The Role of Cortisol in the Regulation of the Fetal Cardiovascular System

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Abstract

There is epidemiological evidence of an association between low birth weight and the development of hypertension, non insulin dependent diabetes mellitus and obesity in adult life. Recent evidence from animal models has suggested that exposure of the fetus to excess glucocorticoids may play a key role in the onset of these adult diseases. Endogenous glucocorticoids play a major role in the normal growth and development of a number of key organ systems including the cardiovascular system and the hypothalamic-pituitary-adrenal axis.

Infusion of metyrapone, an inhibitor of endogenous cortisol synthesis, at 125d gestation, a gestational age when circulating cortisol concentrations are low, resulted in a significant decrease in fetal arterial blood pressure. This study provides evidence supporting the hypothesis that endogenous glucocorticoids play a key role in the development of arterial blood pressure at 126-127d gestation.

Metyrapone infusion at 137d gestation did not result in a significant fall in fetal arterial blood pressure which is in contrast to metyrapone infusion at 125d which resulted in a decrease in arterial blood pressure. Arterial blood pressure increased with increasing gestational age in fetuses infused with either metyrapone or vehicle from 125d and that there was no difference in arterial blood pressure at 137-139d gestation in these two groups of fetuses. Furthermore, infusion of metyrapone from 125d gestation did, however, result in a significant blunting of the fetal arterial blood pressure responses to increasing doses of angiotensin II (Ang II) but a transient suppression of fetal cortisol biosynthesis at 137d gestation did not alter the fetal arterial blood pressure responsiveness to increasing doses of Ang II at 138/139d

gestation. Taken together these findings suggest that there may be a critical window before the start of the prepartum increase in cortisol, when endogenous fetal cortisol contributes to the regulation of arterial blood pressure and to the development of vascular responsiveness to Ang II. These findings have implications as to understanding the possible mechanisms behind the link between glucocorticoid exposure in utero and the development of hypertension later in adult life. Metyrapone administration in the late gestation sheep fetus also resulted in increases in steroidogenic enzymes consistent with a stimulation of the adrenal cortex by ACTH and an increase in the intracellular metabolism of cortisol. This may represent a unique model which allows the dissociation of the relative actions of ACTH and cortisol on fetal adrenal steroidogenesis and growth during late gestation.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Date : 7th July 2006

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Publications Arising From This Thesis

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Warnes KE, McMillen IC, Robinson JS & Coulter CL 2004: Differential actions of metyrapone on the fetal pituitary-adrenal axis in the sheep fetus in late gestation. *Biology of Reproduction*, 71: 620-628.

Related Publications

Warnes KE, Robinson JS, McMillen IC & Coulter CL 2004: Metyrapone infusion stimulates adrenal growth without activating the cell cycle or the IGF system in the late gestation fetal sheep. *Endocrine Research*, 30: 535-539.

Commonly Used Abbreviations

ACE	angiotensin converting enzyme
ACTH	adrenocorticotrophic hormone
Ang I	angiotensin I
Ang II	angiotensin II
ANOVA	analysis of variance
AT1R	angiotensin type 1 receptor
AT1R	angiotensin type 2 receptor
AVP	arginine vasopressin
BCP	1-bromo 3-chloropropane
11βHSD1	11 beta hydroxysteroid dehydrogenase type 1
11βHSD2	11 beta hydroxysteroid dehydrogenase type 2
3βHSD	3 beta hydroxysteroid dehydrogenase
bpm	beats per minute
CBG	cortisol binding globulin
cDNA	complementary deoxyribonucleic acid
CRH	corticotropin releasing hormone
CYP11A1	cytochrome P450 cholesterol side chain cleavage enzyme
CYP11B1	cytochrome P450 11β hydroxylase enzyme
CYP17	cytochrome P450 17α hydroxylase enzyme
CYP 21A1	cytochrome P450 21α hydroxylase enzyme
d	day(s)

DAB	diaminobenzidine substrate
EDTA	ethylenediamine tetraacetic acid
EP3	prostaglandin E_2 type 3 receptor
EP4	prostaglandin E_2 type 4 receptor
F	female
GR	glucocorticoid receptor (type II)
h	hour(s)
Hb	haemoglobin content
HPA axis	hypothalamic pituitary adrenal axis
IGF-I	insulin-like growth factor 1
IGF-II	insulin-like growth factor 2
IGFBP2	insulin-like growth factor binding protein 2
L-NAME	N G-nitro-L-arginine methyl ester
LNNA	N-omega-nitro-L-arginine
M	male
MC2-R	melanocortin type 2 receptor
min	minutes
mmHg	millimetres of mercury

MR	mineralocorticoid receptor (glucocorticoid receptor type I)
mRNA	messenger ribonucleic acid
n	number
PCO_2	partial pressure of carbon dioxide
PGE_2	prostaglandin E_2
PO_2	partial pressure of oxygen
POMC	pro-opiomelanocortin
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RPP	rate pressure product
s	seconds
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
sO2	oxygen saturation
SPSSX	Statistical Package for Social Sciences
SSC	saline sodium citrate
StAR	steroidogenic acute regulatory protein
TBS	tris buffered saline
TGFβ1	transforming growth factor beta 1

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1 Literature Review

1.1 INTRODUCTION

There is epidemiological evidence of an association between low birth weight and the development of hypertension, non insulin dependent diabetes mellitus and obesity in adult life (Phillips, 1998; Huxley et al., 2000; Parsons et al., 2001). This evidence has given rise to the 'early origins of adult disease' hypothesis which states that a fetus or embryo when subjected to a suboptimal intrauterine environment makes physiological adaptations which enable it to survive in the immediate term and that such changes increase the risk of the development of cardiovascular disease and other adverse health outcomes in adult life (Barker & Osmond, 1986; Barker, 1989). Fetal adaptations to a suboptimal intrauterine environment include a decrease in birth weight, an increase in fetal cortisol concentrations and elevated adrenaline and noradrenaline concentrations (Robinson et al., 2000). Recent evidence from animal models has suggested that exposure of the fetus to excess glucocorticoids may play a key role in the onset of adult diseases.

Endogenous glucocorticoids play a major role in the normal growth and development of a number of key organ systems including the cardiovascular system and the hypothalamic-pituitary-adrenal (HPA) axis. In this review I will discuss the evidence which has shown that fetal glucocorticoid exposure may result in hypertension in postnatal life. I will then focus on how glucocorticoids regulate fetal arterial blood pressure and the importance of the role of glucocorticoids for the normal development of the fetal cardiovascular system. I will then review what is known about the normal development of the fetal cardiovascular system and the

development of the HPA axis, specifically how it is able to have the capacity to respond to an adverse intrauterine environment.

1.2 IMPACT OF AN ADVERSE INTRAUTERINE ENVIRONMENT ON THE CARDIOVASCULAR SYSTEM AND HPA AXIS

Epidemiological studies have demonstrated that low birth weight is associated with an increase in blood pressure in adult life (Barker, 1989), and is also associated with markers of increased activities of the HPA axis. The birth weight of men aged 59-70 years, was highly inversely correlated with blood pressure and plasma cortisol concentrations, such that those men who were lighter at birth tended to have higher blood pressure and higher plasma cortisol concentrations at 59-70 years of age (Phillips et al., 1998). In addition, in a study on men aged between 66 and 77 years, birth weight was found to be negatively correlated with the cortisol response to a bolus injection of adrenocorticotropin (ACTH) 1-24. There was also a positive relationship between peak mean plasma cortisol concentrations in response to ACTH 1-24 injection and arterial blood pressure in these adults (Reynolds et al., 2001). These data suggest that adults, who were born small, tend to have higher blood pressure and a more active HPA axis during adult life. An understanding of the mechanisms by which glucocorticoids regulate the development of the cardiovascular system in the normally grown fetus before birth is essential if we are to appreciate the impact of an adverse intrauterine environment on the cardiovascular system and thus on long-term health outcomes. In this review, I will now outline the evidence from clinical studies that exposure to synthetic glucocorticoids alter the development of the cardiovascular system, and evidence

that glucocorticoids play a role in the programming of cardiovascular outcomes which occur after exposure of the fetus to an adverse intrauterine environment.

1.2.1 Impact of synthetic glucocorticoids treatment before and after birth

In pregnant women at risk of preterm delivery, synthetic glucocorticoids, such as dexamethasone and betamethasone, are used widely to improve the maturation of fetal lungs (Liggins & Howie, 1972). Dexamethasone and betamethasone act at the type II glucocorticoid receptor (GR) as agonists and are 25 times more potent than the endogenous glucocorticoid, cortisol (Schimmer & Parker, 2001). Recently, it has become apparent that treatment of preterm infants with betamethasone or dexamethasone may also affect the infants cardiovascular system. Whilst few studies have specifically measured infant arterial blood pressure in preterm infants and compared them to placebo controls, Demarini and coworkers demonstrated that preterm infants from mothers treated prenatally with corticosteroids had significantly higher mean arterial blood pressure from the first 3h of life than untreated controls (Demarini et al., 1999). A recent study has shown that infants treated in utero with multiple doses of corticosteroids had significantly higher arterial blood pressure in the first week of life than the normal published ranges and the blood pressure of babies that were only exposed to a single treatment of corticosteroids (Mildenhall et al., 2005). Interestingly this study also showed that the cardiac wall thickness was higher than the mean expected for birth weight and gestation (Mildenhall et al., 2005), suggesting that treatment of corticosteroids may change the myocardium and hence may have implications for adult life.

Postnatal glucocorticoids have been used in premature infants with very low arterial blood pressure in order to increase blood pressure and potentially mature their

cardiovascular system. In a randomised control trial, it was demonstrated in premature infants born less than 28 weeks gestation, that a single injection of dexamethasone (0.2mg/kg) at 2h of life significantly increased blood pressure for the first 5d of life when compared to saline treated infants (Kopelman et al., 1999). There is some controversy over the effects of administration of multiple doses of glucocorticoids on neonatal blood pressure. In preterm infants delivered at 23-28 weeks gestation, administration of dexamethasone from 8d of life for 32d resulted in a significant increase in systolic and diastolic blood pressure. Similar results were found in a study where premature infants were administered dexamethasone for 42d, or at 2d intervals for 60d, also resulted in a significant increase in systolic, diastolic and mean arterial blood pressure (Bloomfield et al., 1998). Whilst these data suggest benefits for postnatal administration of glucocorticoids there are data to suggest that the exposure of preterm infants to multiple doses of glucocorticoids may not be beneficial to the development of the cardiovascular system. A report from Stark and colleagues (2001) demonstrated that very low birth weight infants given dexamethasone postnatally, were also more likely to be treated for hypertension (systolic blood pressure >80mmHg) (Stark et al., 2001). This is consistent with a study by Papile and coworkers (1998), who found that infants given multiple doses courses of dexamethasone from 3-4 weeks of life were more likely to be treated for hypertension (systolic blood pressure > 75 mmHg) than infants who received dexamethasone treatment between 1-2 weeks of life (Papile et al., 1998). The specific effects of exogenous glucocorticoids are unclear due to the conflicting reports on administration and effects on the cardiovascular system.

There are few studies which have investigated the longer term consequences of the use of antenatal glucocorticoids. One study measured arterial blood pressure in preterm children at 14 years of age. It was found that children who had received a single course of betamethasone before delivery had higher arterial blood pressure at age 14 than unexposed controls (Doyle et al., 2000) whilst at 20 years of age there appears not to be a difference in arterial blood pressure between the two groups (Dessens et al., 2000). More recently, in a large randomised controlled trial for a single course of antenatal betamethasone (2 injections, 24 h apart), the offspring exposed to antenatal betamethasone, at 30 years of age, had similar blood pressure, plasma cortisol and plasma lipids to those offspring who received placebo. Interestingly though, offspring exposed to betamethasone had higher insulin levels during a glucose tolerance test than that of offspring exposed to placebo, suggesting that antenatal exposure to betamethasone, may result in insulin resistance in the adult offspring (Dalziel et al., 2005b). In offspring from the same randomised controlled trial, there was no difference in the cognitive function, working memory or psychiatric morbidity between the betamethasone or placebo groups at 31 years of age (Dalziel et al., 2005a). Whilst this study showed no adverse effect of antenatal glucocorticoid exposure, on cognitive function, the tendency of individuals, exposed to antenatal betamethasone, to be insulin resistant highlights the relevance of understanding the role glucocorticoids in cardiovascular function later in life, and that it is still critical to determine the impact and or mechanisms in animal models. The mechanisms by which glucocorticoids alter arterial blood pressure in the infant and the long term consequences are unclear.

1.2.2 Synthetic glucocorticoids and the fetal arterial blood pressure

It appears that the age and mode of administration of synthetic glucocorticoids is important for the regulation of arterial blood pressure. An injection of dexamethasone (12mg) into the pregnant ewe at 128-133d gestation, caused a significant increase in fetal mean arterial blood pressure at between 2 and 4h post injection and blood pressure returned to control levels by 24h after the injection (Bennet et al., 1999). Administration of dexamethasone or betamethasone ($10\mu g.h^{-1}$) into the fetal sheep at 125d gestation, also resulted in a significant increase in fetal arterial blood pressure (Derks et al., 1997). During a 2d infusion of betamethasone, arterial blood pressure remained elevated compared with pre infusion levels and the ewes went into labour by 24h after the end of the infusion (Derks et al., 1997). Whilst, there was no change in the fetal heart rate during the infusion period, fetal heart rate was higher in the dexamethasone treated group at the end of the infusion (Derks et al., 1997). It therefore appears that both the timing and duration of synthetic glucocorticoid treatment are important in the regulation of fetal arterial blood pressure and heart rate.

There is also evidence that exposure to synthetic glucocorticoids may also have long term consequences on the regulation of arterial blood pressure. The offspring of pregnant rats treated with dexamethasone during the last trimester of gestation had significantly higher systolic and diastolic blood pressure and higher corticosterone concentrations than the offspring of vehicle treated control rats. These data provide evidence that glucocorticoids are important in the programming of postnatal arterial blood pressure (Levitt et al., 1996). In addition, fetal sheep exposed to dexamethasone early in gestation had significantly higher arterial blood pressure at

6 months, 1 and 2 years of age (Dodic et al., 2001). These studies provide evidence that adverse exposure to glucocorticoids in utero may have 'long term' consequences in the regulation of arterial blood pressure.

A fetus may be exposed to elevated endogenous levels of glucocorticoids through a suboptimal intra uterine environment, which may therefore result in postnatal hypertension. There have been a series of animal studies which investigated the effects of poor maternal nutrition on fetal growth and on the development of the cardiovascular system and HPA axis.

1.2.3 Effects of maternal undernutrition in the rat

Pregnant rats fed a low protein diet throughout pregnancy, have offspring which as adults, have significantly higher systolic blood pressure than offspring from mothers fed a control diet (Langley & Jackson, 1994; Langley-Evans et al., 1996; Langley-Evans, 1997). Whilst there was no difference between the birth weights of the two treatment groups, fetuses from mothers fed a low protein diet, had larger placentas, and lower placental expression of the enzyme, 11 beta hydroxysteroid dehydrogenase (11βHSD) type 2. 11βHSD2 is important as it converts corticosterone (or cortisol in sheep and humans) to the inactive dehydrocorticosterone (or cortisone in sheep and humans) in the placenta, thus providing the fetus with protection from exposure to high maternal corticosterone concentrations. One possibility is that in mothers fed a protein restricted diet, the reduced expression of placental 11βHSD2 allowed a greater transfer of maternal corticosterone into the fetal circulation (Langley-Evans et al., 1996). Such exposure may then have acted to 'program' the development of high blood pressure in the animals, whose mother was fed a protein restricted diet. This theory is supported by

a second study by Langley-Evans and coworkers, in which pregnant rats, fed a low protein diet, were concomitantly treated with metyrapone, an inhibitor of glucocorticoid synthesis. The adult offspring from this group of mothers, had similar blood pressure to the systolic blood pressure of those from control well fed mothers (Langley-Evans, 1997) suggesting that fetal exposure to maternal corticosterone leads to an increase in blood pressure in the offspring of undernourished rats. Interestingly, the emergence of high blood pressure in the offspring of protein restricted rats can be abolished by treatment with the angiotensin converting enzyme (ACE) inhibitor, captopril (Langley-Evans & Jackson, 1995). Taken together, these data suggest that the low protein diet fed to pregnant rats, caused an increase in the fetal exposure to maternal glucocorticoids which may modulate the renin-angiotensin system, to result in an increase in blood pressure in adult life.

1.2.4 Effects of maternal undernutrition in the sheep

The role of endogenous glucocorticoids in the development of the cardiovascular system has also been investigated using large animal models, such as the sheep and pig. Large animal models are perhaps more appropriate for determining the underlying mechanisms of high glucocorticoids leading to high blood pressure in later life, because gestational length is longer and the development of the fetus is more comparable to the human. It has been demonstrated that a period of exposure of the fetus to undernutrition as a result of feeding mothers a diet of 50% less than recommended requirements during late gestation (115-145d) in the sheep, significantly increases fetal arterial blood pressure (Edwards & McMillen, 2001). Whilst there was no significant change in fetal cortisol concentrations over this period, there was, however, a negative correlation between fetal arterial blood pressure and fetal ACTH concentrations in the fetuses of undernourished ewes but

not in the well fed animals (Edwards & McMillen, 2001). Interestingly, maternal undernutrition resulted in an increase in maternal cortisol concentrations from 5d after the start of the undernutrition protocol (Edwards & McMillen, 2001). During this time in gestation, it has been demonstrated that most of the cortisol in the fetal circulation is derived from transplacental transfer from the maternal circulation (Hennessy et al., 1982). Edwards and coworkers therefore, suggested that fetuses were exposed to high maternal cortisol concentrations, which decreased fetal ACTH concentrations and acted on the fetal cardiovascular system to result in an increase in fetal blood pressure (Edwards & McMillen, 2001). This effect on fetal arterial blood pressure persisted during late gestation, such that fetal arterial blood pressure at 135d gestation, was higher in undernourished fetuses than in fetuses of well fed ewes. The conclusion therefore, was that the exposure of the fetus to higher cortisol concentrations at 115d, as a result of feeding the mother a diet of 50% less than recommended requirements, resulted in a long term increase in fetal arterial blood pressure (Edwards & McMillen, 2001).

There is evidence that maternal undernutrition between 60d prior to and 7d after conception significantly reprogrammed the fetal cardiovascular system and HPA axis, regardless of the nutritional status throughout gestation (Edwards & McMillen, 2002a; Edwards & McMillen, 2002b). In these studies ewes were assigned to either a maintenance diet or a diet which was 70% of the maintenance diet, for 60d before conception until 7d after conception. At 8d gestation, ewes then either continued on the same diet or were switched to either the maintenance or reduced diet. These authors found that fetal arterial blood pressure at 115d gestation was higher in twins from ewes which were undernourished in the periconceptional period independently

of whether they had been exposed to either control or undernutrition for the remainder of gestation (Edwards & McMillen, 2002b). It was also shown that the fetal plasma cortisol response to a corticotropin releasing hormone (CRH) challenge was higher in fetuses undernourished during the periconceptional period when compared with controls, suggesting that these fetuses also had an increased responsiveness of the fetal adrenal to ACTH stimulation (Edwards & McMillen, 2002a; Edwards & McMillen, 2002b). This work is supported by an earlier study in which ewes were fed a diet of 85% of requirements from 14d prior to conception to 70d gestation. Whilst fetal sheep at 130d gestation did not have altered mean arterial blood pressure, there was an increase in basal femoral vascular resistance in the fetuses of feed restricted ewes (Hawkins et al., 2000a). Furthermore, lambs subjected to the same nutritional environment in utero, had significantly higher mean arterial blood pressure at 85d after birth, suggesting that changes in the femoral vascular resistance *in utero* persisted into postnatal life (Hawkins et al., 2000a). This study also demonstrated that these lambs had an increased pituitary-adrenal response to an acute injection of CRH and arginine vasopressin (AVP). It can be concluded therefore, that exposure of the fetus to a suboptimal intrauterine environment early in gestation, can have long term consequences on both the cardiovascular system and the HPA axis (Hawkins et al., 2000a; Edwards & McMillen, 2002a; Edwards & McMillen, 2002b).

1.2.5 Effects of fetal exposure to glucocorticoids in early gestation

From the evidence reported it appears that exposure of the fetus to a suboptimal intrauterine environment early in development led to hypertension and a more responsive HPA axis in fetal and/or postnatal life (Hawkins et al., 2000a; Edwards & McMillen, 2002a; Edwards & McMillen, 2002b). It has also been demonstrated that

exposure of the fetus to exogenous natural or synthetic glucocorticoids early in pregnancy, results in hypertensive adult offspring. Adult sheep, exposed to 2d of dexamethasone or cortisol treatment between 22-29d gestation had significantly higher mean arterial blood pressure than animals that were not exposed (Dodic et al., 1998; Dodic et al., 2002a). Interestingly, when fetuses were exposed to 2d of dexamethasone between 55-59d gestation, there was no difference in the blood pressure between the two groups when measured during adult life (Dodic et al., 1998). It appears therefore, that the timing of the exposure to either exogenous or endogenous glucocorticoids during pregnancy and in the newborn period is important. Thus it is necessary to understand the role that endogenous glucocorticoids play in the maturation of the cardiovascular system.

Clearly, fetal exposure to glucocorticoids at different times during gestation changes the development of fetal cardiovascular system, however the role of endogenous glucocorticoids during the normal development of the fetal cardiovascular system is unclear. It is therefore, critical to understand the development of the fetal cardiovascular system, and also the capacity of the fetal HPA axis to produce and regulate cortisol concentrations during different stages of gestation.

1.3 THE FETAL CARDIOVASCULAR SYSTEM

During fetal life, the lungs do not have a respiratory function as arterial oxygen and carbon dioxide transfer occurs via the placenta. Thus, there are a number of adaptations of the fetal cardiovascular system to optimise the supply of oxygen to developing tissues before birth (Thornburg, 1991). Oxygenated blood flows from the placenta to the fetus via the umbilical vein. Approximately 45% of the blood from the

umbilical vein flows into the fetal liver and blood from the left side of the liver then returns to the inferior vena cava and ductus venosus via the left hepatic vein, and blood from the right side of the liver is returned to the inferior vena cava via the right hepatic vein (Rudolph, 1985). The remainder of the umbilical venous blood bypasses the liver and flows via the ductus venosus into the inferior vena cava (Rudolph, 1985). Blood from the inferior vena cava, now a mixture of well and poorly oxygenated blood, travels to the heart where some blood streams into the right atrium. Most of the blood, however, is preferentially shunted through to the left side of the heart via the foramen ovale, a hole in the septum between the right and left atria, which closes after birth. Blood from the left ventricle is pumped preferentially to the brain via the brachiocephalic and carotid arteries. Most of the blood which enters the right ventricle of the heart will travel through the ductus arteriosus, instead of the pulmonary artery, as pulmonary arterial resistance is relatively high *in utero*. The ductus arteriosus connects the pulmonary artery to the descending aorta, so blood from the right ventricle is pumped directly to the fetal organs and placenta (Thornburg, 1991).

To enable the newborn to adapt to the extrauterine environment, a number of changes occur in the cardiovascular system within the first 48h after birth. These changes include the closure of the foramen ovale, as pulmonary ventilation significantly decreases the pulmonary arterial resistance, causing a pressure gradient between the right and left atria which is then responsible for the closure of the foramen ovale. Other adaptations include closure of the ductus arteriosus and many studies have investigated the mechanisms which underlie the closure of the ductus arteriosus. Prostaglandin E2 (PGE2), acts to maintain a patent ductus

arteriosus through vasodilation through the actions via one of the PGE2 receptors (EP4) (Smith, 1998). One mechanism which has been postulated to be involved in the closure of the ductus arteriosus is that the increase in oxygen tension which occurs at birth results in a decrease in the vasodilatory effect of PGE2 acting through the EP4 receptor and an increase in the vasoconstrictor action of PGE2 at the EP3 receptor (Smith, 1998). After birth there is a dramatic fall in circulating concentrations of PGE2. The loss of PGE2 from the circulation is attributed to an increase in blood flow to the lungs, a major site of prostaglandin catabolism and the loss of the placenta, a major site of prostaglandin synthesis (Smith, 1998).

As in the adult, fetal cardiac output is determined by stroke volume and heart rate, however the distribution of cardiac output (combined ventricular output) is different in the fetus than the adult. The placenta receives approximately 40% of the fetal cardiac output and around 35% is distributed to the fetal carcass. The fetal brain, heart and gut each receive ~5%, the fetal lungs receive ~4%, while the fetal kidneys, liver and spleen each receive ~2% of the cardiac output (Rudolph, 1985; Thornburg, 1991). In utero cardiac output is at the upper limit of its functional capability, and hence a redistribution of blood flow is the major response to hypoxia (Gilbert, 1988). A change in fetal blood pressure, in response to an insult, such as hypoxemia is therefore, controlled primarily by changes in peripheral vascular resistance.

A number of studies in the sheep have shown that fetal arterial blood pressure is higher after 130d gestation than before 120d (Boddy et al., 1974; Blanco et al., 1988; Tangalakis et al., 1992; Daniel et al., 1996), whereas fetal heart rate is lower in older fetuses when compared to younger animals (Daniel et al., 1996). More

recently, in a study where blood pressure was measured continuously from 120d gestation until spontaneous delivery at 147d gestation, it was demonstrated that there is a significant increase in mean arterial blood pressure in the sheep fetus between 120d (35.2 ± 1.7 mmHg) and 143d gestation (47.4 ± 2.4 mmHg) (Unno et al., 1999). Taken together, these studies demonstrate there is an increase in fetal arterial blood pressure and a decrease in fetal heart rate between 120d and 140d gestation.

The mechanism(s) by which fetal arterial blood pressure increases and heart rate decreases during late gestation is unclear. A number of systems are known to regulate fetal arterial blood pressure and perhaps one or a combination of these, ultimately lead to the reciprocal changes in arterial blood pressure and heart rate during late gestation. These systems include the autonomic nervous system, factors secreted by endothelial cells including nitric oxide and the renin-angiotensin system. This review will firstly discuss the autonomic control of fetal blood pressure, briefly describe the role of nitric oxide in the control of regional blood flow and then discuss the role of the renin-angiotensin system in regulation of fetal arterial blood pressure.

1.3.1 Autonomic regulation of the fetal cardiovascular system

In the fetal sheep, arterial baroreceptor activity is detected by the firing rate of baroreceptor afferent fibres and is present from 90d gestation. In addition, carotid chemoreceptor activity is detected by the firing rate of individual chemoreceptor afferent fibres and can be measured from 100d gestation (Blanco et al., 1984). It therefore, appears that these afferent pathways are functional in the sheep fetus from as early as 90d gestation.

Segar and coworkers (1994) found a significant positive relationship between renal sympathetic nerve activity, mean arterial blood pressure, and heart rate in the fetal sheep. Renal sympathetic nerve activity is a measure of the activity of the sympathetic nervous system, such that high renal sympathetic nerve activity was associated with high mean arterial blood pressure and heart rate which suggests that the sympathetic nervous system plays a key role in the regulation of arterial blood pressure (Segar et al., 1994b). This is supported by evidence that, when a ganglionic blocker, trimethaphan, was infused intrafetally, there was a significant attenuation of both heart rate and blood pressure variability (Segar et al., 1994b).

In fetal sheep at 121-141d gestation, infusion of adrenaline, which acts via adrenergic β2 receptors, increases fetal heart rate without altering blood pressure (Jones & Robinson, 1975). In contrast, fetal infusion at 120d gestation, of noradrenaline, which acts via the adrenergic α receptors, increases fetal arterial blood pressure and lowers heart rate when infused at doses greater than 3.9μg.min^{-1} (Cheung & Brace, 1988; McMullen et al., 1998), however such high doses mimic physiological conditions seen only during stress such as acute hypoxia. Furthermore, in response to an infusion of phentolamine, a specific α-adrenergic antagonist, fetal arterial blood pressure was unchanged but heart rate was increased (Giussani et al., 1993). Following denervation of the carotid sinus nerve in the fetal sheep, it has also been demonstrated that arterial blood pressure and heart rate are similar when compared with intact controls under basal conditions (Giussani et al., 1993). Whilst complete inhibition of sympathetic nerve activity decreases fetal arterial blood pressure and heart rate, it can be concluded that basal vascular tone is only in part regulated by the α adrenergic receptors of the sympathetic nervous

system, as secretion of adrenaline from the adrenal medulla, acting via β adrenergic receptors is also important in the maintenance of blood pressure under basal conditions.

The role of the autonomic nervous system in the regulation of fetal arterial blood pressure is more evident when the fetus is challenged, such as during exposure to acute hypoxia. In normal fetuses, the cardiovascular response to an acute hypoxic event is an initial fall in fetal heart rate and an increase in blood pressure, which is associated with increased femoral vascular resistance and decreased femoral blood flow (Giussani et al., 1993). Importantly, there is also an increase in the carotid artery blood flow associated with a decrease in carotid vascular resistance, in order to maintain blood flow to the brain during a period of hypoxia (Boddy et al., 1974). There is an increase in fetal heart rate once normoxia is restored (Boddy et al., 1974; Giussani et al., 1993). When the carotid sinus nerve is sectioned, fetuses do not exhibit the initial bradycardia, and do not have an increased femoral vascular resistance in response to acute hypoxia, suggesting that the carotid chemoreceptors detect and regulate the response of the fetal cardiovascular system to low PaO2 (Giussani et al., 1993) and regulate the redistribution of fetal blood flow in order to ensure the fetus survives the acute hypoxia episode. The coordinated cardiovascular response to acute hypoxia occurs within seconds, which suggests that it is under reflex control. In addition, there is a significant increase in the circulating concentrations of both adrenaline and noradrenaline in response to acute hypoxia (Jones et al., 1988; Simonetta et al., 1996) suggesting that hormonal factors such as increased secretion of adrenaline from the adrenal medulla may regulate the longer term responses of the cardiovascular system. If during the period of

acute hypoxia, the fetuses are treated with phentolamine, an α adrenergic receptor antagonist, the increase in femoral vascular resistance is abolished and there is a marked decrease in the blood pressure response to hypoxia. These data suggest that the sympathetic nervous system, including the increased secretion of adrenaline from the adrenal medulla, play a role in the maintenance of fetal arterial blood pressure during exposure to hypoxia (Giussani et al., 1993).

1.3.2 Nitric oxide and the fetal cardiovascular system

Nitric oxide is synthesised by endothelial nitric oxide synthase, located in the vascular endothelial cells, where it acts as a potent endogenous vasodilator (Fleming & Busse, 1999). In fetal sheep between 90-100d gestation, infusion of a nitric oxide synthase inhibitor, N-omega-nitro-L-arginine (LNNA), significantly increased mean arterial blood pressure and decreased cardiac output without a change in fetal heart rate (Fan et al., 1998). In late gestation (128-139d), whilst infusion of LNNA also increased fetal arterial blood pressure by a similar amount as in the younger fetuses, there was a dramatically decrease in fetal heart rate (Fan et al., 1996). In addition, there was a greater decrease in gastrointestinal blood flow in response to LNNA infusion in fetal sheep aged 90-100d than in the older fetuses. These data suggest that nitric oxide plays a greater role in the regulation of blood flow earlier in gestation, at a time when endogenous cortisol concentrations are low.

Similarly, infusion of a different nitric oxide synthase inhibitor, N G-nitro-L-arginine methyl ester (L-NAME), also resulted in an increase in fetal arterial blood pressure in the late gestation sheep fetus (Green et al., 1996). This was associated with an increase in both femoral and carotid vascular resistance (Green et al., 1996). Interestingly, when these fetuses were then exposed to a concomitant episode of

acute hypoxia during the L-NAME infusion, there was no further increase in fetal arterial blood pressure. In addition, carotid vascular resistance continued to be higher and carotid blood flow lower, in response to acute hypoxia in the fetuses infused with L-NAME. L-NAME infusion significantly increased femoral vascular resistance during normoxia but after the induction of hypoxia there was no further increase in femoral vascular resistance (Green et al., 1996). These data suggest that nitric oxide plays an important role in the redistribution of blood flow during an acute hypoxia episode (Green et al., 1996).

1.3.3 Renin-angiotensin system and the fetal cardiovascular system

In the adult, the renin-angiotensin system plays an important role in the maintenance of blood pressure. Angiotensin II (Ang II) regulates both blood volume and vascular resistance (DeGasparo et al., 2000). The renin-angiotensin system is functional before birth. Plasma renin, Ang II, ACE and angiotensin receptors (AT1R and AT2R) have all been measured in fetal sheep (Rosenfeld et al., 1993; Segar et al., 1994a; Forhead et al., 1998; Forhead et al., 2000). Infusion of angiotensin I (Ang I) for 3d, into the sheep fetus significantly increased mean arterial blood pressure by 15mmHg (Moritz et al., 1997). Infusion of Ang II for 2d at 140d increased fetal mean arterial blood pressure to 150% above control values (Poore et al., 1998a). Bolus doses of Ang II at either 103-120d or at 135-145d gestation increased fetal systolic and diastolic blood pressure by 25 and 10 mmHg respectively (Tangalakis et al., 1992). Plasma ACE concentrations are also measurable before birth and positively correlate with gestational age and fetal arterial blood pressure (Forhead et al., 1998). It is likely therefore, that the fetus or placenta are capable of synthesising ACE and hence Ang I can be converted to the vasoactive Ang II.

The actions of Ang II are mediated by two types of receptors AT1R and AT2R. AT1R and AT2R are members of the seven transmembrane domain superfamily of receptors (Shanmugam & Sandberg, 1996). AT1R and AT2R have been found in a number of different tissues including heart, kidney, adrenals and the vasculature of the adult and fetus (Shanmugam & Sandberg, 1996; DeGasparo et al., 2000). In the adult, it is well established that the AT1R mediates the actions of Ang II in the maintenance of blood pressure homeostasis, whereas one role of the AT2R appears to be to inhibit the actions of AT1R (Inagami et al., 1999). In the pregnant ewe, there is also evidence that AT2R may inhibit the vasoconstrictor effects of Ang II on AT1R. In pregnant sheep uterine artery segments *in vitro* blockade of AT2R by a selective antagonist significantly increased the vasoconstrictor response to Ang II suggesting that, as in the non pregnant state, AT2R may inhibit the vasoconstrictor effect of Ang II mediated by AT1R (McMullen et al., 1999). In addition, in the fetus, there is evidence that AT2R may play a role in cell growth and differentiation (Shanmugam & Sandberg, 1996).

In the fetal sheep, Rosenfeld and coworkers (1993) demonstrated that Ang II receptor binding activity in the fetal aorta did not change during gestation, between 107-111d and 125-134d, although binding density of Ang II was significantly higher in the fetal arteries compared with the maternal uterine artery (Rosenfeld et al., 1993). As the relative proportions of the AT1R and AT2R in the fetal arteries were not studied, it is not possible to predict the physiological effect of Ang II via the AT1R and AT2R. Recently, with the development of more specific antagonists, it has been demonstrated that in fetal aortae and carotid arteries, there is an increase in the AT1R binding affinity with increasing gestational age, with a concomitant decrease in

AT2R binding affinity (Burrell et al., 2001). Taken together, these data suggest that the increase in the ratio of AT1R to AT2R with increasing gestation may play a role in the increased vasoconstriction of Ang II via AT1R thus contributing to the increase in blood pressure in late gestation.

