ENERGY BALANCE AND LEPTIN IN THE FETUS

A thesis submitted for the degree of Doctor of Philosophy

To

The University of Adelaide, Australia

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Dedicated to the founding fathers of modern science.

Man can not discover new oceans,

til we have the courage to lose sight of the shore.

-Author Unknown-

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ABSTRACT

Leptin is a 16 kDa protein that is synthesised and secreted by adipocytes. It is well established that in the adult, circulating leptin is altered by fluctuations in energy intake and that leptin acts on centrally and peripherally located leptin receptors to regulate energy homeostasis and related neuroendocrine functions. During pregnancy, the nutritional demands of the conceptus increase and during late gestation, fetal growth rate slows. Circulating leptin is positively related to newborn weight whilst growth restricted newborns are not only hypoglycaemic but also hypoleptinemic. Furthermore, newborns from diabetic pregnancies are hyperinsulinemic and hyperleptinemic. It has therefore been suggested that leptin may be a signal of the nutritional environment of the fetus. Little is known about the role of leptin before birth, and the aim of this series of studies was to investigate whether leptin is expressed in fetal adipose tissue and is nutritional regulation, and to determine the actions of leptin before birth.

It was found that the relative abundance of leptin in fetal adipose tissue increased with gestation. There was also an association between leptin expression in fetal adipose tissue and fetal weight, and this relationship changed as gestation progressed. These findings suggested that fetal leptin could be a signal of fetal nutrient supply or fetal growth. In order to determine whether leptin synthesis and secretion in fetal adipose tissue could be altered by changes in fetal nutrient supply, the effect of a moderate restriction of maternal food intake on leptin synthesis and secretion in the adipose tissue of the sheep fetus was investigated. Whilst maternal undernutrition decreased plasma glucose and insulin levels in fetal sheep, there was no change in circulating leptin concentrations or the relative abundance of leptin in the adipose tissue of fetuses of undernourished ewes.

There was, however, a positive relationship between leptin expression in fetal adipose tissue and circulating leptin concentrations in late gestation. There is also recent evidence that there is a positive relationship between circulating leptin and the relative mass of unilocular fat, which suggests that leptin, may act as a signal of lipid storage in the late gestation sheep fetus. Hence, the impact of an experimentally induced increase in circulating leptin concentrations on the structural and functional characteristics of fetal adipose tissue was investigated. Leptin administration between 136 and 141 days gestation increased circulating leptin concentrations ~4-5 fold and resulted in a significant increase in the proportion of smaller lipid locules present within fetal adipose tissue. This was also associated with a significant increase in the proportion of multilocular tissue and a significant decrease in the proportion and relative mass of unilocular tissue in fetal adipose tissue. The relative abundance of leptin mRNA in fetal adipose tissue was significantly lower in the leptin infused group and there was a positive correlation between the relative abundance of leptin mRNA and the proportion of unilocular tissue in fetal perirenal adipose tissue. Finally, the amount of uncoupling protein-1 protein tended to be higher in leptin infused compared with saline infused fetuses. This is the first demonstration that leptin could act to regulate the lipid storage characteristics, leptin synthetic capacity and potential thermogenic functions of fat before birth.

Recent studies have also suggested that there may be a functional interaction between circulating leptin and the fetal hypothalamic-pituitary-adrenal, (HPA) axis in late gestation. It is well established in the sheep that the prepartum increase in cortisol is required for the differentiation and maturation of key fetal organs such as the fetal lung, liver, kidney and brain, for the normal timing of parturition and the successful transition

to extrauterine life. Given the potential role of leptin as a circulating signal of fetal adiposity in late gestation it is important to determine whether, as in the adult, an endocrine negative feedback loop exists between adipose tissue and the HPA axis in the fetus during late gestation. We have investigated whether leptin can suppress the prepartum activation of the fetal HPA axis and delay the timing of parturition. Firstly, we investigated the effects of a 4 d intrafetal infusion of leptin on fetal plasma ACTH and cortisol concentrations starting from 136 d gestation, i.e. at the onset of the prepartum activation of the fetal HPA axis. Whilst circulating leptin concentrations were elevated in leptin infused fetuses and there was no increase in plasma ACTH and cortisol concentrations. Leptin was also infused into fetal sheep from 144 d gestation until delivery to determine the effects of an increase in circulating leptin on the prepartum changes in fetal plasma ACTH and cortisol concentrations and on the timing of parturition. Intrafetal infusion of leptin from 144 days gestation until delivery did not suppress plasma ACTH concentrations, however, fetal plasma cortisol concentrations were suppressed for an extended period from between 90-42 h before delivery. Whilst plasma cortisol concentrations were reduced by ~40% in leptin infused fetuses, there was no difference in the timing of parturition between the leptin and saline infused groups.

In summary the studies in this thesis demonstrate that leptin is expressed in adipose tissue of fetal sheep in late gestation and that the expression of leptin mRNA in the tissue is directly related to circulating leptin. An increase in circulating leptin results in changes in the structural and functional characteristics of fetal fat and changes in the fetal HPA axis. Thus leptin may act as a signal of adiposity before birth.

DECLARATION

This body of scientific work contains no material that has been accepted for the award of any other degree or diploma in any other University or Tertiary Institution. To the best of my knowledge and understanding, this thesis contains no material previously published or written by any other person, except myself and where due reference is made in the text.

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

Bernard S.J. Yuen

January, 2004

CONTRIBUTION OF COAUTHORS

The experimental results contained within this thesis were conducted as part of my PhD candidature and have been published or submitted for publication. This research has yielded three publications and one submitted manuscript and whilst I am the principal investigator of this work, other researchers have also made a significant contribution. They have been formally acknowledged with coauthorship on the appropriate publications or submitted manuscript.

For the purpose of submitting this thesis, as part of my PhD candidature a declaration of their contribution to this body of work is required by the University of Adelaide, Australia. A detailed summary of the contribution of each coauthor to my experimental chapters (2-5) is given below, whilst an abbreviated summary of their contribution will also appear at the beginning of each experimental chapter.

Prof I Caroline McMillen

Currently with the Discipline of Physiology, School of Molecular and Biological Sciences, University of Adelaide, Australia. Prof McMillen is a co-supervisor of my PhD research program and provided funding for me to conduct my experiments. Furthermore, she made a significant contribution to intellectual debates, data analysis and interpretation, and had an input into the writing process of each publication and submitted manuscript.

Dr Philip C Owens

Previously from the Department of Obstetrics and Gynaecology, School of Health Science, University of Adelaide, Australia. Dr Owens is a co-supervisor of my PhD research program and provided funding for me to conduct my experiments. Furthermore, he made a significant contribution to intellectual debates, data analysis and interpretation, and had an input into the writing process of each publication and submitted manuscript.

Dr Michael E Symonds

From the Academic Division of Child Health, School of Human Development, University of Nottingham, England. Dr Symonds is an expert in the field of adipose tissue development and thermogenesis. Furthermore, his laboratory has the expertise to measure the abundance of proteins in the mitochondria and as a result, tissue samples were sent to him for these analyses. He also contributed to intellectual debates, the interpretation of data and was actively involved in the final editorial process of each manuscript.

Dr Jim R McFarlane

Currently at the Department of Physiology, School of Biomedical, Biological & Molecular Sciences, University of New England, Australia. His laboratory was one of the first to develop an assay to measure detectable concentrations of leptin from sheep plasma. Plasma samples from pregnant ewes and their fetuses were sent to his laboratory for analysis. He also contributed to the final editorial process of manuscripts.

Dr Claire T Roberts

Presently at the Department of Obstetrics and Gynaecology, School of Health Science, University of Adelaide, Australia. Dr Roberts is an expert in anatomy, and in adipose and placenta tissue development. She provides support in matters relating to her fields of expertises. Furthermore, she assisted with the interpretation of histology data and aided in the final editorial process of a manuscript.

Dr Lisa J Edwards

Dr Edwards is a former PhD student of the McMillen laboratory. As part of her PhD research program, she restricted the nutrient intake of pregnant ewes and collected maternal and fetal blood, and fetal tissues. Dr Edwards generously allowed me to determine circulating leptin concentrations in these animals and to determine the abundance of leptin mRNA in the adipose tissue from these fetuses.

Dr Duane H Keisler

From the Department of Animal Sciences, University of Missouri, United States. Without Dr Keisler generously donating an amount of lyophilised ovine leptin, it would not have been possible to determine the actions of leptin on the various physiological systems of the sheep fetus.

Yvonne Evens

Ms Evans is currently a member of Dr Symonds' laboratory. She measured the protein abundance of uncoupling proteins in the adipose tissue of fetal sheep.

Kathleen G Kauter

Ms Kauter is currently with the McFarlane laboratory and was one of the first researchers to develop a leptin ELISA for use in the sheep.

Beverly S Muhlhausler

Ms Muhlhausler is currently a student with Prof McMillen. She conducted the histology on adipose tissue samples from saline and leptin infused fetuses and aided with the interpretation of these data. Furthermore, she was involved in many intellectual debates.

I would like to thank all the coauthors for their contribution to the body of work contained within this thesis. Thank you for providing some of the technical expertise and intellectual input that supported the publication of the work I conducted and which I have presented in this thesis.

ACKNOWLEDGEMENTS

This thesis represents the end of four years of work and would not have been possible without the support of others; in particular my family, friends and supervisors.

For those who know me, this thesis more importantly symbolises the reaching of one mountain summit that has taken over a decade to climb. The road to the summit has been long and windy, and many have walked with me on this journey. Thank you to all those who accompanied me.

PUBLICATIONS ARISING FROM THIS THESIS

Yuen B.S., McMillen I.C., Symonds M.E., Owens P.C. 1999 Abundance of leptin mRNA in fetal adipose tissue is related to fetal body weight. Journal of Endocrinology. 163:R11-R14.

Yuen B.S., Owens P.C., McFarlane J.R., Symonds M.E., Edwards L.J., Kauter K.G., McMillen I.C. 2002 Circulating leptin concentrations are positively related to leptin messenger RNA expression in the adipose tissue of fetal sheep in the pregnant ewe fed at or below maintenance energy requirements during late gestation. Biology of Reproduction. 67:911-916.

Yuen B.S., Owens P.C., Muhlhausler B.S., Roberts C.T., Symonds M.E., Keisler D.H., McFarlane J.R., Kauter K., Evens Y., McMillen I.C. 2003 Leptin alters the structural and functional characteristics of adipose tissue before birth. The FASEB Journal. 10.1096/fj.02-0756fje.

These publications can be viewed in Appendix (Chapter 8).

Manuscript accepted for publication:

Yuen B.S., Owens P.C., Keisler D.H., McFarlane J.R., Kauter K., McMillen I.C. 2004 Effects of leptin on fetal plasma ACTH and cortisol concentrations and the timing of parturition. Biology of Reproduction.

COMMONLY USED ABBREVIATIONS

A B C

ACTH	adrenocorticotrophic hormone
ad libitum	to any desired extent
AGRP	agouti related protein
ANOVA	analysis of variance
ATP	adenosine triphosphate
AVP	arginine vasopressin
bp	base pairs
CART	cocaine- and amphetamine-regulated transcript
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CoA	Coenzyme A
CRH	corticotrophin releasing hormone

D E F G

d	day(s)
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid

EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
g	gravitational acceleration
GDP	guanosine diphosphate
GH	growth hormone

H I J K L

h	hour(s)
Hb	arterial haemoglobin content
HPA axis	hypothalamo-pituitary-adrenal axis
i.c.v.	intracerebroventricular
IGFs	insulin-like growth factors
IGF-I	insulin-like growth factor I
i.v.	intravenous
^{125}I-ACTH	iodinated-adrenocorticotrophic hormone
^{125}I-Cortisol	iodinated-cortisol

M N O

mRNA	messenger ribonucleic acid
min	minute(s)

α-MSH	alpha-melanocortin stimulating hormone
NPY	neuropeptide Y
O_2 content	arterial oxygen content
Ob-R $_{(a-e)}$	leptin receptor isoform (a-e)

P Q R S

PCO_2	arterial partial pressure of carbon dioxide
PO_2	arterial partial pressure of oxygen
POMC	proopiomelanocortin
PAT	perirenal adipose tissue
PVN	paraventricular nucleus
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of the mean
SPSSX	statistical package for social sciences on a vax mainframe computer

T U V W X Y Z

UN	undernutrition

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CHAPTER 1

1. DEVELOPMENT OF ADIPOSE TISSUE AND THE ROLE OF LEPTIN BEFORE BIRTH

1.1 OBESITY: AN EXCESS ACCUMULATION OF ADIPOSE TISSUE

1.1.1 Lipogenesis and lipolysis

Triglycerides (triacylglyercols) are a class of lipids that are synthesised from fatty acids and glycerol. Fatty acids are essential components of cell membranes and are the precursors for the synthesis of steroid hormones. Triglycerides are the form in which fatty acids are stored as an energy reserve. Whilst the conversion of glucose into acetyl-Coenzyme A (CoA) through the glycolysis pathway is often associated with the Krebs cycle and the formation of adenosine triphosphate (ATP), the same end product of glycolysis (acetyl-CoA) is also an important substrate for the synthesis of fatty acids (lipogenesis) (1). Citrate lyase attaches a carbon atom to acetyl-CoA to form malonyl-CoA and from these fatty acid synthase forms fatty acyl-CoA (fatty acid + CoA) (2). These newly synthesised fatty acids are then incorporated with glycerol to form triglycerides. Lipogenesis occurs when there is excess dietary caloric intake. Conversely, during periods of low caloric intake, fatty acids are oxidised to generate energy. Triglycerides are first broken down into glycerol and fatty acids and these fatty acids are then cleaved into multiple acetyl-CoAs in mitochondria, in a process known as β-oxidation. These processes are the main components of lipolysis. Acetyl-CoA enters the Krebs cycle to be oxidised into carbon dioxide and water, and in the process nicotinamide adenine dinucleotide (NAD^+) is phosphorylated to nicotinamide adenine dinucleotide – reduced form (NADH). For the cell to utilise this energy, NADH then undergoes oxidative phosphorylation using a series of electron carriers that are present in mitochondria, which results in the generation of ATP. Thus organisms use lipids as a

form of energy storage (1). Net lipid storage is a balance between lipogenesis and lipolysis. The mechanisms that cause excess storage and prevent the metabolism of lipids are of significant interest because of the link in humans between the excess accumulation of triglycerides i.e. obesity, and a range of morbidities including diabetes and cardiovascular diseases (3, 4).

1.1.2 Adipose tissue

Traditionally adipose tissue has been regarded as either a lipid storage tissue or a tissue that produces heat. It now appears that this view is simplistic. In adult mammals, there are two populations of mature adipocytes with morphologically distinct phenotypes that can be found in adipose depots (5). The prevalence of each type of adipocyte depends on the location of the adipose depot, the age and species of mammal. Historically, adipose tissue has been defined by its anatomical location and by the mitochondrial content of the tissue. When relatively few mitochondria are present in the adipocytes, the tissue has a white appearance and has therefore been called "white adipose tissue". Conversely, if most of the adipocytes are filled with mitochondria the tissue has a brown appearance and therefore is called "brown adipose tissue". Some studies that have used the terms "white" or "brown" to describe adipose tissue need to be treated with caution, as these descriptions may not accurately reflect the cellular heterogeneity of a particular adipose depot (5-9). Therefore, where necessary, I will confine this review of the literature to studies where the cellular composition of the tissue has been reasonably well characterised.

1.1.3 Cellular composition of adipose tissue

The majority of adipose deposits develop in discrete regions of the body (e.g. perirenal, abdominal, omental, pericardial and subcutaneous regions). There are three distinctly different categories of cells present in adipose tissue; adipocytes, capillary endothelial cells and sympathetic neurons (5, 10, 11). Adult adipose tissue is principally comprised of mature white adipocytes, which are characteristically round or hexagonal in appearance, with peripherally located nuclei, few mitochondria and a major or dominant lipid locule. These cells have also been called unilocular adipocytes. The role of these adipocytes is to accumulate lipids in their locule during periods of high caloric intake and release fatty acids in the circulation during periods of low caloric intake (6, 7, 11). Recently, white adipocytes have been demonstrated to synthesise and secrete a hormone, which exerts a regulatory role on central and peripheral systems to control energy homeostasis (12-15). The importance of this hormone, leptin, will be discussed in later sections.

A second type of adipocyte found in adipose tissue is the brown adipocyte. Unlike mature white adipocytes, mature brown adipocytes are primarily present in mammalian adipose tissue during prenatal and early postnatal life although small pockets of brown adipocytes are found in fat depots during adult life (5, 6, 8, 9). These adipocytes have numerous lipid locules (multilocular adipocytes) that are small and many mitochondria with tightly packed cristae (10, 16, 17). These mitochondria contain the mitochondrial protein, uncoupling protein-1 (UCP-1), which is responsible for the generation of heat by uncoupling the electron transport chain during the oxidation of fatty acids (18). The generation of heat through UCP-1 is known as thermogenesis, and this mechanism can be induced in brown adipocytes by lowering the ambient temperature, this mechanism is

known as cold-induced thermogenesis. A similar mechanism is also used to produce heat after eating, i.e. diet-induced thermogenesis. The importance of diet-induced thermogenesis, uncoupling proteins, and the regulation of adipose tissue mass will be discussed shortly.

1.1.4 Evolution and epidemiology of obesity

Over millions of years, mammals have developed mechanisms to cope with periods when food was not readily available. The evolution of the adipose cell, which had the capacity to accumulate triglycerides, enabled animals to store material that had a high energy yield. Adipocytes could store energy in the form of triglycerides during periods when food was abundant and secrete fatty acids into the circulation which could be taken up by cells to produce energy during periods of low caloric intake.

Like other mammals, humans evolved in an environment where food was periodically limited. Over the past century, however, the availability of food for most people has become relatively abundant in developed countries (4, 19, 20). In particular, during the past 30 years, epidemiological studies have reported an increasing trend in the number of people developing obesity and associated metabolic and cardiovascular disorders, such as insulin resistance, type 2 diabetes mellitus, hyperlipidemia and coronary heart disease and an increase in the proportion of deaths associated with these diseases (4, 19, 20).

Studies report that the prevalence of obesity worldwide is steadily rising in many developed countries including the U.S., England and Australia (4, 20, 21). Body mass index (BMI) is a clinical index used to calculate a person's fatness. This index is calculated as weight (kg) divided by height^2 (m^2) of an individual and assumes that most

of the variation in the weight of individuals with the same height is due to fat mass. According to the World Health Organisation definition, a BMI of <18.5 kg/m^2 is below an ideal weight range, a BMI of between 18.5 and 24.9 kg/m^2 is in a healthy weight range, one of between 25.0 and 29.9 kg/m^2 is in the overweight or pre-obese weight range and a BMI greater than 30.0 kg/m^2 is in the obese range (4, 22).

In Australia, a recent Child Obesity Summit (2002) reported that one in five Australian adults were obese (BMI >30 kg/m^2) and confirmed an increase in the prevalence of obesity from 9% of males and 8% of females in 1980 to 19% of males and 22% of females in 2000 (21). This Australian study also reported the incidence of children and adolescent individuals who were obese or overweight, and showed that there was an increase in the prevalence of the disease in all age ranges. Currently, more than 5% of children are obese whilst 14-18% are overweight, and the frequency of overweight and obese children, over the past 10 years, has more than doubled in most of the age groups studied (21). Similar trends in the prevalence of obesity in child, adolescent and adult populations have been reported worldwide (4, 20, 23).

The significance of these trends is best reflected in epidemiological studies that have investigated the association between body shape and mortality to certain diseases. The concept that people with a high fat mass have a greater risk of mortality than do lean people has been supported by some studies (24-28). It has also been well documented, however, that a U-shaped relationship exists between adult weight or BMI, and adults mortality (28-34). Several of these studies show a linear relationship prevails when factors such as smoking (30, 35) and weight loss due to illness (25, 27) are taken into

account. Irrespective of whether the relationship between BMI and frequency of mortality is linear or quadratic, people with high BMI are at greater risk of mortality.

1.1.5 Factors influencing obesity and the early onset of obesity

Obesity is a multifaceted disease and is associated with the development of other metabolic and cardiovascular diseases, including diabetes, hypertension and cardiovascular disease. Not surprisingly genetic, behavioural and environmental factors influence the development of obesity. At approximately 21 years of age, male monozygotic twins were exposed to a prolonged period of overfeeding (36). In this study, Bouchard *et al* (36) showed that the difference between the rate of weight gain, the proportion of weight gained and the distribution of fat deposition was more similar within a set of twins than between sets of twins. Bouchard and colleagues (36) concluded that genetics may therefore play an important role in the development of human obesity. Furthermore, there is a close association between the BMI of twins who have been reared apart (37). In humans, mutations in certain genes do cause the development of obesity (38-40), however, these are only found in isolated individuals and large scale genetic screening of obese humans has, so far, failed to reveal a high incidence of genetic defects in obesity related genes (41-43). Whilst genetics undoubtedly plays a critical role in the development of human obesity, the marked increase in the prevalence of obesity in developed countries within a short period of time is most likely best explained by behavioural and environmental factors.

Studies have examined the relationship between physical activity and BMI and showed that reduced physical exercise increases the risk of both children and adults becoming obese (19, 20, 44-48). Studies have also investigated the development of obesity in

minority groups that have migrated to affluent regions or countries. These studies provide the clearest indication that environmental factors play a key role in the development of obesity. In the U.S., studies on ethnic minorities found that Pima Indians, non-Hispanic blacks and Nigerians were on average heavier or had a greater BMI than their fellow kinsmen in their country of origin (4, 49-51). A substantial body of research, however, has centred on the concept that obesity may, in fact, have its antecedents before birth.

Barker and colleagues (52, 53), put forward the fetal origins of adult disease hypothesis which proposed that changes in fetal development as a consequence of a suboptimal uterine environment before or during pregnancy could lead to the onset of cardiovascular and metabolic diseases, including hypertension, obesity, type 2 diabetes and insulin resistance in adult offspring.

The Dutch winter famine of 1944-1945 lasted 5-6 months and provided a unique opportunity in humans to investigate the effects of maternal malnutrition during different stages of gestation. A study examined over 300,000 males born to women exposed to famine at different stages of pregnancy. At 19 years of age, 2.8% of men whose mothers were previously exposed to famine during the first two trimesters of pregnancy were obese compared with 1.5% of men whose mothers were not exposed to famine during anytime in their pregnancy (54). Many studies cite Ravelli's publication as evidence of the impact of maternal undernutrition during pregnancy on the development of obesity in offspring and conclude that this finding supports the hypothesis that early intrauterine nutritional deprivation is associated with adverse health outcomes in postnatal life (52). A follow up study, however, of a cohort of 50 year old age men found no significant

difference in the BMI of the men whose mothers were, or were not exposed to famine during their gestation (55).

An oversupply of nutrients during pregnancy is also thought to lead to the development of obesity in offspring. Studies have shown that fetal exposure to maternal diabetes mellitus, gestational diabetes or mild maternal glucose intolerance during pregnancy results in the offspring having a greater chance of developing postnatal obesity (56-58). At birth, infants from diabetic pregnancies are found to be larger, fatter and have elevated glucose and insulin concentrations compared to infants from non-diabetic pregnancies. Silverman *et al* (57) reported, that 50% of newborns from diabetic pregnancies had a birthweight above the 90th percentile when corrected for length of gestation. When these children from diabetic pregnancies were re-examined at 8 years of age, half were found to still be in the same percentile range as they were when examined at birth. This suggests that, like the restriction of fetal nutrients during pregnancy, oversupply of fetal nutrients during pregnancy can lead to the postnatal development of obesity.

