P labeling of complimentary DNA (cDNA) antisense probes

A labelling kit was used to label cDNA probes with $\alpha$-[32P]deoxy-CTP (Amersham International) using the random priming method (Ready to Go, Pharmacia-Biotech Inc., Baie d'Urfe, Que.). The ovine full length CBG and 11 βHSD1 cDNA sequences have been described previously (Berdusco et al. 1993, 1994, Yang et al. 1992, 1997) and were used as cDNA probes. The cDNA was denatured by heating in boiling water for 10 minutes and cooled on ice for 5 minutes to prevent annealing. The cDNA (~50ng) was added to the reagent mix (Ready to Go, Pharmacia-Biotech), 5 µl of $\alpha$-[32P] deoxy-CTP, Klenow fragment (1µl; Ready to Go, Pharmacia-Biotech Inc., Baie d'Urfe, Que.) and DEPC water to a final volume of 50µl. This solution was incubated for ~1 hour at 37°C. The labelled probe was separated from unincorporated oligonucleotides by passing it through a Sephadex minicolumn (Nick Column; Pharmacia Biotech Inc., Baie d'Urfe, Que.). The labelled probe was added to the hybridization buffer at a concentration of ~1-1.5×10^6 cpm/ml.

4.2.7 Western blot analysis

4.2.7.1 Protein extraction

Frozen liver samples were pulverized with a mortar and pestle under liquid nitrogen and homogenized (PT200 Homogeniser, Polytron, Kinematica AG, Switzerland) for 1 minute on ice in RIPA lysis buffer (50mM Tris-HCl, 1% v/v TritonX-100, 0.1% w/v SDS, 150mM NaCl, 1% w/v sodium deoxycholatic acid), with 100 µM sodium orthovanadate (Na$_2$VO$_3$) and Complete™ Mini EDTA-free Protease Inhibitors (Boehringer Mannheim Biochemicals). Homogenates were transferred to eppendorf tubes and centrifuged at 15 000g at 4°C for 10 minutes. Supernatants were transferred to new eppendorf tubes and stored at -80°C until protein analysis.

4.2.7.2 Protein Assay

Protein concentration of each sample was determined using the Bradford assay (Bradford 1976). Detailed methodology is described in 3.2.4.2.

4.2.7.3 Electrophoresis and protein detection

Protein samples and protein standards (BioRad Laboratories, Mississauga, Canada) were separated by electrophoresis on 8% - 12% polyacrylamide gels at 150 volts for ~1 hour at 4°C. Proteins and standards were electrophoretically transferred onto nitrocellulose membranes (Bio...
Rad Laboratories, Mississauga, Canada) for ~1.5 hours at 110 volts at 4°C. Staining the resultant blots with S-Ponceau (0.1% w/v Ponceau S in 5% acetic acid v/v; Sigma Chemical Co. St. Louis, USA) verified equal loading and transfer. The blots were washed with phosphate buffered saline (PBS) and 0.1% Tween-20 (Sigma Chemical Co. St. Louis USA) and incubated overnight at 4°C in 5% skim milk powder w/v in PBS + Tween-20 on a mechanical shaker to block non-specific binding. Primary polyclonal antibodies (ovine CBG; generated in this laboratory by Berdusco et al. (1993); ovine 11β HSD1 generated by Yang et al. (1995); and human GR; Affinity Bioreagents, Inc.) were diluted in 5% blocking solution (5% skim milk powder w/v in PBS + Tween-20) (CBG, 1:500; 11β HSD1, 1:500; GR, 5μg/ml) and incubated with the blots for 1 hour. Blots were rinsed in PBS + Tween-20 for 5x5 minute washes. Secondary antisera, conjugated to horseradish peroxidase (anti-rabbit Ig horseradish peroxidase, Amersham Life Sciences) were diluted in blocking solution (CBG, 1:2000; 11β HSD1, 1:3000; GR, 1:2000) and incubated with the blots for 1-2 hours, followed by 6x5 minute PBS + Tween-20 washes. Detection of specific protein bands was accomplished using the Amersham Electrochemiluminescence Detection System (ECL; Amersham Life Science). Blots were placed in a 1:1 solution containing detection reagents for 1 minute, allowed to dry slightly and exposed to X-ray film (X-Omat Blue XB-1, Eastman Kodak, Rochester, USA). The signals for the protein of interest were analyzed using an image analysis system (Imaging Research Inc, St. Catharines, Canada) and represented as relative optical densities (ROD).

4.2.8 Statistical Analysis
Changes in cord plasma glucose and insulin levels, and insulin: glucose ratios (I: G) at both 125 and 146 days of gestation were analyzed using the Student’s t-test. The pancreatic content of ir-insulin, islet area, and insulin area and % apoptotic cells were analyzed using the Student’s t-test. Hepatic CBG and 11 βHSD1 mRNA levels were expressed as relative optical density (ROD) mRNA: 18S rRNA ROD ratio and CBG, 11 βHSD1 and GR protein levels were expressed as ROD and all results were analyzed using the Student’s t-test. Statistical significance was determined as P<0.05. All values are presented as mean ± standard error (SEM) (Sigmastat, Jandel Scientific, California, USA).
4.3 RESULTS

4.3.1 Effects of betamethasone on cord glucose and insulin levels and I:G ratios
Maternal betamethasone administration did not alter mean cord plasma glucose levels at 125 days of gestation (figure 4.1a) but significantly decreased cord plasma insulin levels (figure 4.1b; \( P<0.05 \)). The mean I:G ratio was not altered with prenatal betamethasone exposure, possibly due to large variation between the means (figure 4.1c). At 146 days of gestation, cord plasma glucose levels (figure 4.1d; \( P<0.05 \)) were significantly increased following maternal betamethasone administration, without significant alterations in plasma insulin levels (figure 4.1e) or the mean I:G ratio (figure 4.1f).

4.3.2 Effects of betamethasone on pancreatic insulin content and morphology

4.3.2.1 Fetal pancreatic insulin content, insulin staining and islet area
In order to quantify changes in pancreatic insulin content, protein was extracted from the fetal pancreas and analyzed for ir-insulin levels. Fetal pancreatic insulin content (ng/mg of protein) was not significantly different following betamethasone treatment at 125 (figure 4.2a), and 146 days of gestation (figure 4.2b).

Positive ir-insulin staining was observed in fetal pancreatic islets in all groups of fetuses at 125 and 146 days of gestation (figure 4.3a-d), although large irregular immunopositive islets were identified in addition to smaller more conventional type islets. Adjacent sections incubated without primary antibody showed no positive staining for ir-insulin (figure 4.3e). Total islet area and the absolute area stained for insulin were not significantly altered with betamethasone administration at either 125 (figure 4.4a) or 146 days of gestation (figure 4.4c). Insulin area expressed as a percentage of total islet area was not significantly elevated at 125 days of gestation (figure 4.4b) but significantly elevated at 146 days of gestation (figure 4.4d; \( P<0.05 \)).

4.3.2.2 Fetal pancreatic islet apoptotic staining
Figure 4.5 illustrates sections stained for apoptosis in the fetal pancreas at 125 and 146 days of gestation. Apoptotic staining within islet cells were seen at both gestational ages (figure 4.5a-g).
Figure 4.1
Histograms representing cord plasma glucose and insulin levels and insulin: glucose ratios (I:G) at 125 (a, b, c; n=5 saline, n=6 betamethasone) and 146 days of gestation (d, e, f; n=7 saline, n=8 betamethasone) following either saline or maternal betamethasone administration. Values represent mean ± SEM. *P<0.05.
Figure 4.2
Histograms representing pancreatic insulin content at 125 (a; n=5 saline, n=6 betamethasone) and 146 days of gestation (b; n=7 saline, n=8 betamethasone) following either saline □ or maternal betamethasone ■ administration. Values are presented as ng of insulin /mg of extracted protein, mean ± SEM.
Figure 4.3
Photographs of immunohistochemical staining of insulin in fetal pancreas at 125 (a, saline; b, betamethasone) and at 146 days of gestation (c, saline; d, betamethasone). Control slides showed no staining (e). Magnification 200X.
Figure 4.4

Histograms representing fetal pancreatic morphometric analysis at 125 (a, b; n=5 saline, n=6 betamethasone) and 146 days of gestation (c, d; n=7 saline, n=8 betamethasone). Bars represent total islet area □ and ir-insulin area ◇ following saline and total islet area ● and ir-insulin area ■ following maternal betamethasone administration. Insulin area is also expressed as a % of total islet area following saline □ or maternal betamethasone ■ administration. Values are presented mean ± SEM. *P<0.05.
Figure 4.5
Immunohistochemical localization of apoptotic staining (arrows) in fetal pancreatic islets at 125 (a-d) and 146 (e-h) days of gestation following saline (a, b, e, f) or maternal betamethasone (c, d, g, h) administration. Slide stained for apoptosis are in left panels and stained for hemotoxylin and eosin are in right panels. Mouse islet was used as a positive control (i,j). Arrows identify apoptotic cells stained for apoptosis. Magnification at 400X.
The prevalence of apoptotic staining in endocrine islet cells at 125 days of gestation was ~7% of total islet cell number in control fetuses, consistent with previous reports of apoptosis in the postnatal rat pancreas (figure 4.6a; Petrik et al. 1999), but decreased with advancing gestation to ~5% at 146 days of gestation (figure 4.6b). Maternal betamethasone administration resulted in a reduction in the prevalence of apoptotic staining cells at 125 days of gestation but this difference did not reach statistical significance (figure 4.6a; P=0.08). At 146 days of gestation, the prevalence of apoptotic staining cells was similar between groups (figure 4.6b).

4.3.3 Effects of betamethasone on fetal hepatic 11βHSD1, CBG and GR levels
As shown in figure 4.7, a single transcript of 1.8kb for 11β HSD1 was detected in RNA samples from fetal livers. Due to RNA degradation as shown by the 18S (figure 4.7), only 3 saline treated animals were compared to 5 betamethasone treated animals. Maternal betamethasone administration significantly elevated fetal hepatic 11β HSD1 mRNA levels at 125 days of gestation (figure 4.7; P<0.05) and was associated with a significant increase in 11β HSD1 protein levels (figure 4.8a; P<0.05). 11βHSD1 protein levels (34kD) were not different between the groups at 146 days of gestation (figure 4.8b).

A single 1.8 kb transcript for CBG was found in fetal hepatic RNA samples (figure 4.9). Fetal hepatic CBG mRNA levels at 125 days of gestation were significantly elevated following maternal betamethasone administration (figure 4.9; P<0.05), although this was not associated with significant alterations in CBG protein levels (57kD; figure 4.10a). It was previously shown however, (see 3.3.4) that cord plasma corticosteroid binding capacity was elevated significantly in these animals at 125 days of gestation (Sloboda et al. 2000; Chapter 3). CBG protein levels in the fetal liver at 146 days of gestation were unchanged following maternal betamethasone administration (figure 4.10b). Mean GR protein levels in the fetal liver appeared increased at both 125 and 146 days of gestation after betamethasone treatment, but these differences did not reach statistical significance (figure 4.11a,b; P=0.10).

Samples were not available to detect 11β HSD1 and CBG mRNA levels at 146 days of gestation or GR mRNA levels at 125 and 146 days of gestation.

4.4 DISCUSSION
This study has demonstrated that repetitive maternal administration of betamethasone in the sheep resulted in a decrease in cord plasma insulin levels at 125 days and an increase in cord
Figure 4.6
Histograms representing image analysis of apoptotic cells in fetal pancreatic islets at 125 (a) and 146 (b) days of gestation following saline □ or maternal betamethasone ■ administration. Values are presented as apoptotic cells as a % of total cell number and are mean ± SEM.
Figure 4.7

Northern blot analysis of fetal hepatic 11β HSD1 mRNA at 125 days of gestation following either saline (n=3) or maternal betamethasone (n=5) administration. The autoradiograms for 11β HSD1 mRNA (1.8kb) and control 18S rRNA (1.8kb) are shown in the upper panel. The ROD of 11β HSD1 mRNA was expressed as a ratio 11β HSD1 ROD: 18S ROD and are shown in lower panel. Values presented as ROD and are mean ± SEM. *P<0.05.
Figure 4.8
Western blot analysis of fetal hepatic 11β HSD1 protein expression (34K) at 125 days of gestation (a) following either saline (n=5), □ or maternal betamethasone (n=6), ■ administration, and at 146 days of gestation (b) following either saline (n=6) □ or maternal betamethasone ■ (n=8) administration. Values are presented as ROD and are mean ± SEM. *P<0.05
Figure 4.9
Northern blot analysis of fetal hepatic CBG mRNA at 125 days of gestation following either either saline (n=4) or maternal betamethasone (n=6) administration. The autoradiograms for CBG mRNA (1.8kb) and control 18S rRNA (1.8kb) are shown in the upper panel. The ROD of CBG mRNA was expressed as a ratio CBG ROD: 18S ROD and area shown in lower panel. Values are presented as ROD and are mean ± SEM. *P<0.05.
Figure 4.10
Western blot analysis of fetal hepatic CBG protein expression (57K) at 125 days of gestation (a) following either saline (n=5) or maternal betamethasone (n=6), administration, and at 146 days of gestation (b) following either saline (n=6) or maternal betamethasone (n=8) administration. Values are presented as ROD and area mean ± SEM.
Figure 4.11
Western blot analysis of fetal hepatic GR protein expression (97K) at 125 days of gestation (a) following either saline (n=5), □ or maternal betamethasone (n=6), ■ administration, and at 146 days of gestation (b) following either saline (n=6) □ or maternal betamethasone (n=8) ■ administration. Values are presented as ROD and are mean ± SEM.
plasma glucose at 146 days of gestation. This study also demonstrates an increase in immunoreactive insulin staining in fetal pancreatic β cells at 146 days of gestation following maternal betamethasone administration. Furthermore, this is the first study to report the presence of apoptotic staining in fetal sheep pancreatic β cells during development.