Infusion of the AT1R antagonist, losartan, into fetal sheep at 125-132d gestation for 2h significantly decreased mean arterial blood pressure (Stevenson et al., 1996). In a further study, infusion of a specific AT1R antagonist (GR138950) into the fetus at 126d gestation or at 137d gestation also resulted in a significant fall in fetal arterial blood pressure (Forhead et al., 2000). In addition, the hypotensive response of the fetus was greater in older fetuses when compared with younger animals, suggesting that during late gestation there is an increase in the responsiveness of the renin-angiotensin system acting through the AT1R receptor in the maintenance of fetal arterial blood pressure (Forhead et al., 2000).

Infusion of an ACE inhibitor, captopril, which blocks the conversion of Ang II from Ang I, into both pregnant ewes and fetal sheep has also been shown to decrease arterial blood pressure (Lumbers et al., 1992; Forhead et al., 1996). It is clear that captopril crosses the placenta, as demonstrated by a blunted pressor response to Ang I in the fetus at 1h after the start of captopril infusion to the ewe (Lumbers et al., 1992). These data are consistent with the findings of an earlier study which demonstrated that fetal arterial blood pressure fell in response to a captopril infusion to the fetus, and that the magnitude of the decrease in arterial blood pressure was similar in fetuses younger than 120d, older than 130d and in new born lambs (Robillard et al., 1983). The effects of captopril appear to be dose-dependent as

when captopril was infused at half the dose that was used by Robillard (1983) or Lumbers (1992) there was no effect on fetal arterial blood pressure in healthy, normally grown fetuses at a similar gestational age (Green et al., 1998; Edwards et al., 1999). These data suggest that the maturation of the renin-angiotensin system is not dependent on a maturation of the presence of ACE.

Studies have demonstrated that removal of the afferent chemoreflex pathway results in the renin angiotensin system playing a greater role in the regulation of fetal arterial blood pressure in response to acute hypoxia. When fetuses are exposed to acute hypoxia with a concomitant infusion of captopril, the fetal cardiovascular response to acute hypoxia is similar to that measured in vehicle infused controls (Green et al., 1998). In contrast, when fetuses which have undergone carotid sinus denervation and are exposed to acute hypoxia the concomitant infusion of captopril results in a blunting of the increase in mean arterial blood pressure and an absence of the fall in femoral blood flow (Green et al., 1998). There is also evidence that the renin-angiotensin system may play a greater role in the maintenance of fetal arterial blood pressure in the growth restricted (PR) fetus than in normally grown fetal sheep during late gestation. Growth restriction induced by restriction of placental growth results in fetuses which are exposed to chronic hypoxia. An intrafetal infusion of captopril, decreased arterial blood pressure in growth restricted chronically hypoxic, but not normally grown normoxic fetal sheep after 135d but not between 125-135d gestation (Edwards et al., 1999). Taken together, these studies demonstrate that during acute and chronic hypoxia, the renin-angiotensin system plays a key role in the regulation of fetal arterial blood pressure.

1.3.4 Fetal cortisol and the cardiovascular system

Whilst a number of studies have investigated the role of endogenous glucocorticoids in the regulation of arterial blood pressure, there have been conflicting results (Ray et al., 1988; Unno et al., 1999; Segar et al., 2002). Unno and co workers (1999) reported that the small increase in fetal arterial blood pressure which occurs between 120 and 126d gestation in intact fetuses, was not present in bilaterally adrenalectomised fetuses, but was restored with cortisol replacement. One limitation of this study was that fetal plasma cortisol concentrations could not be determined in either the intact or adrenalectomised fetuses as cortisol concentrations were lower than the detection limit of the cortisol radioimmunoassay used. In addition, this study did not compare arterial blood pressure in adrenalectomised and intact fetuses later in gestation, when fetal cortisol concentrations are normally high (Unno et al., 1999). In a more recent study, Segar and coworkers (2002) demonstrated adrenalectomised fetuses at 130d had significantly lower fetal arterial blood pressure at 139-140d when compared with adrenalectomised fetuses concomitantly infused with cortisol. Cortisol concentrations in these two groups of animals were significantly different, (not detectable in adrenalectomised fetuses compared to 59ng/ml in adrenalectomised with cortisol replacement fetuses). Whilst this study demonstrated that cortisol replacement increased fetal blood pressure, the study did not include a third, intact group in which no treatment had been performed. In contrast, in fetal sheep at 119-133d gestation, Ray and colleagues (1987) showed that 4-6 days following adrenalectomised, fetuses had significantly lower cortisol concentrations and similar blood pressure when compared with intact control animals (Ray et al., 1988), although it may be possible that 4-6 days is sufficient for other control mechanisms,

such as an increased activity of the renin-angiotensin system to compensate for the removal of the adrenals. Fetal adrenalectomy also results in the removal of both steroidogenic cells of the adrenal cortex and the catecholamine containing cells of the adrenal medulla and it is therefore unclear, whether changes in arterial blood pressure in adrenalectomised fetuses are as a consequence of the removal of cortisol alone, or of the combined removal of cortisol and adrenaline. Therefore, it is still unclear whether endogenous cortisol concentrations play a role in the regulation of fetal arterial blood pressure during late gestation.

In a series of studies, the effects of exogenous cortisol infusion on the fetal cardiovascular system have been determined. In fetal sheep from 127d to 143d gestational age, infusion of cortisol for 6h resulted in an increase in fetal arterial blood pressure to 11% above control levels and a significant decrease in heart rate (Wood et al., 1987). In sheep fetuses between 103-120d gestation, a longer term infusion of cortisol (24-48h) also resulted in a significant increase in fetal arterial blood pressure (Tangalakis et al., 1992; Dodic & Wintour, 1994). Later in gestation at 130-132d, a 48h infusion of cortisol did not alter mean arterial blood pressure (Robillard et al., 1994). Interestingly, while infusion of cortisol into fetal sheep before 130d gestation, when endogenous cortisol concentrations are low, significantly increased fetal arterial blood pressure, when the same experiment was performed in fetuses of gestation greater than 135d, when endogenous cortisol concentrations are high, there was no change in fetal arterial blood pressure (Tangalakis et al., 1992; Forhead et al., 2000). These data suggest that the timing of exposure to glucocorticoids is important in determining the effect on fetal arterial blood pressure.

In this thesis I have tested the hypothesis that suppression of cortisol biosynthesis from 125d gestation will result in a decrease fetal arterial blood pressure within 24h (Ch 2) and that continued suppression of cortisol biosynthesis from 125d will also result in a decrease in fetal arterial blood pressure later in gestation at 137d gestation (Ch 3).

Thus the aim tested in this thesis was to determine the effect of suppression of cortisol biosynthesis on fetal arterial blood pressure at 126-127d gestation (Ch 2) and again at 137-139d gestation (Ch 3).

In these studies, fetal arterial blood pressure was determined at 126-127d and 137-139d gestation during pharmacological blockade of cortisol production between 125 and 140d gestation.

The mechanism by which synthetic glucocorticoids act to increase fetal arterial blood pressure is unclear. Anwar and colleagues (1999) studied the vascular reactivity after infusion of betamethasone for 48h from 125d gestation (Anwar et al., 1999). Whilst femoral arteries from betamethasone treated fetuses had similar internal diameters when compared with control animals, exposure to increasing concentrations of potassium resulted in a greater tension response in vessels from the betamethasone-treated fetuses than the vehicle infused controls (Anwar et al., 1999). Recently it has been shown that prenatal dexamethasone exposure does not alter the expression of mRNA when measured in femoral muscle from 5 month old lambs and there was also no difference in the immunohistochemical staining for endothelial nitric oxide synthase protein in the endothelial from femoral arteries between the two groups (Molnar et al., 2003). It appears therefore that whilst

glucocorticoids increase arterial blood pressure it is not through the inhibition of nitric oxide synthase expression in the femoral muscle and vasculature.

Another possible mechanism by which glucocorticoids may regulate fetal arterial blood pressure is through the actions of the renin-angiotensin system. Tangalakis and colleagues (1992) demonstrated that infusion of cortisol into fetuses aged 103-120d gestation, significantly increased the systolic and diastolic blood pressure response to increasing doses of Ang II when compared to vehicle infused controls (Tangalakis et al., 1992). In contrast, when the same experiment was performed in fetuses of 130d gestation or older there was no difference in the blood pressure response to Ang II between cortisol and vehicle infused fetuses (Tangalakis et al., 1992). Infusion of the AT1R antagonist (GR138950) at 125d gestation, resulted in a significant decrease in fetal arterial blood pressure whereas, infusion of the AT1R antagonist at 137d gestation (when endogenous cortisol concentrations are higher), resulted in a significantly greater fall in fetal arterial blood pressure in response to the same dose of an AT1R antagonist (Forhead et al., 2000). Interestingly, when fetuses at 125d were infused with cortisol to levels similar to those present in the older animals, the fetal hypotensive response to the AT1R antagonist was significantly greater than that measured in the vehicle infused controls (Forhead et al., 2000). These data imply that the increase in fetal arterial blood pressure during late gestation may be in part due to a cortisol dependent increase in the actions of Ang II through AT1R in the fetal vasculature (Forhead et al., 2000). It has been demonstrated previously, that at 130d gestation, a 2d cortisol infusion results in a significant increase in the expression of AT1R mRNA in the left atrium (Robillard et al., 1994). Interestingly, PR fetal sheep which have been subjected to placental

growth restriction and hence fetal growth restriction have significantly higher fetal plasma cortisol concentrations when compared with controls (Phillips et al., 1996b). PR These fetal sheep also have significantly higher AT1R mRNA and protein in the vascular smooth muscle from femoral, renal and carotid arteries when compared to normally grown controls (Rosenfeld et al., 2003). These data provide further evidence cortisol may regulate arterial blood pressure via AT1R in the fetal vasculature and the peripheral renin-angiotensin system.

The hypothesis tested in this thesis is that suppression of cortisol synthesis will decrease fetal arterial blood pressure responsiveness to Ang II in the late gestation fetal sheep.

The aim of this thesis was to determine if suppression of cortisol biosynthesis from 125d gestation decreased the fetal arterial blood pressure responsiveness to Ang II later in gestation after 135d gestation.

Cortisol biosynthesis was suppressed from 125d or 137d gestation and the fetal arterial blood pressure responses to increasing doses of Ang II were measured at 137-139d gestation.

There is also evidence that glucocorticoids may act in concert with catecholamines to modulate fetal arterial blood pressure. It has been demonstrated that a 48h infusion of dexamethasone at 125-128d gestation, resulted in a significant increase in fetal arterial blood pressure and in fetal plasma adrenaline concentrations (Fletcher et al., 2002). These elevated adrenaline concentrations may contribute to the elevated heart rate measured in dexamethasone infused animals (Fletcher et al., 2002). In addition, the studies investigating the effect of adrenalectomy on fetal

arterial blood pressure are not conclusive as removal of the adrenal also removes the adrenal medulla, a site that is important in the synthesis and secretion of adrenaline and noradrenaline.

Therefore, it is unclear whether catecholamines modulate the actions of glucocorticoids on fetal arterial blood pressure. An aim of this thesis therefore was to determine if suppression of endogenous cortisol synthesis from 125d would decrease the fetal arterial blood pressure responses to increasing doses of noradrenaline in late gestation.

To understand the effect endogenous glucocorticoids may play on the development of the fetal cardiovascular system it is important to review what is known about the development of the HPA axis and the capacity of the fetal adrenal to secrete cortisol during different stages of development.

1.4 DEVELOPMENT OF THE HPA AXIS

In the sheep fetus, it is well established that there is a prepartum rise in circulating cortisol concentrations, which is essential for the maturation of key fetal organs, such as the lungs, liver, heart and brain and the initiation of parturition (Bassett & Thorburn, 1969; Challis & Brooks, 1989). Destruction of the fetal pituitary (Liggins & Kennedy, 1968; Jacobs et al., 1994; Poore et al., 1998b) or lesions of the hypothalamic paraventricular nuclei, abolish the prepartum rise in ACTH, cortisol, and result in prolonged gestation (McDonald & Nathanielsz, 1991).

The synthesis and secretion of cortisol from the fetal adrenal gland during development is dependent on an intact HPA axis. Hypothalamic secretagogues,

CRH and AVP are synthesised by cells in the paraventricular nucleus which project neural connections to the median eminence. CRH and AVP are released into the hypophyseal portal circulation and act at the corticotroph cells in the anterior pituitary (Challis & Brooks, 1989). CRH and AVP stimulate the synthesis of the ACTH precursor, proopiomelanocortin (POMC) and the secretion ACTH and other products of POMC including proγACTH, γMSH, βendorphin and βlipotropin in both the adult (Pradier et al., 1988; Schwartz & Vale, 1988) and fetus (Mulvogue et al., 1986).

1.4.1 Development of the fetal adrenal

Growth of the Fetal Adrenal

The growth and maturation of the fetal adrenal is coordinated and yet very complex, in order to ensure that key organs are exposed to circulating cortisol concentrations at appropriate times during development. From 60d gestation, two distinct adrenal zones in the cortex can be distinguished, along with the inner mass of chromaffin cells of the adrenal medulla (Robinson et al., 1979; Wintour, 1984). Between 60-80d gestation, the outer cortical zone increases in width (Robinson et al., 1979) due to an increase in the cell number of the zona fasciculata (Boshier & Holloway, 1989). Between 90-120d there is a decrease in the number and volume of steroidogenic secretory cells within the zona fasciculata, with a concomitant increase in the volume of individual cells suggesting that during this time there is predominantly cellular hypertrophy (Boshier & Holloway, 1989), which coincides with a period of steroidogenic quiescence within the adrenal gland (Wintour et al., 1975). During the last two weeks of gestation the fetal adrenal doubles in weight (Boshier et al., 1980) predominantly due to an increase in cell growth and cell division in the zona fasciculata (Boshier & Holloway, 1989). This coincides with an increase in plasma

ACTH concentrations, secreted from the fetal pituitary. ACTH is known to play a crucial role in the functional maturation of the fetal adrenal gland. Administration of ACTH to fetal sheep at 120d results in an increase in adrenal cortical growth (Robinson et al., 1983; Tangalakis et al., 1990) and stimulation of the maturation of cortical cells, which are not present in the adrenals of vehicle infused control fetuses (Robinson et al., 1983). Conversely, the adrenals from hypophysectomized fetuses were significantly lighter and the size of the zona fasciculata was substantially smaller (Robinson et al., 1983). These data confirm that the growth of the fetal adrenal gland and particularly the cortex is dependent on the presence of ACTH concentrations.

Activation of the Fetal Adrenal and Steroidogenesis

The prepartum increase in circulating cortisol concentrations is dependent on the activation of a number of key proteins within the fetal adrenal by fetal ACTH. In the adult adrenal, ACTH binds to its receptor, (melanocortin type 2 receptor; MC2-R), which activates cAMP dependent protein kinase A to increase the synthesis of steroidogenic acute regulatory protein (StAR). StAR mediates the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane where cytochrome P450 side chain cleavage (CYP11A1) is localised, the enzyme which catalyses the conversion of cholesterol to pregnenolone (Kallen et al., 1998; Miller & Strauss, 1999; Stocco, 2001). In addition, studies have demonstrated that coincident with the prepartum increase in adrenal responsiveness to ACTH and steroid output there is an up-regulation of ACTH induced adenylate-cyclase activity and expression of StAR mRNA and mature StAR protein in the fetal sheep adrenal in late gestation (Durand et al., 1985; Fraser et al., 2001; Coulter et al., 2002a)

which may play a role in augmenting the actions of ACTH on adrenal steroidogenesis.

At 40-60d gestation, the ACTH receptor, MC2-R and steroidogenic enzymes, responsible for the conversion of cholesterol to cortisol, CYP11A1, cytochrome P450 17α hydroxylase (CYP17), 3 beta hydroxysteroid dehydrogenase (3βHSD) and cytochrome P450 21α hydroxylase (CYP21A1) mRNA are present in the fetal adrenal (Riley et al., 1992; Tangalakis et al., 1994; Wang et al., 2004). This coincides with ovine fetal adrenal cells *in vitro*, being capable of secreting the highest level of cortisol per gram of tissue, in response to the addition of ACTH (Wintour et al., 1975).

Between 90-120d gestation, there are a relatively low number of ACTH binding sites coinciding with the quiescent phase of cortisol production by the fetal adrenal (Saez et al., 1984). In addition, the expression of steroidogenic enzymes CYP11A1 and CYP 17 mRNA is lower than earlier in gestation (Tangalakis et al., 1989) whilst the expression of CYP21A1 mRNA is unchanged (Tangalakis et al., 1989). The expression of 11β hydroxylase (CYP11B) mRNA, the last enzyme in the cortisol synthetic pathway, is present during this time (John et al., 1987; Myers et al., 1992). The quiescent phase is characterised by the limited ability of the fetal adrenal to secrete cortisol in response to ACTH stimulation. In addition to the limited number of binding sites for ACTH, another possible candidate for the decreased sensitivity of the fetal adrenal to ACTH concentrations to induce adrenal synthesis and secretion of cortisol may be the presence of an inhibitor of adrenocortical steroidogenesis, such as transforming growth factor beta-1 (TGFβ1). In the adult adrenal, addition of

TGFβ1, *in vitro*, inhibits the synthesis and secretion of cortisol under basal conditions and when stimulated by ACTH through regulation of CYP17 (Rainey et al., 1988; Perrin et al., 1990; Rainey et al., 1990). In the fetus, TGFβ1 decreases cortisol synthesis and secretion in response to ACTH and there is evidence that this is through a decrease in the protein expression of CYP11A1 and CYP17 (Rainey et al., 1991; Lebrethon et al., 1994a; Naaman-Reperant et al., 1996). In addition, TGFβ1 mRNA is present in the fetal sheep from 100d gestation and progressively decreases as gestation increases, whilst adrenal expression of CYP17 mRNA is relatively low from 100d gestation and progressively rises at term (Coulter et al., 2003). These data suggest that TGFβ1 may play a role during the quiescence phase of fetal adrenal development.

During late gestation, there is an increase in the expression of MC2-R mRNA and ACTH binding (Durand et al., 1985; Fraser et al., 2001). StAR mRNA has been detected in fetal sheep adrenals from as early as 82d gestation and adrenal StAR mRNA and protein significantly increase between 125d and 141d gestation (Coulter et al., 2002a). These data suggest that StAR may play a role in the up-regulation of cortisol secretion during late gestation in the fetal sheep and this may be due to ACTH having a direct effect on StAR. The expression of CYP11A1 mRNA is increased from 120d gestation until term (Tangalakis et al., 1989; Phillips et al., 1996a), there is an increase in the metabolism of pregnenolone to 17α hydroxy-pregnenolone in adrenal cells from newborn animals when compared with adrenal cells from 120d fetuses (Durand et al., 1985) and expression of CYP17 mRNA is higher at term compared to before 140d gestation (Tangalakis et al., 1989; Phillips et al., 1996a; Simmonds et al., 2001). The expression of 3βHSD is also increased at

term when compared with 132d gestation (Simmonds et al., 2001) and the other enzymes of the cortisol synthetic pathway including, CYP21A1 and CYP11B1 are all present during the last third of gestation but the expression of these enzymes remain relatively unchanged (Tangalakis et al., 1989; Myers et al., 1992; Phillips et al., 1996a; Simmonds et al., 2001). The fetal adrenal progressively secretes more cortisol into the circulation, which ultimately initiates the cascade of events culminating in parturition in the sheep (Challis & Brooks, 1989).

There are conflicting reports regarding the ability of ACTH to regulate the expression of its receptor. The expression of MC2-R mRNA was unchanged after fetal hypophysectomy or intra fetal infusion of ACTH (Simmonds et al., 2001; Carter et al., 2002). More recently however, hypothalamic-pituitary disconnection (HPD) in fetal sheep has been shown to result in decreased expression of MC2-R mRNA expression and *in vitro* exposure to ACTH reversed the expression of MC2-R mRNA (Valego et al., 2005). The discrepancy may be between the effect of ACTH *in vitro* on cultured adrenal cells and the effect of ACTH *in vivo*.

ACTH is known to be the key mediator of cortisol synthesis and secretion from the fetal adrenal. ACTH has been shown to stimulate the expression of StAR mRNA in adrenal cells *in vitro* and *in vivo* via a cyclic AMP dependent mechanism (Penhoat et al., 1989; Lebrethon et al., 1994b; Stocco, 1999; Le Roy et al., 2000; Feltus et al., 2002) and therefore may play a role in the mechanism ACTH stimulating cortisol synthesis of the adrenal. ACTH specifically acts on key steroidogenic enzymes to increase cortisol synthesis and secretion. The expression of CYP11A1 mRNA is increased in adrenocortical cells from fetuses of 40-60d gestation after incubation

with ACTH (Tangalakis et al., 1990). The expression of CYP11A1 mRNA and CYP17 mRNA expression is also increased in adrenocortical cells, from 100-120d fetuses, when incubated with ACTH but which disappears if adrenals were harvested 3-4 days post infusion (Tangalakis et al., 1990). In addition, the expression of CYP11A1, CYP17 and 3βHSD mRNA levels are lower in hypophysectomized fetuses but are restored after ACTH replacement (Tangalakis et al., 1990; Simmonds et al., 2001). In contrast, infusion of ACTH into the fetus does not stimulate adrenal expression of CYP21A1 mRNA (Tangalakis et al., 1990) or CYP11B1 mRNA levels. ACTH stimulation of the fetal adrenal, from 130d gestation until term is increased and results in an increase the fetal adrenal secretion of cortisol (Wintour et al., 1975). In summary, it is clear that ACTH regulates cortisol synthesis and secretion predominantly through its actions on CYP11A1 and CYP17 enzymes. Whilst this does not rule out an increase in activity of the other steroidogenic enzymes, most of the evidence supports that CYP11A1 and CYP17 are the key enzymes which are decreased during the period of relative adrenal quiescence, and increased during the period of reactivation in late gestation.

Exposure of the fetus to cortisol may also depend on the cortisol metabolising enzyme, 11βHSD. There are two isoforms, 11βHSD1 interconverts cortisol to cortisone, but *in vivo* predominantly converts the inactive cortisone to the active cortisol and 11βHSD2, which is a unidirectional enzyme that catalyses cortisol to cortisone (Agarwal et al., 1994; Langlois et al., 1995). In the fetal sheep adrenal, there is a significant decrease in relative expression of 11βHSD2 during late gestation (McMillen et al., 2000). In addition, cortisol infusion at 109-116d gestation (a stage of gestation when endogenous cortisol concentrations are low) results in a

premature and significant decrease in the relative expression of 11βHSD2 in the fetal sheep adrenal (Ross et al., 2000). These data suggest that cortisol decreases the expression of adrenal 11βHSD2 mRNA during late gestation. This may further increase intracellular adrenal concentrations of cortisol by decreasing the intracellular metabolism of cortisol resulting in adrenal cortical cells being exposed to very high cortisol concentrations during late gestation. The role of intra adrenal cortisol in the prepartum increase in fetal adrenal growth associated with differentiated function remains to be established.

1.4.2 Negative feedback between the fetal adrenal and the hypothalamic-pituitary axis

It is intriguing that although a complex and sensitive negative feedback system exists between fetal cortisol and ACTH concentrations, during late gestation there is a concomitant increase in fetal ACTH and cortisol concentrations. There is some controversy as to the mechanisms by which both fetal ACTH and cortisol rise in parallel during late gestation. POMC synthesis and secretion of ACTH is higher in bilateral adrenalectomised fetal sheep when compared to intact controls (Wintour et al., 1980; McMillen et al., 1990), suggesting that plasma cortisol normally acts to suppress the synthesis and secretion of ACTH from the pituitary.

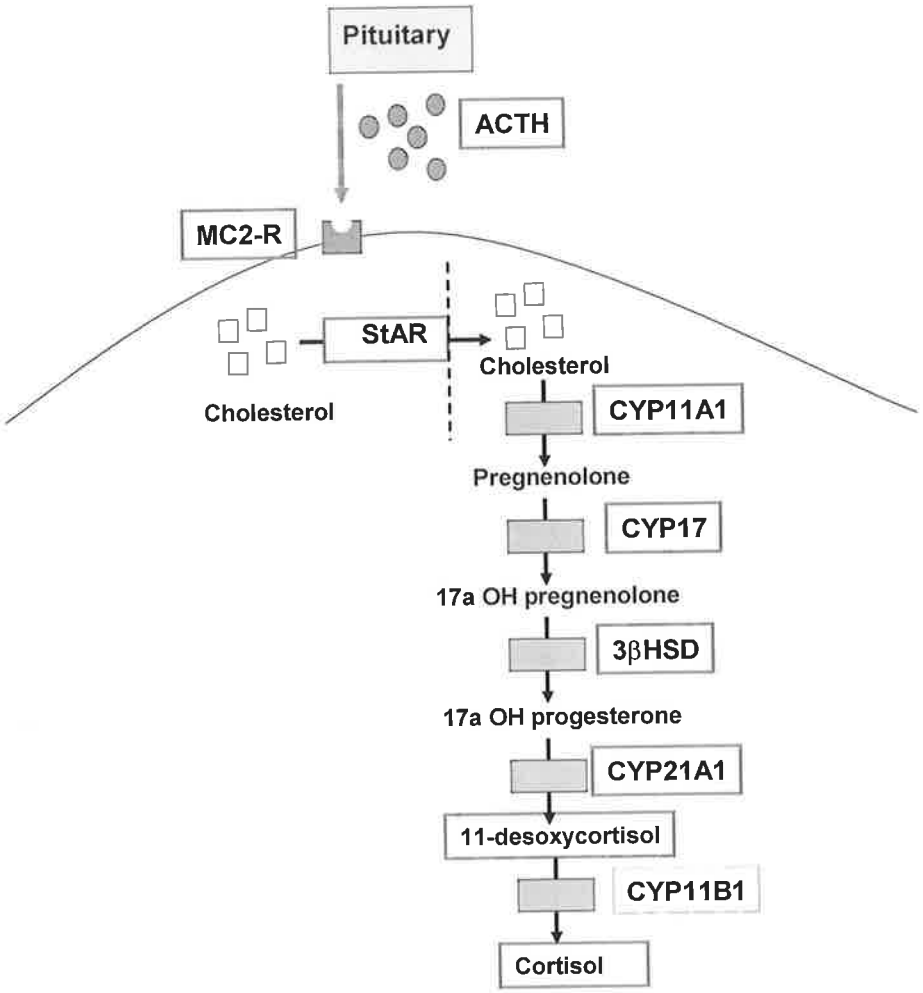

Figure 1.1: A schematic picture of the adrenal cortisol synthetic pathway after stimulation by plasma ACTH.

The hypothalamic secretagogue, CRH is also known to play a role in the negative feedback mechanism in the fetal sheep. Injection of CRH significantly increases fetal ACTH concentrations and this increase is abolished by the concomitant injection of cortisol demonstrating that fetal ACTH secretion is suppressed by fetal cortisol during late gestation (Rose et al., 1985). In another study, basal ACTH concentrations were suppressed by a 4h infusion of cortisol after, but not before, 138d gestation (Ozolins et al., 1990) suggesting there is a developmental maturation of the negative feedback of cortisol on ACTH secretion. In addition, cortisol infusion before and after 138d also abolished the CRH and AVP stimulated increase in ACTH concentrations (Ozolins et al., 1990). Interestingly this study demonstrated that surgical disconnection of the neural/vasculature of the pituitary from the hypothalamus abolished the suppressive effect of cortisol on basal ACTH secretion during late gestation, implying that an intact HPA axis is required for the negative feedback actions of cortisol during late gestation. Cortisol has also been shown to decrease the secretion of ACTH in response to stimulation by CRH in pituitary cells, *in vitro* (Matthews & Challis, 1997).

Pre-exposure of the fetal sheep to exogenous cortisol significantly reduces the fetal ACTH response to an additional acute stress (Wood & Rudolph, 1983; Wood, 1987). Cortisol infusion at 121-131d gestation significantly inhibited the fetal ACTH response to an infusion of the vasodilator, nitroprusside (Wood & Rudolph, 1983). When the same experiment was performed in older fetuses (138-142d gestation), however, there were similar ACTH responses to nitroprusside in both cortisol and vehicle infused fetuses (Wood, 1987), suggesting that cortisol during late gestation

does not inhibit ACTH secretion, perhaps due to a decrease in the negative feedback response.

Other studies have suggested that factors such as corticosteroid binding globulin (CBG) or the enzyme 11βHSD act to modulate the effects of cortisol and hence may contribute to the mechanisms leading to an increase in ACTH and cortisol during late gestation. Around 90% of the total amount of circulating cortisol is bound to the carrier protein, CBG. The binding capacity of CBG progressively increases from 75d gestation and there is also a prepartum increase in CBG concentrations (Ballard et al., 1982). CBG is predominantly synthesised in the fetal liver and the hepatic CBG mRNA levels are highest at 140d gestation in the sheep fetus (Berdusco et al., 1995). Pulsatile injections of ACTH into fetal sheep significantly increased plasma corticosteroid binding capacity although did not alter the expression of hepatic CBG mRNA at 126-130d gestation (Jacobs et al., 1991). In addition, cortisol infusion at 124-129d significantly increased fetal plasma ACTH concentrations with an increase in hepatic CBG mRNA (Jeffray et al., 1998). Taken together, one possible mechanism by which there is a concomitant increase in both fetal ACTH and cortisol during late gestation, is through an increase in the corticosteroid binding capacity of CBG.

The level of 11βHSD activity may also be important in the concomitant rise in both ACTH and cortisol during late gestation. In the fetal pituitary the dehydrogenase activity of 11βHSD1 is higher in term fetuses than in younger animals (Yang et al., 1995). A higher activity of 11βHSD1 may result in an increased conversion of cortisol to cortisone which may lead inturn to a decrease in bioactive cortisol within

the pituitary cell and this may contribute to the concomitant increase in ACTH and cortisol during late gestation.

The hypothesis tested in this thesis was that suppression of cortisol biosynthesis from 125d gestation would increase the stimulation from the fetal pituitary resulting in increased plasma ACTH concentrations.

The aim of Chapter 4 was to determine the effect of suppression of cortisol biosynthesis on the pituitary adrenal axis, specifically pituitary ACTH synthesis and secretion and the capacity of the adrenal to secrete cortisol.

From the literature reviewed the role of endogenous cortisol during late gestation on the development of the fetal cardiovascular system is unclear. Whilst it has been demonstrated that synthetic glucocorticoids and exogenous cortisol act to increase fetal arterial blood pressure and mediate the actions of the fetal renin-angiotensin system, the role of endogenous cortisol in the regulation of arterial blood pressure is unclear. The aims of Chapter 2 & 3 therefore, were to investigate the effect of metyrapone, a competitive inhibitor of CYP11B1, the last enzyme in the cortisol synthetic pathway, on fetal arterial blood pressure, at two ages, 125d when fetal cortisol concentrations are normally low, and at 137d, when fetal cortisol concentrations are increasing.

From the literature reviewed it is also apparent that the relationship between fetal plasma ACTH and cortisol concentrations during late gestation is not fully understood. In Chapter 4, therefore we aimed to investigate this relationship

between fetal pituitary ACTH secretion and adrenal cortisol secretion during late gestation by determining the effect of metyrapone infusion from 125d on the capacity of the adrenal to synthesise cortisol.

1.5 AIMS

The aims of this thesis are:

Chapter 2 Aims

2.1 To determine the effect of metyrapone infusion at 125-128d gestation, when fetal cortisol concentrations are low, on:

- **fetal plasma cortisol, 11-desoxycortisol and ACTH concentrations at 126-128d gestation.**
- **fetal arterial blood pressure and heart rate at 126-128d gestation.**

Experimental Strategy

To investigate the role of endogenous cortisol on fetal arterial blood pressure, cortisol synthesis was suppressed by the infusion of metyrapone. Metyrapone is a competitive inhibitor of CYP11B1, the last enzyme in the cortisol synthetic pathway. Metyrapone was infused into fetal sheep from 125d gestation. Plasma samples were collected daily throughout the experiment to determine plasma ACTH, 11-desoxycortisol and cortisol concentrations. Fetal arterial blood pressure and heart rate were measured at 24-48h after the start of the infusion, at 126-127d gestation.

Chapter 3 Aims

3.1 To determine the effect of metyrapone infusion between 137-140d gestation on:

- **fetal plasma ACTH, 11-desoxycortisol and cortisol concentrations.**

- **fetal arterial blood pressure during the first 6-24h after the start of the infusion**
- **fetal arterial blood pressure responses to increasing doses of Ang II at 138-139d gestation**

3.2 To determine the effect of metyrapone infusion between 125 and 140d gestation on:

- **fetal arterial blood pressure at 137-139d gestation.**
- **fetal arterial blood pressure responses to increasing doses of Ang II and noradrenaline at 137-139d gestation**

Experimental Strategy

Metyrapone was infused from 137-140d gestation to suppress fetal cortisol concentrations. Fetal arterial blood pressure is measured 3h prior to the start of the infusion and the first 6h of the infusion. The fetal arterial blood pressure responses to increasing doses of Ang II were measured 24-48h after the start of the metyrapone infusion. Metyrapone was infused into fetal sheep between 125d and 140d gestation. Fetal arterial blood pressure was measured at 137-139d gestation. In addition the fetal arterial blood pressure response to increasing doses of Ang II and noradrenaline was measured at 137-139d gestation.

Chapter 4 Aims

4.1 To determine the effect of metyrapone infusion from 125-140d gestation on:

- **fetal plasma ACTH, 11-desoxycortisol and cortisol concentrations.**
- **the relative expression of pituitary POMC mRNA.**

- **the relative expression of the MC2-R mRNA and StAR mRNA**
- **the relative expression of CYP11A1 mRNA, CYP17 mRNA, 3βHSD mRNA, CYP21A1 mRNA and 11βHSD2 mRNA in the fetal adrenal gland.**
- **The growth of the fetal sheep adrenal at 140d gestation**

Experimental Strategy

Metyrapone was infused from 125d until 140d gestation. Daily plasma samples were taken to determine plasma concentrations of ACTH, cortisol and 11-desoxycortisol. The relative expression of POMC mRNA in the pituitary and MC2-R mRNA, StAR mRNA, CYP11A1 mRNA, CYP17 mRNA, 3βHSD mRNA, CYP21A1 mRNA and 11βHSD2 mRNA in the adrenal were measured by Northern blot analysis. The cell density of the zona glomerulosa, zona fasciculata and medulla of the adrenal were measured and the thickness of each zone of the adrenal was measured using morphometric techniques.

2 Effect of Infusion of Metyrapone on Fetal Arterial Blood Pressure at 126-127d Gestation

2.1 INTRODUCTION

A range of studies have demonstrated that administration of either synthetic or native glucocorticoids in the sheep fetus during late gestation results in an increase in fetal arterial blood pressure (Tangalakis et al., 1992; Derks et al., 1997; Forhead et al., 2000; Fletcher et al., 2002), an increase in the vascular resistance of the fetal femoral arterial circulation (Derks et al., 1997; Fletcher et al., 2002) and in changes in the responsiveness of isolated fetal femoral arteries to excess potassium, acetylcholine and bradykinin (Anwar et al., 1999). Fetal cortisol infusion also results in an increase in the vascular sensitivity to exogenous Ang II (Tangalakis et al., 1992) and a greater hypotensive response to administration of an AT1R specific antagonist (Forhead et al., 2000).

A 50% reduction in maternal food intake between 115-125d gestation increased maternal plasma cortisol concentrations within the first 10d after the onset of undernutrition with a concomitant increase in fetal arterial blood pressure and in the fetal arterial blood pressure response to increasing doses of Ang II (Edwards & McMillen, 2001). In this study, these authors also found there was a negative correlation between plasma ACTH concentrations and arterial blood pressure in the fetuses of undernourished ewes. They therefore, reasoned that during the first 10d of maternal undernutrition there was transplacental transfer of maternal cortisol resulting in an increase in fetal cortisol concentrations which both suppressed fetal

ACTH secretion and increased fetal arterial blood pressure (Edwards & McMillen, 2001).