More recently, it has been speculated that it is not only the prenatal but also the postnatal environment that can predetermine the onset of adult diseases (59, 60). This notion is consistent with work recently published by Parsons and colleagues (59). They demonstrated that there is an association between birthweight, catch up growth during childhood and adult BMI. Men who were small at birth (in the lowest birth weight quintile) had a low BMI at age 33 and men in the highest birthweight quintile had a high BMI at age 33. Parson's found that variation in the low and high BMI groups could be better explained when postnatal growth rate was taken into account. In men that were

heavy at birth, the relationship between birthweight and adult BMI was independent of growth rate during the first 7 years of life. Interestingly, men who were small at birth and who had a faster growth rate during the first 7 years of life were found to have a similar incidence of obesity in adult life as those men who were born heavy. It appears, therefore, that there is an interaction between the pre- and post-natal environment that determines the risk of developing obesity in adult life.

In summary, the mechanisms that are used to store triglycerides in adipose tissue to increase the chances of survival during periods of low food intake may now be implicated in the excess accumulation of adipose tissue and the associated morbidities, which occur in populations with unlimited access to food and low levels of physical activity. Furthermore, these mechanisms may be implicated in the association between intrauterine growth, postnatal growth and adult obesity.

1.2 DEVELOPMENT OF ADIPOSE TISSUE: MESODERMAL CELLS TO ADIPOSE TISSUE

As the development of adult obesity can be related to prenatal and postnatal growth, it is only appropriate to review the development of adipose tissue during prenatal and neonatal life.

Development of mammals begins when a spermatozoan fertilises an oocyte to form a single cell embryo, the zygote, which subsequently develops into the placenta and fetus. The fertilised egg can be considered as a totipotent stem cell that has the ability to replicate and differentiate into any cell of the body and extraembryonic membranes. Shortly after fertilisation this cell undergoes replication (cleavage) and differentiation to

form a ball of cells known as a morula. Before the conceptus implants in the endometrium, a fluid filled cavity develops within it at which time it is known as a blastocyst. The cells of the blastocyst differentiate to form an outer cell layer (trophoblasts) and a group of cells within the blastocyst known as the inner cell mass (ICM). The trophoblasts and some of the ICM cells form the placenta and other extraembryonic membranes, whilst the remaining ICM cells eventually give rise to three germ layers, the endoderm, mesoderm and ectoderm. These three cell layers differentiate to form the fetus (61-63). Adipose tissue is derived from the mesoderm (61, 62).

Much of our understanding of adipocyte development (adipogenesis) comes from *in vitro* studies, which have specifically examined the development of the white, rather than the brown, adipocyte. Mesodermal stem cells can terminally differentiate into adipocyte, myocyte, or chondrocyte cells. The development of fetal adipose tissue begins when some of the stem cells from the mesodermal layer differentiate into adipoblasts (Figure 1.1). This differentiation process occurs when DNA methylation is inhibited in the mesodermal cells (64-66). Adipoblast proliferation then occurs until cell-to-cell contact at confluent density inhibits mitosis (66). As adipoblasts reach confluence, they arrest at the G_0/G_1 cell cycle boundary, presumably waiting until all adipoblasts in the depot reach the same cell cycle stage. The cells at this arrested stage of development are known as preadipocytes (5).

The cellular events that occur between the adipoblast and the emergence of the preadipocyte are poorly understood. It has been previously thought that mature white and brown adipocytes were derived from the same preadipocyte and that differentiation of the separate adipocyte lineages occurs at this point of adipogenesis. When preadipocytes

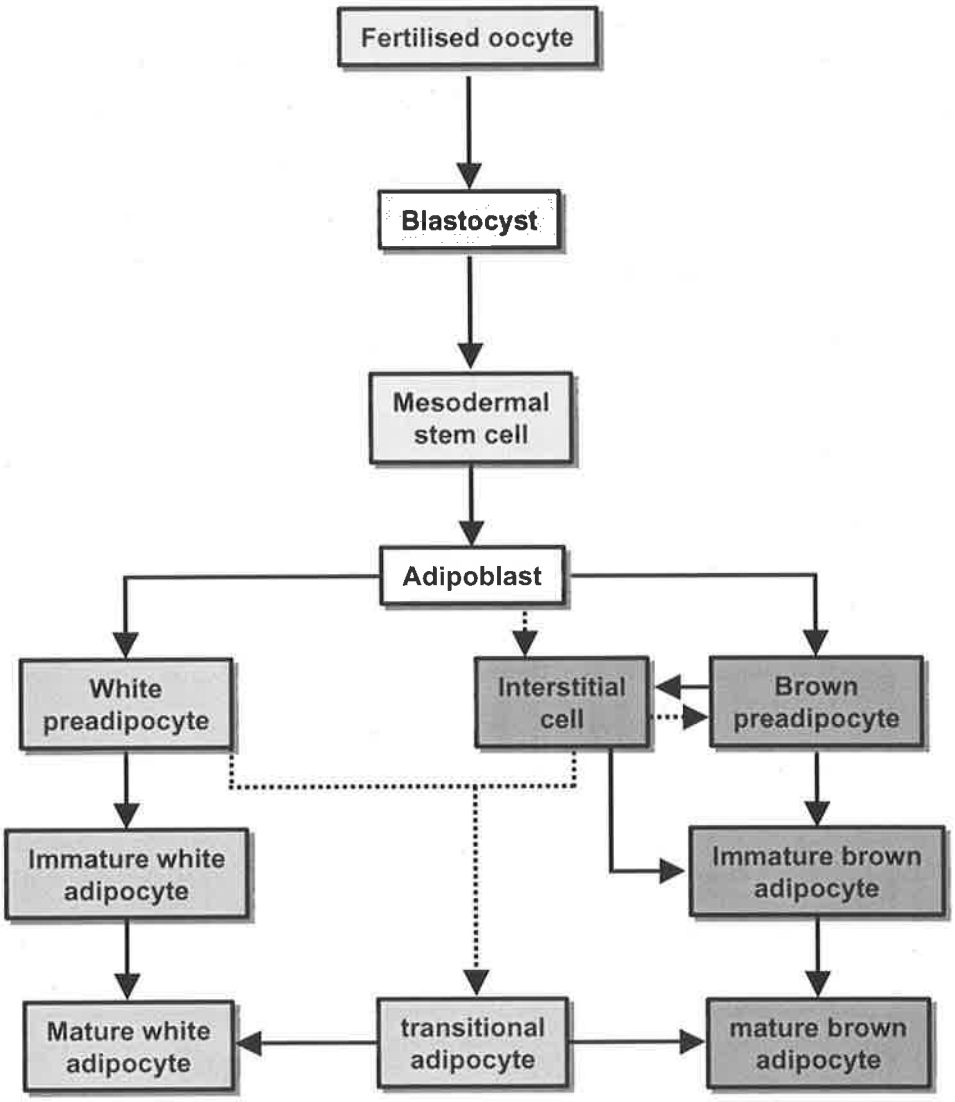


Figure 1.1 Schematic diagram summarising adipogenesis.

The differentiation pathways of adipocytes: from stem cell to white and brown adipocytes. Solid lines define known differentiation pathways. Dotted lines represent cell differentiation pathways that have been speculated and circumstantial evidence exists for these pathways. Blue coloured lines represent cell differentiation pathways that are known and have been abbreviated for the purpose of this schematic. Based on Ailhaud *et al* (5), Klaus (6), Himms-Hagen *et al* (8) and Bukowiecki *et al* (67).

were isolated from the stroma-vascular region of both white and brown adipose tissue and grown under the same conditions, it was found that the preadipocytes preferentially terminally differentiated into either mature white or brown adipocytes, respectively (68, 69). Since then studies have shown that these two types of preadipocytes express a range of different genes (70, 71). It has been concluded from these studies that mature white and brown adipocytes originate from different preadipocytes.

1.2.1 White adipocyte lineage

The developmental pathway of the white preadipocyte to the mature white adipocyte is well defined (Figure 1.1). Cells in the stroma-vascular region of adipose tissue can proliferate and fully differentiate into mature white adipocytes, and mature white adipocytes are unable to replicate *in vivo* (5). Extensive studies on 3T3 cell lines show that these cells display white preadipocyte characteristics and when treated with glucocorticoids, insulin or insulin-like growth factor-I (IGF-I), they differentiate into cells that accumulated lipid in a single locule and possess the morphological and biological characteristics of mature white adipocytes (72-75).

White preadipocytes undergo mitotic division (76). In cell culture, clonal expansion of the white preadipocytes slows when the preadipocytes reach the growth and arrest phase of cell replication. At this point, the expression of adipocyte specific proteins increases (77-79), and subsequently these preadipocytes undergo terminal differentiation forming immature white adipocytes. Maturation of these adipocytes is completed once these cells accumulate lipid in their cytoplasm (5).

1.2.2 Brown adipocyte lineage

Unlike many of the studies performed on white adipocyte development, much of the work conducted on the brown adipocyte lineage has used *in vivo*, rather than *in vitro*, techniques. Bukowiecki and colleagues (10, 67) focused on four cell types, namely: mature brown adipocytes, brown preadipocytes, interstitial cells and capillary endothelial cells (Figure 1.1). When examined under a microscope, mature brown adipocytes were multilocular, packed with mitochondria and had a relatively large nucleus. Brown preadipocytes were smaller, had very few lipid locules and few mitochondria. Some of these mitochondria had highly folded cristae whilst others had very few cristae (10). Interstitial cells were very small, with no lipid locules, an elongated nucleus and were localised in the interstitial spaces between adipocytes, and between adipocytes and capillary endothelial cells. Bukowiecki (67) showed that some brown preadipocytes, like interstitial cells, were localised in the stroma-vascular region of adipose tissue.

Capillary endothelial cells are not the progenitor cells of mature brown adipocytes (67), as some studies had previously speculated (80-82). Endothelial cells are found, however, to promote preadipocyte proliferation (83). Bukowiecki and colleagues (67) showed that interstitial cells differentiated firstly into preadipocytes and then into mature brown adipocytes (67). Furthermore, they suggested that interstitial cells were dormant stem cells that were always present in adipose tissue with a brown appearance. These cells were ready to proliferate and differentiate into mature brown adipocytes when subjected to appropriate stimuli. Geloen *et al* (10) showed that cytoplasmic lipid locules were found to form in one but not both daughter cells from the mitotic division of an interstitial cell. When dormant stem cells divide, some daughter cells differentiate whilst the other daughter cell remains undifferentiated (10). It is unclear whether interstitial

cells represent a preadipocyte precursor cell such as the adipoblast that can differentiate into mature white and brown adipocytes, or whether it represents an intermediate stage in adipogenesis between the adipoblast and the preadipocyte (5).

1.2.3 Development of fetal adipose tissue

The development of adipose tissue has been well described in prenatal and neonatal sheep. At approximately 70 days gestation, lipid locules begin appearing in cells in the perirenal region of the sheep fetus (term ~150 days) (84). These locule containing cells have few mitochondria with few cristae (84), and have a similar morphological description to the brown preadipocyte reported by Bukowiecki (67).

Between 70 and 90 days gestation, there is a progressive increase in the number of locule containing cells and the size of these locules in the perirenal region of fetal sheep (84). Some cells contained one dominant locule whilst others also contained several smaller locules (84). From 90 days gestation, the number of mitochondria and the frequency of cristae parallelled the increase in locule size. By term, some of these adipocytes from the perirenal adipose depot had a distinct unilocular appearance, others had a dominant locule and several smaller locules, and others had a distinct multilocular appearance (Figure 1.2 & Figure 1.3). All adipocytes were found to contain mitochondria. Nerve fibres began to appear in the adipose tissue at approximately 130 days gestation and their numbers progressively increased with gestation (84). The perirenal depot represents over 85% of the total adipose tissue mass in the sheep fetus (Muhlhausler, unpublished data), whilst as a proportion of body weight, the mass of the perirenal adipose depot remains relatively constant between 90 and 140 days gestation (85) (Yuen, unpublished data).

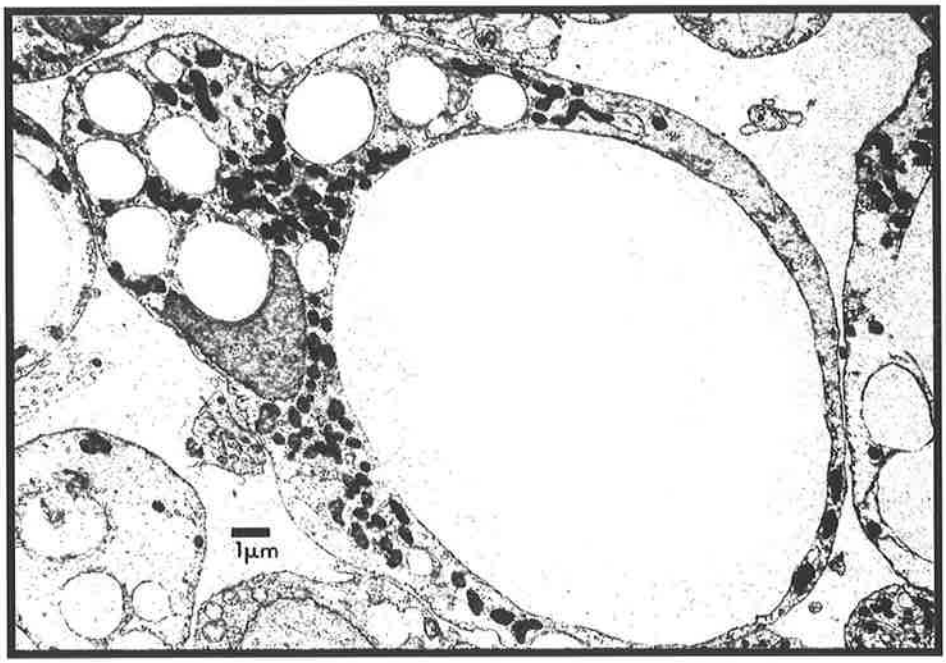


Figure 1.2 The ultrastructure of an adipocyte with a dominant lipid locule and several smaller locules from a sheep fetus.

Horizontal dark bar represents 1 µm. Photograph taken from Gemmell *et al* (84).

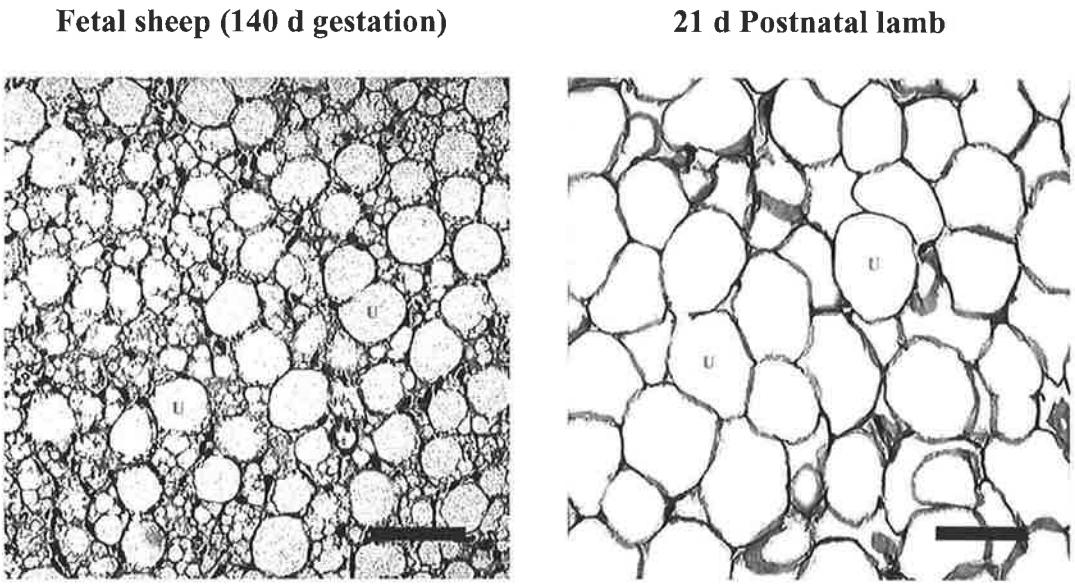


Figure 1.3 Photomicrograph images of perirenal adipose tissue in fetal and neonatal sheep.

Adipocytes with a dominant lipid locules in the perirenal adipose tissue are initially small and circular in fetal life and then increase in size and become hexagonal in postnatal life. There is also a loss of multilocular tissue within the perirenal adipose depot with advancing age. Examples of tissue defined as unilocular (U) or multilocular (M) adipose tissue are indicated on the section. The horizontal dark bar represents 50 μm. (Thanks to J Duffield for providing a sample of perirenal adipose tissue from a 21 d postnatal lamb).

A small proportion of adipose tissue is also found in the subcutaneous region of the fetal sheep. Like the perirenal adipose depot, at 70 days gestation small locule containing cells were observed in the subcutaneous adipose depot of the fetus (84). As gestation advances, characteristic differences begin to emerge between adipocytes from the perirenal and subcutaneous adipose tissues. At term, most of these adipocytes in the subcutaneous depot possess only one large locule and have few mitochondria. These subcutaneous cells have similar morphology to terminally differentiated white adipocytes. Multilocular cells were also found in subcutaneous adipose tissue and their appearance was similar to adipocytes found in the perirenal region. It has repeatedly been demonstrated that adipocytes from rodent adipose tissue with a white appearance express UCP-1 and that some of these adipocytes are multilocular and mitochondrial rich (8, 86, 87). This suggests that brown adipocytes are present in white adipose depots.

1.2.4 Development of adipose tissue in the neonate

At birth, maintenance of core body temperature is essential for the survival of newborn mammals, especially at low ambient temperatures (88). Core body temperature is maintained through the activation of thermogenesis. It has been found that more than 60-70% of non-shivering thermogenesis is generated in adipose tissue with a brown appearance (89). Mature brown adipocytes are packed with mitochondria that possess the brown adipocyte specific protein, UCP-1. This protein reduces the efficiency of the electron transport chain and in the process generates heat. During neonatal life, the expression of UCP-1 declines and the thermogenic function of adipose tissue is lost (90-92).

This loss of thermogenic activity is accompanied by cellular changes that occur in adipose tissue during the neonatal period. As previously mentioned, at term, perirenal adipocytes in newborn lambs are comprised of multilocular adipocytes, and unilocular adipocytes with several smaller locules. Independent of locule size, many locules are circular or oval in shape. By 21 days after birth, perirenal adipocytes in lambs are clearly hexagonal – a shape commonly associated with mature white adipocytes (93) (Figure 1.3). During this same period there is a significant increase in vascularisation and sympathetic innervation of adipose tissue and a concomitant accumulation of lipid (90).

1.2.5 Appearance of brown adipocytes in white adipose tissue

Whilst it has been demonstrated that white and brown adipocytes are derived from different preadipocyte cell lineages (70), it is well established that the proportion of adipose tissue occupied by brown adipocytes during fetal and early neonatal life is substantially more than in adulthood. There is a rapid increase, however, in UCP-1 abundance in the adipose tissue of adult rodents exposed to a low ambient temperature (94, 95). It was suggested that this increase was too rapid to be a consequence of preadipocyte proliferation and differentiation, rather it was speculated that some unilocular adipocytes could convert to multilocular – mitochondrial rich adipocytes that expressed UCP-1 (8). Little is understood about the mechanisms that cause the change in the appearance of adipose tissue from primarily a multilocular tissue to a distinctly unilocular tissue and vice versa, although several hypotheses have been put forward. Firstly, it is possible that brown adipocytes undergo apoptosis and subsequently proliferation of white adipocytes occurs. Secondly, it is postulated that despite separate lineages, brown adipocytes can give rise to white adipocytes (6, 9, 65). Thirdly, it has been speculated that white adipocytes can revert to brown adipocytes when appropriately

stimulated and these cells have been referred to by some researchers as "transitional" adipocytes (5, 8, 86, 96, 97).

Using the knowledge that the UCP-1 gene is specifically expressed in brown, but not white adipocytes, Moulin *et al* (9) manipulated the Cre/loxP DNA-recombinant system to attain specific tissue expression of UCP-1 and investigated the development of adipose tissue in mice. A transgenic strain of mice was bred to contain in their genome an insert where the UCP-1 promoter region (pUCP) was attached to the Cre gene and another insert where a constitutively expressed promoter (pCMV) was attached to locP stop sequences and finally to a lacZ gene (9).

When the UCP-1 promoters were activated in a cell, transcription of UCP-1 and Cre genes occurred, and the expression of the Cre enzyme resulted in the deletion of the locP stop sequences. Consequently, the lacZ gene was constitutively expressed. Moulin and colleagues hypothesised that if the brown preadipocyte differentiated into a brown adipocyte and finally a white adipocyte, then brown adipocytes would first express UCP-1, Cre and lacZ, but later when differentiated into a white adipocyte, lacZ expression would remain, however, UCP-1 and Cre expression would be lost. The alternative hypothesis was that adipocytes that expressed neither UCP-1, Cre or lacZ mRNA would therefore be derived from white preadipocytes. Moulin showed that lacZ mRNA expression was present in adipose depots typically described as brown adipose tissue (intrascapular) and absent in depots typically described as white adipose tissue (inguinal). Low levels of lacZ mRNA expression were detected in perirenal and retroperitoneal adipose tissue. The study clearly demonstrates that white adipocytes are derived from white rather then brown preadipocytes. In this study, perirenal and

retroperitoneal adipose depots also expressed relatively low levels of UCP-1 mRNA indicating the presence of brown adipocytes in these tissues suggesting that these depots can be comprised of both white and brown adipocytes (9).

Himms-Hagen *et al* (8) recently demonstrated that white adipose tissue contains small clusters of multilocular-mitochondrial rich adipocytes. When a known stimulator of brown adipocyte proliferation, a β_3-adrenergic receptor agonist, was administered to rats there was an increase in the numbers of these multilocular cells in white adipose tissue. This increase was a consequence of a sub-population of unilocular adipocytes that converted to multilocular-mitochondrial rich adipocytes. Furthermore, the increased abundance of new mitochondrial proteins in the adipose depot confirmed that the proliferation of multilocular-mitochondrial rich adipocytes was not derived from pre-existing multilocular adipocytes, i.e. transitional adipocytes. Together the Moulin *et al* (9) and Himms-Hagen *et al* (8) studies demonstrate that a population of unilocular adipocytes are derived from white, but not brown preadipocytes, and a second population of adipocytes with a unilocular appearance can convert to multilocular-mitochondrial rich adipocytes when appropriately stimulated, i.e. transitional adipocytes.

In 1994, a significant breakthrough was made in the understanding of the regulation of body adiposity. It was discovered that adipocytes could synthesise and secrete a hormone, which exerts a regulatory role in the central and peripheral systems to control energy homeostasis (12-15). This finding has resulted in an associated increase in our knowledge of the functions of adipose tissue, beyond their lipid storage capacity.