Previous studies have demonstrated that fetal glucocorticoid exposure can potentially program pancreatic development (Phillips 1996, Seckl 1997, Hill 1999, 2000), although almost all evidence is derived from data collected on the rat. GR are present in the β cells of the adult pancreas (Fischer et al. 1990) and glucocorticoids have been shown to regulate pancreatic development (Rall et al. 1977, Hill 1999). Although the effects of prenatal glucocorticoid exposure on fetal pancreatic remodeling are unclear, many studies have demonstrated that prenatal undernutrition alters fetal pancreatic development and it has been suggested that glucocorticoids regulate these effects (Lindsay et al. 1996, Seckl 1997, Phillips 1998, Hill 1999, 2000). Indirect evidence points towards fetal glucocorticoid overexposure mediating β cell dysfunction. Transgenic mice overexpressing GR in the β cells exhibited impaired glucose tolerance and decreased insulin response to a glucose load due to impaired insulin release in vivo (Delauney et al. 1997). Furthermore, it has been shown previously that glucocorticoids inhibit insulin secretion from cultured adult mouse islets (Lambillote et al. 1997). It is unknown in the present study whether insulin secretion is altered, although the observed decrease in cord plasma insulin levels at 125 days of gestation may be a reflection of an alteration in pancreatic insulin secretion. This difference however, was no longer significant at 146 days of gestation. Furthermore, there was a trend to elevated pancreatic insulin content and insulin area of β cells was significantly elevated following maternal betamethasone at 146 days of gestation. These observations may be a reflection of a reduction in insulin secretion or an increase in clearance. A reduction in circulating insulin levels in this study may be related to previous observations that maternal betamethasone administration resulted in a ~20% decrease in fetal weight (Sloboda et al. 2000, Chapter 3). It has been previously shown that intrauterine growth restriction correlates with fetal insulin levels (Fowden et al. 1989) and small for gestational age infants have reduced plasma insulin levels (Economides et al. 1991). Furthermore, exogenous destruction of pancreatic β cells in ovine fetuses resulted in fetal hypoinsulinemia with a related 20% decrease in body weight (Philipps et al. 1991).
Glucocorticoids regulate the proliferation of ductal epithelial cells as well as β cells and also alter important growth factor expression levels that participate in fetal pancreatic development (Price et al. 1992, Li et al. 1998, Hill 1999). Most islet growth (in humans and rats) takes place during the perinatal period. In rodents, neogenesis of islets continues in neonatal life although it decreases as a function of age, and β cells undergo a “wave” of apoptosis during the postnatal period (Finegood et al. 1995, Hill 2000). This is thought to result in a fundamental change in the phenotype of pancreatic β cells whereby a new population of cells is formed that is better suited for adult metabolic control (Scaglia et al. 1997, Hill 1999). Therefore, any alterations in this remodeling event may alter the ability of the offspring to handle metabolic stress (Hill 1999, 2000). Physiological levels of glucocorticoids appear to play a modulatory role in the development of the rat pancreas, however very little is known regarding the development of the ovine pancreas. There is evidence that a wave of apoptosis occurs in human pancreatic development during the perinatal period (Kassem et al. 2000), although it is presently unknown if the fetal ovine pancreas undergoes similar remodeling. One study has described in detail the appearance of large islet bodies that stained positively for insulin, in addition to conventional islets of Langerhan present in the neonatal ovine pancreas. These large bodies were considered to represent immature β cells with persisting fetal characteristics (Titlbach et al. 1985), although functional analysis of these islets was not performed. Titlbach et al. (1985) described regressive changes in these large islet bodies in the first 10 days of postnatal life, where large bodies were no longer evident in young adult sheep. It is possible that the apoptotic remodeling “event” occurs late in gestation or in early postnatal life in sheep. In the present study, apoptotic staining was observed in fetal pancreatic islets as early as 125 days of gestation and was still present at 146 days, but further investigation into the ontogeny of apoptotic staining and the presence of factors regulating neogenesis in the fetal sheep pancreas are required. Although it has been proposed that glucocorticoid exposure may regulate fetal pancreatic apoptosis, we did not observe significant changes in apoptotic staining following maternal betamethasone administration. However, alterations in mean islet area following betamethasone administration resulting in more numerous, smaller islets would not contribute to alterations in apoptotic staining, but may contribute to alterations in glucose handling. It is possible, that effects of maternal betamethasone administration on pancreatic remodeling occurred at times in gestation other than 125 and 146 days, and probably early in the neonatal sheep.

This study has demonstrated an increase in fetal hepatic 11β HSD1 mRNA and protein and CBG mRNA expression levels at 125 days of gestation following repetitive maternal
administration of betamethasone. These observations are consistent with previous studies that have reported that fetal growth restriction (McMillen et al. 2000) and fetal dexamethasone infusion significantly elevated fetal hepatic 11βHSD1 (Yang et al. 1994) and CBG (Berdusco et al. 1993, Zhao et al. 1997) mRNA expression late in gestation. Furthermore, Yang et al. (1994) showed that elevations in 11βHSD1 mRNA expression levels were associated with a two-fold increase in enzymatic activity, supporting the idea that glucocorticoids regulate functional activity of this enzyme. In the present study, 11βHSD1 and CBG mRNA levels were significantly increased at 125 days of gestation and not at term (146 days). It has been shown previously that fetal hepatic 11βHSD1 and CBG expression levels normally increase in the ovine fetus from d120-125 to term (d140-145) (Langlois et al. 1995, Berducso et al. 1995, McMillen et al. 2000). Although direct comparison between fetuses at 125 and 146 days of gestation was not performed in this study, it is possible that an increase in basal 11βHSD1 and CBG levels from 125 to 146 days of gestation in control fetuses, eliminated any significant differences between the groups after betamethasone treatment at 146 days of gestation. Whether the alterations in expression levels in this study have a functional significance is uncertain, however, given the previous established relationship between 11βHSD1 and CBG mRNA expression and functional activity (Yang et al. 1994, Berducso et al. 1994) it appears likely that 11βHSD1 activity and corticosteroid binding in these animals has increased following maternal betamethasone administration. In support of this, previous observations (Sloboda et al. 2000, Chapter 3) demonstrated that maternal betamethasone administration resulted in an increase in plasma corticosteroid binding capacity (CBC) at 125 days of gestation with associated increases in cord plasma ACTH and cortisol later in gestation (146 days). It appears therefore likely that the increases in mRNA and protein expression reflect functional changes in the fetal liver.

The role of hepatic 11βHSD1 facilitating an increase in local glucocorticoid concentrations in the liver has been suggested by many studies (Yang et al. 1992, 1994, Langlois et al. 1995, Jamieson et al. 1999, 2000). In vivo, hepatic 11βHSD1 acts primarily as a reductase enzyme, converting inactive cortisone/deoxycorticosterone to biologically active cortisol/corticosterone (Seckl & Chapman 2000, Penning 1997). 11βHSD1 is present in the fetal sheep liver as early as day 60 of gestation and increases dramatically at term (140-147 days) (Yang et al. 1992). Reductase activity is detectable from 85 days of gestation and also increases with advancing gestation (Langlois et al. 1995). Furthermore, 11βHSD1 and GR have been colocalized in the liver of the rat (Whorwood et al. 1991) suggesting that 11βHSD1 may
regulate ligand access to GR (Whorwood et al. 1991, Seckl & Chapman 2000, Jamieson et al. 2000). Glucocorticoids in turn regulate 11β HSD1 expression and activity (Voice et al. 1996, Liu et al. 2001). In the same respect, hepatic CBG mRNA is present in the liver of fetal sheep as early as day 100 and increases dramatically at term (Ballard et al. 1982, Berdusco et al. 1993). CBG is primarily produced in the fetal liver (Ballard et al. 1982, Berdusco et al. 1993) and has been shown in many species to actively bind 65-80% of circulating cortisol (Seralini et al. 1996, Gayrard et al. 1996), suggesting that CBG regulates local and circulating levels of cortisol, resulting in an overall decrease in bioactive glucocorticoids (Berdusco et al. 1995, Challis et al. 1995). Many studies have reported that glucocorticoids regulate both CBG expression levels and activity (Schlechte & Hamilton 1987, Berdusco et al. 1993, 1994, Challis et al. 1985, 1995, Jeffray et al. 1995). It seems therefore likely that in these study elevations in hepatic 11β HSD1 and CBG levels work together, to regulate circulating and local glucocorticoid levels.

It has been previously demonstrated that increasing levels of plasma cortisol correlate positively with the activity of key hepatic enzymes involved in gluconeogenesis, such as PEPCK and glucose-6-phosphate (Fowden et al. 1990, 1993). Furthermore, in 11β HSD1 knockout mice, gluconeogenic enzyme expression levels are reduced (Koteletsev et al. 1997) indicating the potential influence this enzyme has on liver metabolic processes. Therefore, inappropriate increases in intrahepatic levels of glucocorticoids, through a change in hepatic 11β HSD1 or CBG expression levels, could influence metabolic regulation. Previously, Nyirenda et al. (1998) have demonstrated that maternal dexamethasone administration to rats (100μg/kg per day in the last week of pregnancy) resulted in a decrease in birth weight, impaired glucose tolerance and a significant increase in hepatic PEPCK and GR mRNA expression in adult offspring. Similar results were found in offspring of mothers fed a low protein diet (Desai et al. 1997). In the present study however, fetal GR protein levels in the present study were not significantly different (P=0.1). It may be that differences in hepatic GR, as the result of betamethasone treatment may not become evident until postnatal life. Inappropriate increases in hepatic 11β HSD1 activity could therefore be contributing factors in liver glucose production, since increased activity would increase glucose production through gluconeogenesis (via an increase in PEPCK) (Jamieson et al. 1999). Cord plasma glucose levels were significantly elevated at 146 days of gestation, perhaps in response to an increase in fetal hepatic gluconeogenesis. These observations are important in light of current evidence that demonstrates an association between intrauterine growth restriction and postnatal alterations in HPA and metabolic function (Lithell et al. 1990,
Phillips et al. (1998) demonstrated that raised basal cortisol levels were related to lower birth weights and associated with higher blood pressure, basal plasma glucose and triglyceride levels and insulin resistance in adult men.

The observations made in this study are important in light of current evidence linking low birth weight, prenatal undernutrition and glucocorticoid exposure to the increased risk of developing postnatal insulin resistance and diabetes (Langely et al. 1994, Desai et al. 1995, Phillips 1996, Barker 1998). Many studies have reported the association between birth weight, impaired glucose tolerance and diabetes in adults (Osmond et al. 1993, Hales et al. 1991, Lithell et al. 1996). Although the direct mechanisms linking these factors are unknown, studies have provided strong evidence that prenatal undernutrition and fetal glucocorticoid exposure regulate both peripheral insulin resistance and central liver and pancreatic metabolic function, resulting in alterations in postnatal glucose tolerance and insulin resistance (Langley et al. 1994, Lindsay et al. 1996, Nyirenda et al. 1998, Hill 2000). Future studies are therefore essential to further the understanding of the mechanisms underlying intrauterine programming of metabolism.
CHAPTER 5

5 The Effect of Prenatal Betamethasone Administration on Postnatal Ovine Hypothalamic-Pituitary-Adrenal Function

5.1 INTRODUCTION

During intrauterine development, the fetus may potentially be exposed to a number of adverse circumstances that lead to an increase in circulating glucocorticoids. Several studies have implicated fetal glucocorticoid overexposure as a key component of fetal programming and the increased risk of developing adult cardiovascular and metabolic diseases (Seckl 1997, 2000, Langley-Evans et al. 1997, Challis et al. 1999). Placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) normally prevents trans-placental transfer of maternal glucocorticoids (Burton & Wadell 1999, Stewart et al. 1994). However, alterations in the activity and/or the expression of this protective enzyme may expose the fetus to increased levels of maternal glucocorticoids, resulting in the programming of homeostatic mechanisms that could potentially lead to later disease (Edwards et al. 1993, Stewart et al. 1995, Seckl 1997, Langley-Evans et al. 1996). Furthermore, prenatal stress resulting in the elevation of maternal corticosteroid levels has been associated with alterations in postnatal endocrine function (Takahashi et al. 1991, Weinstock et al. 1992, Copper et al. 1996).

Synthetic glucocorticoids, such as betamethasone, are poor substrates for the 11β-HSD2 enzyme (Seibe et al. 1993), and pass relatively freely across the placenta into the fetal circulation. As a result, synthetic glucocorticoids are administered to women at risk of preterm delivery, to enhance fetal lung maturation and to reduce neonatal mortality from respiratory distress syndrome (Liggins & Howie 1972, Ballard & Ballard 1995). Despite the obvious advantages of prenatal synthetic glucocorticoid administration, relatively little is known regarding the effects of clinical glucocorticoid administration on either the developing fetal endocrine axes or long term postnatal endocrine function. Recent studies have begun to investigate the potential consequences of chronic fetal exposure to synthetic glucocorticoids. Synthetic glucocorticoid administration to the pregnant rat (100μg/kg per day for one week) late in gestation resulted in significant elevations in basal corticosterone levels with associated hypertension in adult offspring (Levitt et al. 1996). Fletcher et al. (2000) demonstrated that low dose dexamethasone infusion into the ovine fetus prevented hypoxia-induced increases in fetal plasma ACTH and cortisol levels, but increased the plasma glycemic response. These
observations suggest that fetal glucocorticoid exposure may have differential effects on developing HPA and metabolic axes. It has recently been shown that maternal betamethasone administration at mid-gestation results in elevated postnatal insulin responses to a glucose load in a pattern that resembles type II diabetes (Sloboda et al. 2000; Chapter 6). These observations suggest that prenatal fetal glucocorticoid exposure has an important impact on postnatal glycemic control.

Few studies have examined the effects of clinically relevant doses of glucocorticoids on fetal endocrine development and subsequent postnatal HPA function. It has been shown in sheep that maternal betamethasone administration, at a dose that mimics clinical administration, significantly elevates fetal basal plasma ACTH, cortisol and cortisol binding capacity (Sloboda et al. 2000; Chapter 3). However, it is presently unknown whether this effect persists into postnatal life. In addition, maternal (Jobe et al. 1998, Sloboda et al. 2000), but not fetal betamethasone administration significantly reduces birth weight (Newnham et al. 1999, Jobe et al. 1998), and weight at 3 months postnatal age (Moss et al. 2000), suggesting that the route of glucocorticoid administration plays an important role in the outcome of exposure. These observations become clinically relevant if we consider the epidemiological evidence that links a reduction in birth weight to an array of postnatal diseases (Barker 1998). Furthermore, recent studies in humans have shown an association between low birth weight and increased HPA activity in adulthood (Levitt et al. 2000, Phillips et al. 2000, Reynolds et al. 2001). These reports propose that intrauterine glucocorticoid exposure may be the key to the association between fetal growth restriction and HPA hyperactivity. Therefore, the aim of this study was to determine the effects of maternal and fetal betamethasone administration on postnatal ovine hypothalamic-pituitary-adrenal function at six months and one year of age.

5.2 MATERIALS AND METHODS
All experimental procedures were approved by the Animal Experimentation Ethics Committee of the University of Western Australia and at the University of Toronto according to guidelines of the Canadian Council of Animal Care.

5.2.1 Experimental Procedures
Pregnant ewes bearing singleton fetuses were randomly allocated to receive either no treatment (NT) or maternal or fetal injections of saline and/or betamethasone (Table 5.1). All treatment animals were injected intramuscularly with 150mg of medroxyprogesterone acetate (Depo Provera™, Upjohn, Australia) at 100 days of gestation to reduce pregnancy losses due to
glucocorticoid treatment. It has been shown previously that this treatment does not affect the fetal steroidogenesis, but results in the inhibition of delivery (Nathanielsz et al. 1988). Saline-treated animals were injected with normal saline at 104, 111, 118 and 125 days of gestation (MS or FS); animals that received a single betamethasone dose were injected with betamethasone at 104 days of gestation and saline at 111, 118 and 125 days of gestation (M1 or F1); animals that received repeated betamethasone doses were injected with betamethasone at 104, 111, 118 and 125 days of gestation (M4 or F4). Maternal betamethasone (Celestone Chronodose™, Schering Plough, Australia) injections were given intramuscularly in a dose of 0.5mg/kg body weight; saline injections were of a comparable volume (5-6ml). Fetal injections were given using an established technique (Newnham et al. 1999). Briefly, the ewe was held in a supine position, 70% ethanol was applied to the ewe’s abdomen as a coupling medium and the fetus was imaged using a 3.5 MHz sector transducer (Echo Camera SSD-500, Aloka, Japan). Betadine solution (Faulding, Australia) was applied to the injection site and a 21-gauge 9cm spinal needle (Terumo, Australia) was introduced through the maternal abdomen into the muscle of the fetus’ shoulder or rump. Betamethasone (0.5mg/kg estimated fetal body weight; 1.4kg at 104 days, 1.9kg at 111 days, 2.2kg at 118 days, 2.5kg at 125 days) or an equal volume of saline (1ml) was injected into the fetus by an assistant. The needle tip was imaged throughout the entire procedure. Drs. J. Newnham and T. Moss were responsible for the maternal and fetal injections.