Whilst there is evidence that exposure of the fetus to either increases in exogenous or maternally derived glucocorticoids increase fetal arterial blood pressure, the role of endogenous fetal glucocorticoids in the regulation of fetal arterial blood pressure, is less well understood. In the sheep, the prepartum increase in fetal plasma cortisol concentrations occurs in parallel with an increase in fetal arterial blood pressure (Boddy et al., 1974; Tangalakis et al., 1992; Daniel et al., 1996; Fowden et al., 1998; Unno et al., 1999). Unno and coworkers compared the arterial blood pressure between 120 and 126d gestation in bilaterally adrenalectomised and intact fetal sheep. These authors showed that a small increase (~3 mmHg) in fetal arterial blood pressure occurred between 120 and 126d gestation in the intact fetuses, which did not occur in the adrenalectomised group. It is unclear from this study, whether endogenous cortisol concentrations act to maintain fetal arterial blood pressure because in both the intact control and adrenalectomised fetuses, endogenous cortisol concentrations were below the limit of detection of the cortisol radioimmunoassay (Unno et al., 1999). One further limitation of this study is that fetal adrenalectomy results in the removal of both steroidogenic cells of the adrenal cortex and the catecholamine containing cells of the adrenal medulla. It is therefore difficult to conclude from these studies whether changes in fetal blood pressure in adrenalectomised fetuses are as a consequence of the removal of cortisol alone, or of the combined removal of cortisol and adrenaline, specifically as intrafetal infusion of adrenaline increases arterial blood pressure.

The aim of the present study, therefore, was to compare the effects of suppression of endogenous cortisol biosynthesis on fetal arterial blood pressure, at a stage in late gestation, when basal cortisol concentrations are low ie. before the start of the prepartum increase in fetal cortisol. We have used metyrapone, a competitive inhibitor of the steroidogenic enzyme CYP11B1, to inhibit cortisol synthesis in the sheep fetus (Lye & Challis, 1984). CYP11B1 catalyses the formation of cortisol from 11-desoxycortisol and is the last enzyme in the biosynthetic pathway for cortisol. We have infused metyrapone into fetal sheep starting at 125d gestation and measured the fetal plasma cortisol, 11-desoxycortisol, and ACTH concentrations and fetal systolic, diastolic and mean arterial blood pressure and heart rate for 48-72h after the start of the infusion.

2.2 MATERIALS AND METHODS

2.2.1 Animals and Surgery

All experiments were carried out according to the guidelines of the Standing Committee of Ethics and Animal Experimentation, The University of Adelaide Animal Ethics Committee. Twenty one pregnant Merino ewes were used in this study. Ewes were housed individually in crates and kept in a 12h light – dark cycle. Animals were fed once daily and were given water ad libitum throughout the entire length of the protocol.

Ewes were fasted 24h prior to surgery. Antibiotics (2ml i.m; Ilium Penstrep, 250mg.ml^{-1} procaine penicillin, 250mg.ml^{-1} dihydrosteptomycin sulphate, 20mg.ml^{-1} procaine hydrochloride; Troy Laboratories, Smithfield, NSW, Australia) were administered to each ewe prior to surgery. Surgery was performed under general

anaesthesia and aseptic conditions at 119-120d gestation. General anaesthesia was induced using an intravenous injection of sodium thiopentone (0.5ml.kg^{-1} body weight; Pentothal, Rhone Merieux, Pinkenba, Qld, Australia) and maintained throughout surgery with 3-4% halothane (Flurothane, ICI, Melbourne, Vic, Australia) in oxygen (Linde Gas, Pinkeba, Qld, Australia). An incision was made along the abdominal midline, exposing the abdominal cavity of the ewe. The fetal head was located and then the uterus and membranes were opened to expose the fetal head and neck. An incision was made in the fetal neck and single lumen polyvinyl catheters (outer diameter, 1.52mm; inner diameter, 0.86mm; Critchley Electrical Products, Silverwater, NSW, Australia) were inserted into a fetal carotid artery and into a fetal jugular vein. Vascular catheters were secured to the fetal skin at the neck. A catheter was also inserted into the amniotic cavity (outer diameter, 2.7mm; inner diameter, 1.5mm) and was secured to the ear of the fetus. Antibiotics (2ml i.m; Ilium Penstrep, 250mg.ml^{-1} procaine penicillin, 250mg.ml^{-1} dihydrosteptomycin sulphate, 20mg.ml^{-1} procaine hydrochloride; Troy Laboratories, Smithfield, NSW, Australia) were administered to the fetus during surgery. The fetal head was then replaced into the uterus and the uterus and membranes were closed (Vicryl 1, Ethicon, Johnson and Johnson, Sydney, NSW, Australia). Catheters were exteriorised through a small incision on the ewe's flank, the midline incision was then closed (Vicryl 2, Ethicon, Johnson and Johnson, Sydney, NSW, Australia) and the abdominal fat layer was also closed (Vicryl 2, Ethicon, Johnson and Johnson, Sydney, NSW, Australia). Finally the skin of the ewe was closed (Vetafil, 0.3mm; WDT, Garbsen, Germany). Ewes were allowed to recover from surgery for at least 5d before the start of the experimental protocol.

2.2.2 Experimental Protocol

Effect of metyrapone infusion from 125d on fetal arterial blood gas status and plasma hormone concentrations

At 125d gestation metyrapone (65 μmoles.h^{-1}. (kg body weight)$^{-1}$) in 0.6M tartaric acid; n=10; Aldright Chemical Co, Milwaukee, WI, USA) or vehicle (tartaric acid 0.6M.h^{-1}; n=11; BDH Laboratory Supplies, Poole, Dorset, UK) was infused (0.4ml.h^{-1}; Graseby Medical syringe driver M5-10A, Selby Scientific & Medical, Australia) into the fetal jugular vein. Tartaric acid was used to aid the solubility of metyrapone (Lye & Challis, 1984). Fetal weight at 125d gestation (3.08 kg) was estimated using fetal growth curves previously published from our laboratory (Fetal weight=0.0008 x gestational age^2 – 0.1046 x gestational age + 3.6508) (Edwards et al., 1999). The infusion was maintained continuously for 15d until post mortem at 140d gestation. On the first day of the infusion fetal arterial blood (2.5ml) was collected at 1000h (-60 min) and 1100h (0 min) prior to start of infusion. Throughout the infusion period, fetal arterial blood samples (2.5ml) were collected daily, between 0900h and 1100h, for blood gas and plasma hormone analysis. For ACTH determination, blood (1.0ml) was aliquoted into ethylenediamine tetra-acetic acid coated tubes (EDTA, Sarstedt Australia, Inglefarm, SA, Australia), containing aprotinin (10μl, 100,000 KIU ml $^{-1}$, Sigma Chemical Co, St Louis, MO, USA). For cortisol and 11-desoxycortisol determination, blood (1.0ml) was aliquoted into heparin coated tubes (125 IU, Sarstedt Australia, Inglefarm, SA, Australia). Blood samples were centrifuged at 1500 g for 10 min, the plasma was separated and frozen at -20°C until analysis. Blood (0.5ml) was analysed for fetal arterial PO_2, PCO_2, pH, oxygen saturation (sO_2) and haemoglobin content (Hb) using an ABL 520 blood gas analyser (Radiometer, Copenhagen, Denmark).

2.2.3 Blood Pressure Measurements

Effect of metyrapone infusion from 125d on fetal arterial blood pressure at 126-127d gestation

Fetal arterial blood pressure and intraamniotic pressures were measured continuously between 1000 and 1500h, at 126d (n=12) or 127d (n=7) gestation, i.e. between 24 and 48h after the start of the infusion (see Table 2.1). The fetal carotid artery and amniotic catheters were attached directly to displacement pressure transducers and a quad bridge amplifier (ADInstruments, Castle Hill, NSW, Australia). Pressures were recorded using Maclab Chart software on a Power Macintosh computer as described previously (Edwards et al., 1999). Fetal systolic and diastolic blood pressure were calculated after subtraction of the intra-amniotic pressure. A mean value for systolic, diastolic blood pressure and heart rate for 5s was calculated around the first time point of the blood pressure recording and subsequently at 30 min intervals after the first time point in the 5h period. Fetal mean arterial blood pressure was calculated from the formula (diastolic pressure + 0.4 (systolic - diastolic pressure)). The rate pressure product (RPP) was calculated from the formula RPP= systolic pressure x heart rate (Hawkins et al., 2000b).

2.2.4 Radioimmunoassays

ACTH

Fetal plasma immunoreactive ACTH concentrations were measured using a radioimmunoassay kit from ICN Biomedicals (ICN Biomedicals, NSW, Australia) which has been validated previously for use in fetal sheep plasma (McMillen et al., 1990). According to the manufacturer, the cross reactivity of the rabbit anti-human ACTH 1-39 was <1% with β-endorphin, α-melanocyte-stimulating hormone, α-

lipotrophin, and β-lipotrophin. The sensitivity of the assay was 9pg.ml^{-1}. The intra-assay coefficient of variation was <5% and the inter-assay coefficient of variation was <15%.

Table 2.1: Details of the numbers of animals included in each experimental group

	Tartaric Acid infused from 125d gestation	Metyrapone infused from 125d gestation
Surgery	11	10
Singleton (S) / Twin (T) pregnancies	S(9) T(2)	S(10)
Gender	M(3) F(7) unknown (1)	M (4) F(4) unknown (2)
Measurement of arterial blood pressure at 126-127d	9	10
Measurement of fetal plasma ACTH at 125-128d	7	8
Measurement of fetal plasma 11desoxycortisol at 125-128d	7	9
Measurement of fetal plasma cortisol at 125-128d	7	9
Measurement of fetal plasma glucose	8	10

Cortisol

Fetal plasma cortisol concentrations were measured using a radioimmunoassay, validated for use in fetal sheep plasma. Fetal plasma samples from metyrapone and vehicle infused fetuses were extracted and assayed in duplicate. Briefly, cortisol was extracted from the plasma using dichloromethane as described previously (Bocking et al., 1986). The efficiency of the recovery was >85%. Samples were then reconstituted in assay buffer (Tris hydrochloride; bovine serum albumin; sodium azide). Standards were serially diluted in assay buffer, from a stock (100nmol.L^{-1}) solution (range 0.78-100nmol.L^{-1}). Anti-cortisol (100µl; 1:15 dilution; Orion Diagnostica, Turku, Finland) was added followed by ^{125}I-cortisol (100µl ; Amersham, Pharmacia, Biotech, UK). Tubes were vortexed and incubated at 37ºC for 1h before

the addition of goat anti-rabbit serum (initial dilution 1:30; 100µl) and polyethylene glycol (1ml; 20%; BDH Laboratory Supplies, Poole, UK). Tubes were vortexed before centrifugation at 3700g at 4°C for 30 min. The supernatant was aspirated and the precipitate counted on a gamma counter (Packard, Illinois, USA).

Validation of Radioimmunoassay

Anti-cortisol (100µl; 1:15 dilution; Orion Diagnostica, Turku, Finland) was incubated with labelled ^{125}I- cortisol (Amersham, Pharmacia, Biotech, UK). Hydrocortisone (Sigma Chemical Co, St Louis, Missouri, USA) was used as the standard at dilutions of 0.078 – 10pmol.100µl^{-1}. Extracted cortisol from increasing volumes of fetal sheep plasma was diluted in parallel to the standard curve and the cortisol was quantitatively recovered (114.8 ± 7.6%). The relationship between expected concentration of cortisol (x) and the observed concentration of cortisol (y) in increasing volumes of fetal sheep plasma was described by the equation y=1.5x – 0.86 (r=0.983, P<0.003; Figure 2.1).

The sensitivity of the assay was 0.2nmol.L^{-1}, according to the manufacturer, the cross-reactivity of the rabbit anti-cortisol antisera was < 1% with pregnenolone, aldosterone, progesterone and estradiol. The intraassay coefficient of variation was <5% and the interassay coefficient of variation was <20%.

Cross reactivity of anti-cortisol antisera with 11-desoxycortisol

The cross-reactivity of the anti-cortisol with 11-desoxycortisol was determined using a stock solution of 11-desoxycortisol (100µmol.L^{-1}; hydrocortisone, Sigma Chemical Co, St Louis, MO, USA) which was diluted serially in assay buffer (100µmol.L^{-1} – 1nmol.L^{-1}) and these standards were then assayed in the cortisol assay as described above. The cross reactivity of the anti cortisol with 11-desoxycortisol was 3.7% (Figure 2.2).

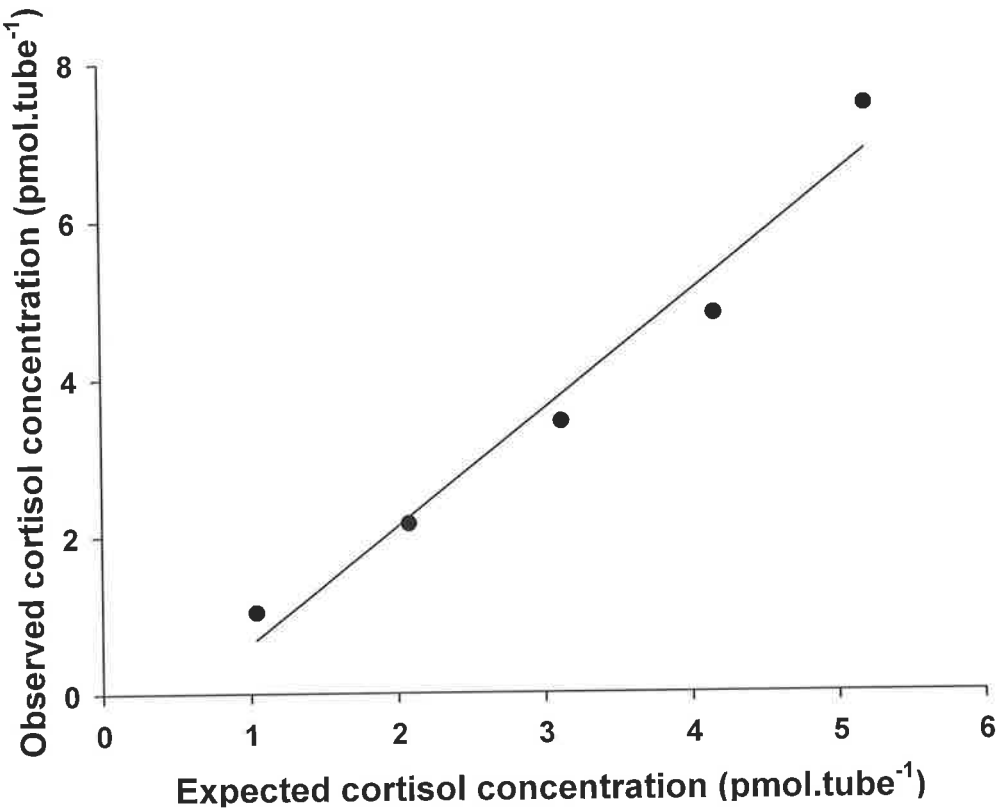

Figure 2.1: Relationship between observed and expected cortisol concentrations in increasing volumes of fetal sheep plasma.

The relationship between observed and expected concentrations of cortisol is described by the linear regression equation y=1.5x-0.86 (r=0.983, $P<0.003$) where x is the expected cortisol concentration and y is the observed cortisol concentration present in increasing volumes of a sample of fetal sheep plasma.

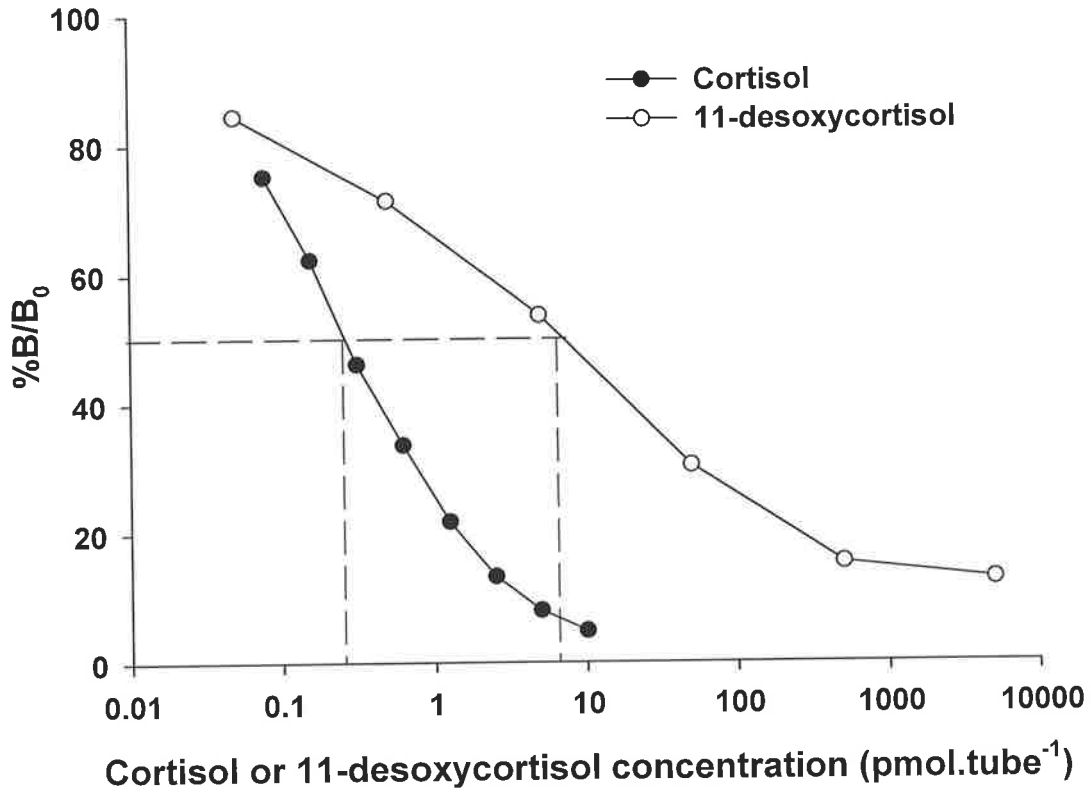

Figure 2.2: The cross reactivity of anti-cortisol with 11-desoxycortisol.

The dotted lines indicate the doses of cortisol (•) and 11-desoxycortisol (○) respectively, which displace I^{125}-cortisol from the anti-cortisol by 50% when compared to the binding of ^{125}I-cortisol and anti-cortisol in the absence of antigen.

11-desoxycortisol

Fetal plasma 11-desoxycortisol concentrations were measured using a radioimmunoassay kit (ICN Biomedicals, NSW, Australia) validated for use in extracted fetal sheep plasma as described below. Due to the higher concentration of 11-desoxycortisol present in the fetal plasma samples collected from metyrapone infused animals, these samples were diluted in assay buffer (1:10; 0.1M PBS; Sigma Chemical Co, St Louis, MO, USA; 0.1% BSA and 0.1% NaN_3; BDH Laboratories, Poole, Dorset, UK) prior to extraction, whereas samples from vehicle infused animals, did not require dilution prior to extraction. 11-desoxycortisol was extracted from standard solutions (10μl), and plasma samples (2-100μl) using dichloromethane method of steroid extraction (Bocking et al., 1986).

Extracted standards and samples were then reconstituted in buffer (10μl), and 100μl of buffer was then added (in place of steroid globulin binding inhibitor (SGBI)) and the remainder of the assay was then performed according to the manufacturers directions (ICN Biomedical, NSW, Australia). The rabbit anti-human 11-desoxycortisol was specified by the manufacturer to have a cross reactivity of <0.3% with progesterone and pregnenolone sulphate. The mean binding of the anti-11-desoxycortisol to ^{125}I-11-desoxycortisol in the absence of antigen was 67.4 ±1.1%. The sensitivity of the assay was 180pg.ml^{-1} (or 0.5nmol.L^{-1}). The intra-assay coefficient of variation was < 5% and the inter-assay coefficient of variation was < 15%.

Extraction Efficiency

The efficiency of recovery of 11-desoxycortisol from fetal sheep plasma using the dichloromethane method of steroid extraction (Bocking et al., 1986) was 96 ± 2%.

Validation of Radioimmunoassay

Extracted 11-desoxycortisol from increasing volumes of fetal sheep plasma resulted in a displacement binding curve which was parallel to the standard curve and 11-desoxycortisol was quantitatively recovered from increasing volumes of plasma (105.4 ± 5.6%). The relationship between expected concentration of 11-desoxycortisol (x) and the observed concentration of 11-desoxycortisol (y) in increasing volumes of fetal sheep plasma can be described by the equation y=1.2x – 2.6 (r=0.999, P<0.001; Figure 2.3).

Cross reactivity of anti- 11-desoxycortisol antisera with cortisol

The cross reactivity of the anti-11-desoxycortisol to cortisol was tested. A stock solution of cortisol (100 µM; hydrocortisone, Sigma Chemical Co, St Louis, MO, USA) was prepared in 100% ethanol (Chem-Supply Pty Ltd, Gillman, South Australia). From the 100µM stock solution, cortisol was serially diluted (100µM – 1nM) and assayed in the 11-desoxycortisol assay. The cross reactivity of anti 11-desoxycortisol with cortisol was 0.25% (Figure 2.4).

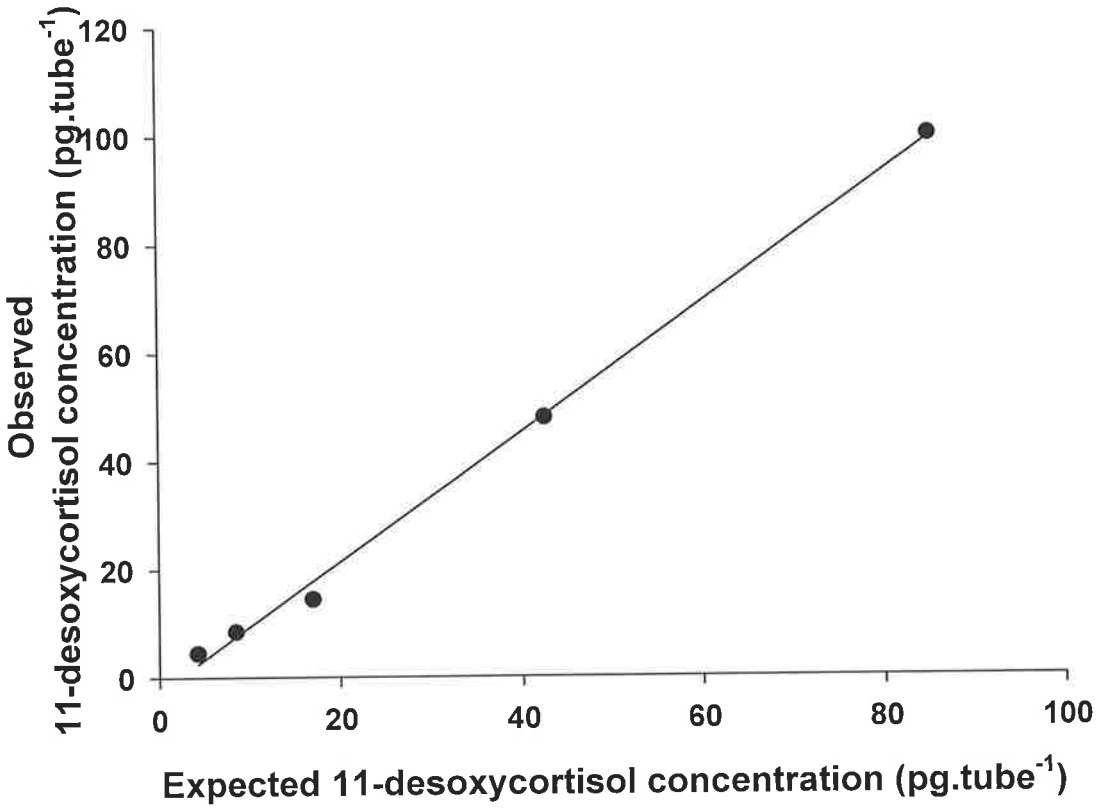

Figure 2.3: The relationship between the expected and observed concentrations of 11-desoxycortisol in increasing volumes of fetal sheep plasma.

The relationship between observed and expected concentrations of 11-desoxycortisol is described by the linear regression equation y=1.2x –2.6 (r= 0.999; P<0.001) where x is the expected 11-desoxycortisol concentration and y is the observed 11-desoxycortisol concentration present in increasing volumes of a sample of fetal sheep plasma.

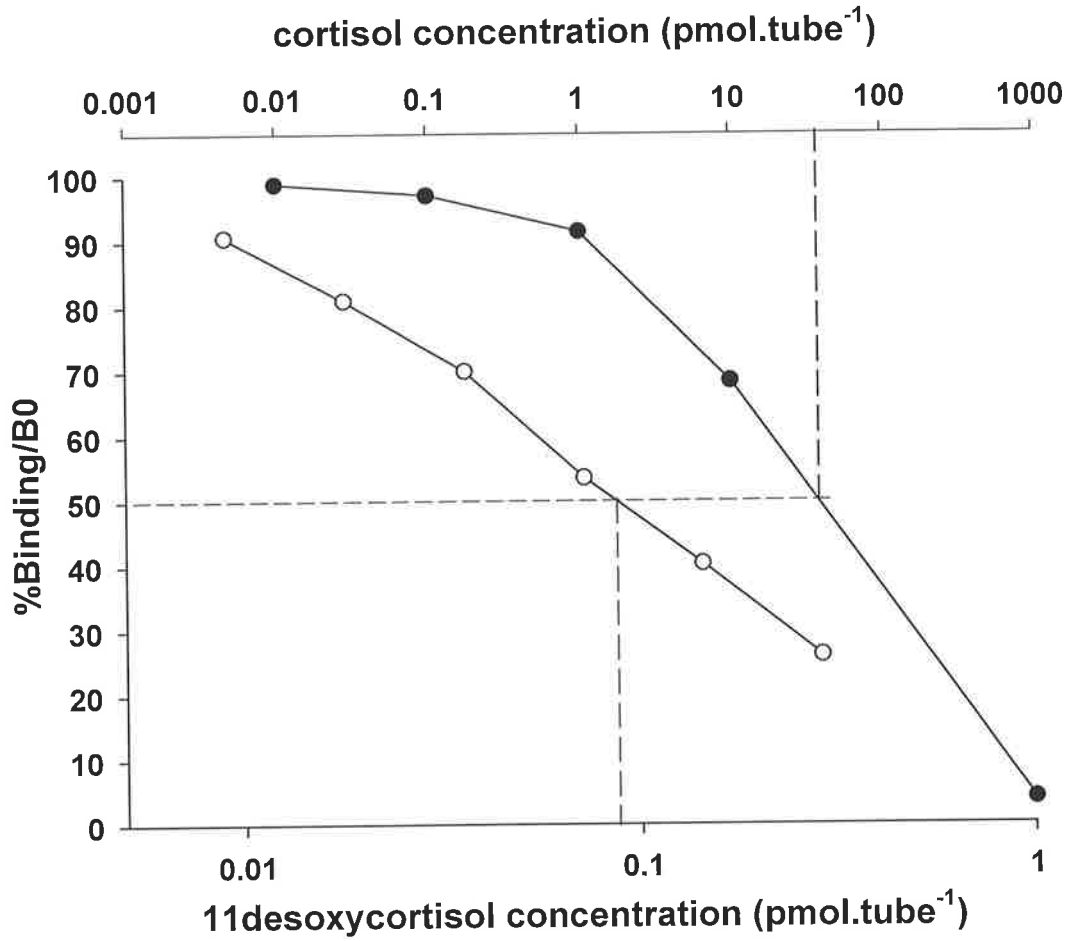

Figure 2.4: The cross reactivity of anti-11-desoxycortisol with increasing doses of cortisol.

The dotted lines indicate the doses of cortisol (•) and 11-desoxycortisol (○) respectively, which displace I^{125}-cortisol from the anti-11-desoxycortisol by 50% of the initial binding (B_0).

2.2.5 Statistical Analysis

Effect of metyrapone infusion from 125d on fetal blood gas status and plasma hormone concentrations at 125-128d gestation.

All data are expressed as mean ± standard error (SEM). In this chapter, the hormonal and blood pressure data from the fetal sheep during the first 72h of metyrapone infusion at 125-128d gestation are reported. The mean values of fetal arterial PO_2, PCO_2, pH, sO_2 and Hb were determined for each fetus from surgery until 72h after the start of the infusion. Mean blood gas and pH values for the fetuses in the metyrapone and vehicle infused groups were compared using a Student's unpaired t-test.

For all plasma samples, the amount of cortisol and 11-desoxycortisol in each sample was then calculated using the following equations.

$x + 0.037\ (y) = a$ $\quad\quad$ $y + 0.0025\ (x) = b$

Where x is the "actual" amount of cortisol, y is the "actual" amount of 11-desoxycortisol, a is the measured value of cortisol in a sample using the cortisol assay and b is the measured value of 11-desoxycortisol in a sample using the 11-desoxycortisol assay.

Prior to infusion, fetal plasma ACTH, 11-desoxycortisol and cortisol concentrations were averaged to derive a mean hormonal value for each metyrapone and vehicle infused fetus during the baseline period. For the first three days of the infusion period, the changes in fetal plasma ACTH, 11-desoxycortisol and cortisol were calculated by subtracting the mean baseline value prior to infusion from the plasma hormone concentration on each day of infusion.

Blood pressure and hormone data were log transformed where required to reduce the heterogeneity of the data prior to analysis. Fetal arterial blood pressure and the change in plasma ACTH, 11-desoxycortisol and cortisol concentrations after the start of the metyrapone or vehicle infusion were compared using a multifactorial Analysis of Variance (ANOVA) with repeated measures using the Statistical Package for Social Sciences (SPSSX, Chicago, IL, USA) on a Vax mainframe computer system. The factors included in the ANOVA were group (metyrapone vs vehicle infused) and either the day of infusion for the hormonal analyses (ie. 126d, 127d and 128d) or time points during the blood pressure recording (ie. every 30 min between 1000h -1500h). Where the ANOVA identified significant differences between groups, a Duncan's multiple range post-hoc test was used to identify differences between mean values. A probability of <5% (P<0.05) was taken to be significant.

2.3 RESULTS

2.3.1 Effect of metyrapone infusion from 125d on fetal blood gas status at 125-128d gestation

There was no difference in the mean fetal arterial PO_2 (mmHg), PCO_2 (mmHg), pH, sO_2 (%) and Hb ($g.dL^{-1}$) (calculated from the day of surgery until 128d), between the metyrapone and vehicle infused groups (Table 2.2).

2.3.2 Effect of metyrapone infusion at 125d on fetal plasma ACTH concentrations between 125-128d gestation

Before the start of the infusion period, there was no significant difference in plasma ACTH concentrations between the metyrapone (92 ± 21.2 $pg.ml^{-1}$) and vehicle (67 ± 9.3 $pg.ml^{-1}$) infused fetuses. At 24-72h after the start of the infusion period, fetal plasma ACTH concentrations were significantly higher ($F = 7.07$; $P<0.05$) in metyrapone infused fetuses when compared with vehicle infused controls (change from baseline at 72h, 44.6 ± 14.2 and 14.6 ± 5.6 $pg.ml^{-1}$ respectively; Figure 2.5A).

Table 2.2: Mean fetal arterial blood gas, pH, sO_2 and Hb values in metyrapone and vehicle infused fetuses between 120 and 128d gestation.

	Vehicle Infused (n=11)	Metyrapone Infused (n=10)
PO_2 (mmHg)	22.4 ± 0.6	22.8 ± 0.4
PCO_2 (mmHg)	46.9 ± 0.7	45.8 ± 0.6
pH	7.403 ± 0.003	7.397 ± 0.003
sO_2 (%)	70.2 ± 1.7	72.0 ± 1.0
Hb ($g.dL^{-1}$)	9.9 ± 0.1	10.4 ± 0.1

2.3.3 Effect of metyrapone infusion from 125d on fetal plasma 11-desoxycortisol concentrations at 126-128d gestation

Before the start of the infusion period there was no difference in the plasma concentrations of 11-desoxycortisol between the metyrapone (0.9 ± 0.07 $nmol.L^{-1}$) and vehicle (0.8 ± 0.06 $nmol.L^{-1}$) infused groups. At 24h after the start of the infusion, plasma 11-desoxycortisol concentrations were significantly higher (5.0 ± 0.9 $nmol.L^{-1}$) in the metyrapone infused fetuses and remained high until 128d gestation

(F= 9.58, P<0.01), when compared with vehicle infused controls. In contrast, there was no change in plasma 11-desoxycortisol concentrations (+24h infusion; 0.9 ± 0.05 nmol.L^{-1}) in the vehicle infused fetuses throughout the infusion period (Figure 2.5B).

2.3.4 Effect of metyrapone infusion from 125d on fetal plasma cortisol concentrations at 126-128d gestation

Before the start of the infusion, there was no significant difference in plasma cortisol concentrations between the fetuses in metyrapone (5.3 ± 1.7 nmol.L^{-1}) and vehicle infused (2.6 ± 0.4 nmol.L^{-1}) groups. Whilst plasma cortisol concentrations tended to fall (-2.7 ± 1.7nmol.L^{-1}) in the metyrapone infused group compared to the vehicle infused fetuses (0.3 ± 0.4nmol.L^{-1}), there were no significant differences between the metyrapone and vehicle infused fetuses in the change in plasma cortisol concentrations during the first 24h of the infusion period (Figure 2.5C). There was also no difference in plasma cortisol concentrations in the fetuses of the metyrapone infused group at 72h after the start of the infusion period (72h; 7.3 ± 6.8 nmol.L^{-1}) when compared with vehicle infused fetuses (72h; 1.3 ± 1.3 nmol.L^{-1}; Figure 2.5C). There was no difference in the mean plasma cortisol concentrations between the metyrapone and vehicle infused fetuses between 125d and 128d gestation.