1.3 DISCOVERY OF A SATIETY FACTOR

In 1940, Hetherington and Ranson lesioned the hypothalami of rats and showed that these rats accumulated fat over time and when allowed to feed *ad libitum* became severely obese (98). These studies showed that the brain, and in particular, the ventromedial region of the hypothalamus was a "satiety centre". Subsequently, Kennedy (99) observed that substantial variations in food intake were associated with changes in body temperature. These findings and others led Coleman and colleagues (100-102) to perform several parabiosis studies, linking the circulation of a normal mouse with one of two types of genetically obese mice in an attempt to demonstrate the presence of a "satiety factor" in the peripheral circulation.

In 1950, Ingalls *et al* (103) used Mendelian selection techniques to breed a massively obese strain of mouse. This mouse was known as the obese or ob/ob mouse and its phenotype was caused by a recessively inherited genetic defect that leads to the early development of obesity. A diabetic (db/db) strain of mice was also bred and these mice were found to be hyperglycaemic and obese. Whilst these strains were genetically distinct, they were both hyperphagic and hypothermic (100, 103). One distinct difference between these strains of mice was the obesity shown by the db/db mouse was less marked than that in the ob/ob mouse (100).

Coleman (101, 102) performed parabiosis studies on normal, obese (ob/ob) and diabetic (db/db) mice in which the circulations of two strains of mice were partially surgically connected. Parabiosis of the db/db and normal mouse caused the death of both mice within 50 days after surgery and in the majority of cases, the normal mouse in the partnership died first. When the mice were separated at post mortem, the average body

weight of the normal mouse had declined (24%) when compared to before the surgery. These normal mice had very little food in their stomachs and little or no deposits of adipose tissue. In contrast, the stomachs of the db/db partners were bulging with food and body weight had increased on average 46%. Coleman concluded that the normal mouse had starved and the diabetic mouse had increased its food intake excessively (101).

A similar result was found when ob/ob mice were parabiosed with db/db mice. Whilst both partners died, the ob/ob partner loss fat mass and appeared to have starved to death (102). Coleman and colleagues suggested that the db/db partner produced, but did not respond to, a blood derived satiety factor that prevented overfeeding (100).

Parabiosis of the two ob/ob mice lead to gains of more than 50% in weight by 4 months after surgery, compared to before surgery in both mice. Interestingly, these mice appeared to be in good health and minimal deaths were observed (102). When the normal and ob/ob mice were parabiosed it also lead to viable unions. The ob/ob partner ate less and gained less weight than ob/ob mice parabiosed with another ob/ob mouse (102). This observation suggested that a humoral factor provided by the normal mouse inhibited food consumption in the ob/ob partner and supported the conclusion that ob/ob mice have a functional "satiety centre" that was capable of responding to the humoral factor produced by normal as well as db/db mice (100). Sixteen years later, Zhang and colleagues (12) discovered the satiety factor that was primarily expressed in adult adipose tissue and named this factor, leptin. Within a year, Campfield, Halaas, Pelleymounter, Weigle, Stephens and colleagues (104-108) had demonstrated the role of the hormone, leptin, in the control of satiety, and Tartaglia (109) reported the expression

of the leptin receptor in the brain. Together these findings confirmed the original hypothesis of Coleman that there was a circulating factor which controlled satiety.

1.4 THE DISCOVERY OF LEPTIN AND ITS PHYSIOLOGICAL ROLE

In 1994, Friedman and colleagues cloned the gene encoding the circulating satiety factor, which Coleman had deduced must exist, and named it leptin (from the Greek *–leptos*, thin) (12). Since then the leptin gene has been isolated in many mammalian species, including humans, mice, rats, sheep, pigs and cows (12). The leptin gene is highly conserved between species (110) and when transcribed yields a ~4.4 kb transcript (12, 111, 112). In humans, the gene spans ~20 kb of which 4240 nucleotides and a poly (A) sequence of ~200 nucleotides are transcribed into messenger RNA (mRNA).

In adult mammals, adipose tissue has the highest abundance of leptin mRNA (12, 113). Mature white adipocytes are the predominant cell in adult adipose depots and when cultured are found to express leptin mRNA and secrete leptin protein (114). Moreover, leptin mRNA abundance in adipose tissue is correlated highly with the concentration of leptin in the circulating which indicates that circulation leptin is derived principally from adipose tissue (115). Not surprisingly, obese mammals have higher levels of leptin mRNA expression and circulating leptin concentrations than lean mammals (113, 116-118). Other tissues also express low levels of leptin mRNA, including liver (119), stomach (120-122), mammary gland (123-125) and skeletal muscle (126) and placenta (127-132). It is unclear, however, what paracrine or autocrine role, if any, leptin plays in these tissues.

The leptin gene of the ob/ob mouse produces mRNA transcripts that contain a substitution mutation creating a premature stop codon. The translated transcript generates a truncated protein, which is then degraded intracellularly i.e. the ob/ob mouse is leptin deficient (12). The obese phenotype in the ob/ob, but not db/db mice, could be reversed when recombinant leptin was administered daily. Less dramatic effects on body weight were observed when leptin was infused into normal mice. The decrease in body fat, which occurred within two days of infusing leptin in leptin deficient and normal mice, was caused by a decrease in appetite and an increase in core body temperature (104-108).

Stephens and colleagues (108) provided evidence to link the actions of leptin to the "satiety centre" in the brain. They showed that large subcutaneous doses (400-4000 μg/kg) of recombinant mouse leptin were required to suppress feeding in leptin deficient mice, however, similar effects could be achieved with lower doses (0.6-40 μg/kg) of leptin when administered directly into the third ventricle of the hypothalamus (108). This is consistent with the hypothesis of Coleman and others, of a peripheral factor acting centrally to regulate energy balance via effects on food intake, adipose tissue mass and thermogenesis.

1.5 REGULATION OF LEPTIN SYNTHESIS AND SECRETION

In the adult human (114, 133, 134), rodent (133) and ruminant (135, 136), there is a close association between body weight or fat mass and both the abundance of leptin mRNA in adipose tissue and circulating leptin concentrations. Energy restriction and fasting each decrease plasma leptin concentrations to a greater extent than can be predicted by the loss of body fat mass (137-140). Boden *et al* (137) showed that whilst fasting for 52 h decreases circulating leptin levels in humans, the percentage of the subject's body fat

remained unchanged during this period. This and other observations suggest that leptin synthesis, secretion and clearance are influenced by other factors besides fat mass.

Depot-specific differences in leptin gene expression have been demonstrated in adipose tissue in the human (141-143), rodent (144, 145) and sheep (115). The abundance of leptin mRNA is higher in adipocytes from the parametrial and perirenal regions than in those from the subcutaneous region of female rats (144). Ehrhardt (146) found, however, that leptin mRNA abundance in subcutaneous adipose tissue was 2-3 times greater than in perirenal adipose tissue in pregnant ewes. Furthermore, Hube *et al* (141) has shown that subcutaneous adipose tissue contained more leptin mRNA than the omental depot in humans. Interestingly, leptin mRNA expression in subcutaneous, epididymal and retroperitoneal adipose depots is positively correlated with adipocyte volume which suggests that the relationship between leptin gene expression and the adipose depot may be related to volume of the adipocytes within the depots (145). Consistent with these findings, Lonnqvist *et al* (114) showed that leptin expression and secretion by subcutaneous adipocytes from lean and obese women was positively correlated with adipocyte numbers and both the size and lipid content of the adipocytes.

1.5.1 Dietary regulation of leptin

Whilst food intake and food deprivation have opposite effects on circulating leptin, it has been speculated that components within the diet may regulate leptin production and secretion. Frederich and colleagues (147) showed that a high fat diet elevated circulating leptin levels in mice. In humans, Raben *et al* (148) found however, that meals that were fat, carbohydrate or protein rich but had similar caloric energy intake and similar effects on circulating leptin concentrations in humans. In another study, humans who consumed

a high fat, but low carbohydrate meal were found to have lower circulating leptin concentrations, 24 h after the meal (149), however prolonged consumption (12 days) of a high fat diet did not alter circulating leptin concentrations (150). Interestingly, rats fed a high fat diet for 5 months had higher circulating leptin concentrations compared to those on a control diet (151).

Irrespective of the contribution that dietary components have on the synthesis and secretion of leptin, Saladin *et al* (152) demonstrated that leptin expression in adipose tissue is lower in fasted than in fed rats and refeeding of fasted rats restored leptin mRNA abundance to that measured in fed rats (152). More specifically, Ahima *et al* (153) showed that in fasted mice, the fall in plasma leptin levels was coincident with a decrease in plasma glucose and insulin concentrations and an increase in plasma glucocorticoid concentrations and it was speculated that diet sensitive hormones or metabolites could regulate circulating leptin.

1.5.2 Glucose regulation of leptin synthesis and secretion

Glucose stimulates leptin production in cultured adipocytes (154, 155). Rat adipocytes perfused with glucose increased leptin production in a dose dependent manner (155). Several glucose-mediated pathways regulate adipocyte synthesis and secretion of leptin. Leptin secretion can be suppressed when cultured adipocytes are either unable to transport glucose intracellularly or are unable to perform glycolysis. These effects, however, are reversible by the addition of glucose, but not insulin (155). In adipocytes and muscle cells, glucose is transported into the cytoplasm where it is converted to glucose-6-phosphate, a rate limiting substrate for glycolysis. The hexosamine biosynthetic pathway receives a small proportion of glucose that is converted to fructose-

6-phosphate. Both UDP-N-actylglucosamine, the end product of this pathway, and glucose increase the abundance of leptin mRNA in adipose tissue and circulating leptin concentrations in rats (126). Indeed, when the rate limiting enzyme of the hexosamine biosynthetic pathway, fructose-6-phophate amidotransferase, is overexpressed in the adipose tissue of transgenic mice both the adipose tissue expression of leptin and circulating leptin concentrations are elevated (156).

1.5.3 Insulin regulation of leptin synthesis and secretion

Infusion of glucose increases circulating leptin, but also results in hyperinsulinemia (157) and therefore the effects of insulin, independently of glucose, on leptin synthesis and secretion cannot be discounted. In *in vitro* experiments, insulin regulates leptin gene expression and secretion independently of glucose (158). Insulin stimulates leptin production in mature white and brown adipocytes in a dose dependent manner (159, 160). In humans, circulating leptin concentrations increased when insulin was administered and euglycemia was maintained (161). Indeed, leptin expression was suppressed in the adipose tissue of pigs treated with streptozotocin, which destroys the β-pancreatic cells of the animals and makes them hypoinsulinemic. Leptin expression could be restored when these animals were treated with insulin (162).

At a molecular level, one of the activators of leptin gene expression is the hetrodimeric transcription factor, adipocyte determination differentiation dependent factor (ADD) 1 and sterol regulatory element binding protein (SREBP) 1. The expression of ADD1 and SREBP1 is suppressed in adipose tissue of fasted rats and is upregulated by insulin. Indeed, Kim *et al* (163) showed that the ADD1/SREBP1 complex binds to the insulin response element (IRE) of the leptin promoter and when the IRE is mutated,

ADD1/SREBP1, is unable to transactivate the leptin gene. Therefore, nutrition may regulate leptin expression through fluctuations in insulin concentration that mediate the levels of the ADD1/SREBP1 transcription factor in adipocytes and consequently alters the abundance of leptin mRNA. Irrespective of the cellular and molecular mechanisms that control the regulation of the leptin gene, nutrition acts through fluctuations in glucose and insulin levels to regulate leptin synthesis and secretion in the adipocytes of mammals.

1.5.4 The role of the SNS in the regulation of leptin synthesis and secretion

It is well established that an increase in sympathetic nervous activity (SNS) results in the mobilisation of fatty acids from adipose tissue and in the activation of thermogenesis (164, 165). Evidence has shown that noradrenergic activity also modulates leptin synthesis and secretion from adipocytes. Individuals with spinal cord injury are at a higher risk of developing obesity than able-bodied individuals (166, 167). Jeon *et al* (168) showed that in able-bodied people, the resting metabolic rate was related to circulating leptin concentrations and that this relationship was not present in a group of people with spinal cord injury. In Siberian hamsters, denervation of adipose depots, which had a white appearance, caused an increase in the mass of adipose tissue and an increase in the number of fat cells in the depot compared to intact animals. Adrenaline and the synthetic β-adrenergic receptor agonist, isoprenaline, decrease leptin expression and circulating leptin concentrations in humans (169-171) whilst in the rodent, the administration of either noradrenaline or isoprenaline also decrease leptin synthesis and secretion (172-175).

Neuronal catecholamine synthesis is suppressed by inhibition of the rate limiting enzyme tyrosine hydroxylase and this causes the depletion of noradrenaline in tissues (174). Infusion of an inhibitor of tyrosine hydroxylase results in an increase in circulating leptin concentrations in normal but not leptin deficient mice (174). The additional infusion of a β_3-adrenergic receptor agonist attenuated the increase in circulating leptin observed in these normal mice. Interestingly, these same receptors in adipose tissue are necessary for the activation of UCP-1 for cold-induced and diet-induced thermogenesis (164, 176, 177).

1.5.5 Biological rhythms in leptin synthesis and secretion

Saladin and colleagues (152) demonstrated that leptin expression in rat adipocytes *in vivo* were lowest during the light phase and increased whilst feeding and were maximal later during the dark phase. Frequent blood sampling of lean and obese human subjects over 24 h period shows that circulating leptin concentrations are lowest between 08:00 and 14:00 and peak between 20:00 and 02:00. Interestingly, the amplitude of the biological rhythm of obese subjects was significantly greater than in lean subjects. These changes in circulating leptin were independent of changes in fatness and food intake as meals were provided at regular 4-5 h intervals (178, 179). Besides the biological rhythm of circulating leptin, small fluctuations in circulating leptin were observed after every meal.

Leptin expression and circulating leptin concentrations decrease in adult mammals exposed to short periods of light, i.e. short photoperiods (180, 181). These effects may occur through prolactin and other pituitary hormones, which are regulated by photoperiod hormones. Interestingly, adipose tissue expresses prolactin receptors (182),

and prolactin concentrations are regulated by photoperiod (183, 184) and can increase circulating leptin concentrations in rats (185).

1.5.6 Sex hormones

Females generally have a higher percentage body fat and also have higher circulating leptin concentrations than males (133). Serum leptin levels are found to be lower in ovariectomised female rats whilst the administration of estradiol can restore circulating leptin concentrations (186). 17-β estradiol also increased adipocyte expression of leptin and these effects could be inhibited with ICI_{182780}, an estrogen receptor antagonist. Dihydrotestosterone, an androgen metabolite of testosterone, suppresses leptin expression in adipocytes but these effects can be restored by the addition of an androgen receptor antagonist, cypoterone acetate. This suggests that sex hormones regulate leptin expression and secretion in males and in females, and that these effects are independent of fat mass and feeding patterns.

1.5.7 Role of glucocorticoids

Glucocorticoids also regulate the synthesis and secretion of leptin by adipocytes (187, 188). In humans, a potential glucocorticoid response element has been identified 1-1.5 kb before the start codon of the leptin gene (189). It remains to be determined, however, whether this potential glucocorticoid response element can transactivate the leptin gene. Irrespective of this, *in vivo* administration of cortisol or the synthetic glucocorticoid, dexamethasone, increases leptin expression in mature white adipocytes and circulating leptin levels in humans (158, 190, 191) and rodents (14). Cortisol also has a synergistic effect on insulin induced leptin secretion by human white adipocytes (158).

Buyse *et al* (160) demonstrated that glucocorticoids also regulate the expression of leptin in mature brown adipocytes. The T37i cell line is derived from a brown fat malignant tumour in the mouse and these cells can be induced to differentiate into mature brown adipocytes that express UCP-1 mRNA (192). In the presence of dexamethasone, insulin induced leptin secretion is suppressed in these mature adipocytes (192). It appears that glucocorticoids exert differential effects on white and brown adipocytes and this may explain some of the inconsistencies in studies relating to leptin expression in which the cellularity of the adipose tissue has not been defined.

1.5.8 Growth hormone and Insulin like growth factor-I

The contribution of growth hormone (GH) and IGF-I to the regulation of leptin expression in adipose tissue has been examined in several studies. Over three months, GH treatment in men decreased serum leptin levels and reduced body fat (193). However, in hypophysectomised rats, where the pituitary is surgically removed producing GH deficiency, leptin expression in adipose tissue was unaffected by GH treatment, but was decreased by IGF-I administration (194). IGF-I infusions in humans reduced plasma leptin concentrations (195). Houseknecht *et al* (196) demonstrated that GH alone was unable to directly stimulate leptin mRNA expression in bovine adipocytes but was able to suppress insulin or dexamethasone induced leptin expression. Castrated male cattle treated with GH had increased adipose tissue expression of leptin and IGF-I mRNA. Interestingly, leptin and IGF-I mRNA abundance were positively correlated in treated animals (196) suggesting a mechanistic pathway for the actions of GH and IGF-I on leptin gene expression in adipose tissue.

1.6 LEPTIN RECEPTOR

Leptin protein binds with high affinity to circulating and membrane bound leptin receptors (109, 197-200). Multiple forms of the leptin receptor (Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re) have been detected in the human, rat, mouse and sheep and there is a high amino acid sequence homology between all leptin receptor isoforms (109, 197, 199, 201-203). The various forms of leptin receptors are generated from a single gene using different splice variants (204). With the exception of one form of the leptin receptor (Ob-Re), the remaining leptin receptor isoforms contain a single membrane-spanning protein, which is homologous and shares similar structural and functional properties with the class 1 cytokine receptor family (199, 205). One form of the leptin receptor, the long form (Ob-Rb), has a cytoplasmic domain of 303 amino acids and contains sequence motifs consistent with the capacity to transduce intracellular signal. The remaining membrane bound leptin receptor isoforms have little or no cytoplasmic motifs capable of intracellular signalling (Ob-Ra, Ob-Rc, & Ob-Rd) (see section 1.6.3) (199, 205).

1.6.1 Long form of the leptin receptor (Ob-Rb)

The long form of the leptin receptor is expressed in a number of mammalian tissues. Ob-Rb mRNA is found in highest abundance in the hypothalamus, specifically in the arcuate nucleus (also known as the infundibular nucleus in ruminants), and in the ventromedial (VMN), paraventricular (PVN) and dorsomedial (DMN) nuclei (15). The unresponsiveness of the db/db mouse to leptin is the result of a mutation in the gene encoding the leptin receptor. This mutuation prevents leptin from signalling intracellularly and hence acting as a satiety factor (105, 106, 201, 202).

Ob-Rb mRNA is also detected at relatively lower levels in a number of other tissues including the pituitary (206, 207), the adrenal cortex (208, 209) and medulla (210), adipose tissue (203), kidney (109), skeletal muscle (109), lung (109, 211), pancreas (212, 213) and placenta (127, 214-216).

1.6.2 Leptin receptor signalling pathway

Cytokines are ligands for the cytokine receptor family and control a variety of biological responses including cell growth, differentiation and apoptosis. Members of the class 1 cytokine receptor superfamily are known to signal intracellularly through Janus kinases (JAK) and signal transducers and activators of transcription (STAT) proteins. Typically, JAK proteins are constitutively associated with the intracellular membrane proximal domain of a cytokine receptor. Binding of the ligand to the receptor causes receptor dimerisation and JAK proteins are autophosphorylated. This induces the phosphorylation of tyrosines on the cytoplasmic domain of the receptor. The phosphorylated intracellular domains provide binding sites for STAT proteins which form homo and hetero dimers that can translocate into the cell nucleus and stimulate gene transcription (217-219). It is this cell signalling pathway which is typically associated with the leptin receptor, appetite regulation and the development of obesity.

Leptin signals intracellularly by binding to Ob-Rb resulting in activation of JAK-2 and recruitment of STAT-3 and STAT-5 (218, 220). STAT-3 proteins are highly abundant in hypothalamic nuclei and other regions within the central nervous system (CNS), suggesting that, at least in the hypothalamus, the actions of leptin on hypothalamic leptin receptors are mediated through STAT-3 (221-223). Bates and colleagues (224) produced a transgenic mouse (s/s) where tyrosine 1138 on the long form of the leptin receptor was

replaced with a serine residue that specifically disrupted STAT-3 signalling. It was shown that whilst differences existed between db/db and s/s mice, relating to fertility, hyperglycaemia and hypothalamic NPY expression, an alternative leptin mediated appetite pathway in the CNS (i.e. melanocortin system) was suppressed in both strains of obese mice. This confirmed that STAT-3 signalling mediated the actions of leptin on the long form of the leptin receptor to alter other neuroendocrine pathways, besides NPY, that controlled body energy homeostasis.

1.6.3 Leptin receptor short forms

The functional roles of the membrane-bound short forms of the leptin receptor (Ob-Ra, c-d) remain to be elucidated. Ob-Ra is the most abundantly expressed form of all leptin receptors and is expressed in a number of tissues including the hypothalamus (109, 211, 225, 226) and placenta (214, 227, 228). Some evidence suggests that this receptor may be involved in cell signalling or the transport of leptin across cell membranes. It is well established that leptin signalling occurs through the JAK-STAT pathway (205), however, unlike Ob-Rb, Ob-Ra lacks the necessary binding sites to recruit STATs and hence this prevents the activation of the JAK-STAT pathway (205, 229). It has emerged that leptin can also act through another signalling pathway, including the Ras/mitogen-activated protein kinases (MAPK) pathway (229), a pathway typically associated with cell growth and differentiation (230). Several studies have shown that Ob-Ra can signal through this pathway (231-233), however, the importance this pathway in relation to the regulation of appetite remains unclear (230).

Studies have suggested that Ob-Ra is involved in the transport leptin across cell membranes (234-242). In rodents, the choroid plexus has been shown to express Ob-Ra

and is thought to actively regulate the availability of leptin to the CNS via a unidirectional transport system (238, 243). Hileman *et al* (240) used a monolayer of Madin-Darby Canine Kidney cells to overexpress Ob-Ra. They showed that >10% of radiolabelled leptin was transported from one side of the monolayer to the other. It can be concluded from these and other studies that this receptor can function to transport leptin across cell membranes.

The Ob-Re is the only leptin receptor isoform that lacks a membrane-spanning domain. Its expression can be detected in placental (214, 244) and hypothalamic tissue (244). The receptor can be detected in the blood of pregnant and non-pregnant mammals and acts as a circulating binding protein (197, 198, 245). It is speculated that the receptor may be produced from proteolytic cleavage of other membrane-associated leptin receptors, such as Ob-Rc and Ob-Rd for which a functional role is yet to be determined (198, 245).

1.7 ACTIONS OF LEPTIN

1.7.1 Action of leptin in the hypothalamus

In the rodent, the arcuate nuclei are located adjacent to each lateral wall of the third ventricle in the mediobasal hypothalamus. Within these nuclei, there are at least two groups of leptin responsive neurons that have opposing actions on food intake; the neuropeptide Y (NPY)-synthesising neurons which also express agouti-related protein (AGRP) (246, 247) and an adjacent population of neurons which co-express proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (248, 249). Axons from the NPY/AGRP and POMC/CART neurons project into both the paraventricular nuclei and lateral hypothalamic areas where they act to regulate

energy balance and neuroendocrine functions. Interestingly, both populations of neurons co-express the long form of the leptin receptor (249-253).