Ewes were permitted to deliver their lambs spontaneously in a field environment and were not disturbed until the time of experimentation. Lambs were raised by their mothers and were observed closely several times each day for the first few postnatal weeks. Two of the lambs in the M4 group were supplemented with powdered milk (Divetalact, Australia) when it became apparent that lactation was affected in these ewes from this group. At approximately 2 months of age lambs were immunized, their tails cropped and the males were castrated. Weaning occurred at 3 months of age. Six lambs from each of the 7 treatment groups were chosen at random as subjects for CRH+AVP challenge tests at 6 months and retested at 1 year of age (table 5.1, 5.2). The remainder of the injected lambs remained in a field environment. At one year, supplementation of numbers in some groups (table 5.2) where possible, was required due to loss (predation) unrelated to experimentation; these additional lambs were obtained from the remaining lambs from the original flock of animals that were not originally allocated to participate in the CRH+AVP challenges. Prior to the experiments at one year of age, the estrous cycles of all female animals were synchronized with intravaginal progesterone sponges (30mg Flugestone Acetate, ChronoGest30, NSW).
<table>
<thead>
<tr>
<th>Gestational Age</th>
<th>No Treatment Control</th>
<th>Maternal intramuscular injections</th>
<th>Direct fetal intramuscular injections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Maternal saline</td>
<td>Maternal 1-beta</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>MPA</td>
<td>MPA</td>
</tr>
<tr>
<td>105</td>
<td>-</td>
<td>Saline</td>
<td>Beta</td>
</tr>
<tr>
<td>111</td>
<td>-</td>
<td>Saline</td>
<td>Saline</td>
</tr>
<tr>
<td>118</td>
<td>-</td>
<td>Saline</td>
<td>Saline</td>
</tr>
<tr>
<td>125</td>
<td>-</td>
<td>Saline</td>
<td>Saline</td>
</tr>
</tbody>
</table>

**Table 5.1 Prenatal treatment protocol.**

MPA (medroxyprogesterone acetate, 150mg), Beta (Betamethasone 0.5mg/kg ewe bodyweight or estimated fetal bodyweight).
<table>
<thead>
<tr>
<th>AGE</th>
<th>No Treatment</th>
<th>Maternal intramuscular injections</th>
<th>Direct fetal intramuscular injections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Maternal saline</td>
<td>Maternal 1-beta</td>
</tr>
<tr>
<td>6 months</td>
<td>M n= 3</td>
<td>M n=3</td>
<td>M n=4</td>
</tr>
<tr>
<td></td>
<td>F n= 3</td>
<td>F n=3</td>
<td>F n=2</td>
</tr>
<tr>
<td>One year</td>
<td>M n= 3*</td>
<td>M n=3*</td>
<td>M n=3</td>
</tr>
<tr>
<td></td>
<td>F n= 2</td>
<td>F n=2</td>
<td>F n=2</td>
</tr>
</tbody>
</table>

Table 5.2 Treatment groups sample size and sex at 6 months and one year.

Beta, betamethasone; M male; F female. * indicates groups that were supplemented at one year.
5.2.2 CRH challenges at 6 and 12 months postnatal age

At both 6 months and one year of postnatal age lambs underwent aseptic surgery to implant femoral arterial and venous catheters (halothane anesthesia, 1-2% in O₂, following induction with ketamine/xylazine). All animals were allowed at least 3 days to recover before undergoing CRH + AVP challenges. Catheters were removed after the completion of experiments. Upon completion of experiments, the animals were returned to the field to await further future testing.

**CRH + AVP challenges:** Food was withdrawn 12h before challenges, but the animals were allowed free access to water. Basal arterial blood samples (5ml) were drawn at 30 (-30) and 15 (-15) minutes and immediately before (0) the administration of an intravenous bolus of 0.5µg ovine CRH + 0.1µg AVP (Bachem, CA, USA) per kg lamb weight followed by a 10ml saline flush. One basal sample (-30min) was used to assess basal blood gases and pH. Arterial samples (5ml) were collected at 5, 10, 20, 30, 60, 90, 120, 180 and 240 minutes after the CRH + AVP bolus. Samples were centrifuged at 1500g for 10 minutes at 4°C; plasma was collected and stored at -80°C until further analysis. All challenges were administered between 0800-0900 h in order to minimize the impact of circadian variability in measurements of plasma ACTH and cortisol.

5.2.3 Measurement of plasma ACTH and cortisol

Plasma immunoreactive (ir)-ACTH concentrations were measured using a commercial RIA kit (Incstar, Stillwater, MN, USA) previously validated for use in the fetal sheep (Norman *et al.* 1985, Jeffray *et al.* 1998). The intra-assay coefficient of variation was 4.5%, and the inter-assay coefficient of variation was 4%. The mean assay sensitivity was 15 pg/ml. Plasma cortisol concentrations were quantified by RIA after extraction with diethyl ether. The intra-assay coefficient of variation was 6%. The inter-assay coefficient of variation was 15%. See 3.2.3.1 for further details.

5.2.4 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). A comparison of group means was made using the two-way analysis of variance (ANOVA, SAS, Cary, NC, USA and S-PLUS, Mathsoft, WA, USA) followed by a Tukey’s post hoc pairwise multiple comparison analysis where differences between the means were significant (P<0.05). In all cases, basal values represent the mean value of three samples drawn at 30 minutes, 15 minutes and immediately prior to the administration of the CRH + AVP intravenous bolus. Due to the small
sample size in each of the treatment groups, the effect of gender on any of the outcome variables was not analyzed. In order to assess the overall effect of prenatal betamethasone on the response patterns to the challenge, the areas under the response curves for each group were calculated between times 5 and 240 minutes. These analyses were performed by Dr. L. Gurrin (University of Western Australia). The formula to calculate the area under both ACTH and cortisol curves is as follows using the trapezoid rule (Salas et al. 1986):

\[ \text{AUC} = 2.5 \cdot t_5 + 7.5 \cdot t_{10} + 10 \cdot t_{20} + 30 \cdot t_{60} + 30 \cdot t_{90} + 45 \cdot t_{120} + 30 \cdot t_{180} + 45 \cdot t_{240} \]

where \( t_x \) = time concentration at time point \( x \).

To examine the effects of both prenatal betamethasone and age on postnatal ACTH and cortisol responses to the CRH + AVP challenge, the observed peak and AUC ACTH and cortisol values at 6 months were compared with those at one year postnatal age within each treatment group, using the Students' t-test (SigmaStat, Jandel Scientific, MD, USA). The effect of betamethasone on age related changes were analyzed using a one-way ANOVA on the incremental ACTH and cortisol responses at 6 months and one year of age, followed by a Tukey’s post hoc analysis where differences between the means were significant (\( P<0.05 \)). The cortisol: ACTH ratio at baseline, 5 and 90 minutes post bolus were calculated at 6 months and one year and compared using a one-way ANOVA followed by a Tukey’s post hoc analysis where differences between the means were significant (\( P<0.05 \)) (SigmaStat, Jandel Scientific, MD, USA). In all cases the level of statistical significance was taken to be \( P<0.05 \).

5.3 RESULTS

5.3.1 Basal blood gases
There was no effect of treatment on pH, PO2 or PCO2 at either 6 months or one year of postnatal age (table 5.3).

5.3.2 Six months of age

5.3.2.1 Effects of prenatal maternal betamethasone administration
At 6 months postnatal age, basal plasma ACTH and cortisol concentrations were similar in all groups (table 5.4). All animals responded to the CRH + AVP challenge with peak ACTH levels occurring approximately five minutes post bolus administration (figure 5.1a). M1 and M4 groups exhibited significantly lower peak ACTH values compared to untreated controls (figure 5.1a; \( P<0.05 \)). The area under the curve (AUC) (inset histogram, figure 5.1a) for the NT group was higher than in all other groups but was only significantly different from that of the M4 group (\( P<0.05 \)). In all maternal administration groups, peak cortisol levels were observed at 20 minutes.
### Table 5.3 Basal blood gas values at 6 months and one year of age

Values are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>6 Months of Age</th>
<th>One Year of Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.49±0.02</td>
<td>7.46±0.03</td>
</tr>
<tr>
<td>PO$_2$ (mmHg)</td>
<td>101.2±4.1</td>
<td>110.8±2.6</td>
</tr>
<tr>
<td>PCO$_2$ (mmHg)</td>
<td>33.0±1.5</td>
<td>36.3±1.3</td>
</tr>
<tr>
<td>MS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.52±0.03</td>
<td>7.44±0.04</td>
</tr>
<tr>
<td>PO$_2$ (mmHg)</td>
<td>96.7±4.3</td>
<td>119.9±5.9</td>
</tr>
<tr>
<td>PCO$_2$ (mmHg)</td>
<td>34.1±0.7</td>
<td>32.5±4.9</td>
</tr>
<tr>
<td>M1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.42±0.02</td>
<td>7.49±0.01</td>
</tr>
<tr>
<td>PO$_2$ (mmHg)</td>
<td>102.5±2.9</td>
<td>113.0±3.0</td>
</tr>
<tr>
<td>PCO$_2$ (mmHg)</td>
<td>29.5±0.5</td>
<td>35.6±3.0</td>
</tr>
<tr>
<td>M4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.50±0.03</td>
<td>7.48±0.03</td>
</tr>
<tr>
<td>PO$_2$ (mmHg)</td>
<td>93.0±4.6</td>
<td>108.0±3.0</td>
</tr>
<tr>
<td>PCO$_2$ (mmHg)</td>
<td>29.9±1.5</td>
<td>38.2±0.6</td>
</tr>
<tr>
<td>FS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.51±0.02</td>
<td>7.43±0.02</td>
</tr>
<tr>
<td>PO$_2$ (mmHg)</td>
<td>95.9±2.0</td>
<td>111.2±5.0</td>
</tr>
<tr>
<td>PCO$_2$ (mmHg)</td>
<td>30.2±3.5</td>
<td>37.3±0.7</td>
</tr>
<tr>
<td>F1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.47±0.02</td>
<td>7.49±0.02</td>
</tr>
<tr>
<td>PO$_2$ (mmHg)</td>
<td>98.3±6.5</td>
<td>116.0±15.5</td>
</tr>
<tr>
<td>PCO$_2$ (mmHg)</td>
<td>30.5±1.5</td>
<td>35.6±1.2</td>
</tr>
<tr>
<td>F4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.48±0.04</td>
<td>7.50±0.05</td>
</tr>
<tr>
<td>PO$_2$ (mmHg)</td>
<td>104.6±5.6</td>
<td>114.4±5.1</td>
</tr>
<tr>
<td>PCO$_2$ (mmHg)</td>
<td>32.0±1.7</td>
<td>34.2±2.9</td>
</tr>
</tbody>
</table>
post bolus administration, following the ACTH peak by approximately 15 minutes (figure 5.1b). The cortisol response pattern to the CRH + AVP challenge and the AUC for cortisol were similar in all groups (figure 5.1b; inset histogram). Table 5.5 represents the cortisol: ACTH ratio at baseline and 5, 20 and 90 minutes post challenge at 6 months of age. No significant differences were observed between the groups (table 5.5).

5.3.2.2 Effects of prenatal fetal betamethasone administration

At 6 months postnatal age, basal plasma ACTH and cortisol concentrations were not different from controls following prenatal fetal betamethasone administration (table 5.4). All animals exhibited similar peak levels of ACTH in response to the CRH + AVP bolus, at approximately 5 minutes after administration (figure 5.2a). The ACTH response patterns and the AUC were similar for all groups (figure 5.2a). All groups exhibited peak cortisol values between 20 and 30 minutes following the administration of CRH + AVP. FS and F4 lambs had the lowest peak values at 20 minutes compared to NT and F1 (figure 5.2b; P<0.05), otherwise for the remainder of the challenge, the cortisol response patterns and the cortisol AUC were similar in all 4 groups (figure 5.2b). No significant differences in the cortisol: ACTH ratios were observed between the groups (table 5.5).

5.3.3 One year of age

5.3.3.1 Effect of prenatal maternal betamethasone administration

At one year postnatal age, basal plasma ACTH concentrations were similar in all groups (table 5.4). Basal plasma cortisol however, was significantly higher in the M1 group compared to NT, MS and M4 groups (table 5.4; P<0.05). All animals responded to the CRH + AVP intravenous bolus at one year with peak ACTH levels at approximately five minutes post bolus administration (figure 5.3a). The ACTH response patterns and the ACTH AUC at one year of postnatal age were not significantly affected by prenatal maternal betamethasone administration (figure 5.3a). All maternal treatment groups exhibited peak cortisol values at either 20 or 30 minutes following the administration of a CRH + AVP intravenous bolus (figure 5.3b). The M1 group exhibited significantly higher 5 and 10 minute cortisol values compared to the NT and M4 groups (P<0.05), and significantly higher peak cortisol levels at 20 minutes compared to all other groups (figure 5.3b; P<0.05). M1 lambs demonstrated significantly higher cortisol AUC compared to the NT and M4 groups (figure 5.3b; P<0.05), but not MS possibly due to large variation between the means. In contrast, the mean cortisol response pattern of the M4 group was lower than in all other groups, but was not statistically significant. Table 5.6 represents the
Table 5.4 Basal ACTH and cortisol levels at 6 months and one year postnatal age.

Values are presented as mean ± SEM. * P< 0.05 M1 versus NT, MS and M4.
Figure 5.1
ACTH (a) and cortisol (b) response to CRH + AVP challenge at 6 months postnatal age in maternally treated groups. Inset histograms represent the area under the ACTH and cortisol response curves for each treatment group. ○ No treatment (NT); ▼ Maternal Saline (MS); ■ Maternal single dose (M1); ◆ Maternal multiple dose (M4). Values are mean ± SEM, * P<0.05. The absence of error bars indicates that the variation in the mean data was numerically contained within the upper and lower limits of the symbols used for the data points.
<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Basal F:ACTH</th>
<th>F: ACTH at 5 min. post bolus</th>
<th>F:ACTH at 20 min. post bolus</th>
<th>F:ACTH at 90 min. post bolus</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT (n=6)</td>
<td>0.62 ± 0.23</td>
<td>0.07 ± 0.01</td>
<td>0.20 ± 0.02</td>
<td>0.25 ± 0.13</td>
</tr>
<tr>
<td>MS (n=5)</td>
<td>0.60 ± 0.26</td>
<td>0.11 ± 0.04</td>
<td>0.20 ± 0.06</td>
<td>0.57 ± 0.17</td>
</tr>
<tr>
<td>M1 (n=6)</td>
<td>0.77 ± 0.26</td>
<td>0.17 ± 0.03</td>
<td>0.35 ± 0.06</td>
<td>0.57 ± 0.17</td>
</tr>
<tr>
<td>M4 (n=6)</td>
<td>0.60 ± 0.23</td>
<td>0.20 ± 0.08</td>
<td>0.34 ± 0.05</td>
<td>0.58 ± 0.14</td>
</tr>
<tr>
<td>FS (n=5)</td>
<td>0.56 ± 0.47</td>
<td>0.09 ± 0.05</td>
<td>0.12 ± 0.06</td>
<td>0.14 ± 0.15</td>
</tr>
<tr>
<td>F1 (n=5)</td>
<td>0.43 ± 0.41</td>
<td>0.13 ± 0.04</td>
<td>0.27 ± 0.05</td>
<td>0.37 ± 0.12</td>
</tr>
<tr>
<td>F4 (n=6)</td>
<td>1.10 ± 0.40</td>
<td>0.13 ± 0.04</td>
<td>0.20 ± 0.05</td>
<td>0.30 ± 0.12</td>
</tr>
</tbody>
</table>

Table 5.5 Cortisol to ACTH ratio at baseline, and 5, 20 and 90 minutes after CRH + AVP bolus administration at 6 months postnatal age.