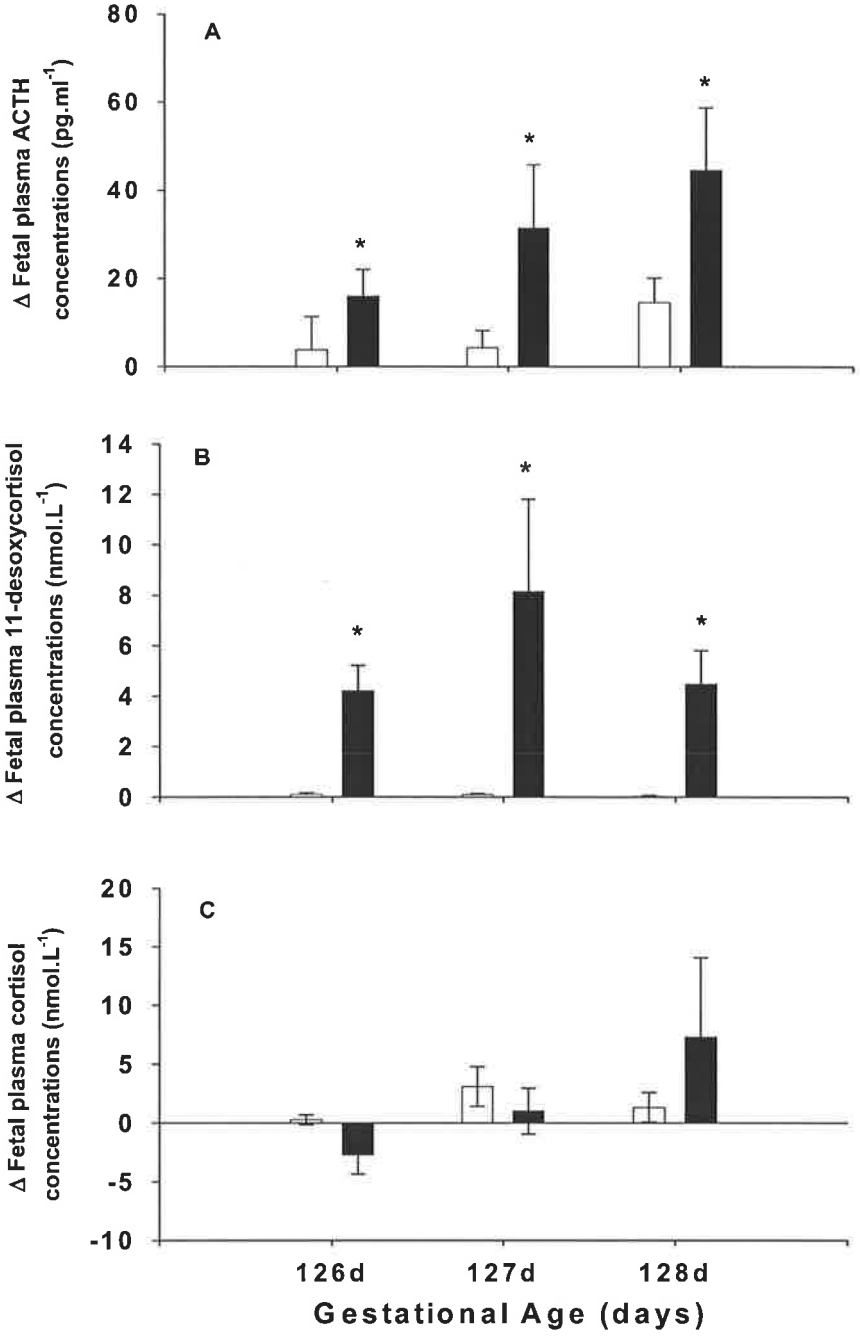

Figure 2.5: Changes in fetal plasma ACTH, 11-desoxycortsiol and cortisol concentrations at 126-128d after the start of infusion of metyrapone or vehicle.

There was a significant increase in both plasma ACTH (A) and 11-desoxycortisol (B) from preinfusion levels in metyrapone (dark bar) infused fetuses and when compared with vehicle (clear bar) infused controls. *indicates hormone values which are significantly higher in the metyrapone than in the vehicle infused group ($P<0.05$).

2.3.5 Effect of metyrapone infusion from 125d on fetal arterial blood pressure and heart rate at 126-127d gestation

Fetal systolic blood pressure was significantly lower (F=6.69; $P<0.02$) in the metyrapone infused group when compared to vehicle infused fetuses throughout the 5h recording period. There was also a significant effect of time of day (F= 2.18; $P<0.05$) on fetal systolic blood pressure, which was present in both the metyrapone and vehicle infused groups i.e. fetal systolic blood pressure was lowest at 1030h and highest at 1200h in both groups (Figure 2.6A).

Fetal diastolic blood pressure was also significantly lower (F= 4.73; $P<0.05$) in metyrapone infused fetuses, when compared to vehicle infused controls. There was no effect of time of day on fetal diastolic blood pressure, however in either metyrapone or vehicle infused groups (Figure 2.6B).

Fetal mean arterial blood pressure was significantly lower (F=6.21; $P<0.05$) in the metyrapone infused group than in the vehicle infused controls. There was also a significant effect of time of day (F=1.95; $P<0.05$) on mean arterial blood pressure in both metyrapone and vehicle infused groups. In both the metyrapone and vehicle infused fetuses, mean arterial blood pressure was lowest at 1030h and highest at 1200h in both groups (Figure 2.6C).

There was no effect of metyrapone infusion on fetal heart rate at 126-127d (Figure 2.7A). The RPP, used as a marker of cardiac work, was significantly lower (F= 6.89; $P<0.05$) in metyrapone infused fetuses than in vehicle infused controls (Figure 2.7B).

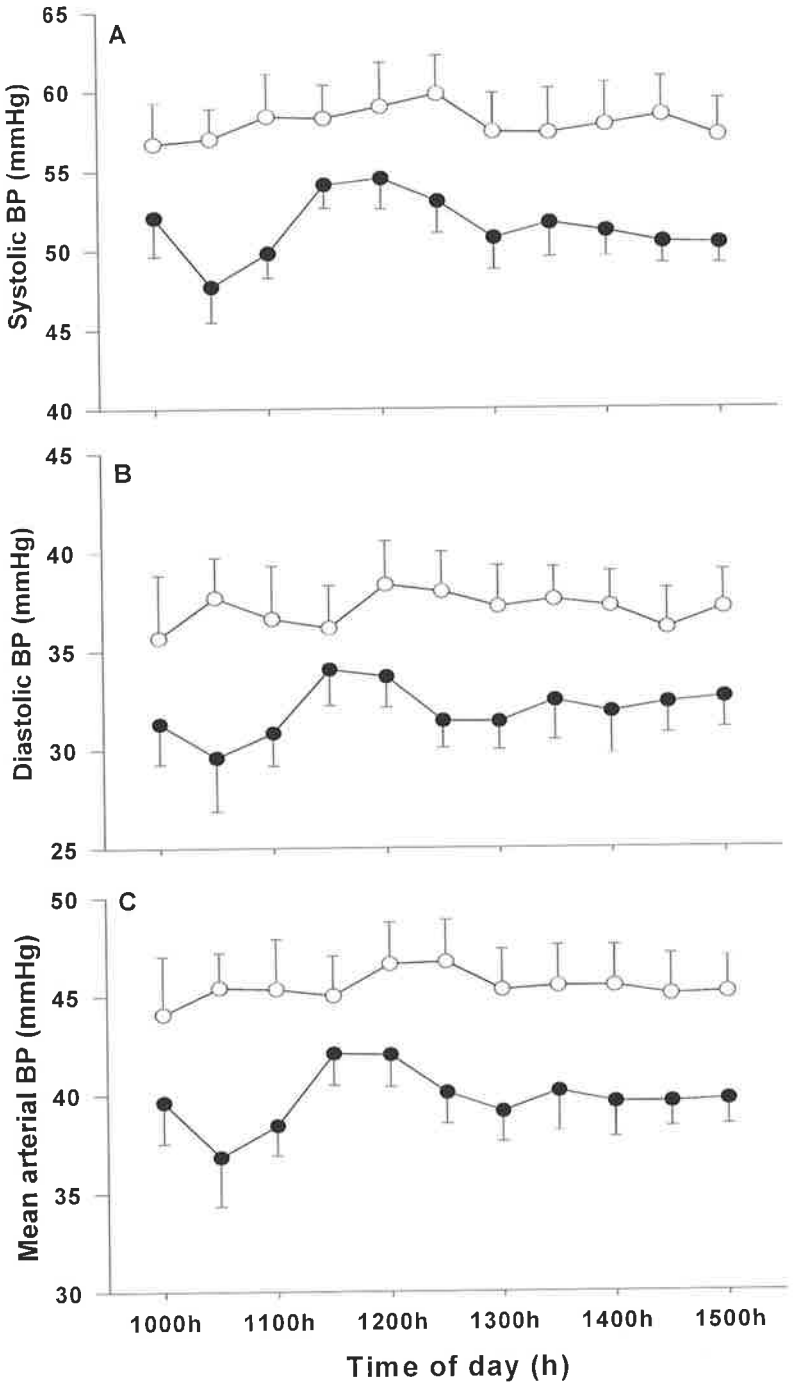

Figure 2.6 : The effect of metyrapone or vehicle infusion from 125d gestation on arterial blood pressure at 126-127d gestation.

Fetal systolic (A), diastolic (B) and mean arterial blood pressure (C) were measured between 1000h and 1500h at 126-127d gestation in metyrapone (closed circles) and vehicle (open circles) infused fetuses. Fetal systolic, diastolic and mean arterial blood pressure values were significantly lower ($P<0.05$) in the metyrapone than vehicle infused fetuses at 126-127d gestation.

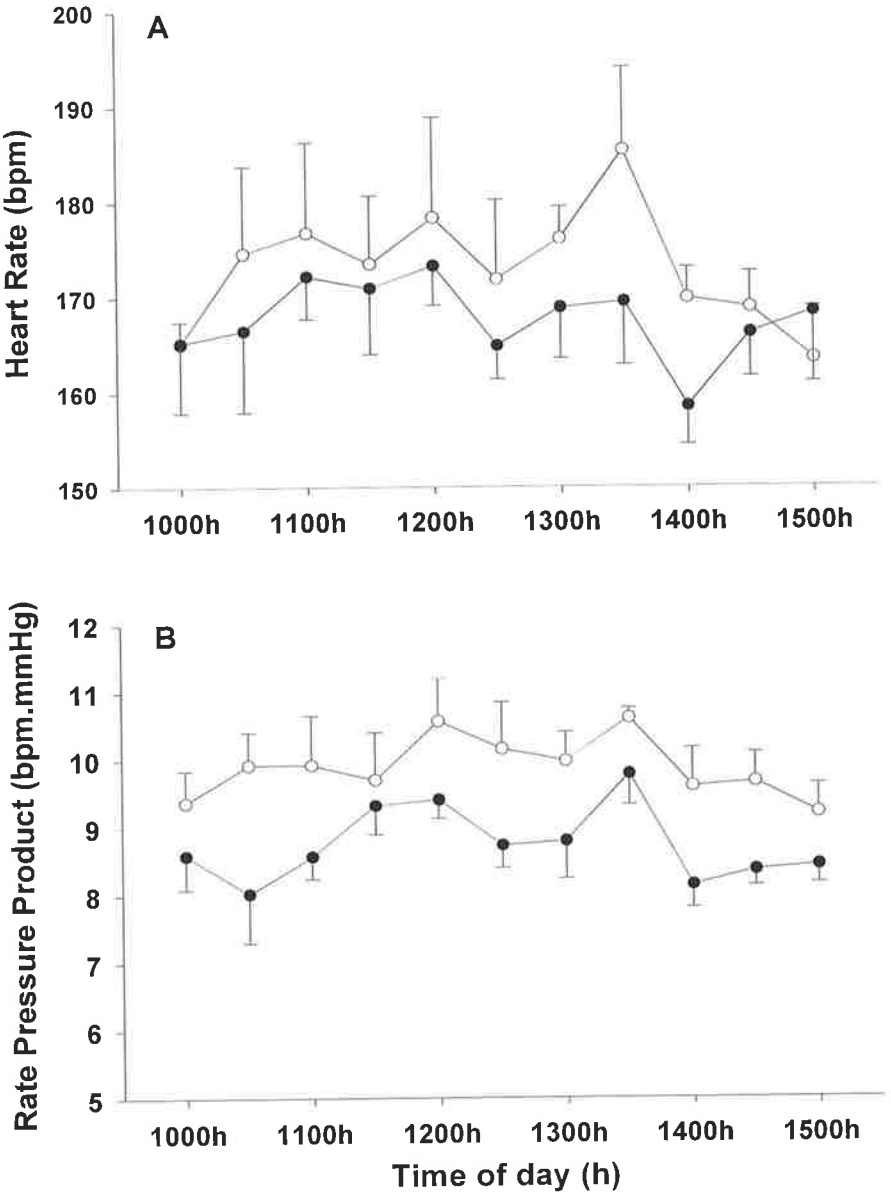

Figure 2.7: The effect of metyrapone or vehicle infusion on heart rate and rate pressure product at 24-48h after the start of infusion.

Heart rate (A) was not different between metyrapone (closed circles) and vehicle (open circles) infused fetuses. The rate pressure product (B) was significantly lower ($P< 0.05$) in metyrapone infused fetuses when compared with vehicle infused fetuses.

2.3.6 Effect of metyrapone infusion from 125d on the relationship between fetal arterial blood pressure and plasma ACTH concentrations at 126-127d gestation

There was a significant inverse relationship between systolic, diastolic and mean arterial blood pressure and plasma ACTH for vehicle infused fetuses (r^2=0.86, P<0.01; r^2=0.93, P<0.005; r^2=0.94, P=0.001 respectively) (Figure 2.8A, B & C), whereas there was no significant relationship between fetal blood pressure and ACTH concentrations in metyrapone infused fetuses.

2.4 DISCUSSION

We have investigated the role of endogenous fetal cortisol in the maintenance of arterial blood pressure in the fetal sheep at 125-128d gestation when fetal plasma cortisol concentrations are relatively low. In the present study, we found that whilst plasma ACTH and 11-desoxycortisol concentrations were higher, cortisol concentrations were not significantly different in the metyrapone infused fetuses compared with controls. One possible explanation may be that cortisol concentrations in the metyrapone infused fetuses, decreased early in the first 24h of the infusion, i.e. prior to the first fetal blood sample which was collected at +24h. It is clear that the fetal HPA axis responded to a fall in cortisol as plasma ACTH concentrations were increased in the metyrapone infused group. The increase in ACTH was maintained throughout the first 72h of the infusion period indicating that higher ACTH concentrations were required to overcome the metyrapone induced block of adrenal CYP11B1 and restore circulating cortisol concentrations to preinfusion values.

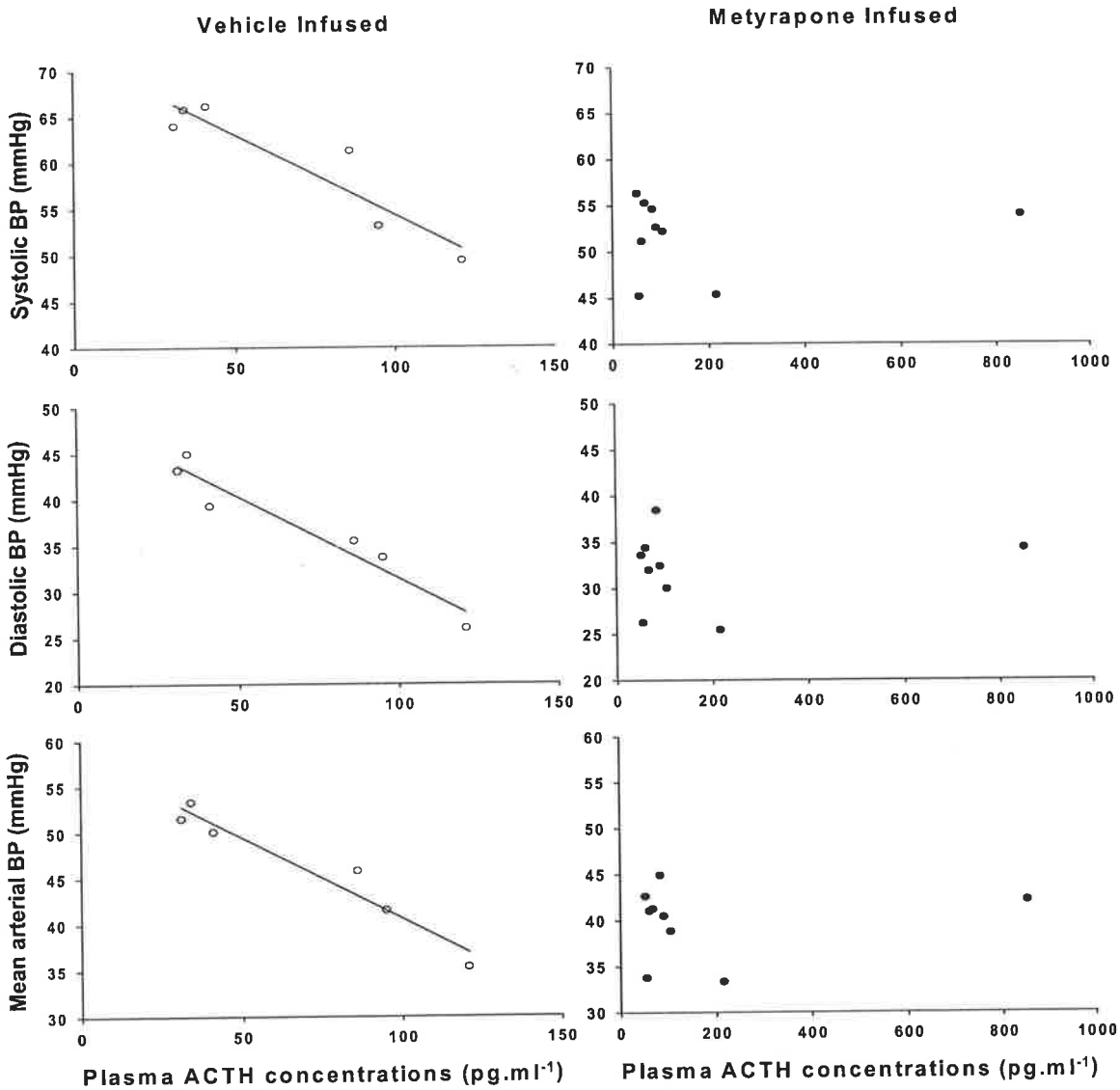

Figure 2.8: The relationship between systolic, diastolic and mean arterial blood pressure and plasma ACTH concentrations in vehicle and metyrapone infused fetuses at 126-127d gestation.

There was a significant inverse relationship between systolic ($y = -0.17x + 72$, $r^2 = 0.86$, $P < 0.01$), diastolic ($y = -0.18x + 49.2$, $r^2 = 0.93$, $P < 0.005$) or mean arterial blood pressure ($y = -0.18x + 58.2$, $r^2 = 0.94$, $P < 0.001$) and plasma ACTH concentrations in the vehicle (open circles) infused fetuses. There was no relationship between fetal arterial blood pressure and plasma ACTH concentrations in the metyrapone (closed circles) infused group (with or without inclusion of the fetal ACTH value of > 800 $pg.ml^{-1}$ in one fetus).

The low levels of fetal cortisol measured during this study, in control fetuses at 125-128d gestation are consistent with that reported in other studies (Wintour et al., 1980; Hennessy et al., 1982; Ross et al., 2000; Edwards & McMillen, 2001; Marsh et al., 2002). Edwards & McMillen (2001) found that plasma cortisol concentrations were ~2-3nmol.L^{-1} at around 115-125d gestation, and Ross and co workers (2000) reported that cortisol concentrations were ~2nmol.L^{-1} at 109-116d. Marsh and coworkers (2002) also demonstrated that plasma cortisol concentrations were ~3nmol.L^{-1} in fetal sheep at 121-122d.

At 125-128d, maternal cortisol has been shown to be a major contributor (37%) to circulating cortisol in the fetus (Hennessy et al., 1982). It has also been shown that circulating cortisol in adrenalectomised fetal sheep is almost entirely derived from the maternal circulation (Wintour et al., 1980). One further possible reason, therefore why metyrapone infused fetuses did not have lower circulating cortisol concentrations is that a significant proportion of the cortisol in the fetus was derived from transplacental transfer.

Systolic, diastolic and mean arterial blood pressure were significantly lower in metyrapone infused fetuses than in the controls. Unno and colleagues (1999) did not find a significant difference in fetal arterial blood pressure between adrenalectomised and intact fetuses at 120-126d, although the small increase (~3 mmHg) in arterial blood pressure which occurred in intact fetuses between 120 and 126d gestation did not occur in the adrenalectomised group. These authors were unable to measure fetal cortisol concentrations in the intact and adrenalectomised fetuses as the cortisol levels were below the sensitivity of their cortisol assay (Unno et al., 1999).

In the present study the mean arterial blood pressure in metyrapone infused fetuses was 5-6 mmHg lower than in vehicle infused controls. Interestingly, in the present study the fetal arterial blood pressure was lowest at 1030h and highest at 1200h in both vehicle and metyrapone infused groups. Brace and coworkers (Brace & Moore, 1991) have reported that there is a diurnal rhythm of arterial blood pressure in fetal sheep and that fetal arterial blood pressure is lowest at 1030h and highest at 2100h. In the present study however, fetal arterial blood pressure was not measured continuously for 24h. Whilst fetal cortisol concentrations show a diurnal variation when ewes are fed once daily, this only occurs in fetal sheep after 135d gestation (Simonetta et al., 1991).

In the current study, there was a significant negative correlation between fetal plasma ACTH concentrations and arterial blood pressure in vehicle infused controls but not in the metyrapone infused fetuses. Edwards & McMillen (2001) found a similar inverse relationship between fetal arterial blood pressure and plasma ACTH concentrations at 115-125d in a group of undernourished ewes (Edwards & McMillen, 2001). Cortisol is measured after extraction of total cortisol i.e. cortisol bound to corticosteroid binding globulin and 'free' cortisol from fetal plasma. One possibility is that in the control group in the present study, increases in circulating concentrations of 'free' cortisol result in a concomitant increase in arterial blood pressure and an inhibition of fetal ACTH secretion. In the metyrapone infused fetuses however, the fetal HPA axis is responding to the metyrapone block of endogenous cortisol synthesis and therefore ACTH secretion from the pituitary is stimulated rather than suppressed. These data in the control group and the decrease in mean arterial blood pressure measured in the metyrapone infused fetuses suggest that cortisol may contribute to the regulation of fetal arterial blood

pressure during this period in gestation when fetal plasma cortisol concentrations are relatively low i.e. 2-10 $nmol.L^{-1}$. Any actions of cortisol at these low concentrations would be through occupancy of the high affinity, type I glucocorticoid receptor (MR), rather than the low affinity, type II glucocorticoid receptor (GR). Whilst MR are expressed in the fetal sheep kidney (Moritz et al., 2002) in late gestation, the presence of the enzyme 11βHSD2, which metabolises cortisol to the inactive cortisone may protect the renal MR from glucocorticoid occupancy (Langlois et al., 1995; McMillen et al., 2000). In the adult, MR and GR are each present in the aorta, mesenteric artery and vascular smooth muscle cells (Lombès et al., 1992; Kornel et al., 1993; Takeda et al., 1995) and there is evidence that glucocorticoids act in vascular smooth muscle to modify the actions of vasoactive agents, including the catecholamines, Ang II, nitric oxide and prostaglandins (Falardeau & Martineau, 1989; Rees et al., 1990; Pirpiris et al., 1992; Walker & Williams, 1992; Wehling et al., 1995; Muto et al., 1996). It has been previously demonstrated that there was a small but significant decrease in fetal arterial blood pressure in response to a specific AT1R antagonist at 128d gestation and that intrafetal infusion of cortisol resulted in a further fall in arterial blood pressure in response to the AT1R antagonist (Forhead et al., 2000). One possibility therefore is that at 126-127d gestation, a transient fall in plasma cortisol results in a decrease in arterial blood pressure as a result of a decrease in the vascular responsiveness to the actions of circulating Ang II.

It is intriguing that fetal arterial blood pressure was lower in the metyrapone fetuses at 24-48h after the start of the infusion while fetal cortisol concentrations were maintained at pre-infusion values by the increase in fetal ACTH. It could be argued that the inverse relationship between arterial blood pressure and plasma ACTH

concentrations in the control group and the association between low arterial blood pressure and high ACTH concentrations in the metyrapone infused group reflect the actions of ACTH, rather than cortisol, on fetal arterial blood pressure. Infusion of ACTH in the pregnant ewe, however, results in an increase, rather than decrease, in maternal and fetal arterial blood pressure and the increase in fetal arterial blood pressure in these studies was directly associated with an increase in fetal cortisol (Lumbers et al., 1998). Furthermore, a short-term infusion of ACTH (24h) in fetal sheep at 125-130d gestation did not significantly alter fetal arterial blood pressure or heart rate (Carter et al., 1993). There is evidence, however, that intrafetal infusion of the cleavage product of ACTH, α-melanocyte stimulating hormone (αMSH), can act to decrease mean arterial blood pressure in the sheep fetus (Llanos et al., 1983). It is possible that an increase in an ACTH-derived peptide suppressed fetal arterial blood pressure at 126-127d gestation.

2.4.1 Summary

In summary the main finding of the present study is that infusion of an inhibitor of endogenous cortisol synthesis, at a gestational age when circulating cortisol concentrations are low, resulted in a significant decrease in fetal arterial blood pressure. I speculate that metyrapone infusion resulted in a biologically significant decrease in fetal cortisol concentrations during the first 24h of the infusion period, which resulted in a change of occupancy of MRs located at either peripheral or central sites and that this resulted in a decrease in arterial blood pressure in the metyrapone infused fetuses. This study provides evidence supporting the hypothesis that endogenous glucocorticoids play a key role in the development of arterial blood pressure at 126-127d gestation.

3 Effect of Infusion of Metyrapone on Fetal Arterial Blood Pressure and the Arterial Blood Pressure Responses to Ang II at 137-139d Gestation

3.1 INTRODUCTION

In Chapter 2, it was reported that infusion of metyrapone from 125d gestation, when fetal cortisol concentrations are low, resulted in a decrease in fetal arterial blood pressure. The first aim of the present study was to determine the effects of suppression of endogenous cortisol biosynthesis after the start of the prepartum increase in fetal cortisol on fetal arterial blood pressure. The hypothesis tested was that inhibition of endogenous cortisol synthesis during this period when fetal cortisol concentrations are increasing would also result in a decrease in fetal arterial blood pressure and in the fetal blood pressure responsiveness to Ang II infusion. Metyrapone was therefore infused into fetal sheep starting at 137d gestation and fetal plasma cortisol, 11-desoxycortisol, and ACTH concentrations and basal fetal systolic, diastolic and mean arterial blood pressure and heart rate and fetal arterial blood pressure responses to increasing doses of Ang II were measured. Secondly, I also investigated the effect of a continuous infusion of metyrapone into fetal sheep between 125 and 140d gestation ie. from before and during the prepartum increase in cortisol, on basal fetal arterial blood pressure and heart rate and on the arterial blood pressure responses to increasing doses of the vasoactive agents, Ang II and noradrenaline at 137-139d gestation.

3.2 MATERIALS AND METHODS

3.2.1 Animals and Surgery

All experiments were carried out according to the guidelines of the Standing Committee of Ethics and Animal Experimentation, The University of Adelaide Animal Ethics Committee.

Twenty eight pregnant Merino ewes were used in this study. Ewes were housed in single crates and kept in a 12h light – dark cycle. Animals were fed once daily and were given water ad libitum throughout the entire length of the protocol. Ewes were fasted 24h prior to surgery. Surgery to insert vascular catheters was performed as described in Chapter 2.

Effect of metyrapone infusion from 125d on fetal arterial blood gas status and plasma hormones at 137-139d gestation

At 125d gestation, metyrapone (65μmoles.h^{-1}.kg^{-1} in 0.6M tartaric acid; n=10) or vehicle (tartaric acid 0.6M.h^{-1}; n=11) was infused (0.4ml.h^{-1}; Graseby Medical syringe driver M5-10A) into the fetal jugular vein. Tartaric acid was used to aid the solubility of metyrapone (Lye & Challis, 1984) and fetal weight at 125d gestation (3.08 kg) was estimated using fetal growth curves previously published from our laboratory (Fetal weight=0.0008 x gestational age^2 – 0.1046 x gestational age + 3.6508) (Edwards et al., 1999). The infusion was maintained continuously for 15d until post mortem at 140d gestation. On the first day of the infusion fetal arterial blood was collected at 1000h (-60 min) and 1100h (0 min) prior to the start of the infusion. Throughout the infusion period, fetal arterial blood samples (2.5ml) were collected daily, between 0900h and 1100h, for blood gas and plasma hormone analysis. For ACTH determination, blood (1.0ml) was aliquoted into EDTA coated

tubes as described in Chapter 2. For cortisol and 11-desoxycortisol determination, blood (1.0 - 2.0ml) was aliquoted into heparin coated tubes (125 IU, Sarstedt). Blood samples were centrifuged at 1500 g for 10 min, the plasma was separated and frozen at -20°C until analysis. Blood samples (0.5ml) were also collected daily for analysis of fetal arterial PO_2, PCO_2, pH, sO_2 and Hb using an ABL 520 blood gas analyser (Radiometer, Copenhagen, Denmark).

Table 3.1: **Details of the numbers of animals included in each experimental group**

	Tartaric Acid infused from 125d gestation	**Metyrapone infused from 125d gestation**	**Metyrapone infused from 137/8d gestation**
Surgery	11	10	9
Singleton (S) / Twin (T) pregnancies	S(9) T(2)	S(10)	S(7) T(2)
Measurement of arterial blood pressure at 137-139d	6	7	7
Ang II administration at 137-139d	7	7	7
Noradrenaline administration at 137-139d	5	6	Not performed
Measurement of fetal plasma ACTH at 137-140d	6	9	8
Measurement of fetal plasma 11desoxycortisol at 137-140d	6	9	8
Measurement of fetal plasma cortisol at 137-140d	6	9	8

Effect of metyrapone infusion from 137d on fetal blood gas status and plasma hormones at 137 -140d gestation

At 137-138d metyrapone (65µmoles.h^{-1}.kg^{-1}; n=9; see Table 3.1) was infused (0.4ml.h^{-1}; Graseby Medical syringe driver M5-10A) into the fetal jugular vein. Fetal

weight at this age range (4.69 kg) was also estimated using fetal growth curves previously published from our laboratory (Edwards et al., 1999). The infusion was maintained continuously for 4d until post mortem at 140-141d. On the first day of the infusion period fetal arterial blood (3.5ml) was collected at 0900h (-3h) and 1200h (0h) prior to the start of the infusion. Fetal arterial blood samples (3ml) were taken at +6h, +21h, +24h, +45h and +69h after the start of the infusion.

3.2.2 Blood Pressure Measurements

Effect of metyrapone infusion from 137d or 138d on fetal arterial blood pressure

At 137-138d gestation, fetal arterial blood pressure was measured from 0900h (3h prior to the start of the metyrapone infusion) until 1800h (6h after the start of metyrapone infusion). On the second day of the metyrapone infusion period fetal systolic and diastolic blood pressure were measured continuously from 0900h to 1500h (ie. from +21h to +27h after the start of the metyrapone infusion). Fetal arterial and amniotic pressures were measured directly as described above. Fetal systolic and diastolic blood pressure were calculated after subtraction of the intra amniotic pressure. A mean value for systolic and diastolic blood pressure and heart rate was determined for a 5s interval for every 15 min between –3h and the start of the infusion (time 0). Mean 5s values for blood pressure were also determined at 15 or 30 min intervals between 0 and +6h and subsequently at +21 - +27h after the start of the infusion.

Effect of metyrapone infusion from 125d on fetal arterial blood pressure at 137-139d gestation

Fetal arterial blood pressure was measured between 1000h and 1500h at 137-139d gestation, i.e. at 12-14d after the start of the metyrapone infusion at 125d gestation. Due to technical difficulties, including blocked fetal arterial catheters on experimental days, not all fetuses had blood pressure recorded at both gestational age ranges (for details of animal numbers in each protocol, see Table 3.1). Fetal arterial and amniotic pressures were measured directly by attaching the fetal carotid artery and amniotic catheters to displacement transducers and a quad bridge amplifier (ADInstruments, Castle Hill, NSW, Australia). Data were recorded using Maclab Chart software on a Power Macintosh computer as described previously (Edwards et al., 1999). Fetal systolic and diastolic blood pressure were calculated after subtraction of the intra-amniotic pressure. A mean fetal systolic and diastolic blood pressure value was determined from the continuous 5h recording of fetal arterial blood pressure on day 137 - 139d. A mean value for systolic, diastolic blood pressure and heart rate for a 5s interval was calculated at the start of the blood pressure recording and subsequently at 30 min intervals after the first time point.

Effect of metyrapone infusion from 125d on fetal arterial blood pressure responses to noradrenaline at 137-139d gestation

Bolus doses of noradrenaline (0.5μg, 1μg, 2μg, 5μg; Atenolol, Sigma Chemical Co, St Louis, MO, USA) were administered intravenously on a separate day between 137 and 139d gestation into fetuses which had been infused with either vehicle (n=5) or metyrapone (n=6) between 125 and 140d gestation. The fetal arterial and intraamniotic pressures were measured continuously during the experimental

protocol using a MacLab data acquisition system (ADInstruments, Castle Hill, NSW, Australia).

Fetal arterial blood pressure responses to Ang II at 137-139d in both metyrapone infused groups

The fetal arterial blood pressure responses to increasing doses of Ang II were measured at 138-139d gestation in fetuses infused with metyrapone from 137-138d gestation (n=7) and in fetuses infused with either vehicle (n=7) or metyrapone (n=7) between 125 and 140d gestation (see Table 3.1). Bolus doses of human Ang II (0.2μg, 0.75μg, 1.5μg, 3μg, 5μg, 10μg; Peninsula Laboratories Inc, Belmont, CA, USA) were injected intravenously in a random order. There was a 20 min period between the administration of each Ang II dose. Fetal arterial blood pressure and intraamniotic pressure were continuously recorded using a MacLab data acquisition system (ADInstruments, Castle Hill, NSW, Australia). For each fetus, the Ang II doses were calculated relative to fetal weight as determined at post mortem.

3.2.3 Radioimmunoassays

ACTH

Fetal plasma samples were assayed for ACTH using a kit radioimmunoassay (ICN Biomedicals, NSW, Australia) as described previously in Chapter 2.

11-Desoxycortisol

Fetal plasma samples were assayed for 11-desoxycortisol using a kit radioimmunoassay (ICN Biomedicals, NSW, Australia) validated for use in fetal sheep plasma as described in Chapter 2.

Cortisol

Fetal plasma samples were assayed for cortisol using a radioimmunoassay validated for use in fetal sheep plasma as described in Chapter 2.

3.2.4 Statistical Analysis

All data are expressed as mean ± SEM.

Fetal Blood Gas Status

Effect of metyrapone infusion from 125d on fetal arterial blood gas status between 125-140d gestation

The mean values of arterial PO_2, PCO_2, pH, sO_2 and Hb were determined for each fetus from surgery at 119-120d gestation until post mortem at 140-141d gestation. The mean blood gas and pH values fetuses in the metyrapone and vehicle infused groups were then compared using a Student's unpaired t-test.

Effect of metyrapone infusion from 137d on fetal arterial blood gas status at 137-140d gestation

Mean baseline values of arterial PO_2, PCO_2, pH, sO_2 and Hb were determined for each fetus from surgery until the start of the metyrapone infusion. Mean blood gas and pH values were also determined for each fetus from the start of the metyrapone infusion until post mortem. The mean blood gas and pH values during the baseline and infusion periods were compared using a Student's paired t-test.

Arterial Blood Pressure

Effect of metyrapone infusion from 125d and 137d on fetal arterial blood pressure at 137-139d gestation

In all studies, fetal mean arterial blood pressure was calculated using the formula: (diastolic blood pressure + 0.4 (systolic blood pressure - diastolic blood pressure)) (Edwards et al., 1999). The mean systolic, diastolic and mean arterial blood pressure, and heart rate values for each animal before, and during these periods were compared using a multifactorial ANOVA with repeated measures using SPSSX (Chicago, IL, USA) on a VAX mainframe computer.

Blood pressure responses to Ang II and noradrenaline

The fetal blood pressure responses to Ang II or noradrenaline were analysed in relation to the dose of Ang II or noradrenaline expressed relative to fetal weight (as determined at post mortem). The mean systolic or diastolic blood pressure value before the first dose of Ang II or noradrenaline was used as the baseline for responses to all subsequent doses. The maximum systolic or diastolic blood pressure response within 2 min of the injection of each dose of Ang II or noradrenaline was determined. The change in blood pressure from baseline was then calculated by subtracting the baseline recording from the maximum blood pressure response for that dose. The data were then analysed using a multifactorial ANOVA with repeated measures using SPSSX (Chicago, IL, USA) on a VAX mainframe computer.