During periods of low caloric intake, circulating leptin concentrations fall and there is a concurrent increase in NPY and AGRP gene expression and a decrease in POMC and CART gene expression that occurs in the neurons of the arcuate nuclei (15). It is speculated that leptin's actions on food intake and energy expenditure can be explained through changes in NPY. NPY is the most potent, short-term stimulator of appetite known (15). Central administration of NPY stimulates food intake and suppresses energy expenditure in mammals whilst continuous or repeated administration of the neuropeptide leads to the development of obesity in rats (254, 255).

Leptin acts through leptin receptors to decrease NPY gene expression. As previously mentioned, Ob-Rb and NPY are co-expressed in the same neurons in the hypothalamus (250). When leptin is administered either centrally or peripherally, hypothalamic NPY gene expression is suppressed in normal mice. Interestingly, Erickson and colleagues (256) showed after 10 weeks of age, leptin deficient mice and, leptin and NPY deficient (double mutant) mice were significantly heavier than normal mice. Six weeks later, the double mutant males weighed 13% less than leptin deficient male mice whilst the double mutant females weighed 27% less than their leptin deficient counterparts. These increases in body weight in both strains of mice were due to the accumulation of adipose tissue. In an important study, however, Erickson *et al* (257) found that obesity did not develop in NPY knockout mice and furthermore these mice appeared to have a normal metabolic response to fasting. Erickson's findings brought into question the relevance of

the NPY signalling pathway and implicated an alternative neuronal pathway which was responsive to leptin.

1.7.2 Melanocortins and other neuropeptides

Other neuropeptides expressed in the arcuate nuclei, i.e. CART, POMC and AGRP, are regulated by leptin and mediate energy balance. CART is expressed in a number of regions of the brain, in particular, the arcuate nucleus of mice (258) and humans (259) and the infundibular nucleus of sheep (253). Central administration of CART suppresses food intake (260-262), whilst central administration of antisera raised against CART increases feeding in normal mice (260). In leptin deficient mice, hypothalamic CART expression is suppressed and CART mRNA levels can be restored with the administration of leptin (260). Therefore, it appears that CART is an important component of the regulatory pathway through which leptin acts to control appetite.

Another central pathway through which leptin acts to modulate food intake is via the melanocortin signalling system. This system regulates food intake through the agonist, alpha-melanocortin stimulating hormone (α-MSH) and antagonist, AGRP (263-268). POMC is not only synthesised in the corticotrophs of the anterior pituitary but also at relatively low levels in the neurons of the CNS (252). POMC can be cleaved into a number of products including α-MSH and adrenocorticotrophin (ACTH). Central administration of leptin stimulates hypothalamic expression of POMC mRNA in the arcuate nucleus of adult rats (269). The importance of POMC derived peptides on the regulation of appetite is demonstrated by several studies. When α-MSH or the synthetic peptide ACTH (1-24) is centrally administered food intake is inhibited in adult rats (270,

271) and moreover, when rats are fasted for 24 h then presented with food, their appetite is inhibited when ACTH (1-24) is centrally administered (272).

Melanocortin receptors mediate a number of the actions of POMC and POMC derived peptides (Figure 1.4). Melanocortin receptors, MC3-R and MC4-R, are localised in a number of regions of the brain, including the hypothalamus. Like the leptin receptor deficient mouse, the MC4-R knockout mouse is obese, hyperphagic, hyperinsulinaemic and hyperglycaemic (264, 273). Selective agonists and antagonists to the MC4-R are found to mediate the central actions of melanocortins on feeding behaviour (274-279). In rats, leptin's effects on feeding behaviour and body weight can be attenuated with a MC4-R antagonist (HS014) (280). Furthermore, feeding behaviour is not inhibited when α-MSH is administered to MC4-R knockout mice (266, 281). The functional role of MC3-R remains unclear. Some MC3-R knockout mice have been found to be obese whilst other researchers find no increase in adipose tissue accumulation in these mice (282, 283). It remains to be determined what role, if any, the MC3-R plays in the control of energy homeostasis. Agouti protein and AGRP mediate the antagonist arm of the melanocortin systems (284) (Figure 1.4). Agouti can inhibit α-MSH binding to MC1-R in hair follicle melanocytes (285). In rodents, agouti is normally expressed in skin and regulates skin pigmentation, but ubiquitous expression of agouti is found to lead to the development of obesity, hyperphagia and hyperinsulinaemia (286). Whilst in humans, agouti is expressed in a number of tissues including adipose tissue, gonads and heart, it does not appear to play a role in skin pigmentation (287, 288). In humans, its biological function remains to be elucidated. Interestingly, when leptin is administered, agouti and AGRP abundance can be inhibited in the hypothalamus (289, 290).

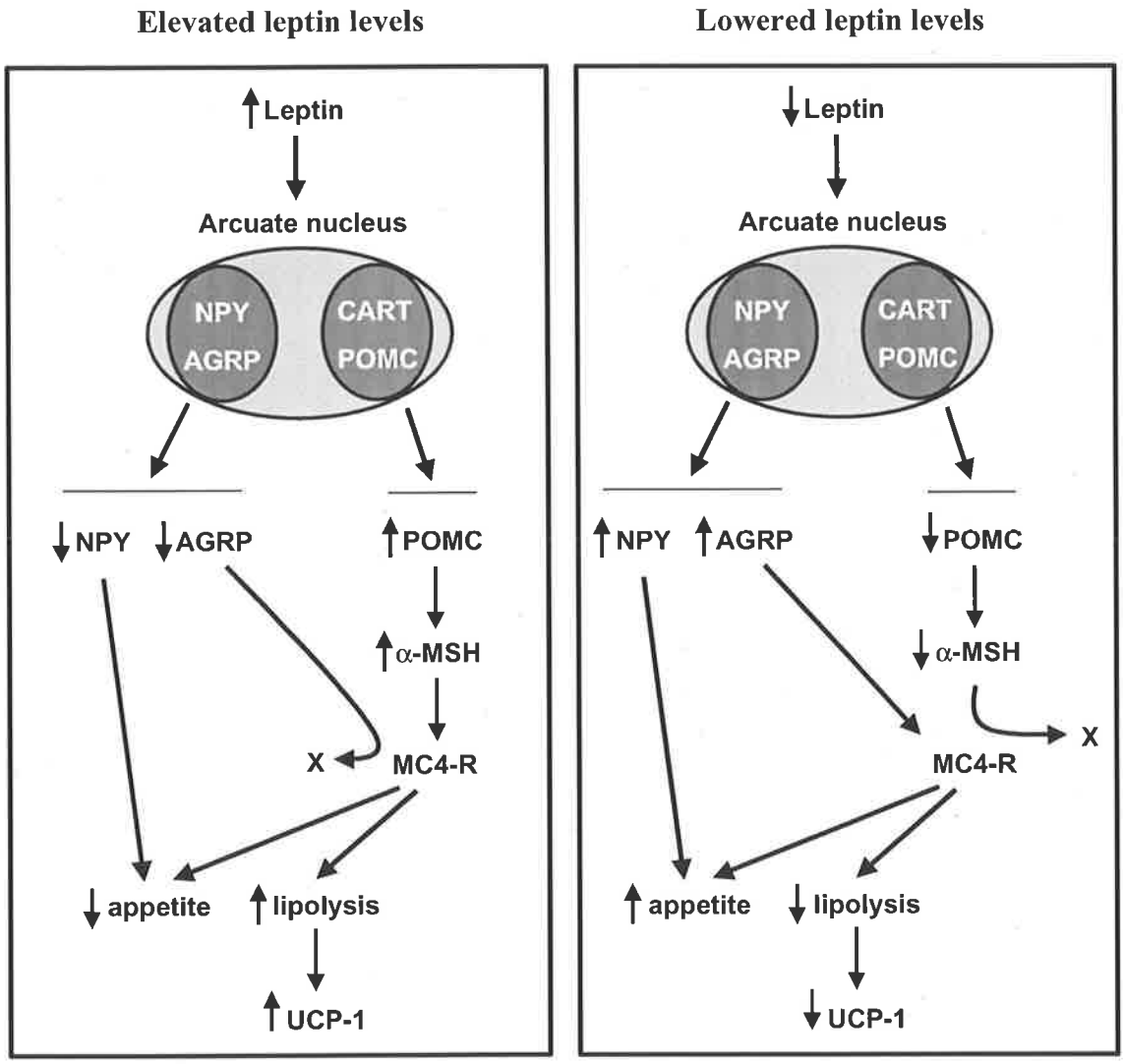


Figure 1.4 Actions of leptin on the melanocortin system

Schematic diagram representing the effect of leptin on neuropeptides in the hypothalamus. Leptin acts on the leptin receptors in the arcuate nucleus in the hypothalamus to alter the melanocortin system to regulate energy homeostasis. "X" represents when the neuropeptide is unable to bind to its receptor. Modified from Schwartz *et al* (15).

A structurally related protein, known as AGRP, is expressed both peripherally and centrally (284, 291) and inhibits the centrally expressed melanocortin receptors, MC3-R and MC4-R. Overexpression of AGRP in transgenic mice results in hyperphagia (286). Central administration of AGRP and another MC3-R/MC4-R antagonist (SHU9119) inhibits feeding in rats (292). Interestingly, SHU9119 can also attenuate the leptin induced increase in UCP-1 abundance in the mitochondrial protein suggesting that the melanocortin system may play a role in the activation of thermogenesis (293). Consistent with this finding, central administration of a MC3-R/MC4-R agonist (melanotan-II) stimulates sympathetic output to adipose tissue with a brown appearance (293).

Hence, leptin's effects on food intake and diet-induced thermogenesis are mediated by changes in neuropeptides such as NPY, AGRP, POMC and CART which are expressed in the hypothalamus. These neuronal signals are integrated and transmitted to an independent second order neuronal signalling pathway in the PVN and lateral hypothalamus that secretes corticotrophin releasing hormone (CRH), orexin, melanocortin releasing hormone (MCH) and thryrotropin releasing hormone (TRH) which directly regulate energy balance in the adult.

1.7.3 Regulation of hypothalamic-pituitary-adrenal function

In adult mammals, leptin interacts with the hypothalamo-pituitary-adrenal (HPA) axis to regulate food intake and fat storage in response to nutritional stresses (Figure 1.5). The pituitary-adrenal axis is controlled by the hypothalamic PVN which synthesises and secretes CRH, and other ACTH secretagogues, including arginine vasopressin (AVP), from nerve terminals in the median eminence, into the hypophyseal portal circulation. The PVN itself is regulated by a diverse set of signals from surrounding nuclei in the

hippocampus and hypothalamus, including the arcuate nucleus. Central administration of NPY increases AVP and CRH secretion into the hypophyseal portal veins (294, 295). These neuropeptides bind to AVP and CRH receptors on corticotrophin cells in the anterior pituitary stimulating POMC mRNA synthesis. POMC is then cleaved, processed and secreted into the circulation. ACTH is one cleavage product of this process and it subsequently induces the synthesis and secretion of glucocorticoids by the adrenal cortex (296-298).

The evidence linking the actions of leptin to the suppression of hypothalamic NPY expression is well described. Recently, leptin has been shown to alter the hypothalamic release of CRH (299). Hypoglycaemia induced release of CRH could be suppressed by leptin in isolated rat hypothalami *in vitro*. Moreover, plasma ACTH and glucocorticoid concentrations were elevated in restrained rats but when leptin was peripherally administered, circulating concentrations of these hormones decreased (299). This implies that leptin can act at the hypothalamus to suppress NPY and consequently the CRH and AVP induced stress response involving ACTH and glucocorticoids, and explains how the converse occurs during fasting when circulating leptin decreases and plasma cortisol concentrations increase (153).

1.7.4 Leptin and the adrenal gland

The long form of the leptin receptor has been detected by RT-PCR in cultured adrenocortical cells from a number of species including humans, rats, mice, sheep and cows (208, 209). Whilst leptin is able to regulate the glucocorticoid stress response through the hypothalamo-pituitary axis, recent evidence has suggested that leptin also has a direct action on the adrenal cortex (208, 300) (Figure 1.5).

Leptin directly inhibits ACTH mediated cortisol release in a dose dependent manner in isolated rat, bovine and human adrenocortical cells (208, 300). Leptin suppresses cortisol production by inhibiting several key rate-limiting enzymes that are involved in the conversion of cholesterol to cortisol (Figure 1.6). These include the steroidogenic enzymes cytochrome P450, C21-hydroxylase, the side chain cleavage enzyme, C17α-hydroxylase and steroidogenic acute regulatory protein (StAR). The expression of each of these enzymes in cultured adrenocortical cells can be inhibited by leptin (208, 300, 301), which demonstrates that leptin can directly influence the synthesis and secretion of cortisol by inhibiting the enzymes involved in the conversion of cholesterol to cortisol.

Recent evidence suggests that leptin also regulates the secretion of catecholamines. The stimulation of the nicotinic receptors in adrenal medullary chromaffin cells increases the synthesis and secretion of catecholamines by these cells. Like the adrenal cortex, the Ob-Rb has been detected in the adrenal medulla of adult mammals. In humans, leptin stimulated catecholamine synthesis in cultured bovine and porcine adrenal medullary cells via activation of phosphorylation of tyrosine hydroxylase (302).

1.7.5 Action of leptin on lipolysis

Leptin increases oxidation of fatty acids, inhibits lipogenesis in adipocytes (126, 303) and stimulates the release of lipids by adipocytes (304). Leptin also induces the release of glycerol from adipocytes (305). The administration of leptin elevated circulating fatty acid concentrations in leptin deficient but not leptin receptor deficient mice (105, 107). Scarpace *et al* (306) showed that lipoprotein lipase mRNA is increased in adipose tissue with a brown appearance and that this effect was independent of sympathetic innervation

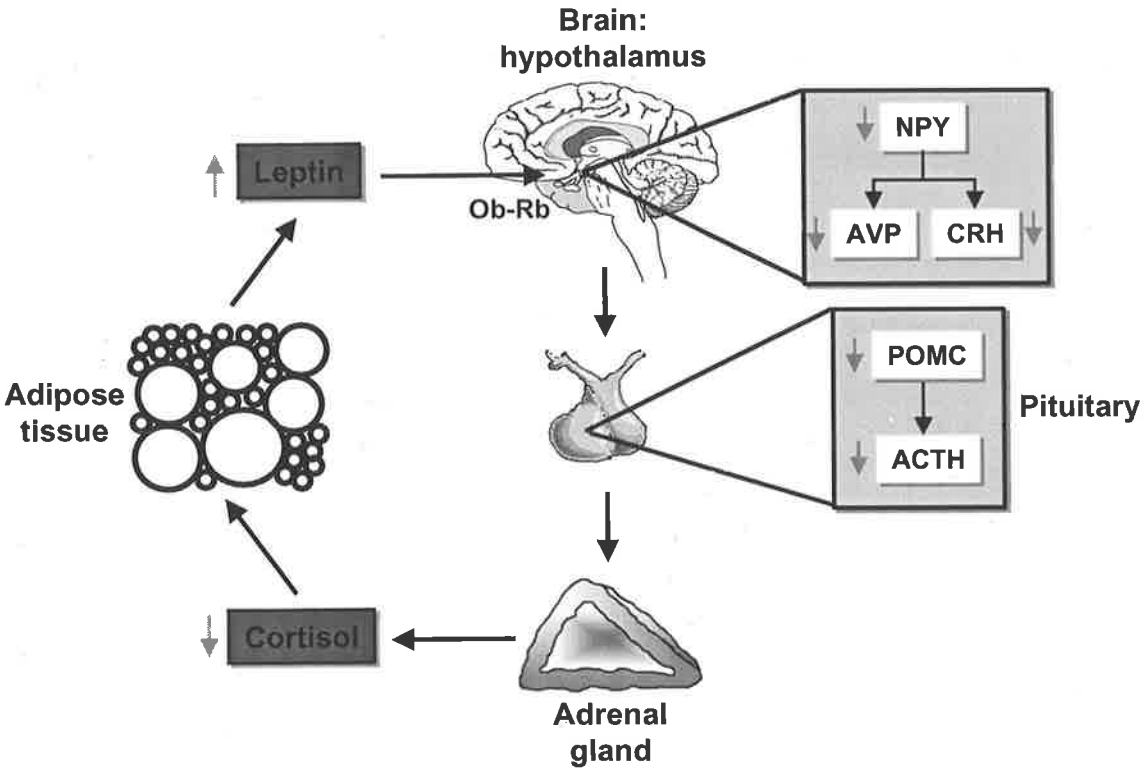


Figure 1.5 Action of leptin on adrenal functioning

Diagram represents the actions of leptin on the adrenal via potential direct and indirect pathways. Leptin has been shown to suppress the activation of the HPA axis and reduce cortisol secretion from the adrenal (indirect pathway). Leptin is also able to directly suppress adrenal steroidogenesis independently of changes in ACTH concentrations (direct pathway).

and suggests that leptin is directly acting on adipocytes via leptin receptors. When fully differentiated brown adipocytes from lean rats were treated with leptin for 24 h, there was also an increase in lipoprotein lipase mRNA (1.9 fold). However, administration of leptin to cultured adipocytes from leptin receptor deficient rats did not change lipoprotein lipase gene expression (305).

1.7.6 The regulation of thermogenesis

When leptin deficient and normal mice are repeatedly injected with leptin, their body weight declines over a period of weeks. This is, in part, a consequence of an increase in energy expenditure that occurs through the process known as thermogenesis. As previously discussed, thermogenesis is the process by which heat is generated by uncoupling proteins in brown and transitional adipocytes. Thermogenesis is induced in response to feeding and low ambient temperature.

Over the past 40 years, there have been a number of key findings that have significantly advanced the study of thermogenesis and these have been summarised below. In 1967, Miller and colleagues (307, 308) discovered that in humans thermogenesis is induced in response to overfeeding. It was shown that diet-induced thermogenesis is associated with brown adipose tissue (309) and that impaired thermogenic function in brown adipose tissue could be linked to the development of obesity (310, 311). The importance of brown adipose tissue in the production of heat was finally explained with the discovery of the adipose tissue derived proteins, known as uncoupling proteins, that could uncouple the electron transport chain and generate heat (312-315). Recently, the advances in molecular techniques and, in particular, the discovery of leptin and its role in the

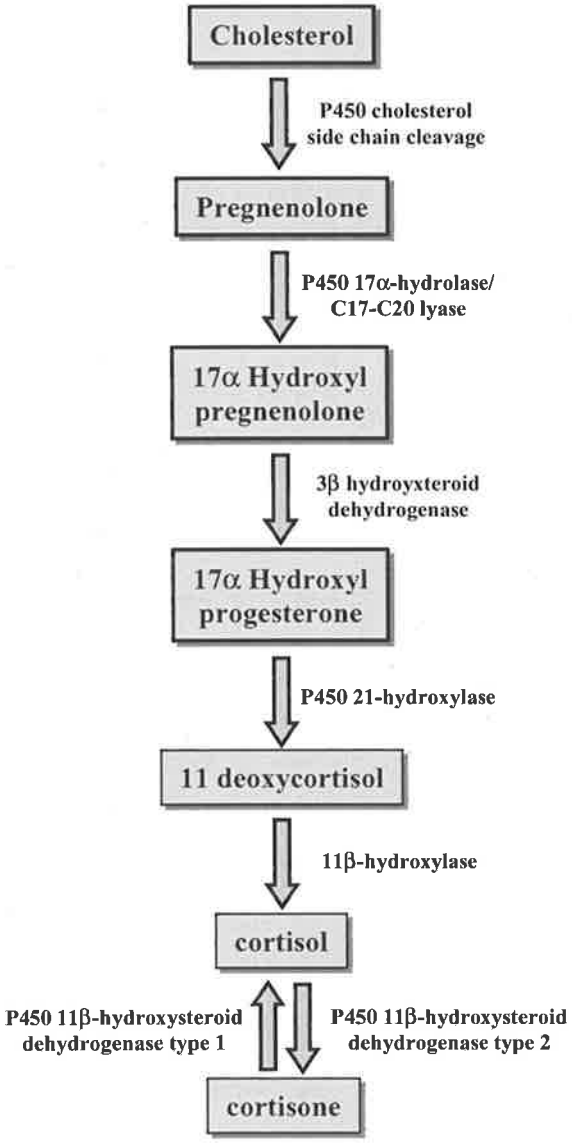


Figure 1.6 The steroidogenic pathway

The conversion in the adrenal of cholesterol to cortisol and the enzymes used to convert the intermediates of this pathway.

regulation of energy homeostasis in mammals, have rekindled the interest in the mechanisms that control thermogenesis.

At least 5 different uncoupling proteins (UCP), including UCP-1, UCP-2, UCP-3, have been discovered and isolated from various tissues including adipose tissue, skeletal muscle, lung and liver (18, 315-318). According to Mitchell's chemiosmotic model of oxidative phosphorylation, the capture of useful energy by the cell is via ATP synthesis along the mitochondrial respiration chain. The driving force for this synthesis is linked to the transport of protons across the inner mitochondrial membrane using the enzyme ATP synthase (319). Uncoupling proteins disrupt the proton electrochemical gradient between the mitochondrial matrix and the inner membrane space preventing the coupling of proton transport to the synthesis of ATP and as a result energy is generated in the form of heat. This heat production is known as thermogenesis (320).

The sympathetic nervous system stimulates lipolysis in adipose tissue causing the breakdown of lipids into fatty acids. It is these fatty acids that activate uncoupling protein activation in brown adipocytes (18, 321, 322). The importance of the sympathetic nervous system in the activation of uncoupling proteins in adipose tissue has been highlighted by a number of studies (323). These authors showed that bilateral sympathetic denervation of interscapular adipose tissue in lean mice leads to decreased thermogenesis and an increase in adipose tissue deposition. In the same year, Arch *et al* (324) discovered that the β_3-adrenergic receptor was expressed by brown adipocytes. Since this finding, treatment with β_3-adrenergic receptor agonists have been shown to increase oxygen consumption, decrease white adipose tissue mass and increase the

density of brown adipocytes expressing UCP-1 in white adipose tissue of adult mammals (325-329).

1.7.6.1 Uncoupling protein-1

The most abundant uncoupling protein in mammals is UCP-1, which is predominantly localised in brown adipocytes (330, 331). When brown adipose tissue was abolished from rodents it resulted in marked obesity (332). Similar findings are demonstrated when using molecular technology to investigate the role of UCP-1. When a constitutive adipocyte specific promoter was attached to the UCP-1 gene in transgenic mice there was a reduction in adipose tissue mass (333, 334). Together these studies suggest that, in mice, UCP-1 is critical in thermogenesis and plays an important role in the resistance to obesity. Leptin also appears to play a role in the regulation of UCP-1. Peripheral and central administration of leptin can increase UCP-1 mRNA expression in adipose tissue and thermogenesis of mammals (335-337). Scarpace and others (164, 306) have determined that leptin's ability to upregulate UCP-1 mRNA expression is dependent on sympathetic innervation of the adipose depot through β_3-adrenergic receptors.

Although the stimulus is different, the mechanisms involved in diet-induced thermogenesis are also involved in cold-induced thermogenesis. Low ambient temperature and overfeeding induce UCP-1 expression in adipose tissue and activate thermogenesis (89, 338-342). It was estimated that adipose tissue comprised predominantly of brown adipocytes, produces ~60-70% of total heat generated from cold-induced thermogenesis (343, 344). Like diet-induced thermogenesis, UCP-1 expression and mitochondrial biogenesis increase in response to low ambient temperature (94). The importance of UCP-1 in mediating cold-induced thermogenesis is

demonstrated in UCP-1 deficient mice. When these mice were acclimatised at thermoneutral temperature, then exposed to low ambient temperature, it caused a significant decrease in core body temperature. Furthermore, when UCP-1 deficient mice were acclimatised to 18°C then moved to a 4°C chamber for a period of 6 months only 9% of the UCP-1 deficient mice survived compared to 100% of the normal mice that survived (345).