All values are expressed as mean ± SEM in ng/pg. F; cortisol.
Figure 5.2
ACTH (a) and cortisol (b) response to CRH + AVP challenge at 6 months postnatal age in fetally treated groups. Inset histograms represent the area under the ACTH and cortisol response curves for each treatment group. ○ No treatment (NT); ▼ Fetal Saline (FS); □ Fetal single dose (F1); ● Fetal multiple dose (F4). Values are mean ± SEM. The absence of error bars indicates that the variation in the mean data was numerically contained within the upper and lower limits of the symbols used for the data points.
Figure 5.3
ACTH (a) and cortisol (b) response to CRH + AVP challenge at one year postnatal age in maternally treated groups. Inset histograms represent the area under the ACTH and cortisol response curves for each treatment group. ○ No treatment (NT); ▼ Maternal Saline (MS); ■ Maternal single dose (M1); ○ Maternal multiple dose (M4). Values are mean ± SEM, * P<0.05. The absence of error bars indicates that the variation in the mean data was numerically contained within the upper and lower limits of the symbols used for the data points.
cortisol: ACTH ratios at baseline and 5, 20 and 90 minutes post challenge at one year of age. The M1 lambs exhibited significantly higher cortisol: ACTH ratios at baseline, 20 and 90 minutes post challenge compared to NT, MS and M4 groups (table 5.6, P<0.05).

5.3.3.2 Effects of prenatal fetal betamethasone administration

At one year postnatal age, basal plasma ACTH concentrations were similar in all groups (table 5.4). All animals responded to the CRH + AVP challenge with peak ACTH values at either 5 or 10 minutes post bolus administration (figure 5.4a). The F1 and F4 groups exhibited the lowest peak ACTH values and their ACTH response patterns remained lower than that of the NT and FS groups but did not reach statistical significance. Consistent with the response pattern, the ACTH AUC for the F1 and F4 groups were significantly lower than that of the FS group (figure 5.4a, inset histogram; P<0.05). Peak cortisol levels were observed at either 20 or 30 minutes following the administration of CRH + AVP (figure 5.4b). Cortisol levels were significantly higher in the F1 group at 30 minutes post challenge (P<0.05), but were similar throughout the rest of the challenge period. The cortisol AUC was similar in all groups (figure 5.4b). Prenatal betamethasone administration did not alter the cortisol: ACTH ratio in any of the fetal treatment groups (table 5.6).

5.3.4 Comparison of the ACTH and cortisol responses (6 months and one year of postnatal age)

The peak ACTH values after CRH + AVP were significantly higher in NT, MS, and FS lambs at one year of age compared with values at 6 months of age (figure 5.5a and 5.6a; P<0.05). M1 and M4 lambs exhibited higher one year peak ACTH values compared with those at 6 months, although this difference was only significant in M1 lambs (figures 5.5a; P<0.05). In contrast, fetal betamethasone treatment attenuated the developmental increase observed in peak ACTH responses in the NT and FS groups. F1 and F4 lambs had similar ACTH peak responses at one year compared to 6 months of postnatal age (figures 5.6a).

Absolute ACTH AUC was significantly higher in MS, FS and M1 lambs (figures 5.7a and 5.8a; P<0.05). Although the ACTH AUC was elevated at one year of age in NT and M4 lambs, these differences did not reach statistical significance (figures 5.7a and 5.8a; P=0.07 M4 6months versus one year). Consistent with peak values, ACTH AUC for F1 and F4 lambs was similar at one year of age compared with those at 6 months of age (figure 5.8a). Following CRH + AVP challenge, the peak cortisol values at one year were similar to the values at 6 months of
Figure 5.4
ACTH (a) and cortisol (b) response to CRH + AVP challenge at one year postnatal age in fetally treated groups. Inset histograms represent the area under the ACTH and cortisol response curves for each treatment group. ○ No treatment (NT); ▼ Fetal Saline (FS); ■ Fetal single dose (F1); ● Fetal multiple dose (F4). Values are mean ± SEM, * P<0.05. The absence of error bars indicates that the variation in the mean data was numerically contained within the upper and lower limits of the symbols used for the data points.
### Table 5.6 Cortisol to ACTH ratio at baseline, and 5, 20 and 90 minutes after CRH+AVP bolus administration at one year postnatal age.

All values are expressed as mean ± SEM in ng/pg. F; cortisol. * P<0.05 M1 versus NT, MS and M4 groups.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Basal F:ACTH</th>
<th>F: ACTH at 5 min. post bolus</th>
<th>F:ACTH at 20 min. post bolus</th>
<th>F:ACTH at 90 min. post bolus</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT (n=5)</td>
<td>0.16 ± 0.04</td>
<td>0.07 ± 0.04</td>
<td>0.13 ± 0.02</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>MS (n=5)</td>
<td>0.16 ± 0.07</td>
<td>0.04 ± 0.06</td>
<td>0.08 ± 0.06</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>M1 (n=5)</td>
<td>0.42 ± 0.07*</td>
<td>0.17 ± 0.06</td>
<td>0.34 ± 0.06*</td>
<td>0.30 ± 0.04*</td>
</tr>
<tr>
<td>M4 (n=5)</td>
<td>0.12 ± 0.08</td>
<td>0.08 ± 0.06</td>
<td>0.16 ± 0.06</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>FS (n=4)</td>
<td>0.15 ± 0.07</td>
<td>0.07 ± 0.04</td>
<td>0.10 ± 0.12</td>
<td>0.17 ± 0.90</td>
</tr>
<tr>
<td>F1 (n=5)</td>
<td>0.28 ± 0.07</td>
<td>0.08 ± 0.04</td>
<td>0.35 ± 0.12</td>
<td>0.19 ± 0.99</td>
</tr>
<tr>
<td>F4 (n=4)</td>
<td>0.18 ± 0.09</td>
<td>0.12 ± 0.04</td>
<td>0.20 ± 0.14</td>
<td>0.27 ± 1.11</td>
</tr>
</tbody>
</table>
Figure 5.5
Peak ACTH (a) values at ● 6 months and ○ one year postnatal age and cortisol (b) values at ▼ 6 months and ▽ one year of postnatal age following CRH + AVP challenges in animals receiving maternal treatments. Values are mean ± SEM, * P<0.05 6 months versus one year. The absence of error bars indicates that the variation in the mean data was numerically contained within the upper and lower limits of the symbols used for the data points. No treatment (NT; n=5); Maternal Saline (MS; n=5); Maternal single dose (M1; n=5); Maternal multiple dose (M4; n=4).
Figure 5.6
Peak ACTH (a) values at ● 6 months and ○ one year postnatal age and cortisol (b) values at ▼ 6 months and ▽ one year of postnatal age following CRH + AVP challenges in animals receiving fetal treatments. Values are mean ± SEM. * P<0.05 6 months versus one year. The absence of error bars indicates that variation in the mean data was numerically contained within the upper and lower limits of the symbols used for the data points. No treatment (NT; n=5); Fetal Saline (FS; n=4); Fetal single dose (F1; n=5); Fetal multiple dose (F4; n=4).
Figure 5.7
ACTH AUC (a) and cortisol AUC (b) values at 6 months and one year postnatal age following CRH + AVP challenges in animals receiving maternal treatments. Values are mean ± SEM, * P<0.05 6 months versus one year. No treatment (NT; n=5); Maternal Saline (MS; n=5); Maternal single dose (M1; n=5); Maternal multiple dose (M4; n=4).
Figure 5.8

ACTH AUC (a) and cortisol AUC (b) values at ■ 6 months and □ one year postnatal age following CRH + AVP challenges in animals receiving fetal treatments. Values are mean ± SEM, * P<0.05 6 months versus one year. No treatment (NT; n=5); Fetal Saline (FS; n=5); Fetal single dose (F1; n=4); Fetal multiple dose (F4; n=4).
age in NT, MS, M4 and F4 lambs (figure 5.5b, 5.6b). A single dose of prenatal betamethasone increased peak cortisol responses at one year compared to those at 6 months, although this difference was statistically significant in the F1 groups only (figure 5.5b; P=0.07 M1 peak cortisol one year versus 6 months of age; figure 5.6b P<0.05). FS lambs did exhibit a modest, although significant increase in peak cortisol responses at one year of age compared with that at 6 months of age (figure 5.6b; P<0.05).

Although the M1 lambs demonstrated higher cortisol AUC at one year compared to 6 months of age, this difference did not reach statistical significance (figure 5.7b; P=0.057). F1 lambs however, demonstrated significantly higher cortisol AUC at one year of age compared with that at 6 months of age (figure 5.8b; P<0.05). In all other groups, cortisol AUC was similar at one year of age compared with that at 6 months of age.

5.4 DISCUSSION

This study has shown that prenatal glucocorticoids administered either to the mother or directly to the fetus significantly alter postnatal ovine HPA responses, and that these alterations are dependent upon the dose and route of glucocorticoid administration. In addition, the responses between 6 months and one year of postnatal age were altered with age and these age related changes were influenced by prenatal betamethasone administration.

In the present study administration of 0.5mg/kg prenatal betamethasone resulted in alterations in postnatal ACTH and cortisol responses at one year of age to an intravenous bolus of CRH + AVP. This method tests both pituitary and adrenal responses to hypothalamic neuropeptides and has been shown previously to elicit a substantial ACTH and cortisol response in ovine fetuses (Norman & Challis 1987, Hawkins et al. 1999) and in adult sheep (Brooks & Challis 1989).

5.4.1 Administration of Medroxyprogesterone Acetate (MPA)

Due to the constraints of glucocorticoid treatment in pregnant sheep, the administration of MPA was necessary to minimize the occurrence of preterm labour and abortion induced by glucocorticoids (Liggins 1968, Ikekami et al. 1997, Jobe et al. 1998). Synthetic progestins, such as MPA, have been shown to have high affinity for GR (Selman et al. 1996) and have shown to suppress HPA axis activity (Lang et al. 1990, Selman et al. 1997). Therefore, the potential effect of MPA administration on HPA axis function in this study cannot be ignored. Previous studies however, involved prolonged treatment (from days to weeks) with high doses of MPA (80mg–
Furthermore, in vitro studies have shown that treatment of human JEG-3 cells (choriocarcinoma cell line) with MPA did not alter P450scc activity, MPA treatment in human adrenal microsomes did not affect P450C17 activity, but MPA treatment did suppress 3βHSD activity in yeast microsomes (Lee et al. 1999). Few studies have evaluated the effect of MPA administration on fetal sheep HPA axis activity. Nathanielsz et al. (1988) set out to determine the effects of exogenous progesterone administration on endogenous progesterone and estrogen biosynthesis in the sheep. Maternal administration of 250mg of MPA, 24 hours before the start of fetal cortisol infusion, resulted in the inhibition of delivery without alterations in fetal plasma DHEA, DHEAS, progesterone and 17α-hydroxyprogesterone suggesting that exogenous progesterone when administered to the pregnant sheep is unable to gain access to the fetal compartment and alter steroidogenesis (Nathanielsz et al. 1988). It appears that MPA prevents the activation of the myometrium and therefore prevents preterm labour. In some cases in this study where animals treated with betamethasone were significantly different than NT animals (that did not receive MPA), it would appear that these differences were not likely due to MPA treatment, but due to betamethasone treatment. Furthermore, there were no significant differences between NT and MS or FS lambs during any of the experiments at either age.

5.4.2 Maternal betamethasone administration

A single dose of maternal betamethasone administration significantly elevated the basal and stimulated cortisol levels at one year of postnatal age without associated alterations in ACTH concentrations. These data are consistent with previous studies that have reported elevated basal corticosterone levels in rats treated prenatally with maternal dexamethasone late in gestation (Levitt et al. 1996). Furthermore, a recent study has shown that exposure of the sheep fetus to an adverse intrauterine environment (acute hypoxia) resulted in elevations in fetal basal and stimulated cortisol but not ACTH levels (Gardner et al. 2001). Uno et al. (1994) have shown that prenatal treatment with maternal dexamethasone resulted in elevated basal and post-stress cortisol levels in juvenile monkeys. These observations were associated with a dose dependant reduction in the numbers of fetal hippocampal pyramidal cells (Uno et al. 1990, 1994). Many studies have reported significant alterations in hippocampal glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA levels following prenatal glucocorticoid exposure (Levitt et al. 1996, Uno et al. 1990, Dean et al. 1999), linking glucocorticoid exposure with alterations in HPA negative feedback at the level of the hippocampus.
The mechanism regulating the observed elevation in basal and stimulated cortisol concentrations in this study following prenatal betamethasone administration however, is difficult to relate to alterations in negative feedback function since plasma ACTH levels were unaltered in the M1 lambs. It has been reported previously that administration of only one dose of 0.5mg/kg of dexamethasone to the pregnant rhesus monkey significantly depleted fetal hippocampal neurons (Uno et al. 1990). In the present study however, animals were not sacrificed, therefore it can only be hypothesized that one dose of prenatal betamethasone administered at a critical time point in hippocampal or hypothalamic development may alter the GR and/or MR pattern of expression and alter the set point of HPA negative feedback function. The amount of betamethasone given appears to alter the response as; multiple doses of maternal betamethasone appear to have different effects. Previously, we have shown that 3 doses of maternal betamethasone significantly increased fetal pituitary but not hypothalamic GR mRNA levels (Chapter 3; Sloboda et al. 2000). It is possible that an increase in negative feedback function at least at the level of the pituitary, following multiple doses of maternal betamethasone may result in attenuated cortisol responses to CRH + AVP. This reduction in cortisol response in the M4 lambs however was not significantly different from NT or MS lambs, indicating that this effect is possibly not physiologically relevant. Whether pituitary, hypothalamic or hippocampal GR are altered in this postnatal model is unknown. Further studies are needed to evaluate tissue specific alterations in postnatal HPA function.

Since both basal and stimulated levels of plasma cortisol were elevated in M1 lambs at one year of age without associated changes in plasma ACTH, it is possible that prenatal betamethasone administration resulted in alterations in adrenal responsiveness in these animals. The expression of ACTH receptors and the activity of steroidogenic enzymes determines adrenal responsiveness (Reperant & Durand 1997) and alterations in their expression and/or activity could result in changes in the stimulated stress response. The observation that M1 lambs had increased adrenal responsiveness and M4 lambs appeared to have a modest reduction in responsiveness is paradoxical. The ovine fetal adrenal undergoes a rapid growth period at 40-90 days of gestation and is capable of secreting cortisol at this time (Bosheir et al. 1989, Wintour et al. 1995). This is followed by a period of relative quiescence (90-120 days of gestation) characterized by a decrease in responsiveness until 120 days of gestation (Glickman & Challis 1980), when adrenal sensitivity increases to a maximum at term (Rose et al. 1982, Boshier et al. 1989, Wintour et al. 1995). This increase in responsiveness has been associated with an increase in ACTH receptor mRNA levels (Fraser et al. 2001). Although speculative, it is possible that an
early exposure to prenatal betamethasone at 104 days of gestation may have increased ACTH receptors, receptor signalling and/or steroidogenic enzymes during this period of relative quiescence in a way that permanently increased the set point of adrenal responsiveness. The elevation in the basal and stimulated cortisol to ACTH ratios may be a reflection of this altered adrenal responsiveness. Gardner et al. (2000) have recently shown that cord occlusion for 3 days late in gestation in the fetal sheep resulted in elevated cortisol responses to exogenous ACTH associated with a marked increase in adrenal weight due to an increase in adrenocortical mass. It was suggested that an increase in adrenal mass might result in a greater potential for increased adrenocortical steroidogenic capacity. It has been previously reported that cortisol modulates ACTH activation of fetal adrenal function late in gestation (Challis et al. 1985, Challis et al. 1986, Reperant & Durand 1997). In addition, dexamethasone treatment of ovine adrenocortical cells increased mRNA levels of all 3 ACTH receptor transcripts in a dose dependant manner (Picard-Hagan et al. 1997). Multiple doses of maternal betamethasone resulted in a significant reduction in the ACTH response to CRH + AVP at 6 months of age. This effect is difficult to explain since this difference was only apparent when compared to the NT animals, and was no longer apparent at one year of age.