Blood pressure and hormone data were log transformed, where required, to reduce the heterogeneity of the variance of the data sets prior to analysis. Fetal arterial blood pressure, including responses to Ang II and noradrenaline, and the plasma

hormonal data in the metyrapone and vehicle infused groups at 137-139d gestation were compared using a multifactorial ANOVA with repeated measures using SPSSX (Chicago, IL, USA) on a VAX mainframe computer system. The factors included in the ANOVA were group (metyrapone infused vs vehicle infused), dose (<0.1 $\mu g.kg^{-1}$ – 4.0 $\mu g.kg^{-1}$), time points (ie. every 30 min between 1000h -1500h or day ie. between 137d, 138d and 139d). When a significant interaction between major factors was identified by ANOVA, the data were split on the basis of the interacting factor and reanalysed.

Where the ANOVA identified significant differences between groups, a Duncan's multiple range post-hoc test was used to identify the differences between mean values. A probability of <5% (P<0.05) was taken to be significant.

3.3 RESULTS

3.3.1 Effect of metyrapone infusion from 125d on fetal arterial blood gas status

There were no significant differences in the arterial PO_2, PCO_2, pH, sO_2 or Hb between fetuses infused with either metyrapone or vehicle throughout the period between 119d and 140d gestation (Table 3.2).

3.3.2 Effect of metyrapone infusion from 125d on fetal plasma ACTH, 11-desoxycortisol and cortisol concentrations at 137-139d gestation

Plasma ACTH concentrations were significantly higher (F=11.9, P<0.01) at 137-139d gestation, in fetuses infused with metyrapone when compared to vehicle infused controls (Figure 3.1A). Plasma 11-desoxycortisol concentrations were also

significantly higher (F=80.9; $P<0.05$) in the metyrapone infused group at 137-139d when compared to vehicle infused controls (Figure 3.1B). Whilst plasma cortisol concentrations tended to be higher in metyrapone infused fetuses at 137-139d, there was no significant difference between the two treatment groups (Figure 3.1C).

Table 3.2: Mean arterial blood gas, pH and Hb values in fetuses infused with either vehicle or metyrapone from 125d gestation. Mean values are calculated between fetal surgery and 140d

	Vehicle Infused (n=11)	Metyrapone Infused (n=10)
PO_2 (mmHg)	21.0 ± 0.8	21.1 ± 0.8
PCO_2 (mmHg)	49.8 ± 1.3	49.5 ± 0.9
pH	7.400 ± 0.004	7.394 ± 0.005
SO_2 (%)	64.3 ± 2.6	63.6 ± 2.1
Hb ($g.dL^{-1}$)	10.9 ± 0.3	11.3 ± 0.2

3.3.3 Effect of metyrapone infusion from 125d on fetal arterial blood pressure at 137-139d gestation

There was no significant difference in the systolic, diastolic and mean arterial blood pressure values or in heart rate at 137-139d between fetuses which had been continuously infused with metyrapone or vehicle between 125 and 140d gestation (Figure 3.2A-D). Mean arterial blood pressure was significantly higher (vehicle; F=28.1; $P<0.0001$; metyrapone; F=127.1; $P<0.0001$) at 137-139d gestation when compared to mean arterial blood pressure at 126-127d independently of treatment (Figure 3.3A&B). Fetal heart rate was also significantly lower in vehicle (F=58.9; $P<0.0001$) and metyrapone (F=55.6; $P<0.0001$) infused fetuses at 137-139d when compared to fetal heart rate at 126-127d gestation (Figure 3.3C&D).

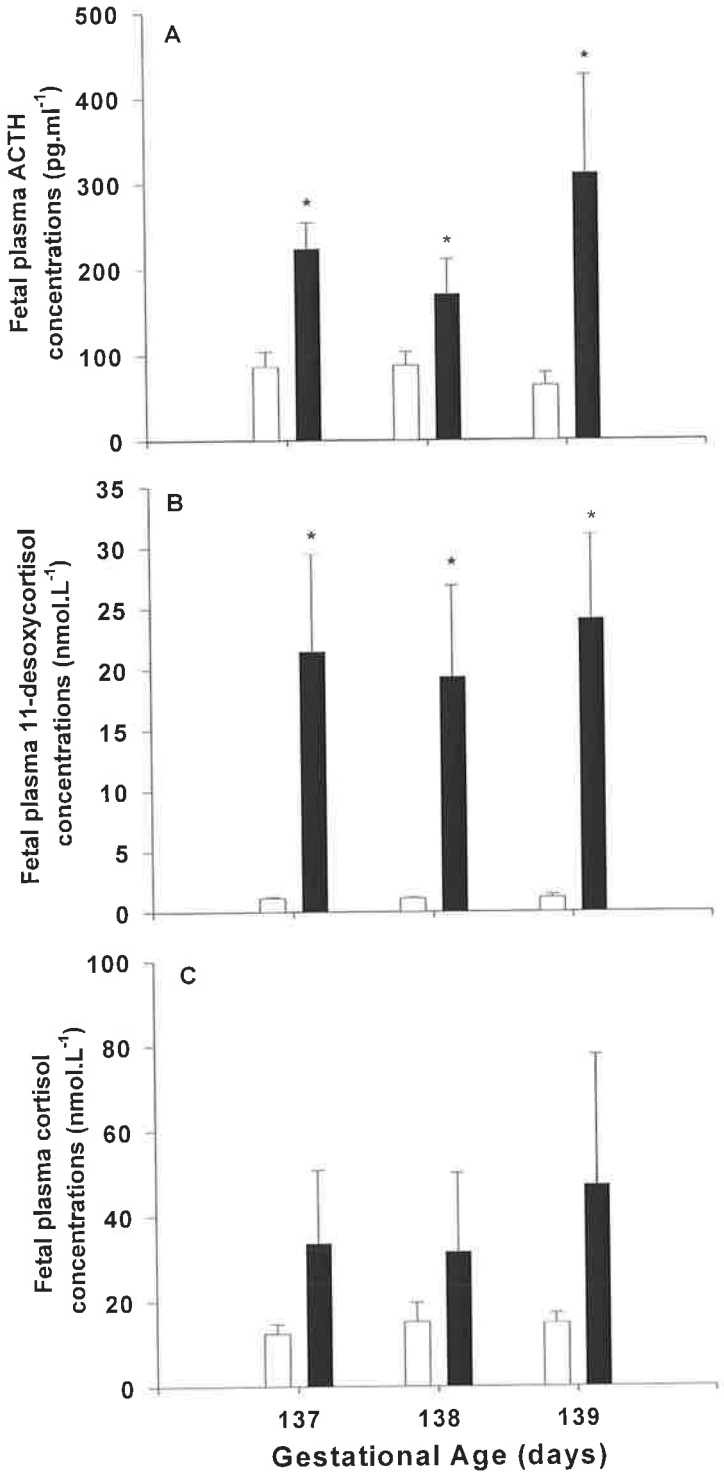

Figure 3.1: The effect of metyrapone or vehicle infusion from 125d gestation on plasma ACTH, 11-desoxycortisol and cortisol concentrations at 137-139d gestation.

* indicates plasma hormones significantly higher in metyrapone (closed bars) than in vehicle (open bars) infused fetuses.

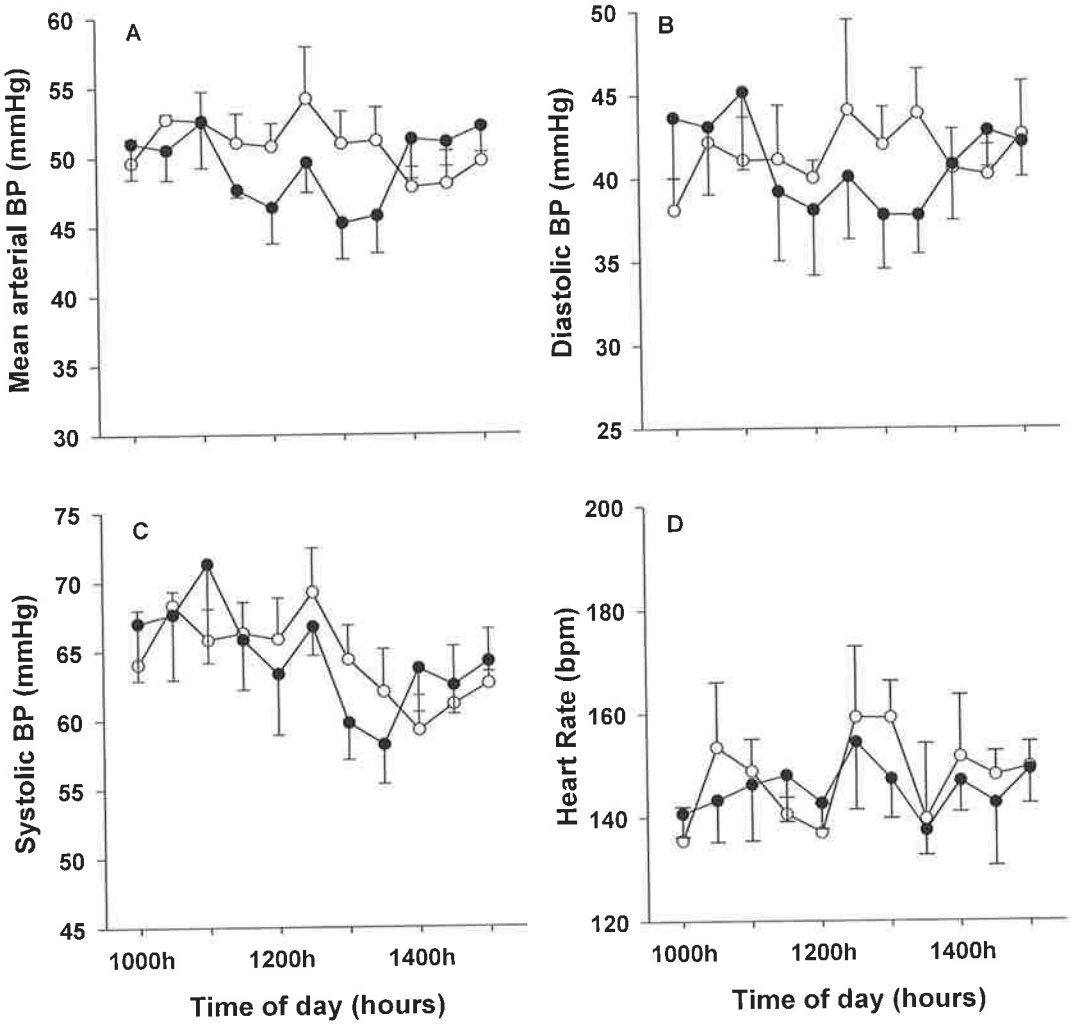

Figure 3.2: The effect of metyrapone or vehicle infusion from 125 to 140d gestation on systolic, diastolic and mean arterial blood pressure and heart rate at 137-139d gestation.

There was no difference in the mean arterial, systolic, diastolic blood pressure or heart rate at 137-139d gestation in fetuses infused with either metyrapone (closed circles) or vehicle (open circles) from 125d gestation.

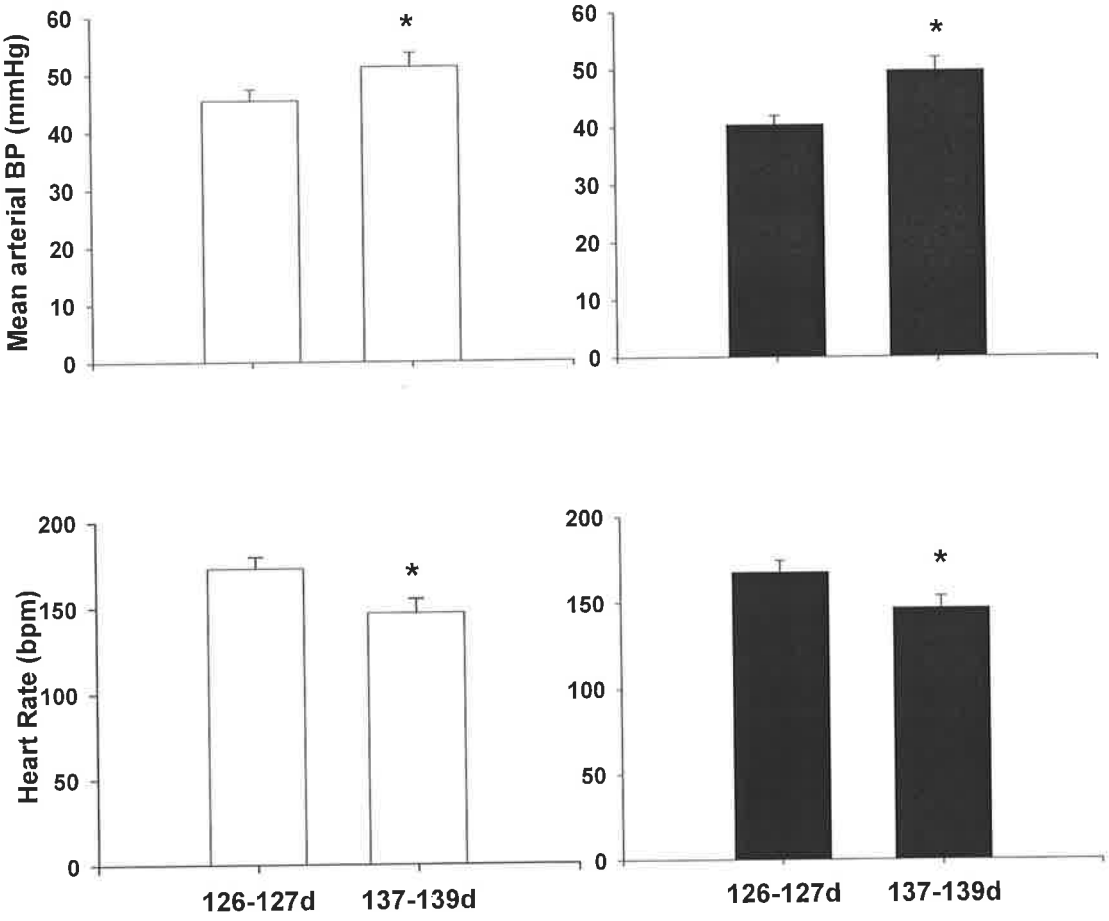

Figure 3.3: Effect of gestational age on mean arterial blood pressure and heart rate in fetuses infused with metyrapone or vehicle from 125d gestation.

Mean arterial blood pressure (top panels) was significantly higher ($P<0.05$) and fetal heart rate (bottom panels) was significantly lower ($P<0.05$) in both metyrapone (dark bars) and vehicle (open bars) treatment groups at 137d when compared to 126d gestation. * Indicates significant differences between mean values.

3.3.4 Effect of metyrapone infusion from 137d on fetal arterial blood gas status

There were no significant changes in fetal arterial PO_2 (preinfusion, 21.3 ± 0.7 mmHg; infusion, 20.3 ± 1.1mm Hg), PCO_2 (preinfusion, 48.6 ± 0.6 mmHg; infusion, 50.1 ± 1.1 mmHg), pH (preinfusion, 7.401 ± 0.004; infusion, 7.396 ± 0.006) and Hb (preinfusion, 9.9 ± 0.5 $g.dL^{-1}$; infusion, 10.3 ± 0.6 $g.dL^{-1}$) during the first 72h of the metyrapone infusion when compared with preinfusion values. There was a small but significant decrease ($P<0.05$) in arterial sO_2 (preinfusion, 65.5 ± 2.2 %; infusion, 57.5 ± 3.8 %) during the metyrapone infusion period.

3.3.5 Effect of metyrapone infusion from 137d on fetal plasma ACTH, 11-desoxycortisol and cortisol concentrations

There was a significant interaction between the effects of metyrapone and vehicle infusion and day of infusion on plasma cortisol, ACTH and 11-desoxycortisol concentrations. In the vehicle infused group, there was no change in plasma cortisol, ACTH or 11-desoxycortisol concentrations between 137 and 140d gestation. In the metyrapone infused group, however, plasma ACTH concentrations were significantly higher ($F=27.4$; $P<0.001$) than preinfusion values at +6h and remained high for the remainder of the infusion period (Figure 3.4A). Plasma 11-desoxycortisol concentrations were also significantly higher ($F=70.8$; $P<0.001$) at +6h after the start of metyrapone infusion when compared to preinfusion values and were increased even further by +45h (Figure 3.4B). Plasma cortisol concentrations were significantly lower ($F=4.9$; $P<0.01$) at +6h after the start of metyrapone infusion when compared with preinfusion levels at 137d. By +21h however, plasma cortisol concentrations had increased to preinfusion levels and were higher than preinfusion levels at +69h (Figure 3.4C).

3.3.6 Effect of metyrapone infusion from 137d on fetal arterial blood pressure at 137-140d gestation

Fetal systolic, diastolic, mean arterial blood pressure and heart rate did not change significantly during the first 6-21h after the start of the metyrapone infusion (Figure 3.5A-D; Figure 3.6A-D). There was also no significant difference in systolic, diastolic or mean arterial blood pressure or fetal heart rate at 137-139d gestation between fetuses infused with vehicle from 125d, metyrapone from 125d or metyrapone from 137d (Table 3.3).

Table 3.3: Summary of blood pressure and heart rate values for fetuses infused with either vehicle or metyrapone from 125d or with metyrapone from 137d at 137-139d gestation.

	Vehicle Infused 125-140d (n=6)	Metyrapone Infused 125-140d (n=7)	Metyrapone Infused 137-140d (n=7)
Systolic blood pressure (mmHg)	64.7 ± 2.9	64.4 ± 3.3	60.7 ± 3.0
Diastolic blood pressure (mmHg)	41.6 ± 2.1	39.3 ± 2.5	38.8 ± 2.5
Mean arterial blood pressure (mmHg)	50.7 ± 2.0	49.4 ± 2.2	47.6 ± 2.8
Heart Rate (bpm)	148 ± 8	145 ± 7	153 ± 9
RPP (mmHg.bpm)	9.5 ± 0.5	9.4 ± 0.7	9.4 ± 0.7

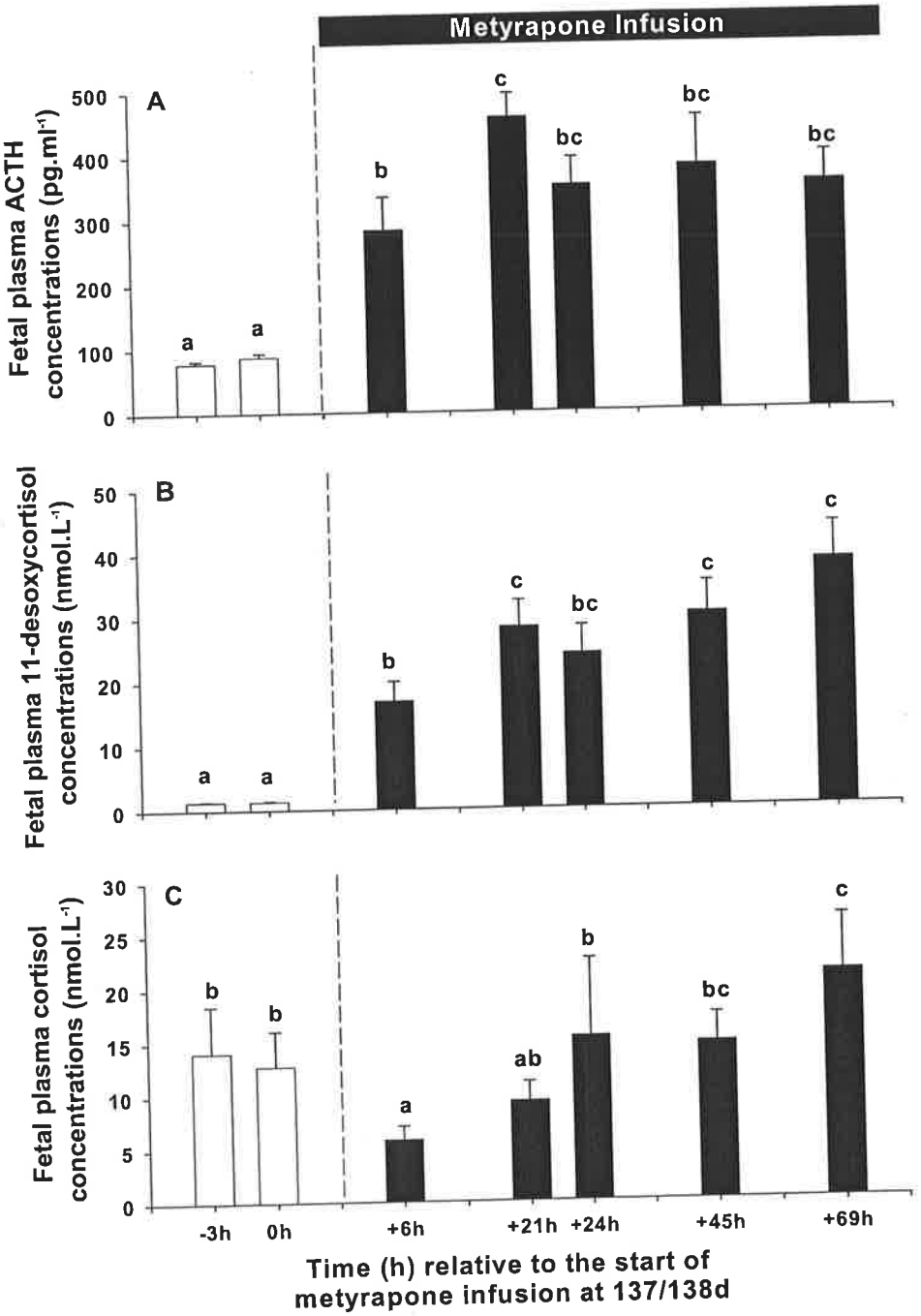

Figure 3.4: The effect of metyrapone infusion from 137d on plasma ACTH, 11-desoxycortisol and cortisol concentrations.

Plasma ACTH (A), and 11-desoxycortisol (B) concentrations before (open bars) metyrapone infusion at 137d were significantly lower than after the start of infusion (dark bars). Plasma cortisol concentration (C) significantly decreased at 6h after the start of metyrapone infusion. The dashed line indicates the start of metyrapone infusion. Different alphabetic superscripts denote mean values, which are different from each other ($P<0.05$).

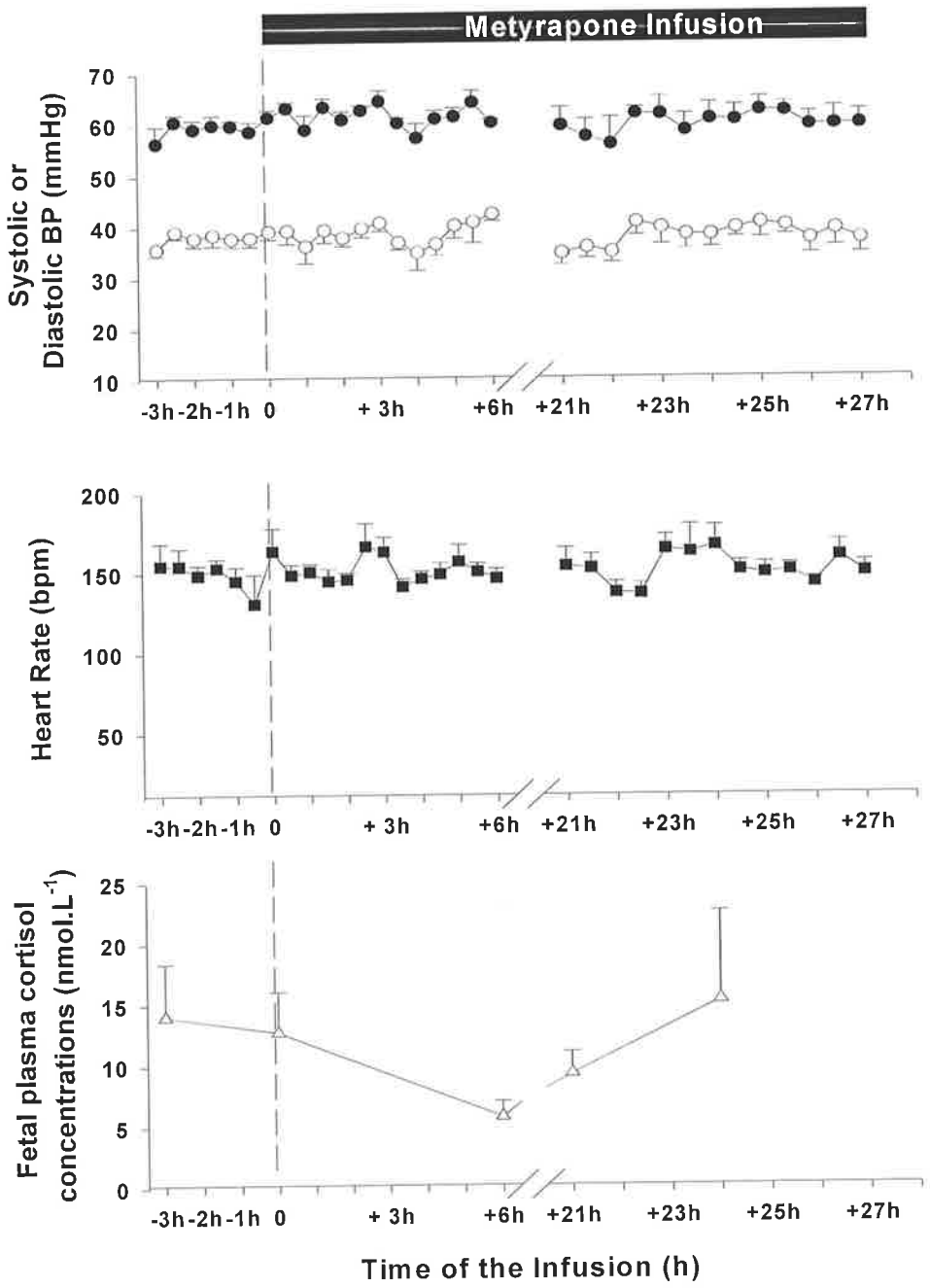

Figure 3.5: The effect of metyrapone infusion from 137d on fetal systolic, diastolic blood pressure, heart rate and plasma cortisol concentrations to 27h after the start of the infusion.

There was no effect of metyrapone infusion (indicated by the solid horizontal bar) from 137d gestation on fetal systolic blood pressure (●), diastolic blood pressure (○), or heart rate (■) despite a decrease in plasma cortisol concentrations (Δ) at 6h after the start of metyrapone infusion.

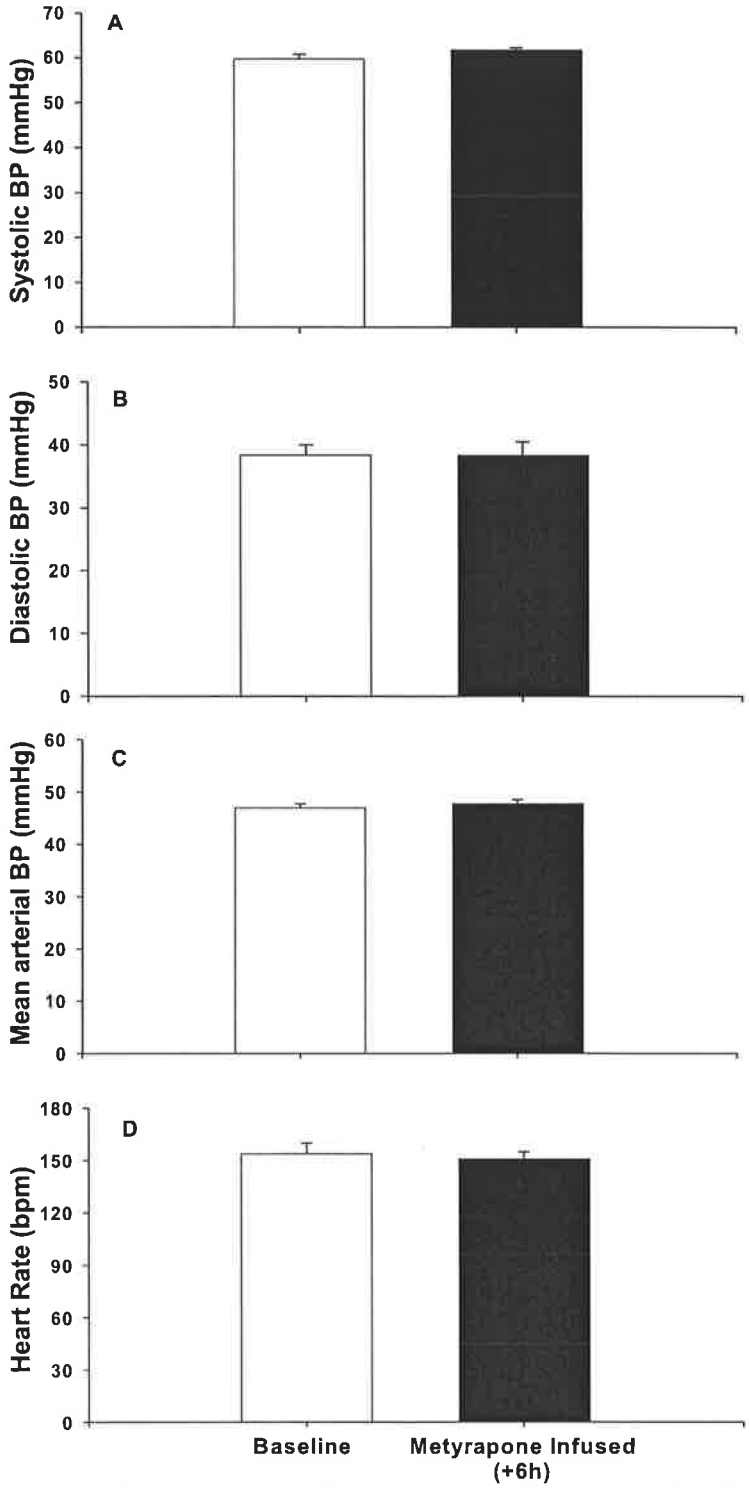

Figure 3.6: The effect of metyrapone infusion from 137d on systolic, diastolic, and mean arterial blood pressure and heart rate.

There was no difference of systolic (A), diastolic (B) or mean arterial pressure (C) or heart rate (D) before (open bars) and during the first 6h (dark bars) of metyrapone infusion at 137d gestation.

3.3.7 Fetal arterial blood pressure responses to Ang II

The fetal arterial blood pressure response to Ang II at 137-139d gestation was significantly different between the group of fetuses infused with metyrapone from 125d gestation when compared with the groups either infused with vehicle from 125d, or with metyrapone from 137/8d gestation ie. there was a significant interaction ($P < 0.01$) between the effects of treatment group and Ang II. In fetuses infused with either vehicle from 125d, or with metyrapone from 137/8d, fetal systolic, diastolic and mean arterial blood pressure increased with increasing doses of Ang II. In fetuses infused with metyrapone from 125d, however, the fetal arterial blood pressure responses to Ang II did not increase with increasing doses of Ang II at 137 - 139d gestation (Figure 3.8 A&B).

3.3.8 Effect of metyrapone infusion from 125d on fetal arterial blood pressure responses to noradrenaline

There was no effect of increasing doses of noradrenaline on fetal arterial blood pressure at 137-139d gestation in fetuses infused with either vehicle or metyrapone from 125-140d gestation (Figure 3.9 A&B). The effect of increasing doses of noradrenaline on arterial blood pressure in fetuses infused with metyrapone from 137d gestation was not measured as there was no effect in the long term metyrapone infused fetuses.

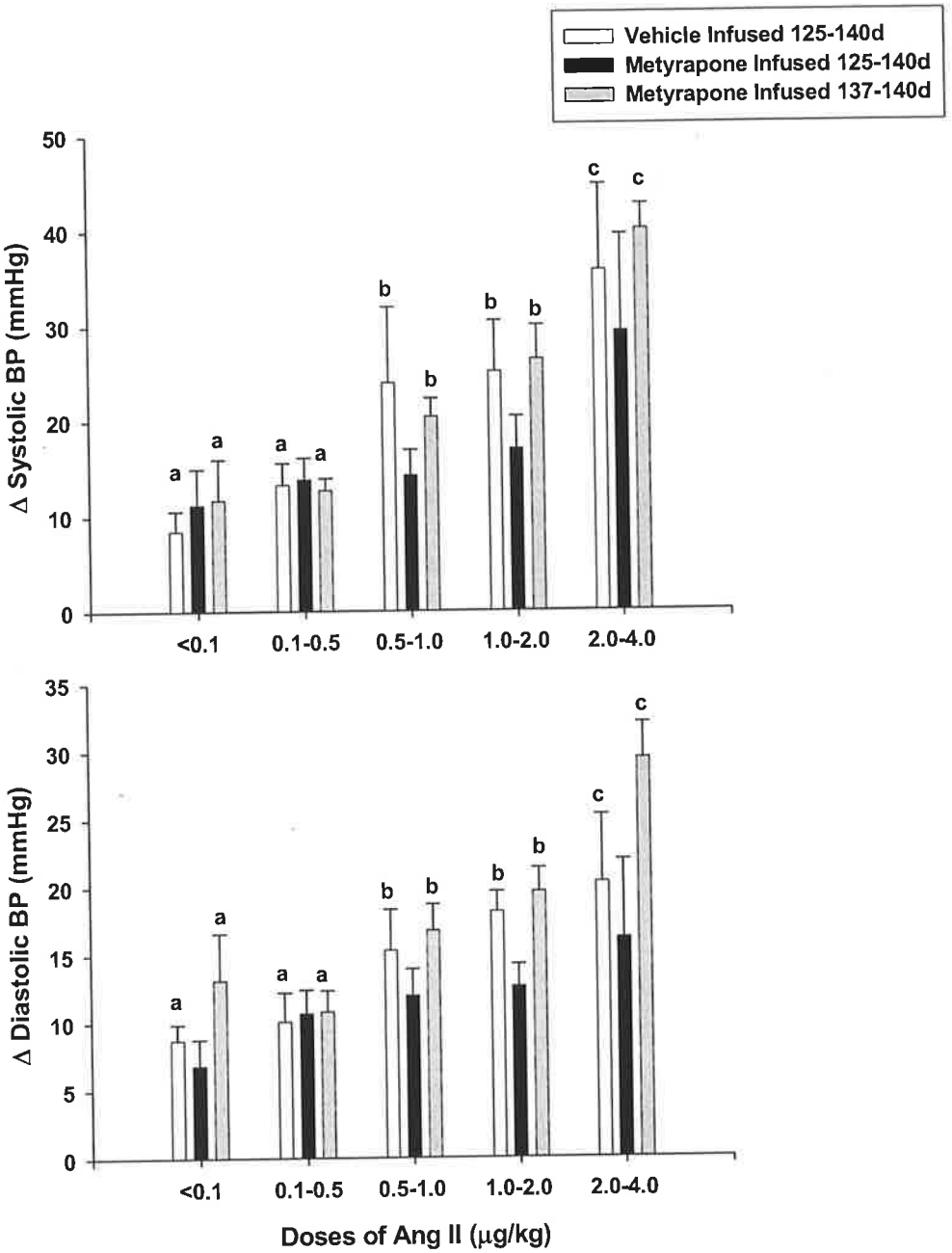

Figure 3.7: Fetal arterial blood pressure responses to increasing doses of Ang II at 137-139d gestation in fetuses infused with vehicle or metyrapone from 125d gestation and fetuses infused with metyrapone from 137/8d gestation.