Whilst UCP-1 plays an important adaptive role when adults are exposed to low ambient temperature, the activation of this protein is also critical during early postnatal life. Newborns that have impaired thermogenic function are at a greater risk of morbidity. If newborns are exposed to low ambient temperature, the rate of decline in UCP-1 expression in adipose tissue is delayed (346). Interestingly, in neonates UCP-1 expression and protein abundance in adipose tissue can be altered by events that occur during pregnancy, including maternal exposure to low ambient temperature (347), maternal overfeeding (348) and administration of glucose to ewes during pregnancy (349).

1.7.6.2 Uncoupling proteins –2 and –3

Many studies have demonstrated that UCP-1 in adipose tissue is central to mammalian thermogenesis. Cold exposure can induce thermogenesis, however, in the adipose tissue of UCP-1 deficient mice, which suggest the recruitment of other forms of uncoupling proteins for the production of heat (330). Whilst, far less abundant than UCP-1, UCP-2 is ubiquitously expressed in over 12 different tissues, including white and brown adipose tissue, lung and spleen, and UCP-3 is only expressed in muscle, and white and brown adipose tissue (18, 316, 335, 336, 350, 351). Like UCP-1, UCP-2 and UCP-3 have the

capacity to uncouple the electron transport chain (352). In UCP-1 deficient mice, adipose tissue abundance of UCP-2 mRNA increased 14-fold whilst UCP-3 mRNA levels were marginally lower than normal mice (353). It can be speculated that in the absence of functional UCP-1, UCP-2 is recruited for thermogenesis. Interestingly, UCP-1, UCP-2 and UCP-3 mRNA expression in rat adipose tissue is increased with leptin administration (354), suggesting that unlike UCP-1 and UCP-2, UCP-3 is differentially activated for cold-induced and diet-induced thermogenesis.

1.7.6.3 The contribution of uncoupling proteins to obesity

Whilst leptin clearly acts to regulate uncoupling proteins to control thermogenesis, the contribution of uncoupling proteins to the regulation of fat mass metabolism and body weight remains unclear. The single knockout studies on the UCP-1, UCP-2 and UCP-3 genes in mice do not support a role of uncoupling proteins in the control of energy balance and substrate metabolism. The body weight of each of these strains of mice are not noticeably altered by the disruption of a UCP gene and these mice do not show major impairments in whole body resting metabolic rate, diet-induced thermogenesis and total energy expenditure nor in substrate metabolism (330, 355-357). Furthermore, whilst some studies demonstrate an association between body weight and sequence variations in the UCP gene locus in humans (358-361), other studies have not observed such a relationship (362-364). Therefore, it remains to be determined whether uncoupling proteins mediate significant changes in body weight and metabolism, and how significant this contribution is to adult energy balance.

1.7.7 Autoregulation of leptin synthesis and secretion in adipose tissue

Leptin is also able to regulate its own gene expression. Peripheral and central administration of leptin suppresses adipose tissue expression of the leptin gene in rodents (306, 365), and suggests that leptin acts on the sympathetic nervous system to regulate leptin expression in adipose tissue. Catecholamines suppress leptin secretion in cultured human adipocytes (366). Moreover, hypersecretion of adrenaline in phenylethanolamine-N-Methyltransferase (PNMT) – overexpressing transgenic mice suppressed circulating leptin concentrations (367).

1.7.8 Action of leptin on the pancreas

Many studies have reported that in both leptin and leptin receptor deficient mice, hyperinsulinemia precedes the development of obesity (368). The long form of the leptin receptor is expressed in the β-pancreatic cells that secrete insulin (212, 213) suggesting that leptin may act peripherally to regulate pancreatic function. In leptin deficient mice, administration of leptin causes a rapid and substantial decrease in plasma insulin secretion (107, 369-371). Furthermore, leptin administration in isolated β-pancreatic cells from leptin deficient mice results in the suppression of insulin expression (369) and insulin secretion (213, 372). This indicates that in the absence of leptin, insulin levels are elevated and can be lowered by the administration of leptin. Leptin has no effect, however, on isolated β-pancreatic cells from leptin receptor deficient mice (201, 202). It can be, therefore, speculated that leptin acts peripherally through the leptin receptor (Ob-Rb) in β-pancreatic cells to stimulate insulin secretion.

Kieffer *et al* (368) reported that there is conflicting evidence on the actions of leptin mediated insulin secretion by the pancreas of normal mice. A number of studies show a

lack of effect of leptin on insulin secretion (373-376). In the majority of cases, however, leptin suppresses insulin secretion from isolated β-pancreatic cells of normal mice independently of glucose concentrations (369, 377, 378). Finally, it is interesting to note that when leptin was administered to a strain of mice that had developed severe insulin resistance, it abolished the insulin resistance suggesting that leptin may play a role in preventing insulin resistance.

1.8 RESISTANCE TO THE ACTIONS OF LEPTIN

In rodents, leptin reduces fat mass in leptin deficient and normal mice (104, 106). Epidemiological studies report that circulating leptin concentrations in humans are positively correlated with measures of body fatness (42, 134, 379, 380). The report that obese humans have higher leptin concentrations than lean people has prompted speculation that there is a decreased sensitivity to the actions of leptin in the CNS of obese individuals. As previously mentioned, however, large scale genetic screening of normal, obese and diabetic humans has failed, to find a high incidence of mutations in the leptin and leptin receptor genes (41-43).

There are isolated examples of families with a genetic mutation in the leptin gene and affected individuals have low circulating leptin concentrations. These people are of normal weight at birth but then became rapidly obese during childhood (38-40). Infusion of leptin into leptin deficient humans significantly reduces fat mass and body weight (381). Interestingly, humans with a mutation in the leptin receptor do not undergo puberty (40). This suggests that leptin, in humans, is critical in the accumulation of adipose tissue and also in the regulation of reproduction.

The administration of leptin into obese humans, who do not have a mutation of the leptin gene, decreases the fat mass of some but not all subjects (382). Other factors besides fat mass, therefore, influences the responsiveness of obese humans to leptin. The observation that most obese humans have the normal form of the leptin and leptin receptor genes and that leptin protein is high in their circulation, suggests that human obesity, in part, arises from a defect in a post receptor event, i.e. reduced responsiveness to the actions of leptin.

One possibility relates to the transport of leptin into the brain. Obese individuals appear to have a reduced capacity to transport leptin from the circulation into the brain. The ratio of leptin in the CSF fluid : circulating leptin is lower in obese compared to lean subjects (243, 383, 384). Another possibility is that leptin resistance is a consequence of the brain's impaired neuronal network beyond the leptin receptor that controls feeding, such as a decreased responsiveness in the neurones that express NPY, CART, α–MSH, AGRP.

In summary, leptin is principally secreted by adipocytes and acts to regulate energy homeostasis, and related neuroendocrine and reproductive functions. In adult mammals, circulating leptin concentrations are positively correlated with body fat content and with body mass index. Leptin acts at central receptors to alter the secretion of neuropeptides in the arcuate nucleus causing a decrease in food intake and can also act to increase fat mobilisation and oxidation via activating thermogenesis in adipose tissue.

1.9 PREGNANCY AND LEPTIN

Leptin deficient males and female mice are infertile (385), however, fertility can be restored with leptin administration. It is found that leptin appears to play an important role during preimplantation and/or implantation but appears to be not critical during the remaining part of pregnancy in leptin deficient mice (386).

It is well established that during pregnancy, females increase their food intake and have a decreased sensitivity to the actions of insulin (387). These adaptive mechanisms stimulate energy intake so that increased consumption of energy occurs to meet the energy demands of the growing conceptus. As leptin is a signal of lipid storage, not surprisingly, circulating leptin concentrations are higher in pregnant compared to non-pregnant women (115, 388-390) and maternal leptin levels are positively associated with maternal BMI during pregnancy (388, 391). In pregnant mice, plasma leptin concentrations are reported to be at least 20 fold greater than non-pregnant levels (390). Leptin concentrations are only 1-2 fold higher, however, in pregnant ewes compared to their non-pregnant counterparts (115). In many studies, circulating leptin in pregnant mammals peaks during the second or third trimester and then either remains high or slightly declines up until birth (115, 390, 392-395).

Elevated plasma leptin in pregnant, normal mice does not suppress food intake. Rather, pregnant females become leptin resistant and this allows food intake to increase and adipose tissue to accumulate (396, 397). Food intake is suppressed, however, in transgenic pregnant mice that overexpress the leptin gene, which suggests that during pregnancy, leptin only functions at relatively high concentrations (387). Whilst the litter size of these transgenic and normal mice are found to be similar, a 13% reduction in the

mean birthweight of litters from transgenic compared to normal mice is observed (387). Similarly, administration of leptin into pregnant normal mice caused a significant reduction in placental and newborn weights (397). With the knowledge that maternal nutritional status before and during pregnancy is one of the major determinants of birthweight (54, 56, 58, 398), it can be concluded, at least in mice, that during pregnancy changes in maternal nutrition alter maternal leptin levels which impacts on fetal growth via modulating maternal food intake. It is interesting to note that at birth, maternal leptin concentrations are negatively associated with placental weight, although no relationship has been reported between maternal leptin and newborn weight (146, 393, 399).

1.10 PLACENTAL TISSUE: LEPTIN AND LEPTIN RECEPTOR

Leptin expression has been routinely detected in the syncytiotrophoblast cells of the placenta of mouse, human and rat (127, 128, 130). In the sheep, leptin protein has been detected in the placenta, however several studies have been unable to find detectable levels of leptin mRNA expression in the sheep placenta between 40 and 140 days gestation (146)(Yuen, unpublished data). The role of placental leptin, however, remains to be elucidated. It has been observed that placental leptin expression is altered in complicated pregnancies, such as diabetic and intrauterine growth restriction (IUGR) pregnancies. Leptin expression is lower in human placentae from IUGR pregnancies compared to normal pregnancies (132), and conversely there is a marked increase in the placental leptin expression in diabetic compared with non-diabetic pregnancies (132).

The leptin promoter has a placenta specific upstream enhancer (400, 401) and studies have determined that placenta-derived and adipose tissue-derived leptin are differentially regulated (128, 402). If placenta-derived leptin significantly contributes to the maternal

or fetal circulation, then changes in placental leptin expression are of particular physiological relevance.

In humans, placenta-derived leptin is primarily secreted into the maternal circulation whilst a minimal amount is secreted into the fetal circulation (403-405). Lepercq *et al* (403) and Linnemann *et al* (404) showed that less that 5% of leptin secreted by the placenta was released into the fetal circulation whereas Hoggard *et al* (405) claimed that in humans, as much as 14% of the total leptin derived from the placenta was secreted into the fetal circulation. The secretion of placenta-derived leptin into the fetal circulation is consistent with the previous finding that demonstrates that leptin concentrations are marginally higher in umbilical venous blood compared to the umbilical arterial blood (406). From these findings, it can be concluded that placenta-derived leptin appears to contribute less than 15% to the circulating pool of leptin in the fetus.

Recently, Smith *et al* (214, 407) injected ^{125}I-leptin into the maternal circulation of pregnant rats and detected radioactive leptin in the fetal circulation. The transport of leptin was greater at 22 days compared to 16 days gestation in the rat. Maternal dexamethasone treatment in these rats decreased the transplacental movement of leptin from the maternal circulation to the fetal circulation. Furthermore, placental expression of Ob-Ra, i.e. the leptin receptor associated with the transport of leptin across membranes, was suppressed by maternal dexamethasone treatment and stimulated by treatment with metyrapone (214) – an inhibitor of maternal corticosterone biosynthesis (408).

In summary, it appears that leptin is not only secreted from the placenta into the maternal and fetal circulations, but also that in the rodent, maternally derived leptin can be transported into the fetal circulation via the placentally expressed leptin receptors, Ob-Ra. In 1997, it was discovered that leptin was present in the blood of human fetuses and that circulating leptin was lower in newborns from IUGR pregnancies and it was suggested that, like in the adult, in the fetus, circulating leptin is a signal reflecting fetal nutrition (406).

1.11 LEPTIN IN THE NEONATE

At birth, the leptin gene is expressed in adipose tissue (173, 409) and leptin protein is found in the blood of newborn mammals (173, 393, 409, 410). It also appears that the leptin induced appetite response is active at birth (173). When newborns are prevented from suckling they are found to have lower plasma leptin levels than whilst they are suckling (173). The ontogenic profile of circulating leptin from birth appears to differ between species. In human infants, plasma leptin concentrations decline within 2-4 days after birth (410, 411). Bispham *et al* (409) showed that in lambs, however, plasma leptin concentrations did not change in the first 4 days of life.

The same disorders of pregnancy that are found to alter maternal leptin are also associated with altered circulating leptin concentrations in newborns. Human neonates from diabetic pregnancies are large at birth and, are hyperglycaemic, hyperinsulinemic and have elevated leptin levels compared to their non-diabetic counterparts (412-415). Conversely, newborns that suffer from IUGR pregnancy, are small at birth, hypoglycaemic and have low levels of IGF-I (416-420). Moreover, these IUGR newborns have lower circulating leptin levels (406, 421).

Several other interesting relationships have been associated with newborn or cord blood levels of leptin. Newborn plasma leptin concentrations are positively correlated with placental weight and, size, fatness and BMI at birth in many species (393, 394, 411, 413, 422, 423). Furthermore, large for gestational age neonates have higher leptin levels than those born small for gestational age (423). Together these studies suggest that during pregnancy nutritional factors affect circulating leptin levels in the fetus and may consequently affect fetal growth.

1.12 LEPTIN BEFORE BIRTH

Prior to 1999, very little was known about leptin in the fetus, i.e. where leptin is synthesised, what regulates leptin expression and what role leptin played in fetal energy balance. In humans, leptin can be detected in the fetal circulation by week 18 of pregnancy (term ~40 weeks) (406, 421). Furthermore, circulating leptin levels increase with gestation (406). In fetal mice, leptin expression was detected in a number of tissues including bone/cartilage and hair follicles at 14.5 days post coitus (term ~21 days). These tissues are known to contain resident leptin-expressing adipocytes (127, 128). Unlike adipose depot derived adipocytes, lipolysis is stimulated in bone marrow adipocytes by factors such as anaemia (424).

The absence of leptin expression in the subcutaneous and perirenal regions of the fetal mouse is a consequence of the fact that mice develop very little or no adipose tissue before birth (397, 425). Conversely, distinct adipose depots can be found in other mammalian species. At term, approximately 16% of body weight is comprised of body fat in newborn humans. As previously mentioned, in the sheep fetus lipid containing

cells appear at approximately 70 days gestation (84) and the perirenal depot is clearly noticeable by 90 days gestation. At term, adipose tissue comprises about 1-2% of total body mass (85, 182, 426) and most of this adipose tissue is found in the perirenal region of the sheep fetus (Muhlhausler, unpublished data).

1.13 EXPERIMENTAL HYPOTHESES

Therefore the general aim of this body of scientific work was to investigate the regulation and actions of leptin before birth. The neuroendocrine and metabolic systems of the sheep fetus are relatively well understood and in particular the maturation of the neuroendocrine system in the sheep fetus closely mimics the development of the same system in humans compared to other non-mammalian primates. As previously mentioned, like in humans, the sheep fetus develops a significant mass of adipose tissue before birth. Furthermore, the size of the ewe and fetus make it possible to infuse solutions into their circulations and to collect repeated blood samples. Hence, for these reasons, the following questions have been examined using the pregnant sheep model.

Leptin is expressed in the adipose tissue of adult and newborn mammals, and there is an increase in the accumulation of adipose tissue in the fetus during late gestation. Furthermore, circulating leptin is found to increase in human fetuses. **We therefore hypothesise that leptin mRNA is expressed in the adipose tissue of the fetus and that leptin mRNA expression increases with gestation in the sheep fetus (Chapter 2).**

As summarised above, low caloric intake suppresses leptin synthesis and secretion in adult and newborn. **We have hypothesised that maternal undernutrition during late gestation will result in a decrease in maternal and fetal plasma concentrations of**

glucose, insulin and leptin and that leptin mRNA expression in fetal adipose tissue will also decrease (Chapter 3).

Thirdly, little is known about the actions of leptin before birth. It is hypothesised that, leptin acts in the fetus, as in the adult, to regulate energy homeostasis. More specifically, **we have hypothesised that leptin administration into fetal sheep reduces adipose tissue mass, increases UCP-1 abundance and decreases leptin mRNA expression in this tissue (Chapter 4).**

Finally, in the sheep it is well established that the normal timing of parturition is dependent on the prepartum activation of the fetal HPA axis (296, 298). The secretion of cortisol by the fetal adrenal is critical for the timing of delivery. The action of leptin to suppress cortisol synthesis via actions at the hypothalamus, pituitary and adrenal in the adult raises the intriguing possibility that in the fetus leptin plays a role in the normal timing of parturition. **Therefore, we have hypothesised that the intrafetal administration of leptin will suppress the normal prepartum activation of the fetal HPA axis and delay the timing of parturition (Chapter 5).**

CHAPTER 2

2. LEPTIN MRNA EXPRESSION IS PRESENT IN THE ADIPOSE TISSUE OF THE LATE GESTATION SHEEP FETUS

2.1 DECLARATION

During the course of my PhD candidature, I conducted experiment work that has been subsequently published by my coauthors and I. The majority of the contents within this chapter can be found in the following publication:

Yuen BSJ, McMillen IC, Symonds ME and Owens PC. Abundance of leptin messenger ribonucleic acid in fetal adipose tissue is related to fetal body weight. J Endocrinol. 1999. 163: R11-14.

2.2 CONTRIBUTION OF COAUTHORS

McMillen, IC

Co-supervised the experimental work contained within this publication, contributed to intellectual debates, assisted with data analyses and interpretation, and aided in the writing of the manuscript.

Signature.....Date.22/07/03

Symonds, ME

Assisted in the interpretation of the data, contributed to intellectual debates and evaluated the manuscript prior to submission.

Signature.....Date.....12/6/03.....

Owens, PC

Co-supervised the experimental work contained within this publication, contributed to intellectual debates, assisted with data analyses and interpretation, and aided in the writing of the manuscript.

Signature.....Date.....15 July 03.....

2.3 ABSTRACT

Leptin mRNA was measured in adipose tissue of fetal sheep by reverse transcription polymerase chain reaction (RT-PCR). Abundance of leptin mRNA relative to β-actin mRNA in fetal perirenal adipose tissue increased ($P<0.02$) with gestation, being higher at 144 d (0.73 ± 0.10, n=5) than at 90-91 d (0.40 ± 0.08, n=6) or 125 d (0.40 ± 0.04, n=5) gestation (term ~150 d). There was a positive relationship between the relative abundance of leptin mRNA (y) and fetal body weight (x) between 90 and 144 d gestation ($r^2=0.27$, $P<0.01$). The slope of the linear dependence of leptin mRNA on fetal weight was 15-fold greater at 90-91 d ($y = 2.81x - 1.1$, n=6, $r^2=0.71$, $P<0.025$) than at between 125-144 d gestation ($y = 0.195x - 0.15$, n=16, $r^2=0.39$, $P<0.01$). Thus the leptin synthetic capacity of fetal adipose tissue appears to increase in late gestation but this is accompanied by constraint of its sensitivity to fetal body weight. We hypothesise that leptin synthesis in fetal adipose tissue is related to fetal nutrient supply and growth rate.

2.4 INTRODUCTION

Leptin is a 16 kDa polypeptide hormone synthesised and secreted by adipocytes that acts to suppress appetite and increase energy expenditure in adults (13, 14). Abundance of leptin mRNA in adipose tissue and plasma leptin concentrations correlate positively with body weight and adiposity in human adults (41, 133). In rodents leptin mRNA is expressed in a number of fetal tissues including placenta (127, 128, 130) and there is an association between circulating leptin and birth weight in humans (393, 423, 427). These data suggest there may be a relationship between growth and leptin synthesis before birth, but there have been no reports of leptin mRNA expression in adipose tissue before birth or of the relationship of the expression of leptin in fetal adipose tissue to fetal

growth. In this Chapter, I have reported that leptin is expressed in perirenal adipose tissue, the major adipose depot in the sheep fetus. I have also investigated the relationship between leptin mRNA and fetal weight before (90-91 days) and after (125-144 days gestation) the development of sympathetic innervation of the perirenal fat depot (84, 428).

2.5 MATERIALS AND METHODS

2.5.1 Animals

The study was approved by the Animal Ethic Committee of the University of Adelaide. Merino ewes (n=51) were mated and provided unrestricted access to feed and water. They were killed between 90 and 146 d gestation with an overdose of sodium pentobarbitone (6.5 g i.v.) and the fetuses were removed and weighed. In one group (n=29) both left and right fetal perirenal fat depots were collected at 90-99 d (n=9) and at 137-146 d (n=20) and weighed. In another group a sample of fetal perirenal adipose tissue was obtained at 90-91 d (n=6), 125 d (n=5), 139-141 d (n=6) and 144 d (n=5), immediately frozen in liquid N_2 and stored at –80°C for measurement of leptin and β-actin mRNA.

2.5.2 Reverse transcription polymerase chain reaction

RNA was extracted (429) from ~100 mg adipose tissue (TriReagent, Prod T9424, Sigma) as recommended. cDNA was obtained by reverse transcription of 2 µg total RNA with random sequence hexanucleotides (Cat RP-6, GeneWorks, Adelaide, Australia) using Super-Script™ RNase H⁻ (Cat 18053-017, GIBCOBRL). A fragment of ovine leptin cDNA was amplified through 26 cycles of 60 sec @ 94°C, 15 sec @ 53°C and 60 sec @

72°C (Hybaid PCR Express, Teddington, UK) from 5 μl of reverse transcription product using Taq DNA polymerase (Biotech International, Bently, Australia) according to the manufacturer's instructions with 5'-GAC ATC TCA CAC ACG CAG-3' and 5'-GAG GTT CTC CAG GTC ATT-3' (GeneWorks) as primers. This produced 183 bp of ovine leptin cDNA (nucleotides 67-249 of the 441 nucleotide cDNA of ovine leptin, Genbank Acc. No. U84247). Sequencing with the ABI PRISM Dye Terminator method (Perkin Elmer Corp) after QIAquick purification (QIAGEN Pty. Ltd., Clifton Hill VIC, Australia) confirmed its identity. A fragment of ovine β-actin cDNA was similarly amplified by PCR with 5'-TG TGA TGG TGG GTA TAT GGG TC-3' and 5'-TAG ATG GGC ACA GTG TGG GT-3'. Products of RT-PCR (8 μl) were electrophoresed through a 1.5% agarose gel. This was stained with ethidium bromide, transilluminated with UV radiation and photographed. Intensities of RT-PCR products were measured on the film negative by laser densitometry. Molecular sizes of PCR products were estimated by comparing their electrophoretic migration with those of fragments of pUC19 ds DNA digested with Hpa II (GeneWorks). Each leptin RT-PCR product had a mobility similar to that of the 190 bp fragment of pUC19, consistent with the predicted size of 183 bp.