It is difficult to make firm conclusions from the M4 one year cortisol response data, since responses in this group were not statistically different from controls. Whether the pattern of diminished plasma cortisol response following maternal betamethasone exposure is important physiologically is unknown. Theoretically, multiple maternal betamethasone injections (M4) may have down regulated ACTH receptors in the adrenal, consistent with a decrease in adrenal responsiveness. Fetal ovine cortisol infusion for 96h resulted in the downregulation of key steroidogenic enzymes (P450 17α-hydroxylase, 3 β-hydroxysteroid dehydrogenase) and a tendency to decrease ACTH receptor mRNA (Fraser et al. 1999). Previous studies have shown that fetal sheep exposed to modest undernutrition early in gestation exhibited a reduction in both pituitary and adrenal responsiveness to intravenous CRH + AVP and to hypoxia late in gestation (Hawkins et al. 1999, 2000). Furthermore, 10 days of maternal undernutrition in the pregnant sheep resulted in the downregulation of steroidogenic enzyme expression (Fraser et al. 1999).

5.4.3 Fetal betamethasone administration

This study is the first to show that fetal betamethasone administration may cause alterations in postnatal ovine HPA function at one year of age. Prenatal fetal betamethasone treatment suppressed pituitary responsiveness to CRH + AVP at one year of age compared to
controls. A reduction in pituitary POMC levels, alterations in POMC post-translational processing, changes in ACTH secretory mechanisms or decreases in CRH receptor number or affinity could all contribute to this reduction in pituitary responsiveness (Zhou et al. 1996, Rabadan-Diehl et al. 1997). Zhou et al. (1996) found that dexamethasone treatment adult rats for 5 days resulted in a 48% reduction in CRH receptor (CRH-R) mRNA levels in the anterior pituitary. In addition, chronic stress in rats has been associated with decreased pituitary CRH-R content (Aguilera et al. 1994). The exact mechanisms regulating pituitary CRH-R however, are not clear (Lou et al. 1995, Zhou et al. 1996, Rabadan-Diehl et al. 1997, Ochedalski et al. 1998). Alternatively, an increase in GR in pituitary corticotrophs could reflect an increase in negative feedback and reduce the secretion of ACTH (Matthews et al. 1997, Sloboda et al. 2000). Since tissue data are unavailable, it is difficult to discern from the response data which of these mechanisms is responsible for a reduction in postnatal pituitary responsiveness. Furthermore, the fact that animals treated with fetal betamethasone (F1 and F4) responded with values of cortisol similar to controls, in the presence of reduced levels of ACTH, suggests increased adrenal responsiveness. Previous reports have shown that prenatal undernutrition in rats can produce a similar reduction in ACTH levels without any alterations in corticosterone levels, suggesting that there is a hyper-responsiveness to ACTH in the adrenal of the offspring (Langley-Evans et al. 1996). Therefore, although the mechanisms differ between maternal and fetal betamethasone administration, these prenatal perturbations produced a similar end result on postnatal adrenal responses to exogenous stimulation.

5.4.4 Developmental alterations in HPA responses

We have shown that stimulated HPA axis function in the sheep undergoes a developmental maturation between the postnatal ages of 6 months and one year. NT, MS and FS animals exhibited a significant increase in peak ACTH responses to exogenous CRH + AVP from 6 months to one year of age. This observation suggests an increase in pituitary responsiveness with age. Puberty occurs in the sheep between 8-10 months of postnatal age (McNatty et al. 1998), suggesting that HPA function is altered at a time in development when the reproductive axis is stimulated. The male and female reproductive systems are regulated by the hypothalamic-pituitary-gonadal axis (Everett 1988). Hypothalamic neurons that produce gonadotropin-releasing hormone secrete the peptide into the hypophyseal portal system to stimulate production of follicle stimulating and lutenizing hormones which in turn activate ovarian and testicular production of estrogens and androgens (Everett 1988, Chrousos et al. 1988).
1998). Moreover, it has been shown previously that male and female HPA axis function is different, in that androgens inhibit and estrogens stimulate HPA activity (Chrousos et al. 1998, Giussani et al. 2000). Due to the small sample size, it was not possible to analyze the effects of gender on ACTH and cortisol responses; however, it is possible that sex differences might exist. It would be pertinent in future studies to either increase the sample size to include gender as an outcome variable or to perform experiments on one sex only.

It is clear however, that prenatal betamethasone administration influenced these developmental changes in ACTH and cortisol responses to exogenous stimulation (figures 5.5-5.8). Prenatal fetal betamethasone abolished the maturational increase in peak ACTH. In contrast to the peak ACTH responses, NT, FS and MS animals did not exhibit any substantial changes in postnatal peak cortisol values from 6 months to one year. This observation may be consistent with increased pituitary responsiveness and/or a reduction in adrenal responsiveness. It is difficult to discern from these data whether pituitary responsiveness has increased due to an increased drive from the hypothalamus, or the result of reduction in cortisol negative feedback. It is clear however, that although multiple doses of prenatal betamethasone have no significant effect, one single dose of betamethasone administered either to the mother or the fetus significantly elevates the one year peak cortisol responses compared to those at 6 months. Mechanisms by which a single dose of prenatal betamethasone would alter the developmental maturation of the sheep HPA axis are unknown, although it is possible that alterations in adrenal structure and function (as described earlier) could play a role. Further studies are required to understand whether these alterations are at the level of the hippocampus, hypothalamus, pituitary and/or the adrenal, with subsequent alterations in HPA function and negative feedback.

5.4.5 Maternal versus fetal administration

The difference between the effects of fetal versus maternal glucocorticoid administration warrants further investigation. It has been shown previously that maternal betamethasone administration resulted in significant reductions in ovine fetal and neonatal weight but direct fetal administration did not (Newnham et al. 1999, Moss et al. 2000). This raises the possibility that glucocorticoids administered to the mother may affect placental function. The ovine placenta secretes prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) into the fetal circulation late in gestation (Challis et al. 1976). It has been shown previously that cortisol treated fetal placental cells secrete significantly more PGE<sub>2</sub> levels in vitro (Martin et al. 2001). In addition, fetal PGE<sub>2</sub> infusion in late gestation resulted in a rapid increase in fetal plasma ACTH and cortisol levels (Louis et al. 1976, Ratter et
suggesting that PGE$_2$ may act directly on the fetal pituitary as well as the adrenal to increase cortisol secretion. Furthermore, studies have reported the presence of POMC, PC1, and PC2 mRNA and ACTH in the sheep placenta (Jeffray et al. 1999). Therefore, the placenta has the potential to contribute to circulating levels of fetal ACTH. Further studies are required to fully investigate this possibility. These observations therefore support the hypothesis that maternal betamethasone may potentially act on the placenta to alter HPA function. This may explain the differential effect of maternal versus fetal glucocorticoid administration on HPA function.

In conclusion, prenatal betamethasone administration results in postnatal alterations in ovine HPA axis function that are dose dependent and persist to adulthood. These observations may be a reflection of the intrauterine programming of the HPA axis in utero, potentially linking HPA function with growth, metabolic function and cardiovascular disease. Recently it has been shown that impaired growth in men was positively correlated with an increased susceptibility to stress (Nilsson 2001). Altered responsiveness to stressful stimuli may play a role in the genesis of adult diseases such as hypertension and diabetes. In light of the number of studies linking alterations in intrauterine environment with the increased risk of adult disease, further studies are critical to understanding the mechanisms linking early fetal events to postnatal health.
CHAPTER 6

6 The Effect of Prenatal Betamethasone Administration on Postnatal Ovine Glucose and Insulin Responses

6.1 INTRODUCTION

A number of epidemiological studies have established the association between early life events in utero and their impact on later adult life (Barker 1995, Ravelli et al. 1998, Phillips et al. 1998, Seckl 1997). These studies have shown in several populations, that low birth weight is strongly associated with the risk of developing hyperinsulinemia, insulin resistance, hypertriglyceridemia, and hypertension later in life. Pregnant women exposed to famine gave birth to offspring that demonstrated lower birthweight and head circumference than those not exposed (Ravelli et al. 1998). In addition, these individuals had higher fasting proinsulin levels and higher 120min plasma glucose and insulin levels as adults. Furthermore, those subjects exposed to famine late in gestation had the greatest degree of impaired glucose tolerance. The connection between fetal growth and glucose tolerance has been examined in many studies (Hales et al. 1991, Phillips et al. 1994, 1996). It was found that the risk of developing glucose intolerance and insulin resistance later in life was greater in men who had reduced weight at birth (Hales et al. 1991). Furthermore, children with intrauterine growth restriction (IUGR) showed consistent insulin resistance, especially evident during pubertal development (Chiarelli et al. 1999). These human observations therefore support the hypothesis that an adverse intrauterine environment may permanently program metabolism and that these effects can persist into adulthood.

Recently, it has been suggested that this ‘programming’ effect may be mediated by excess exposure of the developing fetus to glucocorticoids (Seckl 1997, Seckl 1998, Clark, 1998, Dodic 1999). Glucocorticoid administration to pregnant animals has adverse effects on fetal outcome (Johnson et al. 1981, Seckl 1994, 1997, Uno et al. 1994, Dunlop et al. 1997), although evidence for such an effect in humans is conflicting at this time (Andersen et al. 1999, French et al. 1999, Mirabile et al. 1999). Glucocorticoids reduce fetal weight, and alter organ maturation and function in a variety of animal species including non-human primates (Lindsay et al. 1996, Uno et al. 1990, Newnham et al. 1999, Lingas et al. 1999, Sloboda et al. 2000; Chapter 3). Administration of synthetic glucocorticoids during the last week of pregnancy in rats decreases birthweight and impairs postnatal glucose tolerance (Nyirenda et al. 1998) in addition to
elevating adult offspring blood pressure (Levitt et al. 1996). Maternal treatment with carbenoxolone, a placental 11βHSD inhibitor, allows increased passage of maternal glucocorticoids to the fetus (Whorwood et al. 1993), and resulted in reduced birthweight (Lindsay et al. 1996). Furthermore, adult male offspring demonstrated altered glucose tolerance indicated by higher fasting glucose levels and elevated glucose and insulin responses to a glucose challenge (Lindsay et al. 1996). Similarly, maternal administration of dexamethasone early in pregnancy (40-14 days of gestation) in the sheep, resulted in elevated fetal basal and stimulated insulin levels at 135 days of gestation (Cox et al. 1999). It is unknown however whether these alterations would result in persistent changes in postnatal ovine glucose tolerance.

In current obstetrical practice, synthetic glucocorticoids are administered to women at risk of preterm delivery to enhance fetal lung maturation and in many cases weekly doses of glucocorticoids are administered if delivery has not occurred (Ballard & Ballard 1995, NIH Consensus Conference 1995, Quinlivan et al. 1999). The safety of repetitive dosing however is unknown and in light of animal studies suggesting the existence of discrete intrauterine developmental windows (Dodic et al. 1999, Seckl 1997), it is essential that the effects of repetitive glucocorticoid administration be examined more closely. Previously, maternal administration of clinically relevant doses of synthetic glucocorticoids has been shown to alter ovine fetal and postnatal hypothalamic pituitary function. Moreover, single versus multiple doses had differential effects (Sloboda et al. 2000, Sloboda et al. 2001; Chapters 3, 5). Furthermore, maternal, but not fetal administration of the synthetic glucocorticoids results in fetal ovine growth restriction (Jobe et al. 1998, Newnham et al. 1999, Moss et al. 2000) and has differential effects on postnatal HPA function (Chapter 5). These observations suggest that the route of glucocorticoid administration, in addition to the dose, may play a role in the programming of organ systems. It is currently unknown whether the administration of clinical doses of glucocorticoids alter postnatal glucose metabolism. It was therefore the aim of this study to determine the effects of glucocorticoid administration on postnatal basal and stimulated glucose and insulin responses in the sheep at 6 months and one year of age. Moreover, this study set out to examine if maternal versus fetal glucocorticoid administration had differential effects on postnatal glucose and insulin responses.
6.2 MATERIALS AND METHODS

6.2.1 Animals
The animals in this chapter were the same animals that were experimented in Chapter 5. Detailed information on animals and experimental procedures are given in 5.2.1.

6.2.2 Glucose challenges at 6 and 12 months of postnatal age
At both 6 months and one year of postnatal age, lambs underwent aseptic surgery to implant femoral arterial and venous catheters (halothane anesthesia, 1-2% in O2, following induction with ketamine/xylazine). All animals were allowed at least 3 days to recover before performing glucose challenges. Food was withdrawn 12h before challenges, but the animals were allowed free access to water. Basal arterial blood samples (5ml) were drawn at 30 (-30) and 15 (-15) minutes and immediately before (0) the administration of an intravenous bolus of 0.5g/kg glucose (Baxter, Australia) per kg lamb weight followed by a 10ml saline flush. Arterial samples (5ml) were collected at 5, 10, 20, 30, 60, 90, 120 and 180 minutes after the glucose bolus. One ml of whole blood was collected for glucose determination, and the remainder of the sample was centrifuged for 10 minutes (1500g) at 4°C. Plasma was collected and stored at -80°C for insulin analysis. All challenges were administered between 0800-0900 h in order to minimize the impact of circadian variability in measurements. Catheters were removed after the completion of experiments. All animals were returned to the field upon completion of experiments to wait further testing.

6.2.3 Measurement of plasma glucose and insulin
Whole blood (100μl) glucose was measured on a glucose analyzer by the glucose oxidase method (Bayer, Rapidlab 860, WA, AUS). Ir-insulin concentrations were measured using a commercial radioimmunoassay (RIA) kit (Linco Research, Inc., USA; see 4.2.6.3). The inter-assay coefficient of variation was 6% and the mean assay sensitivity was 0.1ng/ml.

6.2.4 Statistical analysis
Results are expressed as mean ± standard error of the mean (SEM). A comparison of group means was made using a 2-way analysis of variance (ANOVA, SAS, Cary, NC, USA and S-PLUS, Mathsoft, WA, USA), followed by a Tukey’s post hoc pairwise multiple comparison analysis where differences between the means were significant (P<0.05). In all cases, baseline values represent the mean value of three samples drawn at 30 minutes, 15 minutes and immediately prior to the administration of the glucose intravenous bolus (0). Due to the small
sample size in each of the treatment groups, the effect of gender on any of the outcome variables was not analyzed. In order to assess the overall effect of prenatal betamethasone on the response patterns to the challenge, the areas under the response curves for glucose and insulin were determined for each group between times 5 and 180 minutes (see 5.4.2). These analyses were performed by Dr. L. Gurrin (University of Western Australia).

Insulin to glucose ratios were calculated on baseline values and the areas under the insulin and glucose curves and were analyzed using a one-way ANOVA, followed by a Tukey's post hoc pairwise multiple comparison analysis where differences between the means were statistically significant (P<0.05). At 6 months of age basal insulin: glucose ratios were not normally distributed and were therefore analyzed using the Kruskal-Wallis one-way ANOVA on ranks (Sigmastat, Jandel Scientific, California, USA). All values are presented as mean ± standard error of the mean (SEM) and the level of significance was set at P<0.05.