Fetuses infused with vehicle (open bars) or metyrapone from 137d gestation (shaded bars) showed an increase in the blood pressure responses to increasing doses of Ang II. This effect was significantly blunted in fetuses infused with metyrapone from 125d gestation (dark bars). Different alphabetic scripts denote mean values which are significantly different ($P<0.01$) from other values within a treatment group.

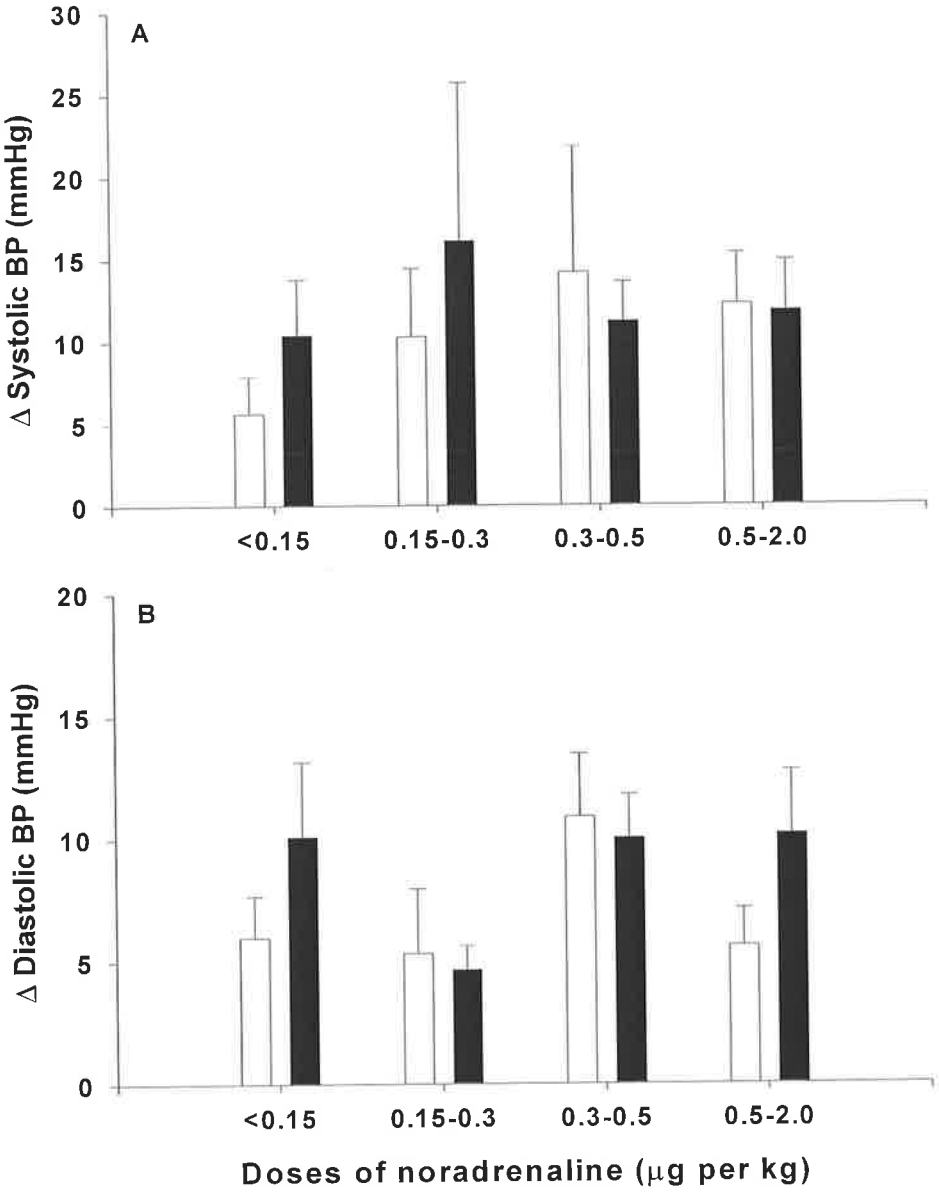

Figure 3.1: Fetal arterial blood pressure responses to increasing doses of noradrenaline at 137-139d gestation in fetuses infused with vehicle or metyrapone from 125d gestation.

There was no difference in the blood pressure responses to increasing doses of noradrenaline in either treatment group, nor in the blood pressure responses to increasing doses of noradrenaline between the metyrapone (dark bars) and vehicle (open bars) infused fetuses.

3.4 DISCUSSION

In this Chapter I have investigated the effect of metyrapone infusion from 125d, or from 137d gestation on fetal arterial blood pressure at 137-139d and the fetal arterial blood pressure responses to increasing doses of Ang II and noradrenaline at 137-139d gestation.

3.4.1 Effect of metyrapone infusion from 137d on plasma hormone concentrations and fetal arterial blood pressure at 137-139d gestation

At 137d gestation there was a significant decrease in plasma cortisol concentrations by 6h after the start of the metyrapone infusion and this decrease was associated with an increase in fetal ACTH and 11-desoxycortisol concentrations. Plasma cortisol concentrations were restored to preinfusion values by 24-48h after the start of the metyrapone infusion and plasma ACTH and 11-desoxycortisol concentrations remained elevated for the remainder of the infusion period. Whilst fetal cortisol concentrations fell to around 5-10 nM, there was no change in fetal mean arterial blood pressure during the first 24h of the metyrapone infusion and neither was there any difference between the two groups at 138-139d gestation. This suggests that the fall in arterial blood pressure observed in fetuses infused with metyrapone from 125d was due to a biologically-significant decrease in cortisol and not due to a non specific or 'toxic' action of metyrapone. A recent study investigated the effect of bilateral fetal adrenalectomised on fetal arterial blood pressure regulation in late gestation (Segar et al., 2002). These authors demonstrated that adrenalectomised fetuses had undetectable circulating cortisol concentrations and that mean arterial blood pressure was significantly lower at 139-140d gestation (44 ± 2 mmHg) when compared with adrenalectomised fetuses which had received cortisol replacement

(53 ± 2 mm Hg) (Segar et al., 2002). It should be noted, however, that this study did not compare the arterial blood pressure in fetuses following adrenalectomised and adrenalectomised with cortisol replacement with arterial blood pressure measured in a group of intact control fetuses. Thus it is not clear whether adrenalectomised alone was sufficient to decrease plasma cortisol and fetal arterial blood pressure. One possible interpretation of the available data from the studies on adrenalectomised fetuses (Unno et al., 1999; Segar et al., 2002) and on fetuses infused with metyrapone from either 125 or 137d, is that a decrease in circulating cortisol to levels below ~ 10 nM is required before there is an impact on mean arterial blood pressure in the sheep fetus. Previously, it has been shown, (Tangalakis et al., 1992; Forhead et al., 2000; Jensen et al., 2002) that fetal or maternal cortisol infusion results in an increase in fetal arterial blood pressure when endogenous cortisol concentrations are low at 120d gestation but not when cortisol concentrations are high at 137-140d gestation and this is evidence that there is a threshold above which fetal cortisol concentrations and mean arterial blood pressure are not directly related during late gestation. Nevertheless, the key question is whether there is a relationship between endogenous cortisol and arterial blood pressure, below that threshold. Edwards and colleagues reported that there was a direct relationship between arterial blood pressure and plasma cortisol concentrations between 135 and 145d gestation when data from fetuses of well nourished and undernourished ewes were combined (Edwards & McMillen, 2001). Forhead and coworkers (2000), however, found that whilst there was an increase in fetal cortisol between 128 and 140d gestation, there were no significant differences between plasma Ang II, renin and angiotensinogen and arterial blood pressure across this age range, although they concluded that this may have been due to wide interanimal variation (Forhead et al., 2000). Whilst I have demonstrated that infusion

of metyrapone resulted in a fall in fetal arterial blood pressure at 125d gestation and not at 137d gestation, it is possible that the fall in fetal plasma cortisol concentrations at 137d gestation was either too transient or of insufficient magnitude to cause an associated decrease in mean arterial blood pressure.

Following infusion of metyrapone between 137-139d gestation, there was a small but statistically significant decrease in arterial oxygen saturation. The oxygen saturation of the fetuses during the metyrapone infusion was still well within the normal physiological range for fetuses in this gestational age range (Edwards et al., 1999) and it is therefore unlikely that this small decrease would have impacted on the fetal hormonal or cardiovascular response to metyrapone infusion.

3.4.2 Fetal arterial blood pressure responses to Ang II

In this Chapter, I found that plasma cortisol concentrations and arterial blood pressure at 137-139d gestation were not different in fetuses which had been infused with either metyrapone or vehicle from 125d gestation. In contrast, I found the fetal systolic and diastolic blood pressure responses to Ang II at 137-139d gestation, were blunted in those fetuses infused with metyrapone from 125d when compared with fetuses infused with vehicle from 125d or with metyrapone from 137d gestation. One possibility is that exposure to a decrease in cortisol at 125d gestation, resulted in a maintained decrease in the expression of the AT1R in the fetal vasculature resulting in a decrease in the responsiveness of the fetal vasculature to Ang II. Cortisol infusion into the fetal sheep at 120d or 128d gestation significantly increases the fetal arterial blood pressure response to increasing doses of Ang II (Tangalakis et al., 1992) and the hypotensive response to an AT1R antagonist (Forhead et al., 2000). An increase in exposure to maternal cortisol also significantly increases the

fetal arterial blood pressure response to Ang II between 115-125d gestation (Edwards & McMillen, 2001). Furthermore, intrafetal cortisol infusion results in an increase in AT1R mRNA expression in the fetal heart (Segar et al., 1995). The blunted responsiveness to Ang II appeared to be specific as there was no difference in the arterial blood pressure responses to increasing doses of noradrenaline between fetuses infused with either vehicle or metyrapone from 125d gestation. It is also the case, however, that fetal arterial blood pressure did not increase with increasing doses of noradrenaline in this study. This result is consistent with a previous study in which demonstrated that arterial blood pressure responses to increasing doses of noradrenaline were not different between cortisol or saline infused fetuses (Tangalakis et al., 1992).

Interestingly despite the blunted response to Ang II in fetuses infused with metyrapone from 125d gestation, there were no differences in fetal mean arterial blood pressure at 137-139d gestation between groups infused with either vehicle or metyrapone from 125d gestation or with metyrapone from 137d gestation. There was also an increase in arterial blood pressure and a fall in heart rate between 126-127d and 137-139d gestation in fetuses infused with either metyrapone or vehicle from 125d gestation. This dissociation of the gestational age increase in arterial blood pressure and the effects of intrafetal Ang II in these three groups of fetuses indicate that the gestational age increase in arterial blood pressure may not be entirely a result of an increased vascular responsiveness to endogenous Ang II. Alternatively there may be other factors, such as catecholamines which have maintained arterial blood pressure despite a decreased response to Ang II in the fetuses infused with metyrapone from 125d gestation.

3.4.3 Summary

In this chapter I have shown that metyrapone infusion at 137d gestation did not result in a significant fall in fetal arterial blood pressure which is in contrast to the data presented in Chapter 2 demonstrating that metyrapone infusion at 125d decreased arterial blood pressure at 126-7d gestation. Furthermore, a transient suppression of fetal cortisol biosynthesis at 137d gestation did not alter the fetal arterial blood pressure responsiveness to increasing doses of Ang II at 138/139d gestation. I have also shown that arterial blood pressure increased with increasing gestational age in fetuses infused with either metyrapone or vehicle from 125d and that there was no difference in arterial blood pressure at 137-139d gestation in these two groups of fetuses. Infusion of metyrapone from 125d gestation did, however, result in a significant blunting of the fetal arterial blood pressure responses to increasing doses of Ang II. Taken together with the data reported in Chapter 2, these findings suggest that there may be a critical window before the start of the prepartum increase in cortisol, when endogenous fetal cortisol contributes to the regulation of arterial blood pressure and to the development of subsequent vascular responsiveness to Ang II.

These findings have implications as to understanding the possible mechanisms behind the link between glucocorticoid exposure in utero and the development of hypertension later in adult life. Previous evidence supports the speculation that exposure to glucocorticoids at inappropriate times during gestation increases the responsiveness to Ang II through a direct effect on the expression of AT1Rs. Cortisol infusion before the prepartum rise in cortisol in fetal sheep increases the responsiveness to Ang II (Tangalakis et al., 1992; Forhead et al., 2000) or increases the expression of AT1R mRNA (Rosenfeld et al., 2003). It is perhaps the exposure

of glucocorticoids *in utero* which permanently sets the number of AT1Rs present in the vasculature which inturn may cause hypertension in adult life.

4 Differential Actions of Metyrapone on the Fetal Pituitary-Adrenal Axis in the Sheep Fetus in Late Gestation

4.1 INTRODUCTION

In Chapters 2 and 3 it was shown that fetal cortisol concentrations contribute to the regulation of arterial blood pressure when endogenous cortisol concentrations are low. The aim of this chapter was to determine the dynamics of adrenal cortisol synthesis and the interaction with the fetal pituitary during late gestation in response to metyrapone infusion. In the sheep fetus, plasma cortisol concentrations increase from around 125d gestation and this prepartum rise in fetal cortisol concentrations is a consequence of a pituitary-dependent increase in fetal adrenal growth and steroidogenesis (Liggins et al., 1967; Barnes et al., 1977; Phillips et al., 1996a). Prior to the pre-partum increase in fetal plasma cortisol concentrations, an intra-fetal infusion of ACTH at 100d gestation significantly increases the steroidogenic capacity of the fetal adrenal by increasing the expression of the adrenal steroidogenic enzymes, CYP11A1 and CYP17 mRNA (Tangalakis et al., 1990). Conversely, the expression of these key branch point enzymes in adrenal steroidogenesis, measured at term, are reduced in the adrenals of fetuses after fetal hypophysectomy, performed at 107-115d gestation, and restored in hypophysectomized fetuses infused with ACTH from 115d gestation to term (Simmonds et al., 2001). These data suggest that cortisol biosynthesis in the fetal adrenal is dependent on ACTH derived from the fetal pituitary. This is further supported by studies which have also shown that intact neurovascular connections between the fetal hypothalamus and pituitary are required for the ontogenetic increase in ACTH (1-39) and adrenal functional development (Antolovich et al.,

1991; Phillips et al., 1996a). After disconnection of the fetal pituitary from the hypothalamus, there was no increase in circulating ACTH (1-39) concentrations or in adrenal CYP11A1, CYP17 and 3βHSD mRNA levels in the adrenal during late gestation (Phillips et al., 1996a).

There is also recent evidence that an up-regulation of expression of mRNA levels of both the ACTH receptor, MC2-R (Fraser et al., 2001; Coulter et al., 2002b; Wang et al., 2004) and StAR in the fetal adrenal (Coulter et al., 2002a) may also play a role in augmenting the actions of ACTH on adrenal steroidogenesis in late gestation. In the adult adrenal, StAR acts to transport cholesterol from the outer to the inner mitochondrial membrane where CYP11A1 is localised (Stocco, 2001) and StAR has been localised within the zona glomerulosa and fasciculata of the fetal sheep adrenal throughout late gestation (Coulter et al., 2002a). ACTH binding, ACTH-induced adenylate-cyclase activity and expression of StAR mRNA in the fetal sheep adrenal each increase coincident with the prepartum increase in adrenal responsiveness to ACTH and steroid output (Durand et al., 1985; Coulter et al., 2002b).

Recent studies have also shown that the action of cortisol in fetal tissues may be regulated via the 11βHSD2 enzyme. 11βHSD2 is a unidirectional NAD-dependent enzyme that catalyses the conversion of the biologically active cortisol to the inert cortisone (White et al., 1997). Interestingly there is a decrease in 11βHSD2 expression in the fetal sheep adrenal between 125 and 141d gestation which is parallel with the increase in circulating cortisol concentrations (McMillen et al., 2000). Furthermore intrafetal infusion of cortisol before 125d gestation also results in a decrease in 11βHSD2 mRNA levels in the fetal adrenal (Ross et al., 2000). This

suggests that cortisol can act through regulation of this enzyme to enhance intracellular exposure to cortisol within the fetal adrenal during late gestation (Ross et al., 2000). It is not clear, however, if an increase in intra-adrenal cortisol is required to mediate the actions of ACTH on adrenal growth and steroidogenesis during the prepartum stimulation of the fetal HPA axis. This study used an *in vivo* model to investigate the effects of high fetal ACTH concentrations on fetal adrenal MC2-R, StAR, 11βHSD2 and steroidogenic enzyme mRNA expression in the presence of an inhibitor of endogenous cortisol biosynthesis. This study infused metyrapone, a competitive inhibitor of the steroidogenic enzyme, CYP11B1, into fetal sheep between 125 and 140d gestation and measured fetal plasma cortisol, 11-desoxycortisol, and ACTH concentrations, pituitary POMC mRNA and mRNA expression of MC2-R, StAR, 11βHSD2, CYP11A1, CYP17, 3βHSD and CYP21A1 mRNA in the fetal adrenal gland.

4.2 MATERIALS AND METHODS

4.2.1 Animals and Surgery

All experiments were carried out according to the guidelines of the Standing Committee of Ethics and Animal Experimentation, The University of Adelaide Animal Ethics Committee. Twenty four pregnant Merino ewes were used in this study. Ewes were housed in single crates and kept in a 12h light – dark cycle. Animals were fed once daily and were given water ad libitum throughout the entire length of the protocol. Ewes were fasted 24h prior to surgery. Surgery was performed to insert vascular catheters into the fetus and ewe as described in Chapter 2.

4.2.2 Experimental Protocol

As described in Chapter 3, at 125d gestation metyrapone (65μmoles.h^{-1}.kg^{-1} or 4.8mmoles.day^{-1} in 0.6M tartaric acid; n=10) or vehicle (tartaric acid 0.6M.h^{-1}; n=11; saline control n=3) was infused (0.4ml.h^{-1}) into the fetal jugular vein. The infusion was maintained continuously for 15d until post mortem at 140d gestation. On the first day of the infusion fetal arterial blood (2.5ml) was collected at 1000h (-60 min) and 1100 h (0 min) prior to start of infusion. Throughout the infusion period, fetal arterial blood samples (2.5ml) were collected daily, between 0900h and 1100h, for blood gas and plasma hormone analysis as described in Chapters 2 and 3. For ACTH determination, blood (1.0ml) was aliquoted into EDTA coated tubes containing aprotinin (10μl, 100,000 KIU ml^{-1}, Sigma Chemical Co, St Louis, MO, USA). For cortisol and 11-desoxycortisol determination, blood (1.0ml) was aliquoted into heparin-coated tubes (125 IU). Blood samples were centrifuged at 1500 g for 10 min; the plasma was separated and frozen at -20°C until analysis. Blood (0.5ml) was analysed for fetal arterial PO_2, PCO_2, pH, sO_2 and Hb using an ABL 520 blood gas analyser (Radiometer, Copenhagen, Denmark).

4.2.3 Radioimmunoassays

ACTH

Fetal plasma immunoreactive ACTH concentrations were measured using a radioimmunoassay kit (ICN Biomedicals, NSW, Australia) which has been validated previously for use in fetal sheep plasma (McMillen et al., 1990) as described previously in Chapter 2.

Table 4.1: Summary of animals used in this study

	Tartaric Acid or saline infused controls	Metyrapone infused from 125d gestation
Surgery	14	10
Singleton (S) / Twin (T) pregnancies	S(9) T(2)	S(10)
Measurement of fetal plasma ACTH at 125-140d	6	7
Measurement of fetal plasma 11desoxycortisol at 125-140d	7	7
Measurement of fetal plasma cortisol at 125-140d	5	6
Measurement of widths of fetal adrenals at 140d	5	5
Measurement of morphometric analysis of fetal adrenals at 140d	6	6
Measurement of fetal pituitary POMC mRNA at 140d	6	8
Measurement of fetal adrenal expression of MC2-R mRNA at 140d	6	8
Measurement of fetal adrenal expression of StAR protein and content	7	7
Measurement of fetal adrenal expression of CYP11A1, CYP17, CYP21A1 and 11βHSD2 mRNA at 140d gestation	8	6
Measurement of fetal adrenal expression of 3βHSD mRNA at 140d gestation	7	7

11-Desoxycortisol

Fetal plasma samples were assayed for 11-desoxycortisol using a kit radioimmunoassay (ICN Biomedicals, NSW, Australia) validated for use in fetal sheep plasma as described previously in Chapter 2.

Cortisol

Fetal plasma samples were assayed for cortisol using a radioimmunoassay validated for use in fetal sheep plasma as described previously in Chapter 2.

4.2.4 Autopsy and Tissue Collection

At 140d ewes were killed using a lethal injection of sodium pentobarbitone (Lethabarb; 25ml at 325mg.ml $^{-1}$; Vibrac Australia, Peakhurst, NSW, Australia). The uterus was removed via hysterectomy and fetus removed by hysterotomy. Fetal weight, and crown rump length were measured. Fetuses were then decapitated and fetal organs including the adrenals and pituitary were removed and weighed. The whole left adrenal, half of the right adrenal and the anterior pituitary, separated from the neurointermediate lobe, were snap frozen in liquid nitrogen and stored at -80°C until Northern and western blot analyses. The other half of the right adrenal was fixed in 4% paraformaldehyde in 0.1M phosphate buffer (BDH Laboratories, Dorset, Poole, UK) for 24 h at 4°C, prior to rinsing in 0.1M phosphate buffered saline (2 x 30 min; Sigma Chemical Co, St Louis, MO, USA), dehydration in 70% ethanol before processing for embedding in paraffin wax for histological analysis.

4.2.5 Immunocytochemical localisation of 3βHSD

To determine the proportion of the adrenal gland comprised of the steroidogenic cells of the adrenal cortex, sections from the mid glandular region of the right adrenal from metyrapone (n=5) and vehicle infused fetuses (n=5) were stained with

antisera raised against 3βHSD. 3βHSD was localised using an immunostaining kit (HistoPlus, Zymed, South San Francisco, CA). The 3βHSD antisera was raised in rabbits against human 3βHSD (Doody et al., 1990) and was used at a concentration of 1:2000. Briefly, paraffin sections were immersed in xylene (2 x 15 min) before rehydration in decreasing concentrations of ethanol (100%, 90%, 70%; 1x 5 min for each). To reduce non-specific binding and background staining, slides were then pretreated with hydrogen peroxide (3%; 20 min; BDH Laboratory Supplies, Poole, UK) before the addition of a serum blocking solution (10 min; Zymed South San Francisco, CA, USA). The sections were incubated with the primary antibody overnight at 4°C. The slides were washed in tris buffer saline (TBS; 0.1M 132.2g Tris HCL, 19.4g Tris Base in 1L; Sigma, MO, USA) and then treated with a broad spectrum biotinylated secondary antibody at room temperature (10 min; Zymed Biotinylated Secondary Antibody, HistoPlus, Zymed, South San Francisco, CA, USA). Slides were then washed in TBS and incubated with a horseradish peroxidase-streptavidin conjugate (Zymed, South San Francisco, CA, USA). To identify positive staining, an immunopure metal enhanced diaminobenzidine substrate (DAB; Pierce, Rockford, IL) was used as the chromagen. DAB was left on each slide for 2 min before rinsing and counterstaining (Mayers Haematoxylin; Sigma Diagnostics, St Louis, USA). Slides were then dehydrated with increasing concentrations of ethanol (90%, 100%) and washed in xylene (3 x 15 min; BDH Laboratories, Poole, UK) before being mounted and coverslipped using DPX mounting medium (BDH Laboratories, Poole, UK).

4.2.6 Morphometric Analysis

Morphometric analysis was performed on sections taken from the mid glandular region of the right adrenal of metyrapone and vehicle infused animals. Serial

sections of each adrenal were cut (3-5μm). Sections were stained with haematoxylin and eosin. Briefly, paraffin wax was removed from the sections in xylene (2 x 15 min; BDH Laboratories, Poole, UK) and then the sections were rehydrated with decreasing concentrations of ethanol (100%, 90%, 75% and 50%). Slides were then stained with haematoxylin (5 min; Sigma Diagnostics, St Louis, MO, USA) to identify the cell nuclei, rinsed, dipped in ammonia, and then rinsed again. Slides were then stained with eosin (1 min; BDH Laboratories, Poole, UK) to identify the cytoplasm of the cells, then dehydrated with increasing concentrations of ethanol (90%, 100%) and washed in xylene (3 x 15 min; BDH Laboratories, Poole, UK) before being mounted and coverslipped using DPX mounting medium (BDH Laboratories, Poole, UK). The density of cell nuclei in the adrenal cortex (zona glomerulosa and zona fasciculata) and medulla were measured in vehicle (n=6) and metyrapone (n=6) infused fetuses using the following method. Firstly the width and length of a defined area were measured in pixels on the image analysis system at 40x magnification on an Olympus BHS microscope using a Panasonic KR222 camera connected to a VideoPro imaging software (Leading Edge, Adelaide, SA, Australia). The width and length were converted to micrometers by the multiplication of 0.2604 (40x magnification) as determined by the use of a haemocytometer to calculate the area. The total number of cell nuclei were counted in a defined area (6860- 17750 μm^2) in 10 random fields of view, at least 1mm apart at a magnification of 40x and analysed using image analysis software (VideoPro, Leading Edge, Adelaide, SA, Australia). For each animal the number of cell nuclei per μm^2 was calculated by the total number of nuclei in 10 defined areas divided by the total area.

The width of the fetal adrenals was measured in four separate sections from each of the vehicle (n=5) and metyrapone (n=5) infused fetuses using an image analysis

system (VideoPro; Leading Edge, Adelaide, SA, Australia). The diameter of the total adrenal was determined by measuring the distance from the edge of the adrenal capsule to the middle of the adrenal vein and multiplying by 2. The width of the adrenal cortex was determined by measuring the distance from the interface of the adrenal capsule and zona glomerulosa to the interface of the zona fasciculata and adrenal medulla. Finally the width of the adrenal medulla was determined by measuring the distance from the interface between the zona fasciculata and adrenal medulla to the edge of the adrenal vein. All data were collected as pixels. The data of the adrenal widths were transformed from pixels to micrometers by the multiplication of 5.1282 (2x magnification), as determined by the use of a haemocytometer.

4.2.7 RNA Isolation

Total RNA was extracted from ~100mg of tissue from each fetal adrenal gland and from the anterior lobe of each fetal pituitary gland using Sigma TriReagent method (TriReagent, Sigma-Aldrich Corp., Castle Hill, NSW, Australia) as described previously (McMillen et al., 2000; Coulter et al., 2002a). Tissue was homogenised in TriReagent (100mg / ml) using a Polytron PT-MR 3000 (Kinematica AC, Switzerland) at 30,000 rpm. Homogenised tissue was then incubated at room temperature for 5 min before 1-bromo 3- chloropropane (BCP; 0.1 ml /1 ml) was added. Samples were mixed and incubated for another 15 min at room temperature. Samples were then centrifuged at 12,000 g at 4°C for 15 min and the top aqueous layer, containing the RNA was transferred into sterile eppendorf tubes. The RNA was precipitated from the aqueous phase by adding isopropranolol (0.5:1 v/v Trireagent) and centrifuging at 12,000 g at 4°C for 10 min. The supernatant was removed and the RNA pellet that remained was washed with 75% ethanol and air-

dried. RNA was reconstituted in 10-20µl of sterile water. The quality and quantity of the RNA was tested using a spectrophotometric analysis at 260 and 280nm (Biophotometer, Eppendorf, Hamburg, Germany).

4.2.8 cDNA and Oligonucleotide probes

POMC mRNA was detected using a 400bp ovine POMC cDNA probe (van de Pavert et al., 1997). Adrenal steroidogenic enzymes were detected using probes to human CYP11A1 cDNA (1.82Kb) (Chung et al., 1986), bovine CYP17cDNA (1.2Kb) (Zuber et al., 1986), human 3βHSD cDNA (435 bp) (Lorence et al., 1990), and human CYP21 cDNA probe (1.14Kb) (Chung et al., 1985). 11βHSD2 mRNA was detected using a 45-mer oligonucleotide probe for ovine 11βHSD2, complementary to nucleotides 1066-1110 (Campbell et al., 1996). StAR mRNA was detected by an ovine StAR cDNA probe (404 bp) (Juengel et al., 1995) and the MC2-R mRNA was detected using an ovine MC2-R cDNA (670 bp), generously donated by Dr Kathleen Mountjoy, University of Auckland. cDNA probes were radiolabelled by the random priming method with α-P^{32} deoxycytidine triphosphate (3000Cimmol.L^{-1}; PerkinElmer Life Sciences, Rowville, Vic, Australia) and Klenow fragment (6.4Uμl^{-1}) using an oligolabelling kit (Pharmacia, North Ryde, NSW, Australia). An antisense oligonucleotide probe complementary to the coding nucleotides of 151-180 of rat 18S rRNA (Chan et al., 1984) was used to confirm equal loading of RNA into each lane. The 11βHSD2 and 18S probes were end-labelled with γ-[^{32}P] ATP (4000Cimmol^{-1}, PerkinElmer Life Sciences, Rowville, Vic, Australia) using T4 polynucleotide kinase (7.9Uμl^{-1}; Pharmacia, North Ryde, NSW, Australia). cDNA and oligonucleotide probes were purified using NICK Sephadex G-50 column (Pharmacia, North Ryde, NSW, Australia).

4.2.9 Northern Blot analysis

Total RNA (20μg) was denatured by adding 2.2M deionised formaldehyde; and 50% deionised formamide and incubating at 55°C for 15 min. RNA was then separated on a 1% formaldehyde agarose gel by electrophoresis with a running solution of 20x buffer (0.1M 3-(N-morpholino) propanesulfonic acid, 40nM sodium acetate, and 5mM EDTA disodium salt; pH 7.0). RNA markers were run in the gel along side total RNA samples to determine the size of the specific RNA bands that was measured. Separated RNA was transferred to Zetaprobe membrane (Biorad, Richmond, CA, USA) via the capillary method with 10 x SSC as the transfer fluid. Membranes were then washed in 10x SSC (1 x SSC: 0.15M Sodium chloride, 15mM sodium citrate) and then baked in the oven at 80°C for 30 min. Membranes were labelled and stored at -20°C until hybridisation.

4.2.10 Hybridisation of membranes

Membranes were prehybridised for 2h prior with a buffer consisting of 50%v/v de-ionised formamide; 5 x SSPE, 7% SDS and 0.1mg.ml^{-1} of denatured herring sperm DNA (Boheringer Mannheim, Mannheim, Germany) at 42°C. A fresh solution of hybridisation buffer was then added to the membrane along with the respective cDNA or oligo probe. Membranes were hybridised for 16h with each cDNA probe before washing the membrane in 1 X SSC, 0.1% SDS solution at 42°C for 30 min. This wash was then repeated with membranes washed in 1 X SSC, 0.1% SDS solution at 42°C for either 15 or 30 min. Membranes were then allowed to dry, sealed in a plastic bag and exposed to a blank Fuji Bas-IIIs Phosphoimager plate (Fuji Photo Co, Tokyo, Japan) for 14-28 days. When probing for 18S, membranes were hybridised at 16h before being washed in 1XSSC, 0.1% SDS solution at 52°C for 30 min to 2h. Membranes were then washed in either 1xSSC, 0.1% SDS

solution at 52°C for 90 min or 0.1XSSC, 0.1%SDS solution at 58°C for 60 min. When required membranes were washed a third time in 0.1XSSC, 0.1%SDS solution at 58°C for 30 min. After washing, membranes were allowed to dry, sealed in a plastic bag and exposed to a blank Fuji Bas-IIIs Phosphoimager plate (Fuji Photo Co, Tokyo, Japan) from 30 min to 2d. Autoradiographs of membranes were visualised using a Fuji-MacBas Phosphoimager (Fuji MacBas, Tokyo, Japan), and intensity of the signal was quantified using Fuji Image Gauge software (V3.46).

4.2.11 Western Blot Analysis for StAR protein

The content of StAR protein in fetal adrenal extracts from metyrapone and vehicle infused fetuses was determined by western blot analysis essentially as described previously (Coulter et al., 1996b; Coulter et al., 2002a). The StAR antibody was generously provided by Dr D.B. Hales (University of Illinois, Chicago, USA) and has been fully characterised and shown to detect the mature 30kDa form of StAR protein by a range of species including sheep, human, rat and mouse adrenals and/or gonads (Juengel et al., 1995; Lehoux et al., 1999; Fritz et al., 2001). In brief, the western blot analysis was performed on adrenal extracts (50ug protein) using a rabbit polyclonal mouse StAR antibody (1:500), overnight at 4C followed by a Horseradish Peroxidase labelled rabbit IgG (1:1000) and detected using an amplified Opti-CN® kit (Bio-RAD, Richmond, CA, USA). The western blot membrane was analysed using a Densitometer (GS-710 Calibrated Imaging Densitometer, Bio-RAD) and quantified using Image-Analysis Software (Quantity-One 4.2.1, BioRAD) and data expressed as arbitrary densitometric units (AU) of StAR protein per µg of total protein.

4.2.12 Statistical Analysis

All data are expressed as mean ± SEM. The ratios of plasma cortisol: ACTH, and plasma cortisol: 11-desoxycortisol at each gestational age were used as a marker to determine the effectiveness of the metyrapone suppression of the 11β-hydroxylase enzyme. Hormone data were log transformed, where required, to reduce heterogeneity of variance as determined by the use of Bartlett and Cochrans tests. Plasma ACTH, 11-desoxycortisol and cortisol values and the ratios of plasma cortisol: ACTH, and plasma cortisol: 11-desoxycortisol between 125d and 140d were analysed using a multifactorial ANOVA with repeated measures, with age and treatment as the specified variables using SPSSX (Chicago, IL, USA) on a VAX mainframe computer system. When a significant interaction between major factors was identified by ANOVA, the data were split on the basis of the interacting factor and reanalysed.

The morphometric measurements of the adrenal glands (cell density, adrenal width, adrenocortical and adrenomedullary widths) were compared between the metyrapone and vehicle treated groups using a Students's unpaired t test.

The ratios of pituitary POMC mRNA : 18S rRNA and adrenal MC2-R mRNA, StAR mRNA, 11βHSD2 mRNA, CYP11A1 mRNA, CYP17 mRNA, 3βHSD mRNA and CYP21A1 mRNA :18S rRNA and amount of adrenal StAR protein were also compared between the vehicle and metyrapone infused groups using a Student's unpaired t-test.

The relationships between plasma ACTH, 11-desoxycortisol and cortisol concentrations, measured on the day closest to autopsy and adrenal CYP11A1

mRNA, and CYP17 mRNA expression were determined using linear regression analysis (SPSSX, Chicago, IL, USA). A probability of 5% was taken to be significant ($P< 0.05$).

4.3 RESULTS

4.3.1 Fetal plasma ACTH, cortisol and 11 desoxycortisol concentrations

Before the start of the infusion, there was no significant difference in plasma ACTH, 11-desoxycortisol and cortisol concentrations between fetuses assigned to the vehicle and metyrapone infused groups. Plasma ACTH concentrations were significantly higher ($F=5.9$; $P<0.05$) in metyrapone than vehicle infused fetuses between 126d and 140d (Figure 4.1a). In both metyrapone and vehicle groups fetal plasma ACTH concentrations were significantly higher ($F=7.2$; $P<0.005$) at 136-140d gestation when compared with values between 126-135d gestation.