2.5.3 Statistics

Results are presented as mean ± SEM. Total perirenal fat mass is the combined weights of the two perirenal fat depots for each fetus. Relative fat mass is the ratio of total perirenal fat mass to body weight of the fetus. Differences between groups were assessed by one- or two-way analysis of variance with Bonferroni's multiple comparison. Associations were evaluated by linear regression (SigmaStat V1, Jandel Scientific).

2.6 RESULTS

2.6.1 Fetal adipose tissue mass

In the first study, which included singletons and twins, the gender of fetuses had no significant effect on fetal weight, perirenal fat weight or relative fat mass. As expected, fetal weight was affected by number of fetuses per pregnancy ($P<0.01$), gestational age ($P<0.0001$) and the interaction between these factors ($P<0.05$). Twin fetuses had lower body weights than singletons and this difference was greater after 137 d than before 99 d gestation. Total perirenal fat weight ($P<0.0001$), but not relative fat mass, increased with gestation and was unaffected by the number of fetuses per pregnancy.

2.6.2 Leptin mRNA expression in fetal adipose tissue

In the second study, which consisted entirely of twins of which one of each of these pair was examined, relative abundance of leptin mRNA in fetal perirenal adipose tissue increased ($P<0.02$) with gestation (Figure 2.1). Relative abundance of leptin mRNA to relative β-actin mRNA (y) in fetal adipose tissue was positively correlated with the weight (x) of the fetus ($y=0.08x + 0.31$, $n=22$, $P<0.01$) such that 27% of the variance in leptin mRNA could be explained by dependence on fetal weight ($r^2=0.27$). The slope of this relationship (Figure 2.2) was greater at 90-91d ($y=2.81x - 1.1$, $n=6$, $r^2=0.71$, $P<0.025$) than at 125-144 d gestation ($y=0.195x - 0.15$, $n=16$, $r^2=0.39$, $P<0.01$).

2.7 DISCUSSION

I have demonstrated that leptin mRNA is expressed in fetal adipose tissue. Lipid accumulation in the perirenal area of fetal sheep occurs from ~70 days gestation and there is a marked increase in mitochondria within this tissue between 80 and 90 days

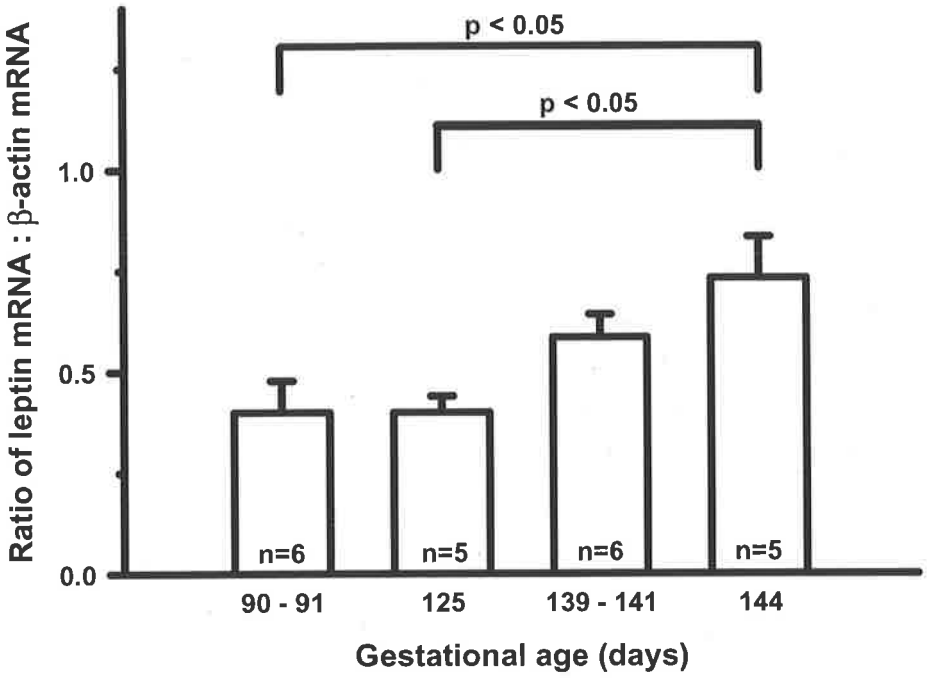


Figure 2.1 Ontogeny of leptin expression in fetal adipose tissue.

The relative abundance of leptin in fetal adipose tissue was significantly greater at 144 d gestation compared with 90-91 d and 125 d gestation ($P<0.02$). Values are present as means ± SEM.

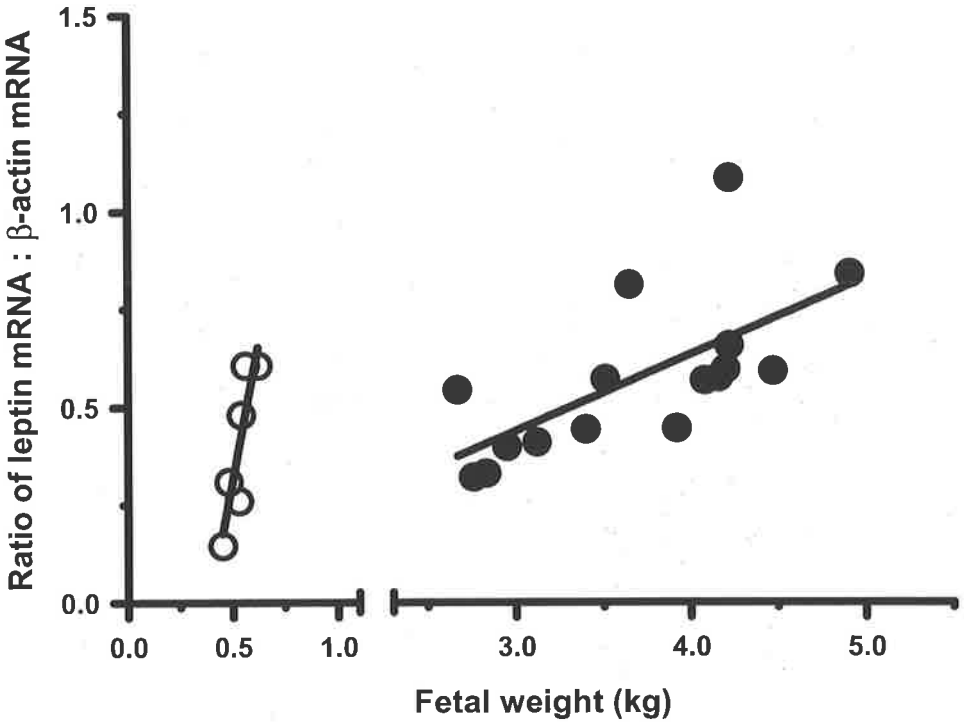


Figure 2.2 Relationship between leptin mRNA and fetal weight.

Expression of leptin in fetal adipose tissue was positively associated with fetal weight at 90-91 d (open circles) and 125-144 d (closed circles) gestation ($R=0.52$, $P<0.01$). The slope of this relationship was greater at 90-91 d ($R=0.84$, $P<0.025$) than at 125-144 d gestation ($R=0.62$, $P<0.01$). Values are present as means ± SEM.

(85). After this age, fetal perirenal fat in the sheep consists of brown adipocytes characterised by many mitochondria with numerous cristae (84). In the rat, leptin is expressed in brown adipose tissue during the first 24 h after birth (430). The present study shows that leptin mRNA is expressed in brown adipose tissue before birth.

I found that there was an increase in the relative abundance of leptin mRNA in fetal adipose tissue during the last 20 days of gestation in the sheep. This may be due to an increase in size of adipocytes in fetal perirenal fat depots, since there is strong evidence that adipocyte cell size is a major determinant of expression and secretion of leptin (114) and the mean cell volume of perirenal adipocytes in fetal sheep increases 3-4 fold between 90 and 144 days gestation (431). Alternatively, increased leptin mRNA expression in fetal perirenal fat in sheep during late gestation may be related to the rise in circulating fetal cortisol concentrations in the two weeks before delivery (432). Glucocorticoids stimulate leptin gene expression in adipocytes *in vivo* (187) and *in vitro* (188). There was a positive relationship between abundance of leptin mRNA in fetal adipose tissue and fetal body weight as early as 90 days gestation. This relationship may also be a consequence of greater leptin synthesis occurring in larger adipocytes in bigger fetal sheep. Greater leptin mRNA expression in larger fetuses might also be a response to stimulation by anabolic hormones such as insulin. Insulin promotes fetal growth in sheep (433) and stimulates leptin synthesis and secretion *in vitro* and *in vivo* postnatally in rats and humans (142, 434).

Despite the greater abundance of leptin mRNA in fetal adipose tissue after 125 days gestation, the nature of its relationship with body weight changed. The slope of the regression between the relative abundance of leptin mRNA and body weight in fetuses of

125 days gestation and older was 15-fold less than at 90-91 days. Thus although the leptin synthetic capacity of fetal adipose tissue appears to increase in late gestation, it is less sensitive to variation in fetal body size than at 90-91 days. This implies that an inhibitory influence appears in late gestation that alters the relationship between leptin expression and fetal size. The lower slope for the relationship between leptin mRNA and fetal weight from 125 days gestation may be associated with the onset of sympathetic innervation of fetal perirenal adipose tissue, which occurs at ~120 days gestation in sheep (84, 428). In cows, the abundance of β_3-adrenergic receptors and UCP-1 in fetal adipose tissue also increases in late gestation (435, 436). Studies in rats and mice have shown that β_3-adrenergic agonists suppress leptin gene expression whilst stimulating UCP-1 expression (437-439). Regardless of the mechanism(s) underlying the age associated change leptin expression, the relationship between leptin mRNA abundance and fetal size is consistent with the hypothesis that leptin synthesis in fetal adipose tissue is related to fetal nutrient supply and fetal growth rate.

In order to determine whether leptin synthesis and secretion in fetal adipose tissue can be altered by changes in fetal nutrient supply, I have investigated the effect a moderate restriction of maternal food intake on leptin synthesis and secretion in the adipose tissue of the sheep fetus.

CHAPTER 3

3. RESTRICTION OF MATERNAL FOOD INTAKE DURING LATE GESTATION DOES NOT ALTER LEPTIN SYNTHESIS OR SECRETION IN THE SHEEP FETUS

3.1 DECLARATION

During the course of my PhD candidature, I conducted experiment work that has been subsequently published by my coauthors and I. The majority of the contents within this chapter can be found in the following publication:

Yuen BSJ, Owens PC, McFarlane JR, Symonds ME, Edwards LJ, Kauter KG and McMillen IC. Circulating leptin concentrations are positively related to leptin mRNA expression in the adipose tissue of fetal sheep in the pregnant ewe fed at or below maintenance energy requirements during late gestation. Biol Reprod. 2002. 67: 911-916.

3.2 CONTRIBUTION OF COAUTHORS

Owens, PC

Co-supervised the experimental work contained within this publication, contributed to intellectual debates, assisted with data analyses and interpretation, and aided in the writing of the manuscript.

Signature.....Date.15 July 03

McFarlane, JR

Dr McFarlane generously agreed to assay maternal and fetal sheep plasma to determine circulating leptin concentrations. Contributed to intellectual debates, assisted with data interpretation and evaluated the manuscript prior to submission.

Signature.....Date.....7/7/03.....

Symonds, ME

Assisted in the interpretation of the data, contributed to intellectual debates and evaluated the manuscript prior to submission.

Signature.....Date.....13/6/03.....

Edwards, LJ

Dr Edwards restricted the nutrient intake of pregnancy ewes and collected maternal and fetal blood and fetal tissues. She generously allowed me to determine circulating leptin concentrations in these samples and to determine the relative abundance of leptin mRNA in these fetal adipose tissue samples. She also evaluated the manuscript prior to submission.

Signature.....Date.....11.6.03.....

Kauter, KG

Ms Kauter was one of the first researchers to develop a leptin ELISA for use in the sheep. This assay system was used to measure the plasma leptin concentrations of the fetuses described in this study.

Signature.....Date.....10/7/03.....

McMillen, IC

Co-supervised the experimental work contained within this publication, contributed to intellectual debates, assisted with data analyses and interpretation, and aided in the writing of the manuscript.

Signature.....Date.....22/7/03.....

3.3 ABSTRACT

I have investigated the effects of maternal undernutrition during late gestation on maternal and fetal plasma concentrations of leptin and on leptin gene expression in fetal perirenal adipose tissue. Pregnant ewes were randomly assigned at 115 days (d) gestation (term ~150 d) to either a control group (n=13) or to an undernourished group (n=16) that received ~50% of the control diet until 144 - 147 d gestation. Maternal plasma glucose but not leptin concentrations were lower in the undernourished ewes. There was, however, a significant correlation between mean maternal plasma leptin (y) and glucose (x) concentrations ($y = 2.9x - 2.4$; $R = 0.51$, $P<0.02$) when the control and undernourished groups were combined. Fetal plasma glucose and insulin, but not fetal leptin concentrations were lower in the undernourished ewes and there was no correlation between mean fetal leptin concentrations and either mean fetal glucose or insulin concentrations. There was, however, a positive relationship between mean fetal (y) and maternal (x) plasma leptin concentrations ($y = 0.18 x + 0.45$, $R=0.66$, $P<0.003$). There was no significant difference in the relative abundance of leptin mRNA in fetal perirenal adipose tissue between the undernourished (0.60 ± 0.09, n=10) and control (0.70 ± 0.08, n=10) groups. Fetal plasma concentrations of leptin (y) and leptin mRNA levels (x) in perirenal adipose tissue were significantly correlated ($y = 1.5 x + 0.3$, $R=0.69$, $P<0.05$). In summary, the capacity of leptin to act as a signal of moderate maternal undernutrition may be limited before birth in the sheep.

3.4 INTRODUCTION

During adult life, plasma concentrations of leptin and the abundance of leptin mRNA in adipose tissue correlate positively with body weight and adiposity and are altered by long

term changes in dietary intake in the rodent, human and sheep (118, 133, 136, 173, 180, 380). There is also a positive relationship between leptin expression in fetal adipose tissue and fetal weight in the sheep (Chapter 2) and leptin concentrations in umbilical cord blood correlate positively with birth weight in the human (393, 413). Since fetal growth rate and body weight at birth are positively affected by nutrition during pregnancy we hypothesised that the synthesis and secretion of leptin may be regulated by fetal nutrient supply. In the present study, we have therefore investigated the effects of maternal undernutrition during late gestation in the sheep on maternal and fetal plasma leptin concentrations and on leptin gene expression in fetal adipose tissue. We have also investigated the relationship between the abundance of leptin mRNA in this fetal tissue and circulating fetal glucose, insulin and leptin concentrations.

3.5 METHODS

3.5.1 Animals and Surgery

All procedures were approved by the Adelaide University Animal Ethics Committee. Surgery was performed on 29 pregnant Border-Leicester Merino cross bred ewes under aseptic conditions between 109 and 113 d gestation (term ~150 days) with general anaesthesia induced by sodium thiopentone (1.25 g i.v., Pentothal, Rhone Merieux, Pinkenba, Qld, Australia) and maintained with 2.5 - 4% halothane (Fluothane, ICI, Melbourne, Vic, Australia) in oxygen. Vascular catheters were implanted in a maternal jugular vein, a fetal carotid artery and jugular vein, and the amniotic cavity, as previously described (440). Catheters were filled with heparinised saline and the fetal catheters exteriorised through an incision made in the ewes' flank. During surgery, ewes and fetuses received a 2 ml intramuscular injection of antibiotics (procaine penicillin 250

mg/ml; dihydrostreptomycin 250 mg/ml; procaine hydrochloride 20 mg/ml Penstrep Illium, Troy Laboratories, Smithfield, NSW, Australia). Ewes were housed in individual pens in rooms with a 12 h light/dark cycle and fed once daily at 1100 h with water provided *ad libitum*. Animals were allowed to recover from surgery for at least 4 d before collection of fetal and maternal blood samples commenced.

1.1.1 Feeding regime

Pregnant ewes were randomly assigned at 115 d to either a control group weighing 56.7 ± 1.9 kg (n=13) that received 19.8 ± 0.2 g/kg of lucerne and 3.0 ± 0.1 g/kg of oats per day or to an undernourished group weighing 53.5 ± 2.3 kg (n=16) that received 10.3 ± 0.1 g/kg of lucerne and 1.6 ± 0.1 g/kg of oats per day. Maternal food allocation was increased in both the control and undernourished groups (lucerne by 15%; oats by 10%) every 10 d until post mortem at 144-147 d pregnancy (440).

1.1.2 Blood sampling protocol

Maternal venous (5 ml) and fetal arterial (3.5 ml) blood samples were collected between 0800-1100 h, before the ewes were fed, 3 times each week between 116 and 140 d gestation. Blood samples were centrifuged at 1500 g for 10 min and plasma separated into aliquots and stored at -20°C for subsequent glucose and hormone assay. There were instances during the 25 day protocol when blood samples could not be collected due to technical problems primarily related to blocked vascular catheters. The number of maternal and fetal blood samples which were available for glucose, insulin and leptin determination are detailed in the subsequent assay sections. Fetal arterial blood (0.5 ml) samples were also collected for the measurement of arterial blood gas status (ABL 520 blood gas analyser, Radiometer, Copenhagen, Denmark).

3.5.4 Tissue collection

Ewes were killed between 144 and 147 d of pregnancy with a lethal overdose of sodium pentobarbitone (Virbac Pty Ltd, Peakhurst, NSW, Australia). Fetuses were delivered by hysterotomy, weighed and killed by decapitation (control group, 12 singletons and 2 twins; undernutrition group, 15 singletons and 1 twin). Fetal perirenal adipose tissue was collected, weighed and a sample was frozen in liquid nitrogen and stored at -80°C.

3.5.5 Glucose assay

Plasma glucose concentrations were determined in 234 maternal plasma samples (control group, 90 samples, n=8 sheep; undernutrition group, 144 samples, n=13 sheep) and 348 fetal plasma samples (control group, 160 samples, n=13 sheep; undernutrition group, 188 samples, n=16 sheep) by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase and measuring the formation of NADH spectrophotometrically at 340 nm (COBAS MIRA automated analysis system, Roche Diagnostic, Basle, Switzerland) (440). The intra- and inter- assay coefficients of variation were both <5%.

3.5.6 Insulin radioimmunoassay

Fetal plasma insulin concentrations were measured in 196 samples (control group, n=88 samples, n=12 sheep; undernutrition, n=108 samples, n=13 sheep) using a commercial kit (Phadaseph radioimmunoassay kit, Pharmacia & Upjohn, Uppsala, Sweden). The detection range of the assay was 1.5-240 μU insulin.ml^{-1}. Guinea pig-anti insulin antisera and ^{125}I-human insulin (100 μl) were added to plasma samples (100 μl) which were incubated for 2 h at room temperature before the addition of 2 ml sheep anti-guinea pig IgG. Samples were allowed to stand at room temperature for a further 30 min before

being centrifuged at 1500 g for 10 min as described previously (440). The inter- and intra- assay coefficients of variation were <10%.

3.5.7 Leptin assay

Plasma leptin concentrations were determined in 119 maternal plasma samples (control group, 50 samples, n=10 sheep; undernutrition group, 69 samples, n=15 sheep) and 99 fetal plasma samples (control group, 44 samples, n=9 sheep; undernutrition group, 55 samples, n=12 sheep) using a competitive ELISA previously validated for sheep plasma (373). The ELISA plate was coated with 6 ng recombinant bovine leptin in 50 µl 0.1 M bicarbonate buffer, pH 9.0 overnight at 37°C. The plate was blocked with 200 µl 5% skim milk in ELISA buffer for 1 h at 37 °C. Samples (100 µl) were assayed in duplicate and added to wells containing chicken anti-recombinant bovine leptin antisera in 100% Triton-X 100, 0.5% SDS and 5% sodium deoxycholate, (50 µl) and the plate was incubated overnight at 37°C. Strepavidin conjugated to alkaline phosphatase (Amrad Biotech, Boronia, Vic, Australia) was added and after incubation for 1 h, the plate was developed with p-nitrophenylphosphate disodium salt hexahydrate. The sensitivity of the assay was 0.25 ng/ml and the inter assay and intra assay coefficients of variation were 15.7 and 11.0% respectively.

3.5.8 Leptin Reverse Transcription-PCR

Perirenal adipose tissue was collected from 20 (control group, n=10; undernourished group, n=10) of the 29 fetal sheep and total RNA was extracted as described in Chapter 2. Briefly, approximately 100 mg of fetal adipose tissue was homogenised with 1 ml of Sigma Trireagent (Sigma Chemical Co., St.Louis MO) and allowed to stand at room temperature for 5 min. This was then mixed with 1-bromo-3-chloro-propane (100 µl),

stood at room temperature for 10 min then centrifuged at 4°C at 3500 g for 10 min. An aliquot of the aqueous layer (500 µl) was recovered and mixed with isopropanol (500 µl). RNA was precipitated by centrifugation at 3500 g for 5 min at 4°C. The pellet was washed in 70% ethanol and allowed to air dry. The RNA pellet was dissolved in sterile water (20 µl) and 1 µl of the solution was diluted in sterile water (500 µl) for the determination of the spectrophotometric absorbance at 260 and 280 nm. The nucleic acid to protein ratio was >1.6 and the RNA yield was 0.44 ± 0.02 µg/mg adipose tissue. Integrity of RNA preparations was evaluated by agarose gel electrophoresis, followed by ethidium bromide staining and identification of ribosomal RNA.

Ovine leptin and β-actin cDNA were amplified by RT-PCR as described in Chapter 2. Both cDNA products from RT-PCR (8 µl) were electrophoresed through a 2.0% agarose gel, stained with ethidium bromide, visualised by ultraviolet transillumination and photographed using a digital camera and quantified using 1D Image Analysis Software Electrophoresis Documentation and Analysis System 120 (Kodak dS Ditigal Science, Kodak, Rochester, NY, USA).

3.5.9 Statistical Analysis

Data are presented as the mean ± SEM. The effects of maternal nutrition on fetal body weight, total perirenal fat mass, mean gestational arterial PO_2, and the relative abundance of leptin mRNA (ratio of leptin mRNA to β-actin mRNA) in fetal perirenal adipose tissue were determined using unpaired Student's t-test. The effects of maternal nutrition on maternal plasma glucose and leptin concentrations were determined by multifactorial ANOVA with repeated measures using feeding group (control vs undernutrition), gestational age (in 5 day blocks) as the specified factors. Similarly the effects of maternal

nutrition and gestation on fetal plasma glucose, insulin and leptin concentrations were also determined using multifactorial ANOVA with repeated measures. Data were transformed where required to reduce heterogeneity of variance. The Duncans new multiple range test was used post ANOVA to identify significant differences between mean values. Linear regression analysis was used to assess the relationship between the mean plasma leptin and the mean plasma glucose concentrations measured in each ewe and fetus during the period 116-140 d of pregnancy. Similarly, linear regression analysis was also used to assess relationships between mean plasma leptin and mean plasma glucose or insulin concentrations measured in each fetus during the period 116-140 d of pregnancy. Relationships between mean fetal plasma leptin concentrations and fetal body weight, fetal fat mass and maternal plasma leptin concentrations were similarly determined. A probability of 5% i.e. $P < 0.05$ was taken as the level of significance in all analyses.