6.3 RESULTS

6.3.1 Basal arterial blood gases
No significant effects of prenatal treatments were observed in arterial pH, PCO₂ and PO₂ at 6 months or 1 year of age (Table 6.1).

6.3.2 Six months postnatal age

6.3.2.1 Effects of maternal betamethasone
At 6 months of age basal blood glucose and plasma insulin concentrations were not different between groups (table 6.2). Blood glucose responses (figure 6.1a) and areas under the glucose response curves (AUC) were similar in all groups (figure 6.1a, inset histogram). The insulin responses in the M1 and M4 groups were consistently higher than NT and MS for the duration of the challenge, significantly elevated in M1 lambs at 10, 20 and 60 minutes and M4 lambs at 10, 20, 60 and 180 minutes following the glucose bolus administration (figure 6.1b; P<0.05 vs. MS). These responses were consistent with the insulin AUC, which was significantly elevated in M1 and M4 lambs compared to MS (figure 6.1b; inset histogram; P<0.05). Table 6.3 represents the glucose to insulin ratios at baseline and the overall AUC for insulin to AUC for glucose to AUC for insulin ratios for all treatment groups. Basal insulin to glucose ratios were not different between groups. The overall AUC for insulin to AUC for glucose ratio however, was significantly elevated in M1 and M4 groups compared to MS (table 6.3; P<0.05, P=0.056 vs. NT).
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>6 Months of Age</th>
<th>One Year of Age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pH$</td>
<td>$PO_2$ (mmHg)</td>
</tr>
<tr>
<td>NT</td>
<td>7.51±0.01</td>
<td>102.2±6.1</td>
</tr>
<tr>
<td>MS</td>
<td>7.48±0.03</td>
<td>95.7±4.1</td>
</tr>
<tr>
<td>M1</td>
<td>7.54±0.02</td>
<td>104.5±2.7</td>
</tr>
<tr>
<td>M4</td>
<td>7.51±0.02</td>
<td>91.0±2.6</td>
</tr>
<tr>
<td>FS</td>
<td>7.53±0.01</td>
<td>93.7±1.0</td>
</tr>
<tr>
<td>F1</td>
<td>7.49±0.01</td>
<td>97.9±6.7</td>
</tr>
<tr>
<td>F4</td>
<td>7.48±0.04</td>
<td>106.7±6.9</td>
</tr>
</tbody>
</table>

Table 6.1 Basal blood gas values at 6 months and one year of age
Values are presented as mean ± SEM.
<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>6 months</th>
<th>One year</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glu (mmol/l)</td>
<td>Insulin (ng/ml)</td>
</tr>
<tr>
<td>NT</td>
<td>4.34 ± 0.28 (6)</td>
<td>0.56 ± 0.09 (6)</td>
</tr>
<tr>
<td>MS</td>
<td>3.78 ± 0.21 (5)</td>
<td>0.85 ± 0.34 (5)</td>
</tr>
<tr>
<td>M1</td>
<td>4.13 ± 0.19 (6)</td>
<td>0.65 ± 0.34 (6)</td>
</tr>
<tr>
<td>M4</td>
<td>4.07 ± 0.19 (6)</td>
<td>0.58 ± 0.31 (5)</td>
</tr>
<tr>
<td>FS</td>
<td>4.09 ± 0.32 (5)</td>
<td>0.48 ± 0.10 (5)</td>
</tr>
<tr>
<td>F1</td>
<td>3.97 ± 0.30 (5)</td>
<td>0.56 ± 0.09 (5)</td>
</tr>
<tr>
<td>F4</td>
<td>4.08 ± 0.30 (6)</td>
<td>0.54 ± 0.09 (6)</td>
</tr>
</tbody>
</table>

Table 6.2 Basal glucose and insulin levels following prenatal betamethasone.

NT no treatment; MS maternal saline; M1 maternal single dose; M4 maternal multiple dose; FS fetal saline; F1 fetal single dose; F4 fetal multiple dose. Values are mean ± SEM. * P<0.05 M4 versus MS. Glu, glucose. ** P<0.05 M4 versus M1, *** P=0.06 M4 versus MS and NT. (x) represents sample size.
Figure 6.1
Glucose (a) and insulin (b) response to a glucose challenge at 6 months postnatal age in maternally treated groups. Inset histograms represent the area under the glucose and insulin response curves for each treatment group. ○ No treatment (NT); ▼ Maternal Saline (MS); ■ Maternal single dose (M1); ◆ Maternal multiple dose (M4). Values are mean ± SEM, *P < 0.05. The absence of error bars indicates that the variation in the mean data was numerically contained within the upper and lower limits of the symbols used for the data points.
<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>6 months</th>
<th>One year</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal I:G</td>
<td>AUC I: AUC G</td>
</tr>
<tr>
<td>NT</td>
<td>0.12 ± 0.01</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>MS</td>
<td>0.23 ± 0.16</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>M1</td>
<td>0.17 ± 0.12</td>
<td>0.26 ± 0.04*</td>
</tr>
<tr>
<td>M4</td>
<td>0.15 ± 0.02</td>
<td>0.30 ± 0.04*</td>
</tr>
<tr>
<td>FS</td>
<td>0.11 ± 0.03</td>
<td>0.18 ± 0.05</td>
</tr>
<tr>
<td>F1</td>
<td>0.14 ± 0.01</td>
<td>0.25 ± 0.06*</td>
</tr>
<tr>
<td>F4</td>
<td>0.13 ± 0.02</td>
<td>0.25 ± 0.04*</td>
</tr>
</tbody>
</table>

Table 6.3 Glucose to insulin ratios.

NT no treatment; MS maternal saline; M1 maternal single dose; M4 maternal multiple dose; FS fetal saline; F1 fetal single dose; F4 fetal multiple dose. All values are mean ± SEM. * P<0.05 M1 and M4 versus MS, * P=0.09 F1 and F4 versus FS. I: G, insulin to glucose ratio; AUC I: AUC G, area under the glucose curve to area under the insulin curve ratio.
6.3.2.2 Effects of fetal betamethasone

At 6 months postnatal age, basal glucose and insulin levels were not different following fetal betamethasone administration (table 6.2). Blood glucose responses and glucose AUC were similar in all groups studied (figure 6.2a). The insulin response patterns are illustrated in figure 6.2b. Although the F1 and F4 lambs showed higher insulin responses to the glucose load compared to MS and NT for the first 60 minutes of the challenge, these differences were statistically significant at only 10 and 20 minutes post challenge (figure 6.2b; P<0.05). The insulin AUC was not different between treatment groups (figure 6.2b, inset histogram). Basal insulin to glucose ratios and overall AUC for insulin: AUC for glucose were not different between groups (table 6.3).

6.3.3 One year postnatal age

6.3.3.1 Effects of maternal betamethasone

At one year postnatal age, basal blood glucose concentrations were significantly higher in M4 lambs compared to MS lambs (table 6.2, P<0.05). Although M4 lambs had similar glucose responses compared to controls (MS, NT), the M1 lambs had significantly higher blood glucose levels at 5, 10, 30 and 60 minutes after glucose bolus (figure 6.3a P<0.05). Consistent with this observation, the glucose AUC was significantly greater in M1 lambs compared to MS lambs (figure 6.3a; inset histogram, P<0.05). Basal plasma insulin levels were higher in M4 lambs compared to NT and MS (P=0.06) and significantly higher than M1 lambs (table 6.2; P<0.05). Although the M4 lambs had consistently higher insulin levels throughout the challenge compared to MS and NT, these differences were significant only at 10, 30 and 180 minutes after the glucose bolus (figure 6.3a; P<0.05 M4 vs. NT and MS). M1 lambs showed no difference in insulin responses compared to MS and NT. Basal insulin to glucose ratios were not different between groups at one year of age (table 6.3). Although the M4 group showed the highest AUC for insulin to AUC for glucose ratio compared to MS and NT, this did not reach statistical significance (table 6.3, P=0.06 vs MS and NT).

6.3.3.2 Effects of fetal betamethasone

Basal blood glucose and plasma insulin levels were similar between the groups at one year of postnatal age (table 6.2). Interestingly, F1 and F4 lambs had significantly lower blood glucose levels at 20, 30, 60 and 90 minutes after the glucose bolus (figure 6.4a; P<0.05).
Figure 6.2
Glucose (a) and insulin (b) response to a glucose challenge at 6 months postnatal age in fetally treated groups. Inset histograms represent the area under the glucose and insulin response curves for each treatment group. ○ No treatment (NT); ▼ Fetal Saline (FS); □ Fetal single dose (F1); ◆ Fetal multiple dose (F4). Values are mean ± SEM. The absence of error bars indicates that the variation in the mean data was numerically contained within the upper and lower limits of the symbols used for the data points.
Figure 6.3
Glucose (a) and insulin (b) response to a glucose challenge at one year postnatal age in maternally treated groups. Inset histograms represent the area under the glucose and insulin response curves for each treatment group. No treatment (NT); Maternal Saline (MS); Maternal single dose (M1); Maternal multiple dose (M4). Values are mean ± SEM, * P<0.05. The absence of error bars indicates that the variation in the mean data was numerically contained within the upper and lower limits of the symbols used for the data points.
The glucose AUC was consistently lower in F1 (P=0.053) and F4 (P=0.098) lambs compared to FS lambs (figure 6.4a; inset histogram), but did not reach statistical significance. The insulin response patterns at one year of age were similar between groups (figure 6.4b). Basal insulin to glucose ratios and AUC for insulin to AUC for glucose ratios were not different between groups at one year of age (table 6.3).

6.4 DISCUSSION
This study has shown that prenatal betamethasone administration alters postnatal ovine glucose and insulin responses to a glucose load that are dependent upon dose (single versus multiple) and route (maternal versus fetal) of administration. At 6 months of age, animals treated prenatally with maternal betamethasone exhibited an elevated insulin response to exogenous glucose, in a manner that is similar to that observed in type II diabetes. This response persisted in the M4 lambs at one year of age. In addition, M4 lambs began to show elevated fasting glucose and insulin levels at one-year postnatal age. This is in contrast to lambs treated with fetal betamethasone. Prenatal fetal betamethasone administration did not result in profound changes in glucose handling at 6 months and at one year of postnatal age.

6.4.1 The sheep as a model for metabolism
Since the sheep is a ruminant species, it seems pertinent to address ruminant carbohydrate metabolism in this discussion. To compensate for the absence of cellulases in their gastrointestinal tract, ruminants have gut diverticulae to provide a compartment for the accommodation of "cellulose-splitting" bacteria and other microorganisms (Leek et al. 1969). Ingested food is subject to microbial degradation where the end products, short chain fatty acids propionate and butyrate, are absorbed through the ruminal epithelium and metabolized in the liver into lactate and pyruvate (Leek et al. 1969, Cook et al. 1965, Bensadoun et al. 1962). Therefore, the primary source of glucose for the ruminant is through hepatic gluconeogenesis (Bensadoun et al. 1962). What role does glucose play then in the stimulation of an insulin response in the ruminant? Experiments in which volatile free fatty acids have been administered into the adult ruminant circulation show conflicting results, in that propionate and butyrate produced either a significant insulin response (Bassett et al. 1972) or no significant insulin
Figure 6.4
Glucose (a) and insulin (b) response to a glucose challenge at one year postnatal age in fetally treated groups. Inset histograms represent the area under the glucose and insulin response curves for each treatment group. ○ No treatment (NT); ▼ Fetal Saline (FS); ■ Fetal single dose (F1); ● Fetal multiple dose (F4). Values are mean ± SEM. The absence of error bars indicates that the variation in the mean data was numerically contained within the upper and lower limits of the symbols used for the data points.
response (Stern et al. 1970). However, more recent studies have described that both the ovine fetus and adult sheep are able to mount a significant insulin response to exogenous glucose administration (Bassett et al. 1973, McCann et al. 1986, Houghton et al. 1989). The fetal sheep is able to respond to intravenous glucose as early as 110 days of gestation through until 145 days (term 150 days) and this response changes postnatally (Bassett et al. 1973, Houghton et al. 1989). Peak glucose and insulin levels are greater in adult sheep than those in fetuses (Houghton et al. 1989), although in vitro, the perifused adult sheep pancreas does not respond to exogenous glucose with the typical 2-phase insulin response (Bassett et al. 1973). This was also evident in vivo in adult sheep following a glucose injection (McCann et al. 1986).

Therefore, the sheep has been proven to be an adequate model in the study of carbohydrate metabolism. The glucose and insulin responses observed in this study following an intravenous bolus of glucose were similar to those observed previously in adult sheep (McCann et al. 1986, Houghton et al. 1989).

6.4.2 Effects of maternal betamethasone

Maternal administration of both single and multiple doses of betamethasone (M1, M4) resulted in an elevated insulin response to exogenous glucose at 6 months of age. The data are consistent with previous studies where prenatal glucocorticoid exposure in rats resulted in offspring with reduced birth weight and altered glucose handling (Lindsay et al. 1996, Nyirenda et al. 1998, Saegusa et al. 1999). It has been previously hypothesized that fetal exposure to glucocorticoids may 'program' glucose metabolism and lead to permanent hyperglycemia and insulin resistance (Lindsay et al. 1996, Saegusa et al. 1999). There are a number of mechanisms that could account for these alterations in glucose handling. In general, glucocorticoids are diabetogenic, altering both glucose uptake (Dimitriadis et al. 1997), and glucose production (Rizza et al. 1982). Dexamethasone administration to adult rats resulted in an inhibition of glucose transporter 4 (GLUT4; insulin sensitive glucose transporter) translocation to the plasma membrane in skeletal muscle cells (Dimitriadis et al. 1997). Treatment of rat pancreatic islets in vitro with dexamethasone, decreased glucose induced insulin secretion (Gremlich et al. 1997). Furthermore, glucocorticoids increase hepatic enzymes involved in glucose production, especially phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in gluconeogenesis (Rosella et al. 1995, Friedman et al. 1997, Nyirenda et al. 1998). PEPCK overexpression in transgenic mice has been shown to impair insulin suppression of hepatic gluconeogenesis (Rosella et al. 1995).
To date, little is known regarding the mechanisms that link fetal glucocorticoid exposure to postnatal glucose intolerance and insulin resistance. Dexamethasone administration to pregnant rats resulted in a reduction in birth weight, glucose intolerance and insulin resistance as well as an increase in hepatic PEPCK and glucocorticoid receptor (GR) mRNA levels in adult offspring (Nyirenda et al. 1998). Furthermore, pregnant rats treated with carbenoxolone, an inhibitor of placental enzyme 11 β-hydroxysteroid dehydrogenase 2 (11 β-HSD2), permitting increased levels of maternal glucocorticoids to reach the fetal circulation, produced offspring with reduced birth weight and postnatal glucose intolerance and insulin resistance (Lindsay et al. 1996, Saegusa et al. 1999). These data suggest that prenatal glucocorticoid exposure may potentially program key hepatic enzymes and receptors involved in glucose metabolism. Previous observations (Sloboda et al. 2001; Chapter 4) demonstrated that fetal hepatic 11 β-HSD1 expression levels were increased following maternal betamethasone administration. Whether this alteration persists postnatally is unknown. In the liver, an increase in 11 β-HSD1 should potentially increase local tissue levels of cortisol. This, in addition to increased levels of GR (Nyirenda et al. 1998) could result in the subsequent stimulation of PEPCK, thereby increasing hepatic glucose production through gluconeogenesis, since previous studies report that PEPCK gene transcription is regulated by glucocorticoids (Friedeman et al. 1997). By one year of age, both M1 and M4 lambs began to exhibit alterations in glucose homeostasis. At one year of age, M1 lambs had post glucose hyperglycemia (as seen in the elevated glucose AUC) without associated increases in insulin levels. M4 lambs had elevated fasting glucose and insulin levels in addition to persistent elevations in post glucose insulin levels. Future studies are required to evaluate tissue specific alterations in glucose production these animals.