After the start of the infusion plasma 11-desoxycortisol concentrations were significantly higher ($F=25.0$; $P<0.0001$) in the metyrapone when compared to the vehicle infused fetuses. In vehicle infused fetuses there was a significant ($F=15.9$, $P<0.001$) and progressive increase in plasma 11-desoxycortisol concentrations with increasing gestational age. In metyrapone infused fetuses plasma 11-desoxycortisol concentrations were significantly higher ($F=27.7$, $P<0.001$) at 136-140d than any other time during the infusion period. (Figure 4.1b).

During the infusion period there was no significant difference between plasma cortisol concentrations in the metyrapone and vehicle infused fetuses between 126d and 140d gestation. Plasma cortisol concentrations in both groups however, increased with increasing gestation (Figure 4.1c).

The ratio of plasma cortisol : 11-desoxycortisol concentrations was significantly lower (F=120.7 ; P<0.0001) in the metyrapone infused fetuses (126-130d, 1.3 ± 0.6; 131-135d, 2.2 ± 1.1; 136-140d, 1.6 ± 0.3) than in the vehicle infused group (126-130d, 4.9 ± 1.0; 131-135d, 8.8 ± 1.8; 136-140d, 12.9 ± 2.2) between 126d and 140d gestation. In the vehicle infused group there was a significant (F= 42.7, P<0.001) increase in the plasma cortisol : 11-desoxycortisol ratio during the infusion period, where plasma cortisol : 11-desoxycortisol ratio was highest at 136-140d than at any other time during the infusion. In addition, the plasma cortisol : 11-desoxycortisol ratio was significantly higher at 131-135d than at 126-130d in the vehicle infused group. In the metyrapone group the plasma cortisol : 11-desoxycortisol ratio was significantly higher (F= 5.9, P<0.005) at 130-140d when compared with earlier in gestation.

There was no difference, in the ratio of the plasma cortisol : ACTH concentrations between the metyrapone and vehicle infused fetuses during the infusion period. The ratio of plasma cortisol: ACTH concentrations was significantly higher (F=58.6; P<0.0001), between 136-140d than at any time between 120-135d gestation in both metyrapone and vehicle infused fetuses (Figure 4.2).

4.3.2 Fetal outcome, adrenal weight and adrenal morphometry

There was no significant difference in mean fetal body weight (Figure 4.3a) or crown rump length between the metyrapone infused (4.48 ± 0.36 kg and 56.3 ± 1.6 cm; n=8) and vehicle infused fetal sheep (4.38 ± 0.18 kg and 55.3 ± 0.9 cm; n=12). The combined adrenal weight was significantly greater (P<0.001) in the metyrapone infused (0.83 ± 0.06g, n=8) than in the vehicle infused (0.43 ± 0.03g, n=14) fetuses. Total adrenal weight as a proportion of body weight was also significantly greater

(P<0.001) in the metyrapone infused than in the vehicle infused fetuses (Figure 4.3b). There was a significant positive correlation between adrenal weight and adrenal diameter in both vehicle and metyrapone infused groups (Figure 4.3d). The adrenal cortex, but not adrenal medulla, was significantly thicker (P<0.05) in the metyrapone infused (n=5) than vehicle infused (n=5) fetuses (Figure 4.4). There were significantly fewer cell nuclei/μm^2 (P<0.01) in the zona fasciculata of adrenals from the metyrapone infused group (n=6) when compared with the vehicle infused controls (n=6,Figure 4.5). There was no difference, however, in the density of the cell nuclei/ μm^2 in either the zona glomerulosa or the adrenal medulla between the metyrapone and vehicle infused groups (Figure 4.5).

4.3.3 Pituitary POMC mRNA

A single transcript (1.4Kb) was detected for POMC mRNA in the anterior pituitary from metyrapone and control fetuses. The relative expression of POMC mRNA: 18S rRNA was 4-5 fold higher (P<0.005) in the anterior pituitary of metyrapone infused fetuses (5120.9 ± 866.0, n=7) when compared to vehicle infused controls (1604.3 ± 485.3, n=8).

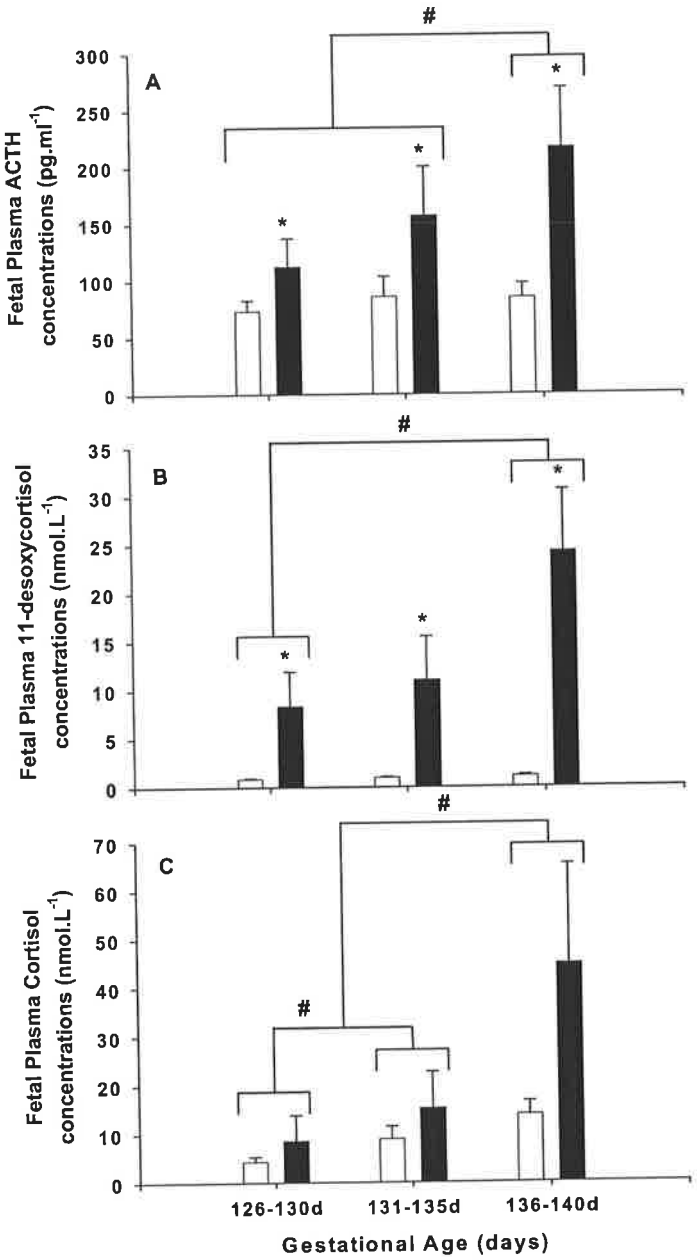

Figure 4.1: The effect of metyrapone or vehicle infusion from 125d on plasma ACTH, 11-desoxycortisol and cortisol concentrations between 126-140d gestation.

Plasma ACTH (A) and 11-desoxycortisol (B) concentrations were higher in metyrapone infused fetuses (dark bars) when compared with vehicle (open bars) infused controls. There was no difference in plasma cortisol (C) concentrations between the two groups. * denotes hormone values which are higher (P<0.05) in metyrapone than in the vehicle infused group. # denotes hormone values between 136-140d gestation which were higher (P<0.05) than values between 126-135d gestation in both groups.

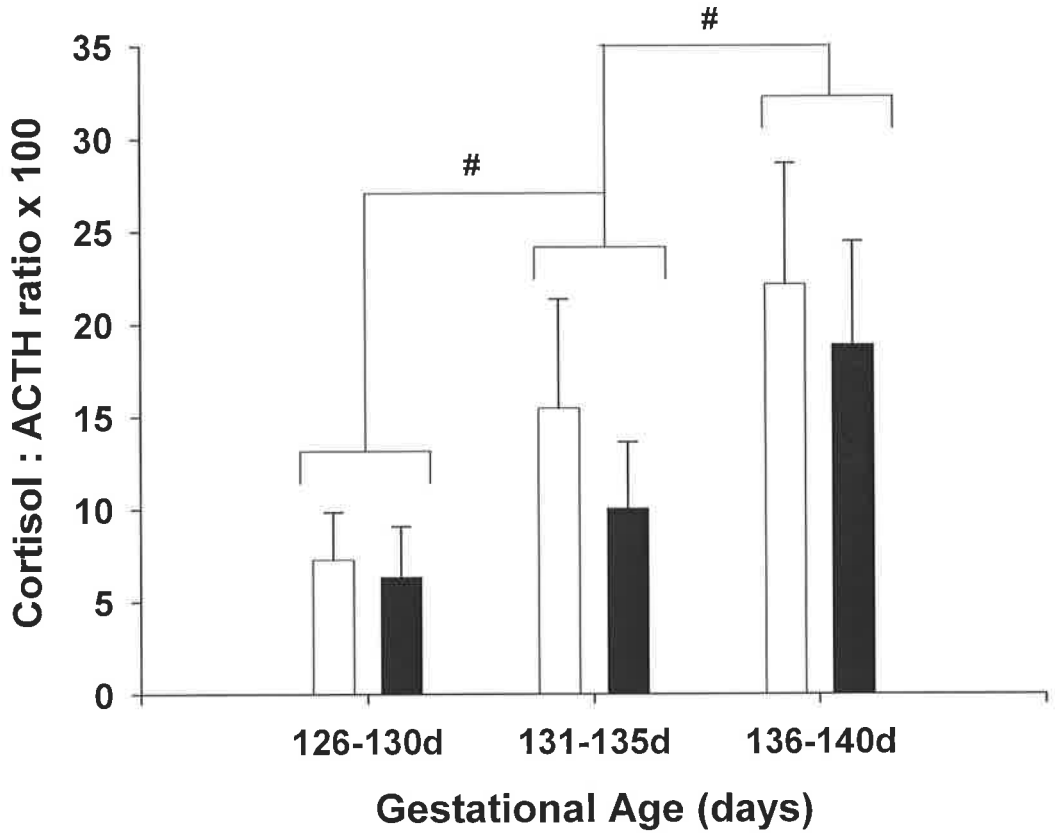

Figure 4.2: The effect of metyrapone or vehicle infusion from 125d on the plasma cortisol : ACTH ratio during the infusion period, from 126 - 140d gestation.

There was a significant increase in the plasma cortisol: ACTH ratio in both vehicle (open bars) and metyrapone (dark bars) infused fetuses. There was no difference between the two groups. # denotes mean ratios which were higher ($P<0.05$) than values at 126-130d gestation.

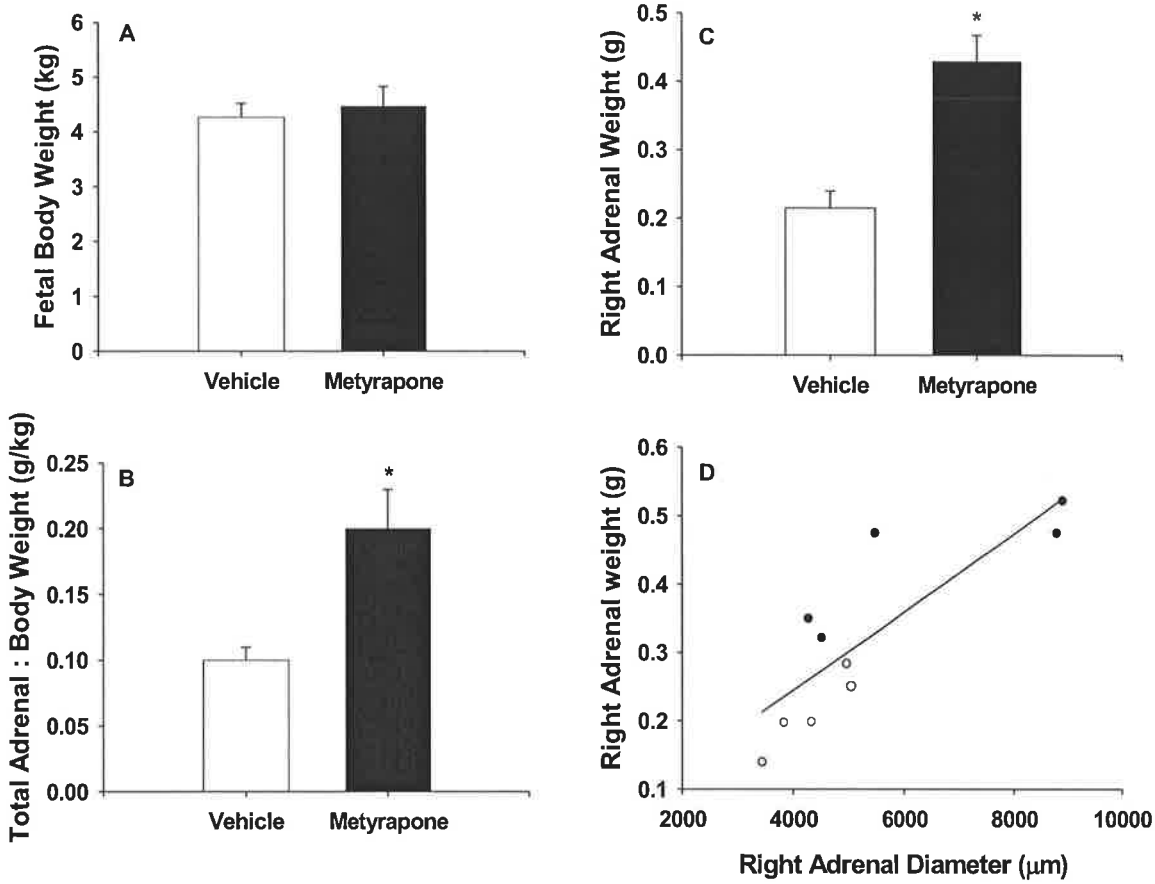

Figure 4.3: The effect of metyrapone or vehicle infusion from 125-140d on fetal body weight, relative combined adrenal weight, right adrenal weight and the relationship between right adrenal weight and diameter in vehicle and metyrapone infused fetuses.

There was no difference between the body weight of metyrapone (dark bars) and vehicle infused (open bars) fetuses. Metyrapone infused fetuses had significantly higher combined adrenal weight (B) and higher right adrenal weight (C) compared to vehicle infused controls. There was a significant relationship between right adrenal weight and diameter (D) in vehicle (open circles) and metyrapone (dark circles) infused fetuses * denotes differences ($P<0.05$) between metyrapone and vehicle infused fetuses.

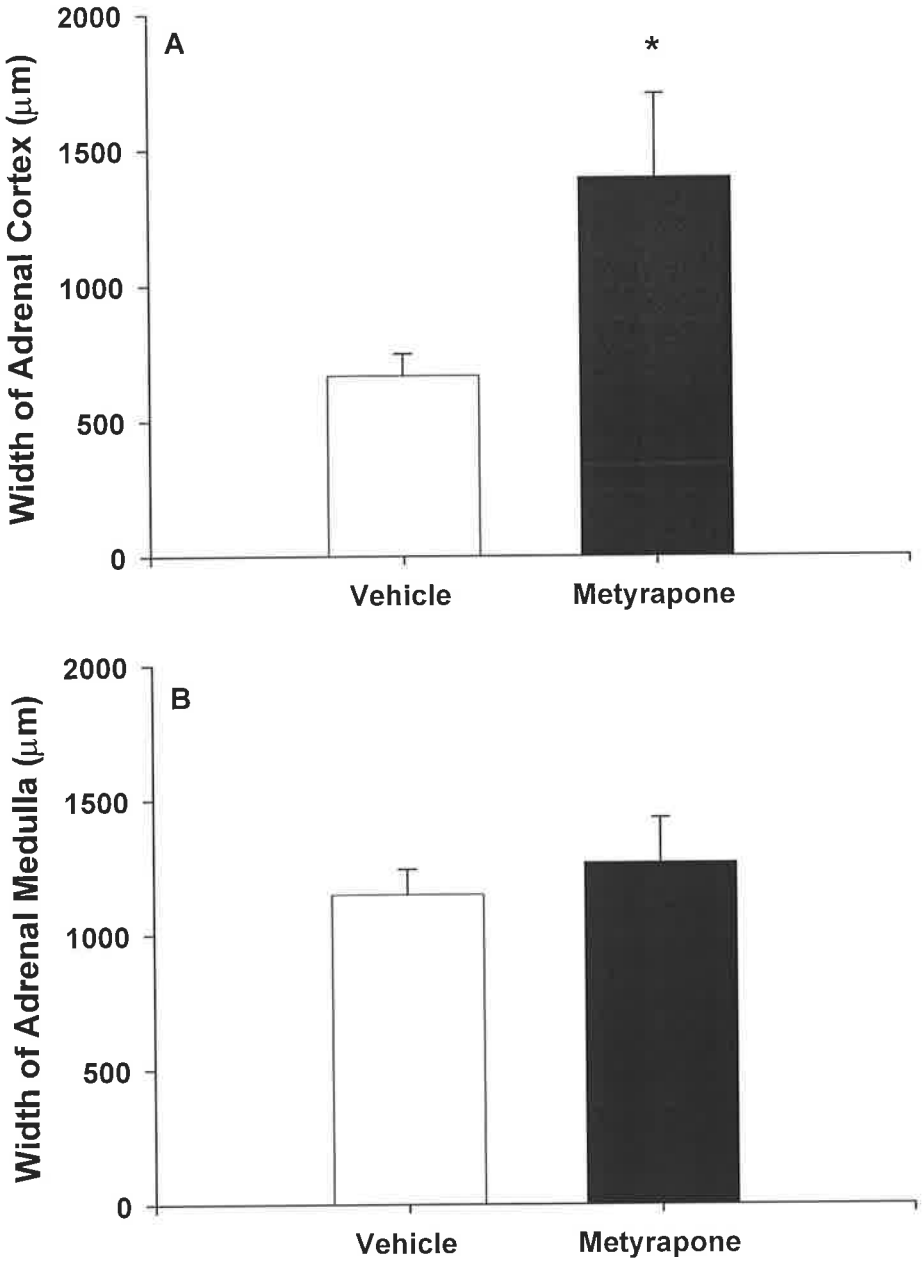

Figure 4.4: The effect of metyrapone or vehicle infusion on the width of the fetal adrenal cortex and medulla.

The width of the adrenal cortex (A) from metyrapone (dark bars) infused fetuses was significantly higher than the width from vehicle (open bars) infused controls. There was no difference in the width of the fetal adrenal medulla (B) between the two groups. * denotes differences ($P<0.05$) between metyrapone and vehicle infused fetuses.

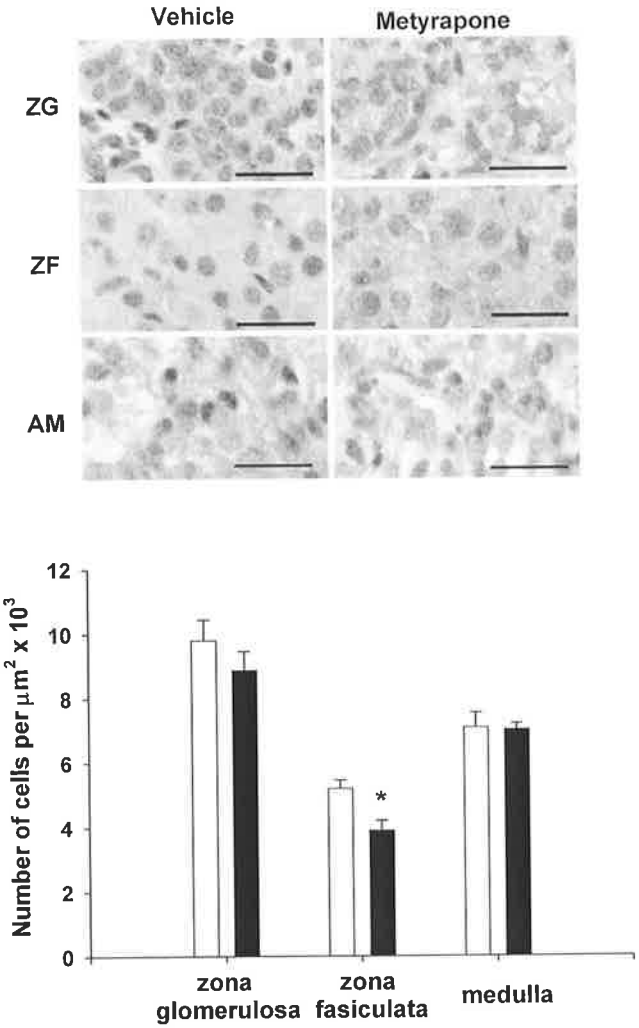

Figure 4.5: The effect of metyrapone or vehicle infusion on the number of cells in the fetal adrenal cortex.

The top panel shows photomicrographs of the zona glomerulosa (ZG), zona fasciculata (ZF) and adrenal medulla (AM) from a vehicle and metyrapone infused fetus. The bottom graph shows the number of nuclei/μm^2 in the zona glomerulosa, zona fasciculata and adrenal medulla of the fetal adrenal in vehicle (open bars) and metyrapone (dark bars) infused fetuses. * denotes differences (P<0.05) between metyrapone and vehicle infused groups. The scale bar represents 25 μm.

4.3.4 Adrenal StAR mRNA and Protein Content

A single transcript (3.0Kb) for StAR mRNA was detected in all fetal adrenals and the relative expression of StAR mRNA was significantly lower ($P<0.05$) in the metyrapone infused group than in vehicle infused controls (Figure 4.6a). The mature 30kDa StAR protein band was detected by western blot analysis in the protein extracts of adrenal glands from metyrapone and vehicle infused fetal sheep (Figure 4.6b). The amount of the mature 30kDa StAR protein in fetal adrenals was significantly greater ($P<0.000$) in the adrenal glands from metyrapone (n=7, 1.06 ± 0.08 arbitrary units (AU)/µg adrenal protein) and vehicle (n=7, 0.47 ± 0.06 AU/µg adrenal protein) infused fetal sheep (Figure 4.6c).

4.3.5 Adrenal MC2-R, CYP11A1, CYP17, 3βHSD, CYP21 mRNA

A major (3.5Kb) and a minor (2.0Kb) transcript were detected for MC2-R mRNA. There was no difference in the relative expression of the major MC2-R mRNA transcript between the metyrapone (72.4 ± 13.8, n=8) and vehicle infused fetuses (53.2 ± 6.8, n=6). A single transcript (1.8Kb) was detected for 11βHSD2 mRNA. The relative expression of 11βHSD2 mRNA : 18S rRNA was significantly higher ($P<0.05$) in metyrapone infused fetuses (n=8) when compared with vehicle infused controls (n=6, Figure 4.7a). Single transcripts for adrenal CYP11A1 mRNA (2.0Kb), for CYP17 mRNA (2.2Kb) and 3βHSD mRNA (1.8Kb) were detected and two major transcripts (2.2Kb and 1.8Kb) were detected for adrenal CYP21A1 mRNA. The relative expression of adrenal CYP11A1 mRNA : 18S rRNA and CYP 17 mRNA:18S rRNA were significantly higher ($P<0.05$) in the metyrapone infused fetuses than in vehicle infused controls (Figure 4.7b&c respectively). There was no difference, however, in the relative expression of 3βHSD mRNA: 18sRNA or CYP21A1 (2.2Kb + 1.8Kb) mRNA : 18sRNA ($P=0.09$) between metyrapone (3βHSD 73.8 ± 3.3, n=7;

CYP21A1 639 ± 80.4, n=6) and vehicle infused fetuses (3βHSD 69.9 ± 6.0, n=7; CYP21A1 451.7 ± 49.1, n=8).

When the groups were combined there was a significant positive correlation between plasma ACTH concentrations and the relative expression of CYP11A1 mRNA (CYP11A1 mRNA = 4.95 (ACTH) + 1397.8; $P<0.0001$, r=0.861) and CYP17 mRNA (CYP17mRNA = 5.77(ACTH) + 781.8; $P<0.005$, r=0.713).

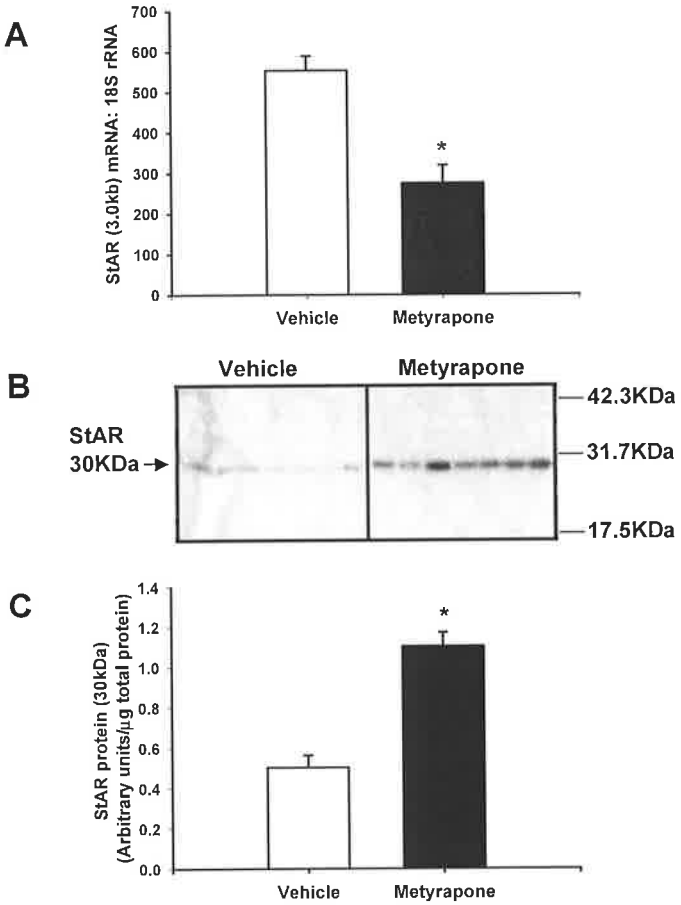

Figure 4.6 : The effect of metyrapone or vehicle infusion from 125d on the relative expression of StAR protein and mRNA.

Panel A shows the relative expression of StAR (3.0Kb) mRNA:18S rRNA in vehicle (n=6; open bars) was higher than the relative expression of StAR mRNA : 18S rRNA in metyrapone infused (n=8; dark bars) fetuses. Panel B shows a western blot analysis of the StAR protein (30kDa) in adrenals from vehicle (n=7) and metyrapone infused (n=7) fetal sheep at 140d gestation. Panel C shows the relative expression of the StAR 30kDa protein in was lower in vehicle (n=7) when compared to metyrapone infused (n=7) fetuses. * denotes differences ($P<0.05$) between metyrapone and vehicle infused fetuses.

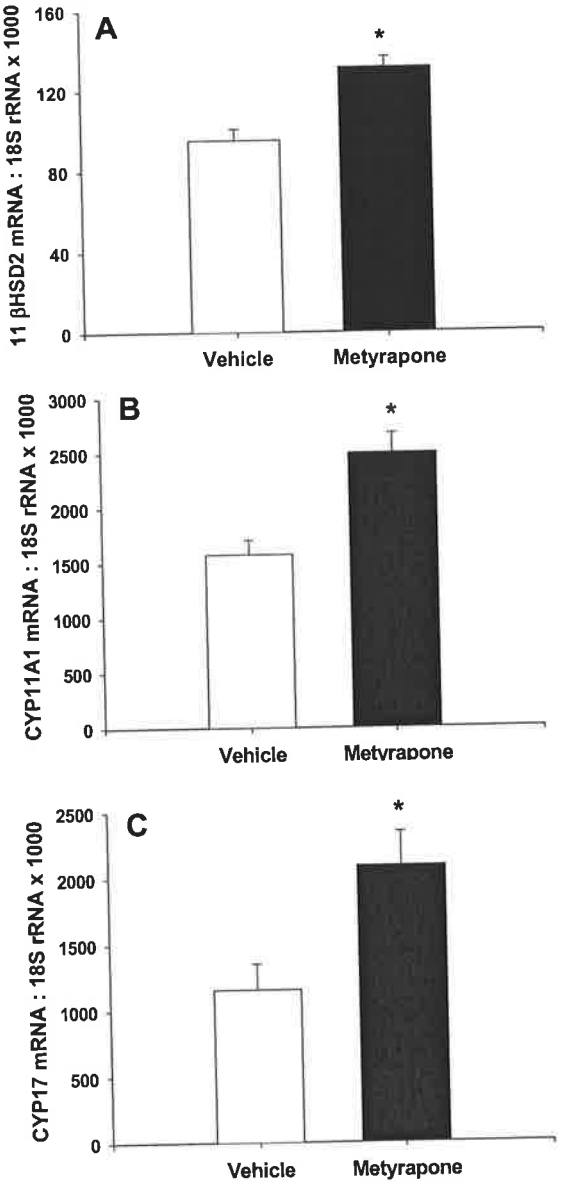

Figure 4.7: The effect of metyrapone or vehicle infusion from 125d gestation on the relative expressions of 11βHSD2 mRNA: 18S rRNA, CYP11A1 mRNA : 18S rRNA and CYP17 mRNA : 18S rRNA in the fetal adrenal at 140d gestation.

The relative expressions of 11βHSD2 (1.8kb) mRNA : 18S rRNA (A) and CYP11A1 (2.0kb) mRNA : 18S rRNA (B) in vehicle infused fetuses (n=6; open bars) was significantly lower than metyrapone infused (dark bars; n=8) fetuses. The relative expression of CYP17 (2.2kb) mRNA : 18S rRNA (C) in vehicle (n=7) infused fetuses was also significantly lower than metyrapone infused (n=8) fetuses. * denotes differences ($P<0.05$) between metyrapone and vehicle infused fetuses.

4.4 DISCUSSION:

In the present study, we have investigated the effects of high fetal ACTH concentrations on fetal adrenal MC2-R, StAR and steroidogenic enzyme mRNA expression and adrenal StAR protein expression in the presence of metyrapone, an inhibitor of endogenous cortisol biosynthesis. Metyrapone infusion from 125d gestation resulted in a significant increase in fetal plasma ACTH concentrations between 126 and 140d gestation and in POMC mRNA expression in the fetal anterior pituitary at 140d gestation. Whilst pituitary POMC mRNA levels were increased and circulating ACTH concentrations were higher throughout late gestation in the metyrapone infused group, fetal plasma cortisol concentrations were not different between metyrapone and vehicle infused fetuses. This indicates that the fetal hypothalamo-pituitary axis has been stimulated to increase pituitary ACTH synthesis and secretion in order to overcome the inhibition of the hydroxylation of 11-desoxycortisol to cortisol within the fetal adrenal. The maintenance of the increased ACTH concentrations in the presence of normal circulating cortisol concentrations is interesting and suggests that the interaction between the fetal pituitary and adrenal in the metyrapone infused fetuses is not in steady state. One possibility is that as ACTH increases to the level required to overcome the steroidogenic enzyme block within the adrenal, fetal cortisol concentrations increase and act in turn to suppress ACTH. In the presence of metyrapone, any fall in ACTH, however, would be followed by a fall in adrenal cortisol output, a restimulation of the fetal pituitary and a consequent increase in fetal ACTH and a restoration of fetal cortisol concentrations.

4.4.1 MC2-R and Steroidogenic enzyme mRNA Expression

Interestingly, we found that there was a gestational increase in the ratio of plasma cortisol : ACTH concentrations in both the metyrapone and vehicle infused fetal sheep after 136d gestation. This indicates that there is an increase in the adrenal responsiveness to the steroidogenic actions of ACTH in both treatment groups. Studies in cultured bovine adrenocortical cells have shown that ACTH stimulates the expression of its own receptor (Le Roy et al., 2000) and there is increased ACTH binding and ACTH-induced adenylate-cyclase activity in the fetal sheep adrenal gland with increasing gestational age (Durand et al., 1985). In the present study, whilst plasma ACTH concentrations were higher in metyrapone fetuses between 126 and 140d gestation, there was no difference in adrenal MC2-R mRNA levels between metyrapone and vehicle infused fetuses. Simmonds and colleagues (Simmonds et al., 2001) demonstrated that the expression of MC2-R mRNA in the fetal sheep adrenal was unchanged after fetal hypophysectomy or intrafetal ACTH infusion. In a previous study, we also showed that there was no significant change in the expression of MC2-R between 125 and 140d gestation (Coulter et al., 2002b). Whilst an increase in adrenal expression of MC2-R mRNA in both metyrapone and vehicle infused fetuses may precede the increase in adrenal responsiveness to ACTH from 136d gestation, it appears that such an increase is not directly related to circulating ACTH concentrations once they exceed those present in the circulation of the vehicle infused group in late gestation.

There were significant increases, however, in the expression of adrenal CYP11A1 and CYP17 mRNA, but not 3βHSD or CYP21A1 mRNA in the metyrapone infused fetuses. Previously, it has been shown that adrenal CYP11A1, CYP17 and 3βHSD mRNA levels decrease after fetal hypophysectomy and are restored following ACTH

replacement (Tangalakis et al., 1990; Simmonds et al., 2001). In the present study, we also showed that plasma ACTH concentrations were correlated significantly with adrenal expression of CYP11A1 and CYP17 mRNA. It is therefore likely that the increases in the mRNA levels of adrenal CYP11A1 and CYP17 in the metyrapone infused fetuses are a consequence of the increase in fetal ACTH concentrations. The lack of a difference between 3βHSD mRNA expression in the metyrapone and vehicle infused fetuses may reflect that maximal stimulation of this enzyme has occurred at the levels of ACTH present in the vehicle infused fetuses in late gestation. This is consistent with evidence that intrafetal ACTH infusion increases the expression of 3βHSD mRNA at 132d but not at term (Simmonds et al., 2001).

We also found no significant difference in the expression of CYP21A1 in the adrenals from the metyrapone and vehicle infused fetuses. It has been shown that intrafetal ACTH infusion does not stimulate adrenal expression of CYP21A1 mRNA indicating that this enzyme may not be responsive to the increase in circulating ACTH which occurs in late gestation (Tangalakis et al., 1990) or after metyrapone infusion. Thus in the metyrapone infused group, the fetal HPA axis is reset to maintain the increase in circulating ACTH concentrations which is required to stimulate fetal adrenal steroidogenesis and overcome the metyrapone induced block of cortisol biosynthesis.

A positive relationship was found between the weight and diameter of the right adrenal. This was expected and validates the methods used to determine adrenal growth. In the metyrapone infused fetal sheep there was a doubling of adrenocortical thickness and a ~20% increase in cell size within the zona fasciculata. Metyrapone treatment for 3-7 days similarly resulted in an increase in

adrenal growth and an increase in adrenocortical cell size of the primate fetus (Coulter et al., 1996a). The most likely factor responsible for the increased adrenocortical growth in the metyrapone infused fetuses is ACTH, although it is also possible that an increase in adrenal steroids proximal to the metyrapone imposed block may have also contributed to increase in adrenocortical growth. Whilst adrenocortical growth was increased markedly in the metyrapone infused group, plasma cortisol concentrations were similar in both groups. This suggests that cortisol production may be relatively decreased from the adrenocortical cells of the enlarged adrenal in the metyrapone treated group.

There is substantial evidence that ACTH mediates its actions on adrenal growth through the insulin-like growth factor (IGF) system (Mesiano et al., 1993). IGFs have found in abundance in the fetal sheep adrenal (Han et al., 1992). Infusion of ACTH or cortisol into fetal sheep from 125d gestation, significantly reduced IGF-II mRNA and protein expression in the fetal adrenal (Lu et al., 1994) suggesting that local cortisol production may decrease IGF-II expression. Results from a further study using animals from this thesis, found there was no difference in the fetal adrenal expression of IGF-II, IGF-I receptor or IGF-BP2 mRNA between vehicle and metyrapone infused groups (Warnes et al., 2004). There was however, a decrease in the expression of the cell cycle rate limiting enzyme cyclin-D1 mRNA. The hypertrophic growth of the metyrapone infused adrenals occurred without an activation of the IGF system.