3.6 RESULTS

3.6.1 Fetal outcome

The mean fetal arterial PO_2 throughout late gestation was not different between the control (21.9 ± 0.5 mmHg) and undernourished (23.4 ± 0.6 mmHg) groups. There was also no difference in fetal body weights (control, 5.02 ± 0.12 kg; undernourished, 4.70 ± 0.16 kg) or relative fat mass (control, 3.89 ± 0.15 g/kg; undernourished, 4.13 ± 0.29 g/kg) between the two groups.

3.6.2 Maternal plasma glucose and leptin concentrations

Maternal plasma concentrations of glucose were significantly lower ($P<0.05$) in undernourished ewes throughout late pregnancy (Figure 3.1). Plasma glucose concentrations were also lower ($P<0.002$) in both control and undernourished ewes after 120 d when compared with earlier in pregnancy (Figure 3.1). There was no significant effect of undernutrition, however, on maternal plasma concentrations of leptin and there was also no significant change in maternal leptin concentrations between 116 and 140 d of pregnancy in either the control or undernourished ewes (Figure 3.1). Mean maternal plasma leptin (y) and glucose (x) concentrations were not correlated within each separate feeding group but they were significantly correlated, however, when data from the control and undernourished groups were combined ($y = 2.9 x - 2.4$; $R = 0.51$, $P < 0.02$, $n=20$) (Figure 3.2). There was no relationship between the mean maternal plasma concentrations of leptin and either maternal body weight at 110-115 d gestation or fetal body weight at 144-147 d gestation.

3.6.3 Fetal plasma glucose, insulin and leptin concentrations

Fetal plasma concentrations of glucose ($P<0.005$) and insulin ($P<0.02$) were significantly lower in the undernourished group (Figure 3.3). Fetal plasma concentrations of insulin were lowest ($P<0.003$) between 131-135 d compared to other gestational periods. There was no significant effect, however, of maternal undernutrition on fetal leptin concentrations and there was no significant change in fetal plasma leptin concentrations between 116 and 140 d gestation in either the undernourished or control groups (Figure 3.3). There was also no difference between plasma leptin concentrations in male and female fetuses.

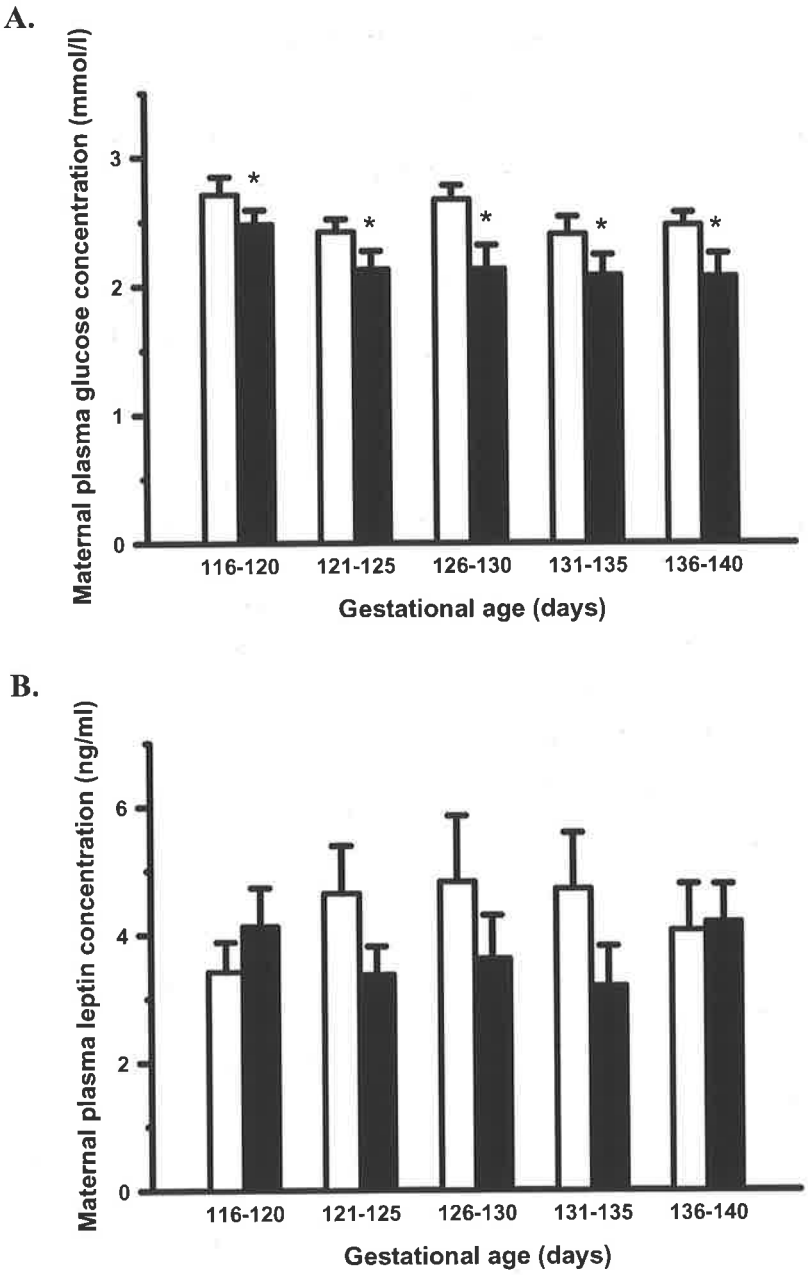


Figure 3.1 Plasma hormone and metabolite concentrations in pregnancy ewes.

Maternal plasma glucose (A) and leptin concentrations (B) in control (open histograms) and undernourished (dark histograms) ewes between 116 and 140 days gestation. The asterisks denote a significant effect of undernutrition on maternal glucose concentrations throughout late gestation.

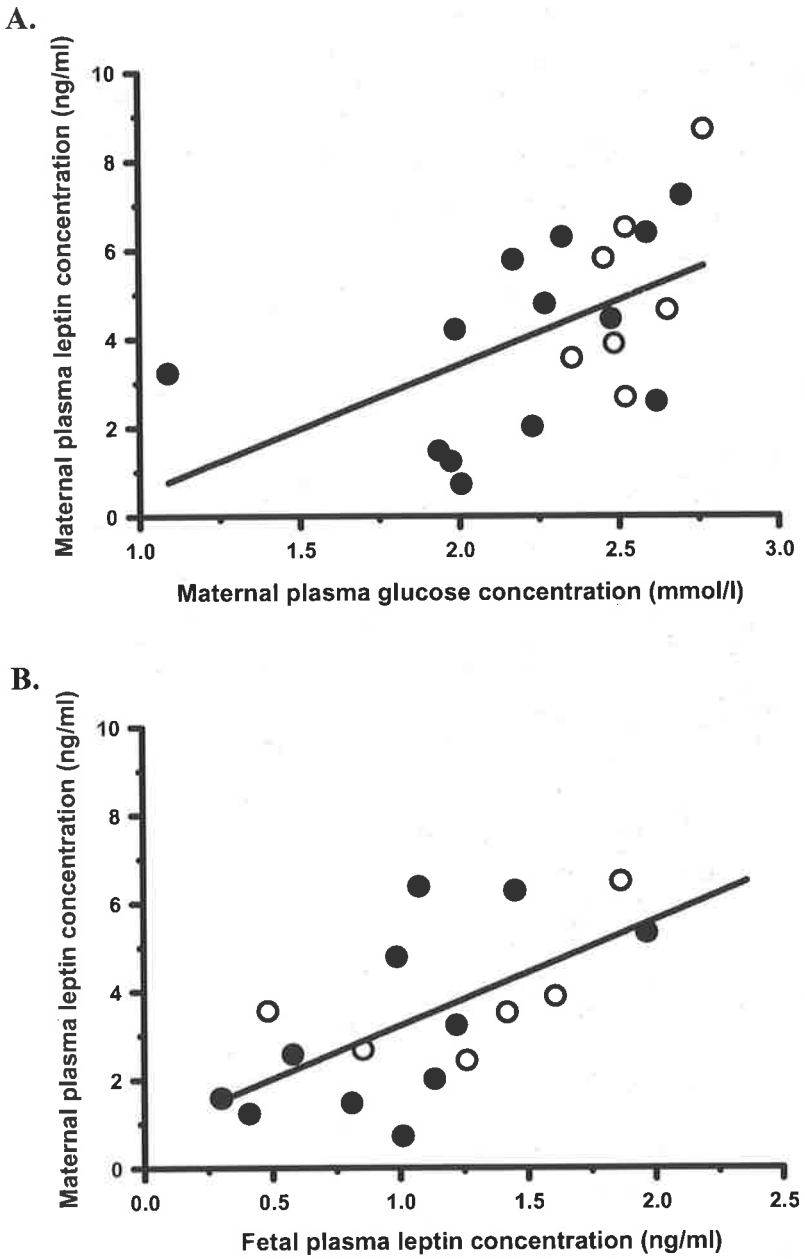


Figure 3.2 Maternal leptin is related to maternal glucose and fetal leptin levels.

The relationship between maternal plasma leptin and glucose concentrations (A) or between fetal and maternal plasma leptin concentrations (B) in undernourished (closed symbols) and control (open symbols) groups.

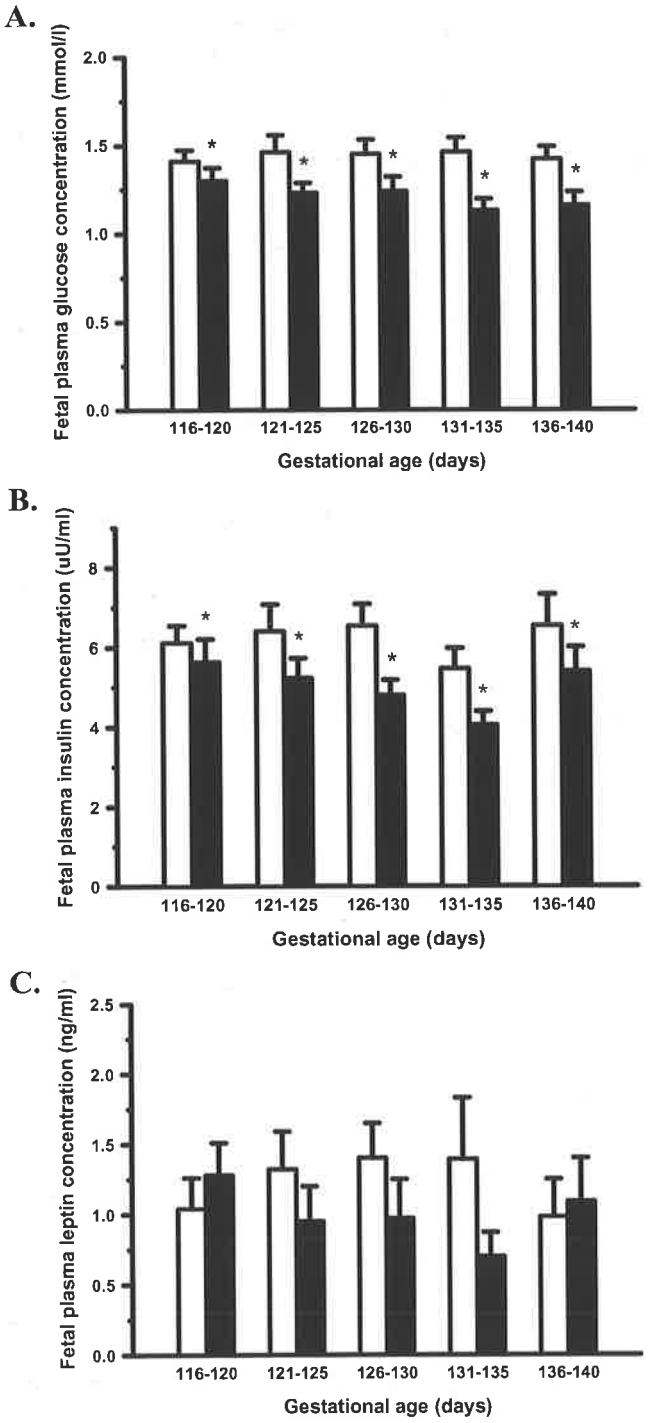


Figure 3.3 Fetal hormone and metabolite levels in the control and undernourished groups.

Fetal plasma glucose, insulin and leptin concentrations in control (open histograms) and undernourished (dark histograms) groups between 116 and 140 days gestation. The asterisks denote a significant effect of undernutrition.

There was no significant correlation between mean fetal plasma concentrations of leptin and either glucose or insulin when data from the undernourished and control groups were combined. Mean fetal (y) and maternal (x) plasma leptin concentrations were significantly correlated ($y = 0.18 x + 0.45$, $R=0.66$, $P<0.003$, $n=17$) (Figure 3.2). The mean fetal plasma leptin concentrations between 116 and 140 d gestation were not correlated with either fetal body weight or with absolute or relative fetal fat mass at 144-147 d gestation.

3.6.4 Leptin mRNA expression in fetal perirenal adipose tissue

There was no significant difference in the relative abundance of leptin mRNA in fetal perirenal adipose tissue between the undernourished (0.60 ± 0.09, $n=10$) and control (0.70 ± 0.08, $n=10$) groups. The mean fetal plasma concentrations of leptin (y) and the relative abundance of leptin mRNA (x) in perirenal adipose tissue were significantly correlated ($y = 1.5 x + 0.3$, $R=0.69$, $P<0.05$, $n=9$) (Figure 3.4). Leptin mRNA expression in fetal adipose tissue was not related to either fetal weight ($P=0.09$), fetal perirenal fat mass, mean fetal glucose or insulin concentrations.

3.7 DISCUSSION

In the present study, there was a positive relationship between mean plasma concentrations of leptin and glucose during the last 30 days of pregnancy when data from

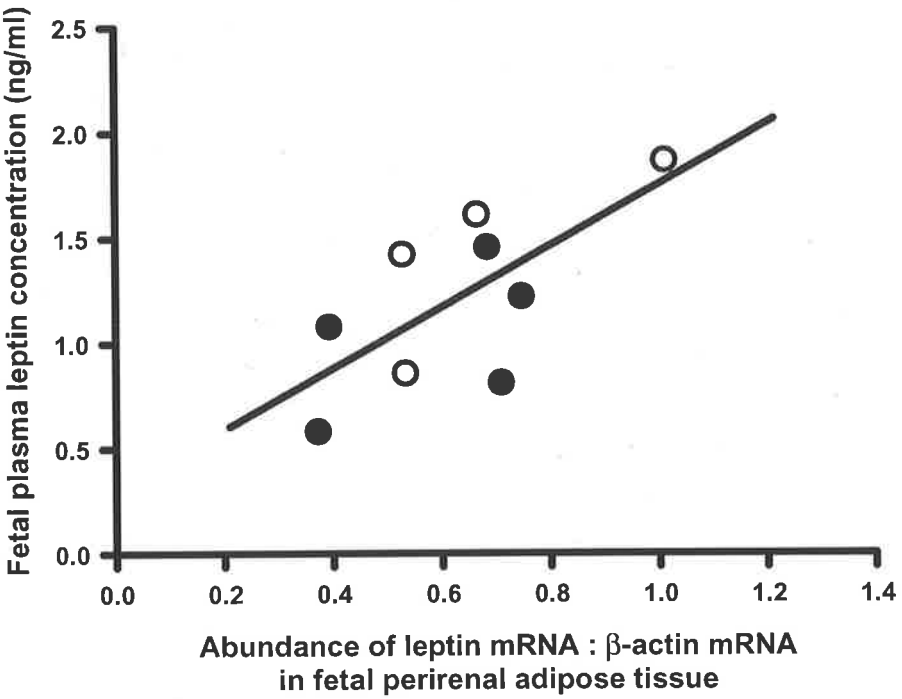


Figure 3.4 Leptin expression is related to circulating leptin levels in the fetus.

The relationship between fetal plasma leptin concentrations and the relative abundance of leptin mRNA in fetal perirenal adipose tissue in undernourished (closed symbols) and control (open symbols).

the control and well fed ewes were combined. Thomas and colleagues (441) recently reported that when the dietary intake of adolescent pregnant ewes was increased from moderate to high, or reduced from high to moderate, at day 50 of pregnancy, circulating maternal leptin concentrations changed within 48 hours of the change in maternal diet. They suggested that this was likely to be a 'direct' nutritional effect. These authors also found that at some 50-90 days after the change in diet, circulating leptin concentrations were correlated with indices of body composition in the pregnant ewe. They were unable, however, to distinguish whether dietary intake or the changed body composition due to the nutritional treatments was the primary factor influencing circulating leptin concentrations. In the current study, it is also possible that differences in maternal body composition, may explain some of the variation in maternal leptin concentrations.

We have also found that maternal plasma leptin concentrations varied between 3 and 10 ng/ml throughout late pregnancy and that there was no significant change in circulating leptin concentrations between 115 days of pregnancy and term in either the control or undernourished adult ewes. These circulating leptin concentrations are similar to those reported by Thomas and colleagues in moderately fed adolescent pregnant ewes from 50 days pregnancy until term (441). These authors also found that there was no significant change in plasma leptin concentrations throughout late pregnancy. The lack of a change in plasma leptin concentrations towards the end of pregnancy in the sheep is in contrast to the increase in plasma leptin concentrations which occurs in mid/late pregnancy in the human (389), rat (392) and mouse (390). Whilst adipose tissue is the main source of circulating leptin in all species, it is unclear to what extent other tissues, such as the placenta, are also a source of leptin in the maternal circulation during late pregnancy. Leptin gene expression is relatively high in the human placenta (128) and is also

detectable in the rodent placenta (127). Species specific differences in the relative level of placental leptin expression may account for the differences in the effect of pregnancy on the maternal plasma concentrations of leptin between sheep and other species. The sheep placenta also expresses the leptin receptor gene (441) and it is therefore possible that maternal leptin may interact with leptin receptors within the placenta to impact on fetal growth and development. Overfeeding the adolescent ewe throughout pregnancy increases maternal growth at the expense of the placenta, leading to growth restriction of the fetus (442). In a cohort of over fed and normally fed adolescent pregnant ewes, there was a negative association between maternal plasma leptin concentrations and birth weight, placental weight and number of placentomes (441). In the present study in the mature ewe, however, I found no significant relationship between maternal plasma leptin concentrations and fetal body weight. It is clear that further work is required to define the relative roles that maternal leptin and nutrients play in placental and fetal growth and development at different stages of reproductive maturity.

Plasma concentrations of leptin in the fetus (<0.3 - 3 ng/ml) were substantially lower than those in the pregnant ewe and there was no effect of either maternal undernutrition or gestational age on circulating fetal leptin concentrations between 116 and 140 days gestation. In a previous study we reported that the abundance of leptin mRNA in fetal adipose tissue increased between 125 and 144 days gestation (443). It may be that leptin concentrations increase in the fetal circulation after 140 days gestation. We also found a positive relationship between fetal and maternal plasma concentrations of leptin during late gestation. One possible explanation is that maternal body composition or fatness at the beginning or during pregnancy determines the leptin synthetic and secretory capacity of both maternal and fetal adipose tissue or the amount of fetal adipose tissue deposited

during late gestation. Whilst there was a positive relationship between circulating fetal leptin and the relative abundance of leptin mRNA in fetal adipose tissue, there was, however, no relationship with either maternal or fetal leptin concentrations or the absolute or relative fetal fat mass. Thus any impact of maternal body composition on circulating fetal leptin concentrations is presumably expressed through the leptin synthetic and secretory capacity of the fetal adipose tissue. An alternative explanation for the close correlation between maternal and fetal plasma leptin concentrations is that the placental leptin receptor may act to mediate the uptake of leptin from the maternal into the fetal circulation. This would be similar to the postulated mode of action of the short isoform of the leptin receptor in the choroid plexus epithelium to transport leptin from plasma into the cerebrospinal fluid (444).

In the same year in which I published this study, three published reports also confirm the expression of leptin in fetal adipose tissue (146, 445) and the presence of leptin in the circulation of the sheep fetus during late gestation (146, 446, 447). These authors reported that fetal plasma leptin concentrations were in the range 0.2-3 ng/ml during late gestation and that there was no change in fetal plasma leptin between 40-140 d gestation. Forhead and colleagues reported, however, that there was an increase in fetal plasma leptin concentrations in a cross sectional study where samples were collected from fetuses at two different stages during late gestation (446). No significant relationship between fetal and maternal plasma leptin was reported in these studies.

In the present study, maternal feed availability was reduced by 50% below maintenance for 29 to 32 days and this was associated with a ~16% fall in maternal glucose concentrations and a 20% fall in fetal plasma glucose and insulin concentrations. There

was no significant effect, however, of this level of maternal undernutrition on the fetal plasma concentrations of leptin or on the relative abundance of leptin mRNA in the perirenal adipose tissue. During the conduct of this study, Erhardt and colleagues also reported that maternal undernutrition for a period of 15 d in late gestation also had no affect on fetal plasma concentrations of leptin (146). It has recently been reported that continuous infusion of insulin into pregnant ewes for up to 34 days resulted in fetal hypoglycaemia and hypoinsulinaemia and a reduction in fetal body weight but that there was no change in the expression of leptin mRNA in fetal perirenal adipose tissue (445). These authors reported however, that if the period of continuous insulin infusion was prolonged beyond 36 days (36-76 days), fetal glucose and insulin concentrations were reduced by 30-50% and leptin mRNA expression was suppressed in fetal perirenal fat (445). Together these studies indicate that the synthesis and secretion of leptin in the sheep fetus is resistant to the changes in fetal glucose and insulin concentrations associated with moderate maternal undernutrition. Fetal leptin synthesis is suppressed, however, in the presence of profound fetal hypoglycaemia and/or hypoinsulinaemia which may occur as a consequence of either pharmacological induction of maternal hypoglycaemia or severe maternal undernutrition.

In the human there are strong positive associations between umbilical cord blood leptin concentrations at delivery and infant body weight at birth, as well as other anthropometric markers of fetal growth including estimates of fetal fat mass (393, 423, 443, 448-450). In Chapter 2, I also reported that the abundance of leptin mRNA in fetal adipose tissue was positively correlated with fetal body weight in a cohort of fetuses at an earlier gestational age than those used in the current study. In the present study, however, whilst the relationship between leptin mRNA expression in fetal adipose tissue

and fetal weight tended to be positive (p=0.09), there was no relationship between circulating leptin concentrations and either fetal weight or relative fat mass. These differences between the sheep and human may be explained in part by the different patterns of fat deposition that occurs in these species during fetal life. In the sheep fetus, fat is deposited at around 0.8 g/kg fetal body weight/day, the proportion of body fat at term is ~0.3-2.0% and the major fat depot is the perirenal adipose tissue which is comprised predominantly of brown fat cells (84). It is not known whether leptin is expressed uniformly in all perirenal adipocytes in the sheep fetus before birth. In contrast, in the human fetus, fat is deposited at a higher rate around 3.5 g/kg fetal body weight/day, the proportion of body fat at term is around 16% and there are subcutaneous fat depots comprised predominantly of white fat cells (437). Despite the differences between sheep and human fetuses in the rate of fat deposition, the leptin synthetic capacity of fat stores and the effect of undernutrition on leptin concentrations during late gestation, it is interesting that perturbations of the intrauterine environment may program the development of postnatal obesity in these and other species. It has been demonstrated that restricted fetal nutrient supply may program alterations in adiposity and/or leptin synthesis beyond the postnatal period in the human (451), sheep (452), rat (453) and pig (454). Further work is required to identify those periods during intrauterine life when changes in the long term development of the adipocyte and leptin signalling system are initiated and to clarify the relative importance of maternal body composition and the level of fetal nutrition in the mechanistic pathway which underlies the association between poor intrauterine growth and postnatal obesity.