M4 lambs exhibited elevated insulin responses post glucose at 6 months of age that persisted up to one year. This pattern of response is indicative of insulin resistance and is similar to that seen in type II diabetes (Bergman et al. 1985, Sacks & McDonald 1996). Cortisol infusion in humans resulted in increased post-absorptive glucose and insulin levels that were associated with a peripheral insulin post-receptor defect (Rizza et al. 1982). The pattern of insulin resistance in the M1 lambs did not persist from 6 months to one year of age. The difference between M1 and M4 lambs is intriguing. It is possible, that M1 lambs at one year were no longer able to secrete adequate insulin to compensate for elevated glucose levels. Pancreatic β cell defects have been associated with incremental hyperglycemia and an insulin response below normal (Bergman et al. 1985). Many studies have reported that prenatal undernutrition results in alterations in both pancreatic morphology and function (Snoeck et al. 1990, Dahri et al. 1991, 158
Maternal protein restriction in rats resulted in a reduction in neonatal β cell proliferation and islet size, in addition to impaired glucose tolerance (Snoeck et al. 1990, Dahri et al. 1991, Berney et al. 1997). Furthermore, prenatal undernutrition has been hypothesized to be of crucial importance in determining fetal growth and the onset of later disease (Phillips et al. 1996, Barker et al. 1998, Hill 1999). Since studies have associated maternal protein restriction and low birth weight with a reduction in placental 11β-HSD2 activity (Langely-Evans et al. 1996, Edwards et al. 1993, Shams et al. 1998) and a deficiency in placental 11β-HSD2 allows increased fetal exposure to maternal glucocorticoids, this would support the hypothesis that glucocorticoids play a key role in mediating the effects of prenatal protein restriction. Firm conclusions however, regarding either peripheral insulin sensitivity or pancreatic function in this study cannot be made since the animals were not sacrificed. Future studies are therefore required to investigate these potential mechanisms.

### 6.4.3 Effects of fetal betamethasone

In contrast to maternal betamethasone administration, fetal administration did not have profound effects on glucose and insulin responses at 6 months of age. Intriguingly, at one year of age, the F1 and F4 lambs appear to have improved their glucose tolerance, as exhibited by a faster clearance of glucose during the challenge and lower AUC values. These values however were not statistically different, suggesting that this response may not be physiologically relevant. The potential mechanism behind these results is unknown. To date there is no literature documenting the effects of direct fetal glucocorticoid administration on postnatal glucose tolerance. Further studies regarding tissue specific alterations would be useful in understanding this phenomenon.

### 6.4.4 Maternal versus fetal administration

It has been shown previously that maternal betamethasone administration resulted in significant reductions in ovine fetal and neonatal weight but direct fetal administration did not (Newnham et al. 1999, Moss et al. 2000). Although the mechanisms are unclear, it appears that route of administration also has an effect on the programming of postnatal glucose and insulin responses. It is possible that glucocorticoids administered to the mother may affect placental function. The ovine placenta secretes prostaglandin E₂ (PGE₂) into the fetal circulation late in gestation (Challis et al. 1976) and it has been shown previously that cortisol treatment in vitro results in increased PGE₂ secretion from fetal placental cells (Martin et al. 2001). Furthermore, PGE₂ infusion into the fetus in late gestation resulted in a rapid increase in fetal plasma ACTH and
cortisol levels (Louis et al. 1976, Ratter et al. 1979, Young et al. 1996). It is possible that maternal administration of glucocorticoids alters placental prostaglandin output prematurely stimulating the fetal HPA axis (Chapter 3) and that elevations in endogenous cortisol concentrations could influence pancreatic development. There are however no studies describing the effects of maternal glucocorticoid administration on placental function and associated alterations in postnatal glucose handling. Further studies are required to fully investigate this possibility.

Another possibility is that the alteration in metabolic function in maternally treated animals is associated with fetal growth restriction. It has been previously reported that maternal glucocorticoid administration in these animals resulted in growth restriction at birth, which persisted to 3 months of postnatal age (Moss et al. 2000). Furthermore, the growth effects were greatest in M4 lambs. Fetal betamethasone administration however, did not result in growth restriction. Given the association between growth restriction and postnatal glucose tolerance and insulin resistance in humans (Hales et al. 1991, Phipps et al. 1993, Phillips et al. 1998, Holeness et al. 2000), this differential effect based on the route of glucocorticoid administration has great importance. The observations in this study suggest that postnatal alterations in glucose handling may be due to secondary effects of growth restriction. Whether the maternally treated lambs in this study were insulin resistant in utero is unknown, although this would be consistent with a reduction in growth since insulin is a major mitogenic factor that regulates fetal growth (Fowden 1989). Therefore, it is clear that the nature and timing of prenatal perturbations are critical factors determining the long term change in the function of the metabolic system.

In conclusion, the results of this study indicate that postnatal glucose and insulin responses to exogenous glucose are influenced by prenatal exposure to glucocorticoids, and that these effects, in some treatment groups, persists to adulthood (1 year of age). These findings are especially important in light of current hypotheses linking alterations in fetal growth with the incidence of developing postnatal glucose intolerance and insulin resistance in many human populations. Even more important perhaps, is the association of insulin resistance with hyperlipidemia, hypertension and coronary heart disease. This study supports the hypothesis that alterations in the fetal environment can influence adult health and that glucocorticoids play a major role in the mechanisms leading to the genesis of disease.
CHAPTER 7

7  General Discussion

7.1  INTRODUCTION-Summary of Results

This study has demonstrated that clinically relevant doses of betamethasone administered to pregnant sheep at 0.7 of gestation resulted in significant reductions in fetal weight at 125 and 146 days of gestation. Betamethasone administration significantly increased cord plasma CBC and (Chapter 3) fetal hepatic CBG and 11βHSD1 mRNA levels at 125 days of gestation (Chapter 4), and significantly elevated basal ACTH concentrations at 146 days of gestation. Cord plasma cortisol concentrations were elevated but the differences did not reach statistical significance. It appears that these changes were not associated with detectable alterations in the mRNA levels encoding key neuropeptides of the HPA axis in the fetal pituitary or the hypothalamus (Chapter 3). Maternal betamethasone administration resulted in a decrease in cord plasma insulin levels at 125 days, an increase in cord plasma glucose at 146 days and an increase in immunoreactive insulin staining in fetal pancreatic β cells at 146 days of gestation. In addition, this is the first study to report the presence of apoptotic staining in fetal sheep pancreatic β cells during development (Chapter 4).

Prenatal glucocorticoids administered to the mother or directly to the fetus significantly altered postnatal HPA responses to exogenous CRH+AVP administration, alterations that were dependent upon the dose and route of glucocorticoid administration. A single dose of maternal betamethasone significantly elevated basal and stimulated cortisol levels at one year of postnatal age without altering ACTH responses. Fetal betamethasone administration resulted in a decrease in pituitary responsiveness to CRH+AVP at one year of postnatal age. In addition, HPA responses to CRH+AVP were altered between 6 months and one year, suggesting a possible maturation of the sheep HPA axis postnatally (Chapter 5). Prenatal betamethasone administration altered postnatal insulin responses to a glucose load that were dependent upon dose and route of administration. Animals treated with maternal betamethasone exhibited elevated insulin responses to exogenous glucose, suggesting insulin resistance. This response persisted to one year of age in lambs that were treated with multiple doses. In addition, these lambs demonstrated elevated fasting glucose and insulin levels at one-year of age. (Chapter 6).
7.2 PHYSIOLOGICAL IMPLICATIONS

Repetitive administration of maternal betamethasone at 0.7 gestation resulted in \(~20\%\) reduction in fetal weight at 125 and 146 days of gestation. The mechanisms altering fetal growth however, are still unknown. Maternal betamethasone treatment resulted in fetal HPA hyperactivity, which persisted until 146 days of gestation as evidenced by elevated basal cord ACTH concentrations. Although mean cord cortisol levels in betamethasone treated animals at 146 days of gestation were 3-fold higher than controls, large variations between individual animals resulted in differences that were not statistically significant. This elevation however still may have physiological significance. Although it is possible that maternal betamethasone administration directly affected fetal growth, alternatively, an increase in fetal HPA activity as a result of the betamethasone treatment may have exposed the fetus to chronically elevated endogenous cortisol, which in turn may have restricted fetal growth. The mechanism may be through an inhibition in fetal IGFII, since previously, it has been shown that cortisol inhibits IGFII expression in the fetal liver (Li et al. 1993).

Betamethasone could have altered HPA function via changes in negative feedback. Negative feedback is an important regulator of cortisol synthesis and secretion through glucocorticoid binding to GR at the hypothalamus and the pituitary. The exact role of GR in the pituitary is still unclear. The increase in GR mRNA levels that was observed in the pars distalis at day 146 after betamethasone treatment is consistent with the increase in pituitary GR seen at term as endogenous cortisol rises (Yang et al. 1990, 1992), but is not consistent with a decrease in negative feedback and HPA hyperactivity. Plasma CBC and hepatic CBG mRNA levels were significantly elevated at 125 days of gestation after betamethasone treatment. It has been shown that a rise in fetal ovine CBG late in gestation in fetal sheep maintains low concentrations of circulating free cortisol and attenuates negative feedback at the pituitary and hypothalamus (Ballard et al. 1982, Berdusco et al. 1994, 1995). Therefore, the increase in CBG production and plasma CBC levels after maternal betamethasone treatment may have decreased circulating free cortisol levels, thereby resulting in a decrease in negative feedback. This could account for the increased ACTH concentrations. In effect, maternal betamethasone may have therefore altered the set point(s) of the fetal HPA negative feedback. These observations are similar to those observed in human populations. Elevated levels of basal cortisol have been associated with decreased birth weight, glucose intolerance, insulin resistance and hypertension in adulthood (Phillips et al. 1996, Levitt et al. 2000, Reynolds et al. 2001). Therefore fetal HPA hyperactivity resulting in chronically elevated fetal cortisol concentrations has potential effects on the growth,
development and function of many fetal organ systems and has long term influences on postnatal HPA and metabolic function (Chapters 5, 6). This model of glucocorticoid administration is therefore useful in studying the mechanisms regulating the association between fetal growth restriction and the increased risk of developing adult disease in humans. Furthermore, as described previously, prenatal stress has also been shown to result in alterations in both HPA and metabolic function. Therefore this model of maternal glucocorticoid administration may serve as an additional model of prenatal stress resulting in increased endogenous cortisol concentrations.

Previous studies have shown that intrauterine growth correlates with fetal insulin levels (Fowden et al. 1989) and small for gestational age infants have reduced plasma insulin levels (Economides et al. 1991). Destruction of pancreatic β cells in sheep fetuses resulted in fetal hypoinsulinemia and a 20% decrease in body weight (Philipps et al. 1991). Previous studies have demonstrated that fetal glucocorticoid exposure can potentially program pancreatic development (Phillips 1996, Seckl 1997, Hill 1999, 2000). Transgenic mice overexpressing GR in the β cells exhibited impaired glucose tolerance and decreased insulin response to a glucose load due to impaired insulin release in vivo (Delauney et al. 1997). Repeated maternal betamethasone administration resulted in a decrease in cord plasma insulin levels at 125 days of gestation and a significant increase in ir-insulin staining of fetal islets (Chapter 4). This may be a reflection of a reduction in insulin secretion. It is possible that maternal betamethasone administration altered fetal pancreatic insulin secretion. Alternatively, an increase in fetal HPA activity could result in chronic fetal exposure to endogenous cortisol, reducing insulin levels and resulting in fetal growth restriction. Little is known regarding the development of the ovine pancreas. There is substantial evidence that a wave of apoptosis occurs in the neonatal rat pancreas (Finegood et al. 1995, Scaglia et al. 1997) but it is presently unknown if the fetal ovine pancreas undergoes similar remodeling. Although apoptotic cells were present at 125 and 146 days of gestation, maternal betamethasone did not alter the prevalence of apoptosis in islets. It remains possible therefore, that apoptotic remodeling occurs postnatally in the neonatal sheep, and that maternal betamethasone may alter this remodeling.

It is also possible that maternal betamethasone treatment has altered locally produced glucocorticoids through changes in 11βHSD1, which in turn affect endocrine function. The role of hepatic 11β HSD1 in facilitating an increase in local glucocorticoid concentrations in the liver has been suggested by many studies (Yang et al. 1992, 1994, Jamieson et al. 1999, 2000). In vivo, hepatic 11β HSD1 acts primarily as a reductase enzyme, converting inactive cortisone to biologically active cortisol (Seckl & Chapman 1997, Penning 1997) and has been colocalized
with GR in the liver of the rat (Whorwood et al. 1991). Repeated administration of maternal betamethasone significantly increased hepatic 11βHSD1 mRNA and protein levels at 125 days of gestation (Chapter 4), suggesting that maternal betamethasone treatment may increase local cortisol levels in the fetal liver. Inappropriate increases in intrahepatic levels of glucocorticoids could influence metabolic regulation. Previously, maternal dexamethasone administration in rats resulted in decreased birth weight, impaired glucose tolerance and significantly increased PEPCK and GR mRNA levels in adult offspring (Nyirenda et al. 1998). Although maternal betamethasone administration did not alter fetal hepatic GR protein levels in the present study, it is possible that changes in hepatic GR levels do not become evident until postnatal life. Inappropriate increases in hepatic 11β HSD1 activity could contribute to elevated liver glucose production, by altering the enzymes responsible for gluconeogenesis. Furthermore, an increase in endogenous cortisol levels may influence 11βHSD1 expression, forming a feed forward loop at the fetal liver. Cord plasma glucose levels were significantly elevated at 146 days of gestation, perhaps suggesting an increase in fetal hepatic gluconeogenesis. Whether these changes persist postnatally is unknown, although maternal betamethasone administration did result in the appearance of glucose intolerance in lambs at one year of age. It remains possible however, that betamethasone administration may have contributed to alterations in the feeding behavior of pregnant ewes. An increase in maternal food intake may have also contributed to alterations in fetal glucose levels late in gestation. This parameter was not measured in these studies.

It is difficult to distinguish between the effects of maternal betamethasone administration on fetal HPA and metabolic development. Many studies have reported that glucocorticoids regulate both CBG and 11βHSD1 expression levels and activity (Schlechte & Hamilton 1987, Berdusco et al. 1993, 1994, Yang et al. 1994). Therefore, it seems likely that in this study, elevations in hepatic 11β HSD1 and CBG levels work together, to regulate circulating and local glucocorticoid levels. Maternal betamethasone administration in the present study resulted in an increase in HPA activity at 146 days of gestation (Chapter 3), in addition to elevations in postnatal basal and stimulated cortisol levels, hyperglycemia and elevated insulin responses to glucose (Chapters 5 and 6). It is possible that maternal betamethasone administration resulted in the programming of key hepatic gluconeogenic enzymes through alterations in hepatic 11βHSD1 and CBG, thereby resulting in long term postnatal alterations in HPA and metabolic function.