The size of the adrenal medulla was not different between the metyrapone and vehicle infused groups. This is consistent with previous studies which have

demonstrated that growth of the fetal adrenal medulla in late gestation is not regulated by the fetal pituitary (Coulter et al., 1989; Coulter et al., 1991).

4.4.2 11βHSD2 mRNA expression

The relative expression of 11βHSD2 mRNA was higher in metyrapone infused fetuses when compared with vehicle infused controls. Previously we reported that 11βHSD2 mRNA expression in the fetal adrenal decreased coincident with the prepartum increase in adrenocortical growth and steroid output and suggested that this would result in an increasing adrenocortical exposure to endogenously generated glucocorticoids (McMillen et al., 2000). Furthermore, infusion of cortisol into fetal sheep at a stage in gestation when ACTH and cortisol concentrations are normally low (ie. between 109 and 116d gestation) resulted in a specific decrease in 11βHSD2 mRNA expression in the fetal adrenal, suggesting that cortisol may act to decrease the expression of the enzyme which regulates its metabolism within the fetal adrenal (Ross et al., 2000). The higher 11βHSD2 mRNA levels in the adrenals of metyrapone infused fetuses compared with the control group would be consistent with a reduction in intracellular cortisol concentrations within the adrenocortical cells of the metyrapone infused fetuses.

4.4.3 StAR mRNA expression and protein content

There was a differential effect of metyrapone administration on adrenal StAR mRNA and protein levels. The regulation of StAR mRNA expression is complex and determined by the balance between transcription and mRNA turnover, each of which in turn are regulated by multiple factors (Jefcoate, 2002). Previous studies have shown that ACTH stimulates the expression of StAR mRNA in adrenal cells *in vitro* and *in vivo* via a cyclic AMP dependent mechanism (Penhoat et al., 1989; Lebrethon

et al., 1994b; Stocco, 1999; Le Roy et al., 2000) and it has also been shown that there is an ontogenetic increase in StAR mRNA and protein in the fetal sheep adrenal between 125 and 140d gestation (Coulter et al., 2002a). *In vitro* studies have also shown, however, that glucocorticoids have a direct role in enhancing the action of phorbol ester on StAR mRNA levels in adrenocortical cell lines (Feltus et al., 2002). Interestingly concurrent infusion of metyrapone with ACTH (1-24) in fetal sheep, *in vivo*, prevented the cAMP accumulation measured following ACTH stimulation of fetal sheep adrenal cells *in vitro* (Lye & Challis, 1984). One possibility therefore is that whilst circulating ACTH concentrations are higher in the metyrapone treated group, the actions of ACTH on adrenal StAR mRNA expression are limited by relatively lower intracellular cortisol concentrations within the fetal adrenal cortex in this group. Whilst there is a decrease in the steady state levels of StAR mRNA levels, there was a significant increase in the 30kD mature form of the StAR protein in the adrenals of the metyrapone infused fetuses. It has been shown that mature StAR 30kDa protein is derived from a 37kDa precursor, which is phosphorylated in response to cAMP and then processed in the mitochondria to the phosphorylated 30kDa form (Jefcoate, 2002). The observation of the dissociation of the effects of metyrapone on StAR mRNA and protein expression *in vivo* is novel and may indicate that the stimulatory actions of ACTH on StAR transcription, mRNA stability and post-translational modification may in part depend on intracellular cortisol concentrations within the adrenocortical cell.

4.4.4 Summary

In summary, metyrapone administration in the late gestation sheep fetus results in changes consistent with a stimulation of the adrenal cortex by ACTH and an increase in the intracellular metabolism of cortisol. This may represent a unique

model which allows the dissociation of the relative actions of ACTH and cortisol on fetal adrenal steroidogenesis and growth during late gestation.

5 Summary and Final Conclusions

This thesis aimed to investigate the role of cortisol in the regulation of arterial blood pressure during late gestation in the fetal sheep. Whilst previous studies have attempted to address this question, the methods used have been limited. This study investigated the specific relationship between fetal plasma cortisol concentrations and fetal arterial blood pressure. It is also the first study to show that the timing and amount of fetal cortisol plays an important role in the normal development of the fetal cardiovascular system. In addition, this thesis used a unique model which allows the dissociation of the relative actions of ACTH and cortisol on fetal adrenal steroidogenesis and growth during late gestation.

5.1 METYRAPONE INFUSION AND FETAL CARDIOVASCULAR SYSTEM

The first hypothesis of this thesis was that suppression of cortisol synthesis from 125d gestation, when endogenous cortisol concentrations are low, would decrease fetal arterial blood pressure. Surprisingly, we found that from 126d gestation, whilst fetal plasma ACTH and 11-desoxycortisol concentrations were elevated, there was no significant difference in fetal cortisol concentrations between the metyrapone and vehicle infused groups. The increased ACTH and 11-desoxycortisol levels are consistent with a decrease in plasma free cortisol concentrations due to increased negative feedback and suggests either that the cortisol negative feedback is sensitive to very small changes in circulating cortisol concentrations and/or that cortisol may have significantly decreased earlier than 24h after the start of the metyrapone infusion. Despite not measuring a significant decrease in plasma cortisol concentrations at 126-128d gestation, fetal arterial blood pressure was

significantly decreased 24-48h after the start of metyrapone infusion from 125d gestation. Fetal plasma cortisol concentrations are normally low at 125d gestation and hence the actions of cortisol on arterial blood pressure would be most likely through the high affinity MR rather than the low affinity GR. I propose that when metyrapone was infused from 125d gestation there was a significant decrease in fetal cortisol concentrations prior to the first post-infusion sample, which acted at the MRs either centrally or peripherally to decrease fetal arterial blood pressure. Interestingly, when arterial blood pressure was measured at 137-139d gestation in the same animals, there was no difference between the two groups, suggesting that either the fall in arterial blood pressure at 126-127d was transient or that other mechanisms were able to maintain arterial blood pressure during late gestation.

In a second experiment, metyrapone was infused from 137d gestation, when cortisol concentrations are increased. In this group of animals there was a significant decrease in plasma cortisol concentrations by 6h into the infusion. By 24h after the start of the metyrapone infusion at 137d however, fetal cortisol concentrations were similar to preinfusion levels. It is interesting that whilst plasma cortisol concentrations were suppressed, there was no change in fetal arterial blood pressure during the metyrapone infusion from 137d gestation. It could be suggested that when metyrapone was administered at 137d gestation, when endogenous cortisol concentrations are higher, the decrease in plasma cortisol was not sufficient to decrease MR occupancy and hence there was no difference in fetal arterial blood pressure before or during metyrapone infusion. It is possible that glucocorticoids regulate fetal arterial blood pressure directly either by acting on the vasculature or indirectly through changes in the central control of arterial blood pressure. In the

adult, MRs are present in the peripheral vasculature (Lombès et al., 1992; Kornel et al., 1993; Takeda et al., 1995) and glucocorticoids are able to modulate the actions of vasoactive agents (Falardeau & Martineau, 1989; Rees et al., 1990; Pirpiris et al., 1992; Walker & Williams, 1992). In addition, there is evidence that MRs in the brain regulate arterial blood pressure. An 8h intracerebroventricular (icv) infusion of a MR specific antagonist significantly decreased systolic blood pressure in conscious rats (Rahmouni et al., 2001). To further understand whether glucocorticoids modulate arterial blood pressure (directly) in the fetus, possible experiments may include determining if metyrapone infusion has changed the expression, number and/or activity of MR and GR both in the vasculature for peripheral effects and in the hypothalamus and/or hippocampus for central effects. Secondly, the effect of central or peripheral infusions of a specific MR antagonist on fetal arterial blood pressure and the fetal arterial blood pressure responsiveness to increasing doses of Ang II would allow the separation of central versus peripheral effects of glucocorticoids.

Previous studies have investigated the role of endogenous glucocorticoids in the regulation of fetal arterial blood pressure during late gestation by removal of the fetal adrenal glands (Unno et al., 1999; Segar et al., 2002). Unno and co workers (1999) reported that the small increase in fetal arterial blood pressure, which occurs between 120 and 126d gestation in intact fetuses, was not present in bilaterally adrenalectomised fetuses, but was restored with cortisol replacement. One limitation of this study was that fetal plasma cortisol concentrations could not be determined in either the intact or adrenalectomised fetuses as cortisol concentrations were lower than the detection limit of the cortisol radioimmunoassay

used. Segar and coworkers (2002) demonstrated adrenalectomised fetuses at 130d had significantly lower fetal arterial blood pressure at 139-140d when compared with adrenalectomised fetuses concomitantly infused with cortisol. Whilst this study demonstrated that cortisol replacement increased fetal blood pressure, the study did not include a third, intact group in which no treatment had been performed or the results were not compared with a third intact control group (Segar et al., 2002). Fetal adrenalectomy results in the removal of both steroidogenic cells of the adrenal cortex and the catecholamine containing cells of the adrenal medulla. It is therefore difficult to conclude from these studies whether changes in fetal blood pressure in adrenalectomised fetuses are as a consequence of the removal of cortisol alone, or of the combined removal of cortisol and adrenaline, specifically as intrafetal infusion of noradrenaline increases arterial blood pressure (McMullen et al., 1998).

Previous studies have shown that cortisol infusion at 120d results in a significant increase in the fetal arterial blood pressure responses to increasing doses of Ang II or an AT1R antagonist (Tangalakis et al., 1992; Forhead et al., 2000). When the same experiment is performed at 135d, there was no difference in the arterial blood pressure responses to increasing doses of Ang II or an AT1R antagonist (Tangalakis et al., 1992; Forhead et al., 2000). My second hypothesis was that a decrease in fetal cortisol concentrations at 125d but not at 137d gestation would decrease the fetal arterial blood pressure responses to increasing doses of Ang II. Infusion of metyrapone from 125d gestation resulted in a significant blunting of the fetal arterial blood pressure responses to increasing doses of Ang II at 138/9d gestation, whilst a transient suppression of fetal cortisol biosynthesis at 137d gestation did not alter the fetal arterial blood pressure responsiveness to increasing doses of Ang II at

138/139d gestation. These findings together with the data reported in Chapter 2, suggest that there may be a critical window before the start of the prepartum increase in fetal cortisol, when endogenous fetal cortisol contributes to the regulation of fetal arterial blood pressure and the development of subsequent vascular responsiveness to Ang II.

In the adult, it is well established that the AT1R mediates the actions of Ang II in the maintenance of blood pressure homeostasis, whereas one role of the AT2R appears to be to inhibit the actions of AT1R (Inagami et al., 1999). In the fetal sheep, Rosenfeld and coworkers (1993) demonstrated that Ang II receptor binding activity in the fetal aorta did not change during gestation, between 107-111d and 125-134d (Rosenfeld et al., 1993). Although the relative proportions of the AT1R and AT2R in the fetal arteries were not studied by Rosenfeld and coworkers, it is not therefore, possible to predict the physiological effect of Ang II via the AT1R and AT2R. Recently, with the development of more specific antagonists, it has been demonstrated that in fetal aortae and carotid arteries, there is an increase in the AT1R binding affinity with increasing gestational age, with a concomitant decrease in AT2R binding affinity (Burrell et al., 2001). Taken together, these data suggest that the increase in the ratio of AT1R to AT2R with increasing gestation may play a role in the increased vasoconstriction of Ang II via AT1R thus contributing to the increase in blood pressure in late gestation. I propose that the decrease in the blood pressure responsiveness to Ang II in the animals infused with metyrapone from 125d gestation may be due to a decrease in the vascular number of AT1R with a possible increase in vascular AT2R. There is a positive relationship with the percentage of AT1R in the fetal carotid artery or aorta with gestational age and a negative

relationship with the percentage of AT2R in the fetal carotid artery or aorta with gestational age (Burrell et al., 2001). In addition, infusion of an AT1R antagonist lowers fetal arterial blood pressure to a greater extent in fetuses aged 140d gestation when compared with 125d fetuses (Forhead et al., 2000). More importantly 125d fetuses infused concomitantly with cortisol and the AT1R antagonist, had a significant fall in arterial blood pressure, which was of similar magnitude as that seen in 140d fetuses (Forhead et al., 2000). The proposed model of the relationship between cortisol and the fetal renin-angiotensin system is summarised in Figure 5.1.

The direct relationship between cortisol and arterial blood pressure through the renin-angiotensin system in the fetus may also have consequences in the development of the fetus. PR fetal sheep have significantly higher plasma cortisol concentrations than normally grown fetuses (Phillips et al., 1996b). PR fetuses also have significantly higher AT1R mRNA expression in the carotid, renal and femoral vasculature when compared with normally grown fetuses (Rosenfeld et al., 2003). This may be taken as evidence that cortisol directly increases the expression of AT1R mRNA in the vasculature. If this is true then it is possible that in the metyrapone infused fetuses there was a change in the relative expression of AT1R and AT2R and that the arterial blood pressure response to Ang II at 126-127d gestation was less in the metyrapone infused when compared with vehicle infused fetuses due to a decrease in the expression of AT1R. In conjunction with the present data it is possible that small alterations of fetal plasma cortisol concentrations in late gestation, before the prepartum rise in cortisol, may have long lasting consequences on the renin-angiotensin system through to adult life.

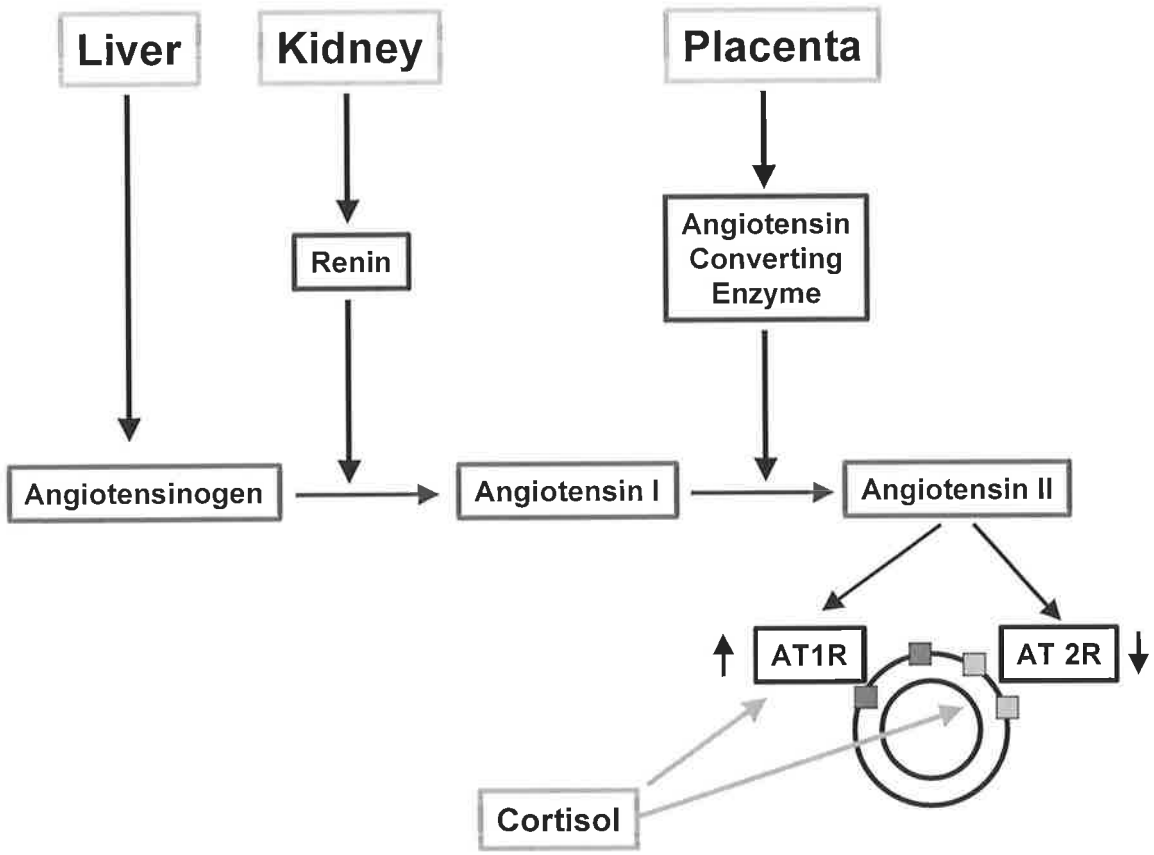

Figure 5.1: Proposed model of the impact glucocorticoids (cortisol) may have on the renin-angiotensin system.

Ang II is produced and acts through the AT1R and the AT2R present on fetal arterial blood vessels. I propose that cortisol may increase AT1R expression and/or decrease AT2R expression.

5.2 METYRAPONE INFUSION AND THE FETAL HPA AXIS

This is the first study to my knowledge, investigating the effects of a long term infusion of metyrapone in the sheep fetus. Metyrapone infusion is a different model of cortisol depletion than adrenalectomy. Adrenalectomy results in a removal of both the adrenocortical cells of the adrenal cortex and the chromaffin cells of the adrenal medulla. In addition, metyrapone infusion allows the relationship between cortisol and ACTH to be investigated in a dynamic *in vivo* system.

I hypothesised that removal of cortisol synthesis at 125d gestation, and hence removal of cortisol negative feedback would be detected by the fetal pituitary and result in an increase of the synthesis of POMC mRNA and secretion of ACTH from the fetal pituitary. I propose that the removal of cortisol negative feedback was also detected by the fetal hypothalamus, although this was not measured in the current study. Whilst metyrapone infusion from 125d gestation did not significantly decrease fetal plasma cortisol concentrations, fetal plasma ACTH concentrations were significantly increased from 126d gestation and remained high throughout the infusion period. At 140d there was also significantly higher expression of pituitary POMC mRNA in metyrapone infused fetuses when compared with vehicle infused controls. I propose that metyrapone infusion transiently suppressed fetal cortisol concentrations, which was detected by the fetal pituitary which, increased the synthesis of POMC and secretion of ACTH. The activation of the pituitary stimulated the fetal adrenal to upregulate cortisol synthesis in an attempt to overcome the metyrapone blockade and maintain cortisol concentrations, as evidenced by high 11-desoxycortisol concentrations. In addition, it is possible that ACTH concentrations

continued to increase as part of the prepartum rise in ACTH concentrations, to maintain the prepartum rise in cortisol, but that the ACTH levels were higher than vehicle infused controls due to the continued infusion of metyrapone.

The current study did not extend to an investigation of the role of the hypothalamus or hippocampus in the regulation of fetal ACTH and cortisol secretion during late gestation. It is known that the hypothalamus is important for the presence of negative feedback after 135d gestation in fetal sheep (Ozolins et al., 1990), although the role of the fetal hippocampus is less well understood. There are little data available investigating exposure of the fetal sheep during late gestation to high levels of cortisol and its effect on the expression of MR and GR in the hippocampus and hypothalamus. Fetal sheep exposed to a 10d infusion of dexamethasone early in gestation, at 27-29d, had significantly lower expression of MR and GR mRNA in the hippocampus when compared with vehicle infused controls, when measured at 130d gestation (Moritz et al., 2002). Interestingly fetal sheep exposed to a shorter, 2d infusion of dexamethasone at 27-29d gestation had significantly higher expression of MR and GR mRNA in the hippocampus when compared with vehicle infused controls, when measured at 130d gestation (Dodic et al., 2002a). Despite the differential effects of the length of exposure to dexamethasone, in both these experiments there was no change in the MR mRNA expression in the hypothalamus or hippocampus, when measured in animals at either 2 months (Moritz et al., 2002) or 7 years of age (Dodic et al., 2002b). Interestingly, in both these studies animals treated with dexamethasone had significantly higher arterial blood pressure as adults when compared with saline treated controls. Nevertheless, it would be interesting to investigate the role of the fetal hippocampus and hypothalamus in the

regulation of the secretion of cortisol and ACTH during late gestation in the metyrapone infused fetal sheep model. I speculate that the biologically-significant fall in fetal cortisol concentrations caused by metyrapone infusion at 125d gestation would increase the expression and number of MR. This would lead to more MR in the hippocampus which may then detect less cortisol relative to amount of MR and inturn signal to the hypothalamus to increase the stimulation of secretion of ACTH from the pituitary, and stimulate an increase in cortisol synthesis to maintain the prepartum rise in cortisol secretion from the fetal adrenal (Figure 5.2) and may permanently alter blood pressure regulation.

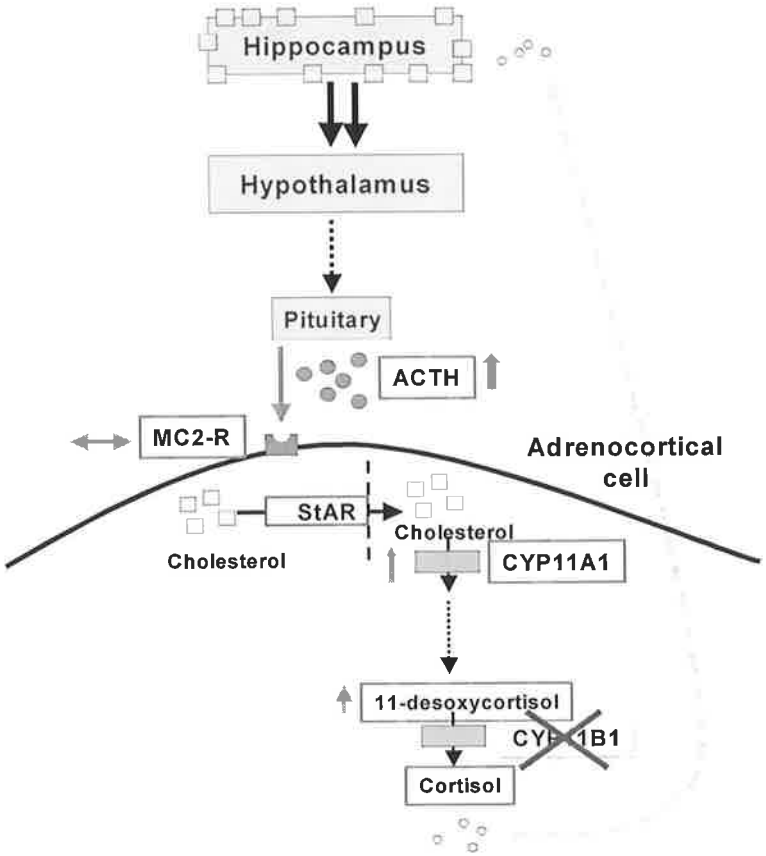

Figure 5.2: Schematic diagram of possible role of MRs in the hippocampus may play in the regulation of cortisol synthesis and secretion during late gestation in the fetal sheep.

An increase in the number of MR (represented by green boxes) in the hippocampus, may result in less cortisol relative to MR number, thus resetting the axis and hence stimulating the HPA axis to increase cortisol synthesis and secretion.

My third hypothesis was that the increase in ACTH concentration, following metyrapone infusion, stimulated the increase in adrenal growth (Tangalakis et al., 1990). Interestingly though, whilst metyrapone infused fetuses had increased adrenocortical growth due to hypertrophy of the adrenocortical cells of the zona fasciculata, there was no difference in the plasma cortisol concentrations between the two groups. These data suggest a decrease in cortisol synthesis per cell in the enlarged adrenocortical cells of the metyrapone infused group. It is not technically feasible however, to measure the cortisol content within individual adrenocortical cells. The concept of low intracellular cortisol concentrations is supported by the presence of higher 11βHSD2 mRNA levels measured in the adrenals from metyrapone infused fetuses. In the fetal sheep adrenal, there is a significant decrease in relative expression of 11βHSD2 during late gestation (McMillen et al., 2000). In addition, cortisol infusion at 109-116d gestation (a stage of gestation when endogenous cortisol concentrations are low) results in a premature and significant decrease in the relative expression of 11βHSD2 mRNA in the fetal sheep adrenal (Ross et al., 2000). In the adrenal from metyrapone infused fetal sheep therefore, a higher expression of 11βHSD2 mRNA suggests a decrease in intracellular adrenal concentrations of cortisol by increasing the intracellular metabolism of cortisol resulting in adrenal cortical cells being exposed to lower cortisol concentrations.

One novel outcome of this thesis is that metyrapone infusion may be a model that can be used to dissociate the effects of long term exposure to high plasma ACTH concentrations with low intracellular cortisol concentrations *in vivo*. The resulting steroidogenic capacity of the adrenal from this study is summarised in Figure 5.3. There was an increase in the mRNA expression of steroidogenic enzymes CYP11A1

and CYP17, but not 3βHSD or CYP21A1. This is consistent with an increase in the exposure of the fetal adrenal to higher plasma ACTH concentrations.

There was also an increase in StAR protein expression, with a decrease in StAR mRNA expression. The differential effect on StAR synthesis and secretion is interesting. Previous studies have shown that ACTH stimulates the expression of StAR mRNA in adrenal cells *in vitro* and *in vivo* via a cyclic AMP dependent mechanism (Penhoat et al., 1989; Lebrethon et al., 1994b; Stocco, 1999; Le Roy et al., 2000). *In vitro* studies have also shown, however, that glucocorticoids have a direct role in enhancing the action of phorbol ester on StAR mRNA levels in adrenocortical cell lines (Feltus et al., 2002). Interestingly concurrent infusion of metyrapone with ACTH (1-24) in fetal sheep, *in vivo*, prevented the cAMP accumulation measured following ACTH stimulation of fetal sheep adrenal cells *in vitro* (Lye & Challis, 1984). One possibility therefore is that whilst circulating ACTH concentrations are higher in the metyrapone treated group, the actions of ACTH on adrenal StAR mRNA expression are limited by relatively lower intracellular cortisol concentrations within the fetal adrenal cortex in this group. One possible way to investigate the role of ACTH and glucocorticoids in the regulation of StAR may be investigating the effect of a metyrapone with cortisol replacement on the regulation of adrenal StAR mRNA and protein. I propose that both StAR mRNA and protein would decrease in response to a metyrapone with cortisol replacement infusion, due to the presence of higher intracellular cortisol concentrations in the cortisol replacement group. In vitro studies measuring intracellular levels of cortisol would also determine the specific role cortisol may play in the regulation of synthesis and secretion of StAR in response to ACTH stimulation.

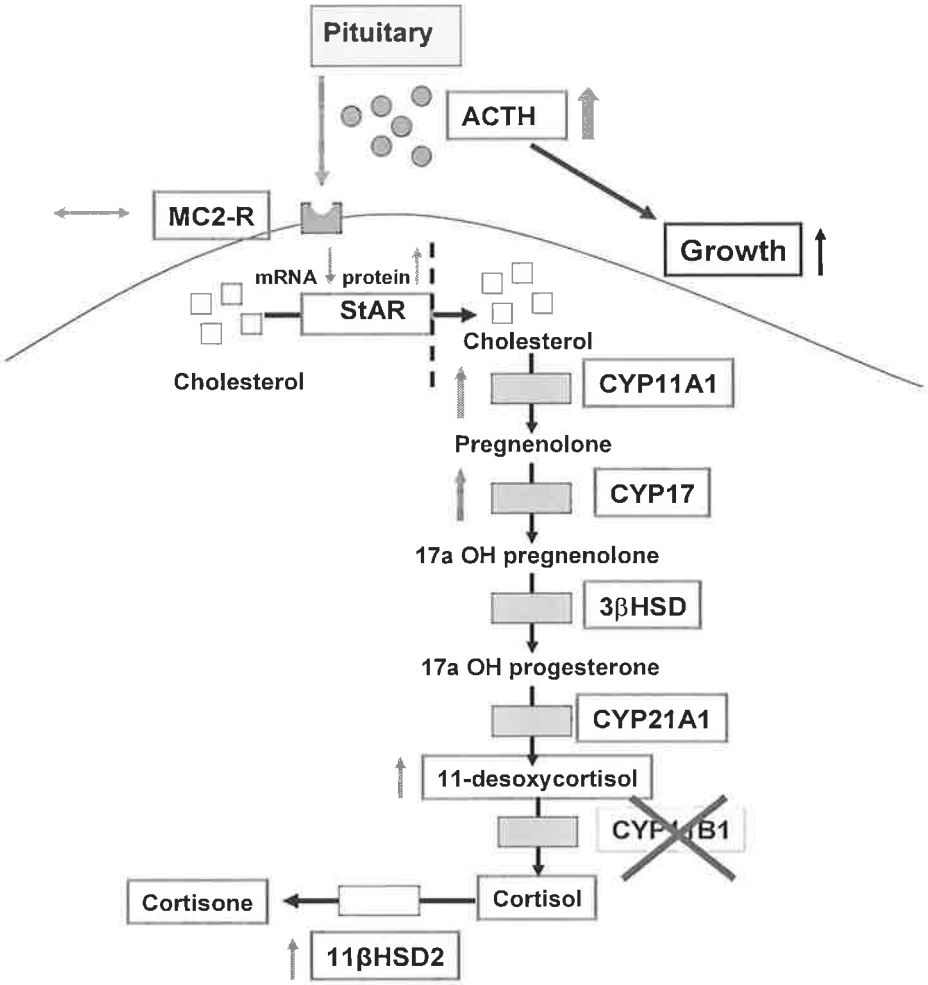

Figure 5.3: A schematic summary of the effect of metyrapone infusion from 125d, on the steroidogenic enzymes and related proteins of the fetal adrenal at 140d gestation.

The parts that are boxed in green are the steroids, steroidogenic enzymes and factors measured in metyrapone infused fetuses from these studies. The direction of the arrows beside each summarise the findings. Metyrapone infusion increased ACTH and 11-desoxycortisol concentrations, increased the expression of CYP11A1, CYP17 and 11βHSD2 mRNA and StAR protein. MC2-R mRNA was unchanged and StAR mRNA expression was decreased.

5.3 FUTURE DIRECTIONS

The findings from this thesis indicate that cortisol is critical for the development of the cardiovascular system and HPA axis during late gestation in the fetal sheep.

It is clear from previous work that cortisol may play a role in the regulation of the cardiovascular system, possibly through the renin-angiotensin system. This thesis found that:

- blood pressure was significantly decreased 24-48h after the start of metyrapone infusion from 125d gestation despite there not being a measurable decrease in plasma cortisol concentrations.
- blood pressure in fetuses infused with metyrapone from 137d gestation did not change despite there being a significant decrease in plasma cortisol concentrations.
- arterial blood pressure responses to increasing doses of Ang II were significantly blunted in those fetuses infused with metyrapone at 125d gestation when compared with both control fetuses and fetuses infused with metyrapone from 137d gestation.

To further understand the role of cortisol in the regulation of the cardiovascular system therefore, there are a number of areas that can be investigated:

- The role of MR and GR in the brain, specifically in the hypothalamus and/or hippocampus and the role these receptors may play in the regulation of arterial blood pressure.

- The role of MR and GR in the periphery, and the possibility that glucocorticoids act directly on the vasculature to regulate arterial blood pressure.
- The specific effect of metyrapone infusion on the proportion of AT1R vs AT2R in the vasculature.

This thesis also investigated the interaction between cortisol and ACTH during late gestation and the steroidogenic capacity of the fetal adrenal in late gestation.

- Metyrapone infusion from 125d gestation significantly increased plasma ACTH and 11 desoxycortisol concentrations whilst maintaining cortisol concentrations.
- Adrenals from metyrapone infused fetuses also had significantly increased expression of CYP11A1 and CYP17 mRNA with a concomitant increase in expression of 11βHSD2 mRNA.
- The zona fasciculata of the adrenals from metyrapone infused fetuses was significantly larger than vehicle infused controls, with evidence of cellular hypertrophy. These data taken together suggest that metyrapone infused fetuses had significantly lower cortisol concentrations within adrenal cells.

To understand the role of peripheral cortisol future studies may include:

- Investigation of the role of MRs and GRs in the hypothalamus and hippocampus in the metyrapone infused model.
- The role of CBG and corticosteroid binding capacity in the metyrapone infused model.

To understand the role of intra-adrenal cortisol future studies may include:

- An investigation into the role of intracellular concentration of cortisol plays in adrenal growth and steroidogenesis during late gestation

In summary, the timing of exposure to endogenous glucocorticoid is critical for the normal development of the fetus. Suppression of circulating cortisol concentrations at 125d gestation, a time when endogenous cortisol concentrations are low, had lasting effects on the fetal cardiovascular system and HPA axis. However, suppression of circulating cortisol concentrations at 137d gestation, when endogenous cortisol concentrations are increasing had no lasting effects on the fetal cardiovascular system. It appears therefore, that just prior to the prepartum rise in cortisol, the fetal cardiovascular system is sensitive to glucocorticoids. In addition, metyrapone infusion from 125d until 140d has been shown to be a unique model, in which the effect of high ACTH concentrations with low intracellular cortisol concentrations can be determined on the development and function of the fetal adrenal gland.

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Amendments:

a) Metyrapone infusions were commenced at 125d gestation or at 137d gestation. At 125d gestation endogenous cortisol concentrations are low. At 137d gestation endogenous fetal cortisol concentrations are increasing. The design of the experiments therefore, was to investigate when endogenous cortisol concentrations are important for the development of the fetal cardiovascular system. This rationale is detailed on page 38.

b) The hypothesis on page 38 states that suppression of cortisol biosynthesis would increase the stimulation from the fetal pituitary resulting in increased ACTH concentrations. It is implied that due to the negative feedback system that exists, that suppression of fetal cortisol biosynthesis will result in a decrease in circulating cortisol levels, which is detected by the fetal pituitary and by trying to maintain fetal cortisol levels, the fetal pituitary will increase plasma ACTH concentrations in order to stimulate the fetal adrenal to increase cortisol concentrations.

c) The dose of metyrapone used was to decrease the synthesis of fetal cortisol, but not too high to be toxic to the fetus. Metyrapone infusion was to suppress cortisol synthesis by the adrenal not to abolish cortisol synthesis.

d) The blood pressure in the fetal sheep was measured at either 126d or 127d. This was due to technical restraints, such as there was not enough equipment to measure the number of animals at 126d in some instances. The blood pressure of some animals therefore was measured at 127d.

e) In Chapter 3 the blood pressure from some animals which had their blood pressure measured at 126-127d, could not be measured at 137-139d gestation. Some reasons for this discrepancy included animals aborting between 127d and 137d gestation, a blocked or partially blocked fetal carotid arterial catheter, or the position of the fetus meant that a true blood pressure reading (with peaks and troughs) could not be obtained.

f) The animals used in the long term infusion of metyrapone group were all singletons. It is therefore unknown from the data whether twin animals reacted differently to metyrapone treatment.

g) There was no difference in the response of fetuses to metyrpaone infusion between males and females. The data therefore was combined for analysis.

h) In Chapter 2, metyrapone infusion from 125d gestation significantly decreased fetal systolic and diastolic blood pressure. For Chapter 3 whilst metyrapone infusion from 137d gestation did not change fetal blood pressure, for consistency systolic and diastolic blood pressure was included as well as mean arterial blood pressure.

i) The numbers of animals used for Figures 2.6-2.8 is n=9 for vehicle and n=10 for metyrapone groups as detailed in Table 2.1 on page 48.

Chapter 3: Materials and Methods

a) The same animals were used for both Chapter 2 and Chapter 3.

b) The vehicle infused animals were used as controls for both metyrapone infused groups. There were two experimental infusion groups (metyrapone infused from 125d and metyrapone infused from 137d gestation).

c) The second paragraph on page 144 is indeed a statement of results and was included to remind the reader of the results from Chapter 4 so that and focus them as to why the future directions