This study has shown that the effects of betamethasone treatment observed in the fetus may in fact be associated with changes in postnatal HPA and metabolic function. Intriguingly,
one dose of maternal betamethasone resulted in elevations in basal and stimulated levels of plasma cortisol at one year of postnatal age without any associated changes in plasma ACTH. Repeated maternal betamethasone administration did not result in profound changes in ACTH and cortisol responses postnatally. Elevated basal and stimulated cortisol levels postnatally are difficult to relate to alterations in negative feedback, since plasma ACTH levels were unaltered. It has been reported previously that administration of one dose of dexamethasone to pregnant rhesus monkeys significantly depleted fetal hippocampal neurons (Uno et al. 1990). In the present study animals were not sacrificed, therefore it can only be hypothesized that one dose of prenatal betamethasone administered at a critical time in hippocampal development might have altered the GR and/or MR pattern of expression and altered the set point of hippocampal negative feedback function. It is also possible that hepatic CBG and plasma CBC levels are altered in these lambs postnatally, contributing to alterations in negative feedback. Alternatively, changes in the expression and/or activity of adrenal ACTH receptors and/or steroidogenic enzymes may result in altered adrenal responsiveness. The ovine fetal adrenal is capable of secreting cortisol at 40-90 days of gestation (Boshier et al. 1989, Wintour et al. 1995), followed by a period of low responsiveness until 120 dGA (Glickman & Challis 1980). Sensitivity then increases to a maximum at term (Rose et al. 1982, Boshier et al. 1989, Wintour et al. 1995), and has been associated with increases in ACTH receptor mRNA levels (Fraser et al. 2001). It is possible that prenatal betamethasone exposure at 104 days of gestation may have increased fetal adrenal ACTH receptors and/or steroidogenic enzymes during this period of relative quiescence in a way that permanently increased the set point of adrenal responsiveness. The increased basal and stimulated cortisol to ACTH ratios could be a reflection of this altered adrenal responsiveness (Chapter 5).

Maternal betamethasone (single and multiple doses) resulted in elevated postnatal insulin responses to exogenous glucose at 6 months of age. This pattern of response is indicative of peripheral insulin resistance and is similar to that seen in type II diabetes (Bergman et al. 1985, Sacks & McDonald 1996). Although the mechanisms are unknown, dexamethasone administration in adult rats resulted in insulin resistance that was associated with an inhibition of GLUT4 translocation in skeletal muscle (Dimitriadis et al. 1997). It is possible that multiple doses of prenatal maternal betamethasone altered peripheral insulin action by altering either glucose transporters or insulin receptors. Furthermore, elevations in endogenous cortisol levels (as seen in the lambs that received one dose of betamethasone) could contribute to a reduction in insulin mediated glucose uptake in the periphery. By one year of age, both maternal groups
began to exhibit alterations in glucose handling. Glucocorticoids affect hepatic PEPCK (Friedman et al. 1997, Nyirenda et al. 1998), and PEPCK overexpression in transgenic mice has been shown to impair insulin suppression of hepatic gluconeogenesis (Rosella et al. 1995). It is possible that increased hepatic 11βHSD1 expression following maternal betamethasone administration (Chapter 4) could elevate local tissue levels of cortisol and result in the subsequent stimulation of PEPCK, thereby increasing hepatic glucose production. This effect may become more prevalent in postnatal life (Chapter 6). However, whether the effect of maternal betamethasone treatment on 11βHSD1 persists postnatally is unknown.

The pattern of insulin resistance seen at 6 months did not persist in lambs treated with one dose of maternal glucocorticoids. It is possible that these lambs were no longer able to secrete adequate insulin to compensate for elevated glucose levels at one year of age. Pancreatic β cell defects have been associated with incremental hyperglycemia and an insulin response below normal (Bergman et al. 1985). Firm conclusions regarding either peripheral insulin sensitivity or pancreatic function in this study cannot be made since the animals were not sacrificed. It is possible that either maternal betamethasone or the resultant elevated levels of endogenous cortisol in these lambs altered pancreatic function. Indeed, repeated maternal administration resulted in a reduction in cord insulin levels at 125 days of gestation (Chapter 4).

In contrast to maternal treatment, prenatal fetal betamethasone administration suppressed postnatal pituitary responsiveness to CRH+AVP at one year of age, without altering responses at 6 months (Chapter 5). A reduction in pituitary POMC levels and in post-translational processing, changes in ACTH secretory mechanisms or decreases in CRH or AVP receptor number or affinity could all play a role in reduced pituitary responsiveness (Zhou et al. 1996, Rabadan-Diehl et al. 1997). Since tissue was unavailable in this study, it is difficult to discern from the response data, which of these mechanisms is responsible for a reduction in postnatal pituitary responsiveness. Furthermore, the fact that animals treated with fetal betamethasone responded with values of cortisol similar to controls in the presence of reduced ACTH levels, suggests increased adrenal responsiveness. Therefore, it appears that fetal betamethasone administration might alter both pituitary and adrenal function. Even more puzzling, is that fetal administration resulted in potential improvement in glucose tolerance at one year of postnatal age, although these results were not statistically different. To date there is no literature documenting the effects of direct fetal glucocorticoid administration on postnatal HPA function or glucose tolerance. Further studies regarding the tissue specific alterations following fetal betamethasone administration are therefore essential.
These studies have shown that programming of postnatal HPA and metabolic activity are influenced by the route of prenatal glucocorticoid administration (maternal versus fetal). Previously, maternal betamethasone administration resulted in significant reductions in ovine fetal and neonatal weight but direct fetal administration did not (Newnham et al. 1999, Moss et al. 2000). It is possible that the observed alterations in metabolic function in maternally treated animals are a direct effect of treatment or an indirect effect of growth restriction. Previously, maternal betamethasone administration in these animals resulted in fetal growth restriction, which persists to 3 months of postnatal age (Moss et al. 2000). Furthermore, the growth effects were greatest in those lambs treated with multiple doses. Fetal betamethasone administration did not result in growth restriction thereby supporting previous associations made between impaired glucose tolerance and fetal growth (Hales et al. 1991, Phipps et al. 1993, Phillips et al. 1998, Holeness et al. 2000).

The differential effects in endocrine function between maternal versus fetal betamethasone administration may be due to alterations in placental function. Previously, fetal PGE₂ infusion in late gestation resulted in a rapid increase in fetal plasma ACTH and cortisol levels in the sheep (Louis et al. 1976, Ratter et al. 1979, Young et al. 1996), suggesting that placental PGE₂ may act on the fetal pituitary and the adrenal to increase cortisol secretion. Furthermore, studies have reported the presence of POMC, PC1 and PC2 mRNA and ACTH in the sheep placenta (Jeffray et al. 1999, Jacobs et al. 1989). Therefore, the placenta has the potential to contribute to circulating levels of fetal ACTH (Keller-Wood 1991). Furthermore, it is possible that maternal betamethasone administration altered placental 11βHSD2 activity. If this were the case, animals treated with maternal betamethasone may in fact be exposed to even higher levels of glucocorticoids, a portion of which could be of maternal origin. There are no studies describing the effects of maternal glucocorticoid administration on placental function in the sheep. Therefore, further studies are required to investigate fully this possibility.

Finally, this study has shown that the sheep HPA axis undergoes a developmental maturation between the postnatal ages of 6 months and one year. A significant increase in peak ACTH responses to exogenous CRH+AVP from 6 months to one year of age was observed in control animals and suggests an increase in pituitary responsiveness with age. In contrast, peak cortisol responses were unchanged from 6 months to one year. It is unclear if increased pituitary responsiveness reflects an increase in hypothalamic drive. At 8-10 months of postnatal age in the sheep however, there is increased activity of the hypothalamic-pituitary-gonadal axis associated with puberty (McNatty et al. 1998). Therefore, it may not surprising that HPA function is altered
at a time in development when the reproductive axis is stimulated. Fetal betamethasone abolished the maturational increase in peak ACTH responses to CRH+AVP, and a single dose of prenatal betamethasone elevated adrenal responsiveness. Future studies are warranted to determine the mechanisms by which betamethasone administration can influence the maturational responses to exogenous CRH+AVP observed in this study.

7.3 CLINICAL IMPLICATIONS

The observations made in this study are important in light of current evidence linking low birth weight and glucocorticoid exposure to the increased risk of developing postnatal diseases such as diabetes, heart disease and hypertension (Langely et al. 1994, Desai et al. 1995, Phillips 1996, Barker 1998). The present studies have shown that prenatal glucocorticoid administration results in alterations in HPA function and the emergence of postnatal glucose intolerance and insulin resistance. These alterations are directly associated to fetal exposure to elevated levels of glucocorticoids. Furthermore, it appears that growth and postnatal glucose handling may be intimately linked, since fetal betamethasone administration does not affect growth or profoundly alter postnatal glucose handling. These results confirm many human observations that low birth weight and an increased risk of glucose intolerance and insulin resistance are strongly associated.

In light of the administration of maternal glucocorticoids in current obstetrical practice, it is essential to consider the impact of long term fetal glucocorticoid exposure. French et al. (1999) provide clear evidence that decreased birth weight ratios of infants of mothers treated with prenatal glucocorticoids are associated with an increased number of glucocorticoid courses (French et al. 1999). This is not to suggest however, that glucocorticoids should not be administered to women at risk of preterm delivery. Administration of multiple doses should be re-evaluated however with large randomized control trials to determine if the benefit of administration of multiple doses of clinical glucocorticoids outweighs the potential detrimental costs to postnatal health.

Recent human data link reductions in fetal growth with alterations in circulating glucocorticoids. Fasting and stimulated plasma cortisol concentrations were inversely related to birth weight in adult men (Phillips et al. 1998). Furthermore, raised plasma cortisol levels were significantly associated with an increase in blood pressure and plasma glucose, and insulin resistance (Phillips et al. 1998, Reynolds et al. 2001). Recently it has been shown that impaired growth in men was positively correlated with an increased susceptibility to stress (Nilsson 2001). Altered responsiveness to stressful stimuli may play a role in the genesis of adult diseases such
as hypertension and diabetes. These data support the hypothesis that an adverse intrauterine environment can permanently reset the developing HPA axis potentially leading to an increased risk of adult diseases. It appears that precocious exposure of the fetus to glucocorticoids creates an adverse intrauterine environment within which these events may occur.

These studies support the hypothesis that alterations in the fetal environment can influence adult health and that glucocorticoids play a major role in the mechanisms leading to the genesis of disease.

7.4 STUDY LIMITATIONS

Due to the increased incidence of preterm labour and abortion in pregnant sheep after glucocorticoid treatment animals were given medroxyprogesterone acetate (MPA) to minimize animal losses (Liggins 1968, Ikekami et al. 1997, Jobe et al. 1998). Synthetic progestins such as MPA, have been shown to have high affinity for GR (Selman et al. 1996) and suppress HPA axis activity (Lang et al. 1990, Selman et al. 1997). Therefore, the potential impact of MPA administration on HPA axis function in this study cannot be ignored. The animals in these studies received one intramuscular dose of MPA (150mg), 5-6 days prior to glucocorticoid administration. Few studies have evaluated the effect of MPA administration on fetal sheep HPA axis activity. However, maternal administration of 250mg of MPA, 24 hours before the start of fetal cortisol infusion, resulted in the inhibition of delivery without significant alterations in fetal plasma DHEA, DHEAS, progesterone and 17α-hydroxyprogesterone (Nathanielsz et al. 1988). These data suggested that exogenous progesterone administered to the pregnant sheep is unable to gain access to the fetal compartment and alter steroidogenesis (Nathanielsz et al. 1988). Therefore, in this study where there were significant differences between animals treated with betamethasone and NT animals only (that did not receive MPA), it would appear that these differences were not due to MPA treatment, but due to betamethasone treatment. Furthermore, there were no significant differences between NT and MS or FS lambs in any of the experiments at any age. However, further studies would be helpful to fully understand the implications of the necessary treatment of pregnant sheep with MPA prior to glucocorticoid administration.

It has been shown previously that gonadal steroids alter HPA axis activity with androgens inhibiting and estrogens stimulating HPA function (Giussani et al. 2000, Chrousos et al. 1998). In the present study, it was not possible to analyze the effects of sex on any of the parameters due to the small sample size. However, one cannot ignore the possibility that sex may have
influenced endocrine function. Therefore, it would be pertinent in future studies to increase the sample size to include sex as a variable or to perform experiments on one sex only.

Since these studies evaluated postnatal HPA and metabolic activity at 6 months and one year of age, it was necessary to castrate males before the onset of estrous in the females to prevent potential pregnancies in the female animals. In addition, since adolescent sheep enter puberty at 8-12 months of age (McNatty et al. 1998), it was necessary to synchronize estrous cycles of females to reduce variability in endocrine responses due to periods of estrous. Studies have shown that HPA responses to stimuli vary in females between luteal and follicular phases (Liu et al. 2001). The potential influence of these necessary actions on the results however, cannot be ignored.

7.5 FUTURE DIRECTIONS

Insulin-like growth factors (IGFs) are generally believed to influence fetal growth by stimulating cell proliferation (D'Ercole et al. 1987), and it has been proposed that a rise in endogenous fetal glucocorticoid regulates IGF-II mRNA expression and thereby regulates fetal growth (Li et al. 1993). It is possible that fetal growth restriction following repeated betamethasone exposure may have been mediated through a reduction in tissue specific IGF-II expression. Further studies investigating the fetal IGF axis would be beneficial in understanding the association between prenatal glucocorticoid administration and growth restriction.

Increases in cord plasma ACTH were not associated with changes in mRNA levels of hypothalamic or pituitary neuropeptides (Chapter 3). A substantial body of evidence exists to suggest that fetal glucocorticoid exposure results in changes in hippocampal GR and MR expression (Sapolsky et al. 1990, Uno et al. 1994, Levitt et al. 1996). Such measurements would be of interest in the context of long-term programming of HPA function by antenatal corticosteroids. It is possible that alterations in hippocampal corticosteroid receptors could result in the observed postnatal hyperactivity (Chapter 5). In addition, further studies are required to understand whether postnatal alterations in HPA function are at the level of the hippocampus, hypothalamus, pituitary and/or the adrenal.

The difference between fetal versus maternal glucocorticoid administration clearly warrants further investigation. It has been shown previously that maternal betamethasone administration resulted in significant reductions in ovine fetal and neonatal weight but direct fetal administration did not (Newnham et al. 1999, Moss et al. 2000). Therefore, it is clear that
the nature and timing of prenatal perturbations are critical factors in determining the long term changes in HPA and metabolic function. This raises the possibility that glucocorticoids administered to the mother may affect placental function. Further studies evaluating the expression levels and activity of placental 11βHSD2, CRH, ACTH in addition to factors regulating placental glucose regulation would be helpful in understanding the mechanisms regulating these differential effects. It is presently unknown whether postnatal alterations are due to direct glucocorticoid exposure or secondary effects due to alterations in placental function.

7.6 CONCLUDING REMARKS

These studies have furthered our understanding of fetal programming and the effects of fetal glucocorticoid exposure on HPA and metabolic function. In conclusion, prenatal betamethasone administration resulted in postnatal alterations in ovine HPA and metabolic function that are dose dependent and persist to adulthood. These observations are a reflection of the intrauterine programming of these axes linking HPA function with growth and metabolic function. These data support the hypothesis that an adverse intrauterine environment can permanently reset developing fetal endocrine axes potentially leading to an increased risk of adult diseases. In light of the number of studies linking alterations in intrauterine environment with the increased risk of adult disease, further studies are critical to understanding the mechanisms linking early fetal events to postnatal health.
Figure 7.1 Summary diagram of the results of the thesis
This diagram represents a summary of the results of the thesis (text in red) and possible physiological implications. Stimulatory (+) and inhibitory (-) actions are shown.
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