In summary, this chapter reports the effect of maternal undernutrition during late pregnancy on maternal and fetal plasma concentrations of leptin and on leptin gene

expression in fetal adipose tissue in the sheep. We have found that maternal plasma concentrations of leptin and glucose are positively correlated across the range of circulating glucose concentrations present in well fed and undernourished pregnant ewes. Interestingly, there was a positive relationship between the fetal and maternal plasma concentrations of leptin during late gestation suggesting that maternal body composition during early pregnancy may determine the leptin synthetic and secretory capacity of maternal and fetal adipose tissue. There was no effect, however, of maternal undernutrition on circulating leptin concentrations or on the abundance of leptin mRNA in adipose tissue in the sheep fetus. The capacity of leptin to act as a signal of moderate maternal undernutrition may therefore be limited in this species before birth.

The McMillen group recently published a report investigating the effects of maternal overnutrition on circulating leptin levels in the sheep fetus. Whilst overfeeding had no effect on fat mass or circulating leptin levels in the fetus, there was however, a positive relationship between circulating leptin and the unilocular proportion of adipose tissue in late gestation sheep fetus (455). The cellular structure of the unilocular adipose tissue in the fetus was similar to that reported in the white adipose tissue of adults. It can, therefore, be concluded that in the fetus, circulating leptin is being secreted from the adipocytes containing dominant lipid locules.

The relationship between leptin mRNA expression in adipose tissue and circulating leptin in the fetal sheep in late gestation in this chapter and the positive relationship between circulating leptin and unilocular fat suggests that leptin may act as a signal of lipid storage in the late gestation sheep fetus. In Chapter 4, I have investigated the impact

of an experimentally induced increase in circulating leptin concentrations on the structural characteristics of fetal adipose tissue.

CHAPTER 4

4. LEPTIN ALTERS THE STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF FETAL ADIPOSE TISSUE

4.1 DECLARATION

During the course of my PhD candidature, I conducted experiment work that has been subsequently published by my coauthors and I. The majority of the contents within this chapter can be found in the following publication:

Yuen BSJ, Owens PC, Muhlhausler BS, Roberts, CT, Symonds ME, Keisler DH, McFarlane JR, Kauter KG, Evens Y and McMillen IC. Leptin alters the structural and functional characteristics of adipose tissue before birth. FASEB J. 2003. 10.1096/fj.02-0756fje

4.2 CONTRIBUTION OF COAUTHORS

Owens, PC

Co-supervised the experimental work contained within this publication, contributed to intellectual debates, assisted with data analyses and interpretation, and aided in the writing of the manuscript.

Signature.....Date.....15 July 03

Muhlhausler, BS

Conducted the histological measurements on the fetal adipose tissue that was collected from saline infused and leptin infused fetuses.

Signature.....Date..17/6/03.....

Roberts, CT

Gave technical advise on the histology techniques used in this publication, assisted with the interpretation of the data and evaluated the manuscript prior to submission.

Signature.....Date..2 Aug 2003.....

Symonds, ME

Assisted in the interpretation of the data, contributed to intellectual debates and evaluated the manuscript prior to submission.

Signature.....Date..13/6/03.....

Keisler, DH

Generously provided recombinant ovine leptin and evaluated the manuscript prior to submission.

Signature.....Date.....

McFarlane, JR

Dr McFarlane generously agreed to assay maternal and fetal sheep plasma to determine circulating leptin concentrations. Contributed to intellectual debates, assisted with data interpretation and evaluated the manuscript prior to submission.

Signature.....Date.....

Kauter, KG

Ms Kauter was one of the first researchers to develop a leptin ELISA for use in the sheep. This assay system was used to measure the plasma leptin concentrations of the fetuses described in this study.

Signature.....Date.....

Evens, Y

Ms Evens conducted the UCP-1 protein measurements in the adipose tissue of saline and leptin infused fetuses.

Ms Evens was unavailable to sign the declaration. Her supervisor (Dr Symonds) has signed on her behalf.

Signature.....Date...12/2/03.....

McMillen, IC

Co-supervised the experimental work contained within this publication, contributed to intellectual debates, assisted with data analyses and interpretation, and aided in the writing of the manuscript.

Signature.....Date...22/2/03

4.3 ABSTRACT

This study aimed to determine for the first time whether leptin can act to alter the structural and functional characteristics of adipose tissue before birth. Leptin (0.48 mg/kg/d) or saline was infused intravenously into fetal sheep for 4 days from either 136 or 137 days gestation (term ~150 days). Circulating leptin concentrations were increased ~4-5 fold by leptin infusion. Leptin infusion resulted in a significant increase in the proportion of smaller lipid locules present within fetal perirenal adipose tissue (PAT) and this was associated with a significant increase in the proportion of multilocular tissue and a significant decrease in the proportion and relative mass of unilocular tissue in fetal PAT. The relative abundance of leptin mRNA in fetal PAT was significantly lower in the leptin infused group and there was a positive correlation between the relative abundance of leptin mRNA and the proportion of unilocular adipose tissue in fetal PAT. The amount of UCP-1 protein tended to be higher (P=0.06) in leptin compared with saline infused fetuses. This is the first demonstration that leptin can act to regulate the lipid storage characteristics, leptin synthetic capacity and potential thermogenic functions of fat before birth.

4.4 INTRODUCTION

In adult mammals, circulating leptin concentrations are positively correlated with body fat content and with body mass index (135, 136, 441, 456). Leptin acts at central receptors to decrease food intake and to increase fat mobilisation and oxidation. Leptin administration suppresses leptin gene expression in white adipocytes, and increases the expression of uncoupling proteins (UCPs) and thermogenesis in brown adipocytes in adult rodents (13, 457). Leptin is also synthesised in a range of uteroplacental and fetal

tissues, including fetal adipose tissue (127, 146, 443, 445) and leptin concentrations in umbilical cord blood are positively correlated with birth weight and with neonatal adiposity (393, 406, 413, 421, 423, 458). There is a significant correlation between fetal leptin concentrations and fetal adiposity in well nourished pregnant ewes and it has therefore been concluded that circulating leptin may be a signal of fat mass in the fetus (455). It is therefore hypothesised that leptin may play a role in the control of substrate utilisation, maintenance of fat mass or regulation of the expression of leptin or UCP-1 in adipose tissue before birth. The first aim of the study was to investigate the effects of leptin administration in the sheep fetus on circulating glucose and insulin concentrations and on the mass and structural characteristics of fetal perirenal adipose tissue (PAT), the major fat depot in the sheep fetus during late gestation. Fetal PAT is comprised of adipocytes that have either a dominant lipid locule (unilocular) or many small lipid locules (multilocular) (84, 85, 455). In adults, unilocular adipocytes are white adipose cells and these cells represent the major sites of lipid storage and of leptin synthesis (459). Ultrastructural studies have demonstrated, however, that both unilocular and multilocular cells in fetal sheep adipose tissue contain an abundance of mitochondria which is a characteristic feature of thermogenic or brown adipose tissue (84). The second aim of the study was to investigate whether leptin infusion alters the proportion of unilocular and multilocular tissue present in fetal PAT and whether there are concomitant changes in the abundance of leptin mRNA, UCP-1 mRNA and UCP-1 protein in this tissue. In order to determine whether leptin has specific effects on mitochondrial UCP-1 expression, I have also measured the expression of two other mitochondrial proteins - VDAC (voltage-dependent anion channel), which is located on the outer mitochondrial membrane and cytochrome *c*, which is present in the mitochondrial space in fetal PAT.

4.5 MATERIALS AND METHODS

4.5.1 Animals and Surgery

The procedures in this study were approved by the University of Adelaide Animal Ethics Committee. Dated pregnant Merino ewes were housed in individual pens in rooms with a 12 h light/dark cycle and fed once daily (~10-12 MJ/kg metabolisable energy) at 1100 h with water provided *ad libitum*. Surgery was performed on 13 pregnant ewes between 110 and 124 d gestation under aseptic conditions as previously described (455). General anaesthesia was induced by an intravenous injection of sodium thiopentone (1.25 g i.v., Pentothal, Rhone Merieux, Pinkenba, Qld, Australia) and maintained by ventilation with 2.5-4% halothane (Fluothane, ICI, Melbourne, Vic, Australia). Catheters were inserted in a maternal jugular vein, a fetal carotid artery and jugular vein, and the amniotic cavity. Catheters were filled with heparinised saline and the fetal catheters exteriorised through an incision made in the ewe's flank. Ewes and their fetuses received a 2 ml intramuscular injection of antibiotics (procaine penicillin 250 mg/ml; dihydrostreptomycin 250 mg/ml; procaine hydrochloride 20 mg/ml Penstrep Illium, Troy Laboratories, Smithfield, NSW, Australia).

4.5.2 Experimental protocol

At 136 or 137 d gestation, fetuses were randomly assigned to either a leptin (n=6) or saline (n=7) infusion group. On the day of the infusion, fetal arterial blood samples (3 ml) were collected at -3 h, -2 h, -1 h and -30 min relative to the start of the infusion at 0 h (1300 h). Either recombinant ovine leptin (250 ìg in 0.5 ml sterile saline) (460) or sterile saline (0.5 ml), were administered into the fetal jugular as a bolus at 0 h (1300 h),

immediately followed by infusion of either leptin (80 μg/0.16 ml/h, i.e. 0.48 mg/kg/d) or sterile saline (0.16 ml/h) for 96 h. Fetal arterial blood samples were collected at +2 min, +30 min, +1 h, +2 h, +4 h and +8 h on the first day of the infusion and at 0900 h, 1300 h and 1700 h on the second and third days and at 0900 h and 1300 h on the fourth day of the infusion. At each sampling time, a fetal arterial blood sample (0.5 ml) was collected for the measurement of fetal blood gases and pH (ABL 520 blood gas analyser, Radiometer, Copenhagen, Denmark). Blood samples were centrifuged at 1500 g for 10 min and aliquots of plasma were separated and stored at -20°C.

4.5.3 Tissue collection

After 96 h of infusion (at 140 or 141 d gestation), ewes were killed with an overdose of sodium pentobarbitone (Virbac Pty Ltd, Peakhurst, NSW, Australia). Fetuses were delivered by hysterotomy, weighed and decapitated (saline infused group: 5 singletons and 2 twins - 3 male and 4 female; leptin infused group: 3 singletons and 3 twins - 2 male and 3 female). Fetal organs were weighed and samples of PAT were collected and either frozen in liquid nitrogen for storage at -80°C or fixed in 4% paraformaldehyde in 0.2 M phosphate buffer at 4°C for 2-3 d.

4.5.4 Leptin ELISA

Plasma concentrations of leptin were measured in 132 fetal samples using a competitive ELISA previously described in Chapter 3 (373). The sensitivity of the assay was 0.5 ng/ml and the inter assay and intra assay coefficients of variation were 11% and 9% respectively.

4.5.5 Glucose assay

Plasma glucose concentrations were determined by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase as described in Chapter 3. The intra- and inter-assay coefficients of variation were both <5%.

4.5.6 Insulin radioimmunoassay

Fetal plasma insulin concentrations were measured using a Phadaseph Radioimmunoassay kit (Pharmacia & Upjohn, Uppsala, Sweden) as described in Chapter 3. The inter- and intra- assay coefficients of variation were <10%.

4.5.7 Adipose tissue histology

After fixation in paraformaldehyde, adipose tissue samples were washed in 4 changes of 0.01 M phosphate buffered saline over 48 h (Sigma Chemical Co., St.Louis MO, USA) and then in 70% ethanol (24 h) before being processed and embedded in paraffin wax. Sections were cut (4 μm) and stained with Hematoxylin and Eosin and then examined using an Olympus BH2 microscope (20x objective and 2.5x NFK) which was connected to a video image analysis system. Images were captured using Video Pro software (Leading Edge, Adelaide, SA, Australia) and standard point counting techniques were used to determine the volume density of unilocular and multilocular tissue in fetal perirenal fat (455, 461). Lipid locules were classified as dominant when their cross sectional area was ≥75 ìm^2. These locules comprised the unilocular component of the adipose tissue depot (455, 461) whereas regions of the adipose depot which contained ^{2}, comprised the multilocular component. One section was randomly selected for each fetus and 8-10 fields (288-360 points), each 1 mm apart, were analysed. The volume density (V_d) of the unilocular tissue in each perirenal adipose depot was

calculated as described previously (455, 461) using the formula: $V_d=N/T$, where N is the number of points falling on unilocular tissue, and T is the total number of points counted.

4.5.8 Measurement of the cross sectional area of the dominant lipid locules within unilocular adipose cells and the number of unilocular cells

The cross sectional areas of the dominant lipid locules (300-700 per fetus) were determined by measuring the area of all locules falling completely within each of the fields of view in a section of PAT from each animal. Areas were calculated using the Area-Pro software program (Video Pro Image Analysis, Leading Edge, Adelaide, SA, Australia). The program was validated by the measurement of a known area using a haemocytometer. The median size of these lipid locules was calculated and the distribution of the size ranges (<200 ìm^2, 201-400 ìm^2, 401-600 ìm^2, 601-800 ìm^2 or > 801 ìm^2) of the dominant lipid locules was determined for each experimental animal. The cell number / μm^2 (i.e. the density of the unilocular adipocytes) in the PAT was calculated for each fetus.

4.5.9 RNA extraction

Total RNA was extracted from PAT samples as previously described in Chapter 2 (443).

4.5.10 Leptin and β-actin mRNA

Ovine leptin and β-actin cDNA were amplified by reverse transcription (RT)-PCR as previously described in Chapter 2 (443). An RT-PCR on the total RNA from the adipose tissue of the leptin and saline infused animals was repeated to confirm the leptin and β-

actin results. The relative abundance of leptin mRNA was calculated by referencing the intensity of the leptin amplicon to the intensity of the β-actin amplicon.

4.5.11 UCP-1 mRNA and 18S rRNA

An oligonucleotide radiolabelling kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA) was used to end-label 1 ng of UCP-1 oligonucleotide (Geneworks, Adelaide, SA Australia) with [^{32}P]-dATP (4000 Ci/mmol; GRA-32U, Geneworks, Adelaide, Australia) according to the manufacturer's instructions. The UCP-1 oligonucleotide, 5'-CGG ACT TTG GCG GTG TCC AGC GGG AAG GTG AT- 3', was complementary to nucleotides 267-298 of the 1194 nucleotide cDNA of rat UCP-1 (Genbank Acc. No. NM 012682) (462, 463). An oligonucleotide complementary to nucleotides 151-180 of rat 18S ribosomal RNA (464) was also end labelled with [γ-^{32}P]-ATP (465).

Samples of total RNA (20 μg) from PAT were electrophoresed through a 1.5% agarose gel containing 2.2 M formaldehyde and 1x northern buffer (containing 0.1 M MOPS (3-(N-Morpholino) propanesulphonic acid) at pH 7.0, 40 mM sodium acetate, 5 mM EDTA (ethylenediaminetetracetate.2H_20 disodium salt pH 8.0). Total RNA was transferred overnight at room temperature onto a Zeta-Probe nylon membrane (Bio-Rad Laboratories, Richmond, CA, USA) by capillary transfer in 10x SSC (saline sodium citrate). Membranes were then washed twice in fresh 10x SSC, dried at 80°C and prehybridised with 100 μg/ml of heat denatured salmon sperm DNA at 52°C for 2 h in 5x SSC, 20 mM NaH_2PO_4, 7% SDS and 5x Denhardt's solution at pH 7.2 (465). This hybridisation solution was then removed. Fresh hybridisation solution, that was preheated to 52°C and that contained the [γ-^{32}P]-ATP labelled UCP-1 oligonucleotide probe, was added to the membrane and allowed to hybridise for 14-16 h at 52°C.

Membranes were then washed in 1x SSC, 0.1% SDS at 52°C for 30 min, and then again being washed in fresh 1x SSC, 0.1% SDS for a further 10 min at 52°C. Membranes were exposed to a phosphorimaging screen for 2.5 d (Fuji-BAS MP2040, Fuji Photo Film Co. Ltd., Tokyo, Japan), and visualised using a Fuji-BAS 1000 phosphorimager (Fuji Photo Film Co. Ltd., Tokyo, Japan). The hybridisation signal was quantified with Fuji-MacBAS software (ver. 2.21). Membranes were then washed in stripping solution containing 0.1x SSC and 0.5% SDS at 85°C for 30 min before being rehybridised with a probe for 18S rRNA. Samples of total RNA were electrophoresed through a second agarose gel and the process repeated to give a duplicate measure of the results. The relative abundance of UCP-1 mRNA was calculated by referencing the intensity of the UCP-1 mRNA band to the intensity of the 18S rRNA band for each fetus.

4.5.12 Mitochondria preparation

Mitochondria were extracted from adipose tissue samples using a previously defined method (337, 347). Briefly, 2 g of PAT from each fetus were thawed and homogenised in 40 ml of 10 mM Tris buffer solution, containing 250 mM sucrose and 1 mM EDTA at pH 7.4. Homogenates were then centrifuged at 800 g for 10 min and lipids were removed by passing the supernatant through two layers of surgical gauze. The supernatant was then centrifuged at 10,000 g for 30 min, the supernatant discarded and the pellet, containing mitochondria, was resuspended in a Tris buffer solution (0.5-1.0 ml) and frozen at -20°C.

4.5.13 Mitochondrial protein measurements

UCP-1, cytochrome *c* and VDAC (voltage-dependent anion channel) proteins were detected in mitochondrial preparations after separation by sodium dodecyl sulphate

polyacrylamide gel electrophoresis by using immunoblotting and enhanced chemiluminescence (ECL, Amersham International, Buckinghamshire, UK) (337, 463). Densitometric analysis was performed on each membrane following image detection using a Fujifilm LAS-1000 cooled CCD camera (Fuji Photo Film Co. Ltd., Tokyo, Japan). All values were expressed as a percentage of a reference sample run in duplicate on all gels.

4.5.14 Statistical analysis

Data are presented as the mean ± SEM. The mean fetal plasma concentration of leptin prior to the start of the infusion was calculated as the mean of the five values during the preinfusion period (i.e. at -3 h, -2 h, -1 h, -0.5 h and 0 h). The effect of leptin infusion on fetal plasma leptin, glucose and insulin concentrations was determined using a multifactorial ANOVA with repeated measures using the Statistical Package for Social Sciences (SPSSX, Chicago, IL, USA) on a VAX mainframe computer. Fetal hormone and metabolite data were logarithmically transformed where required to reduce heterogeneity of variance. Where there was a significant interaction between the effects of treatment and the time of infusion on plasma hormone or metabolite data, the data were split on the basis of the interaction and reanalysed. The Duncan's new multiple range test was used post ANOVA to identify significant differences between mean values.

The mass of unilocular and multilocular fat was calculated by multiplying the mass of the perirenal adipose depot by the volume density of either the unilocular or multilocular tissue and the relative mass of these tissues (g/kg) was then calculated by dividing total fat mass by fetal weight. The effects of leptin and saline infusion on the proportion of

dominant lipid locules present within the different size ranges were compared using 2-way ANOVA. The Student's t-test was used to compare the effects of treatment on fetal body and organ weights, locule density, the volume density and relative mass of unilocular or multilocular fat (g per kg of fetal weight), the relative abundance of leptin mRNA and UCP-1 mRNA, and the abundance of UCP-1, cytochrome *c* and VDAC proteins in PAT. The relationship between the relative abundance of leptin mRNA and the proportion of the perirenal adipose depot composed of unilocular tissue was determined using simple linear regression. The probability of 5% (i.e. $P<0.05$) was taken as the level of significance.

4.6 RESULTS

4.6.1 Plasma leptin concentrations

During the pre-infusion period, there was no difference in the plasma concentrations of leptin between those fetuses assigned to the saline (2.97 ± 0.88 ng/ml) or leptin (4.38 ± 0.99 ng/ml) infusion groups (Figure 4.1). During the 4 d infusion period, there was no change in fetal plasma leptin concentrations in the saline group whereas leptin infusion increased ($P<0.001$) plasma leptin concentrations by ~4-5 fold (+68-96 h: saline infused, 3.74 ± 1.22 ng/ml, leptin infused, 18.44 ± 4.05 ng/ml) (Figure 4.1).

4.6.2 Arterial blood gas status

There was no difference in fetal arterial blood gases or pH between the leptin and saline infused groups, either before or during the 4 d infusion period (Table 4.1).

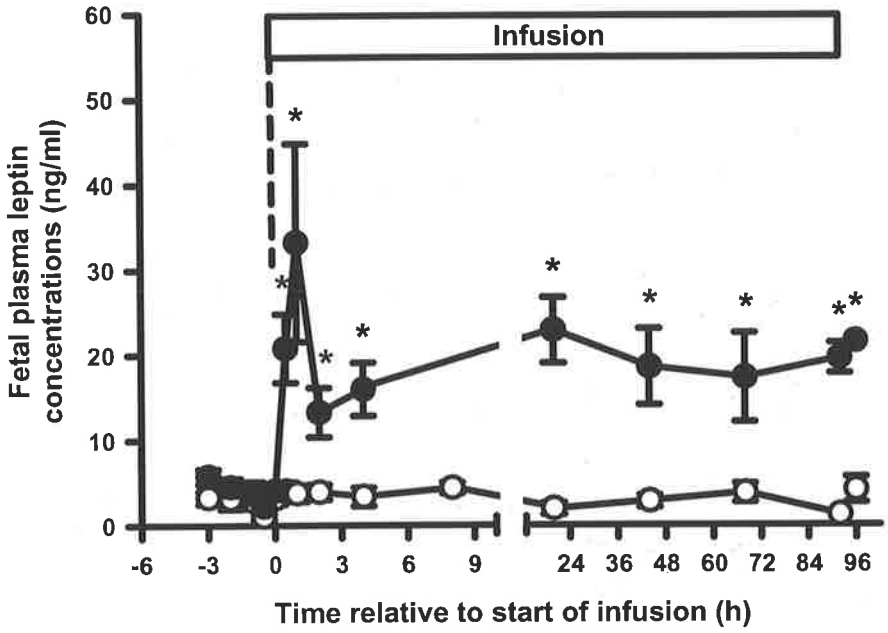


Figure 4.1 Plasma leptin levels in saline infused and leptin infused groups.

Plasma leptin concentrations in fetal sheep infused either with recombinant ovine leptin at 0.48 mg/kg per day (closed circles) or saline (open circles) for 96 h. Asterisks denote a significant difference ($P<0.05$) between leptin concentrations during the infusion period compared to the preinfusion period in the leptin infused group.

Table 4.1 Effect of saline or leptin infusion on fetal arterial blood gas and pH status during the 4 d infusion period.

		Time relative to the start of infusion (h)				
		-3	+20	+44	+68	+92
Saline infused	pO_2 (mmHg)	22.0 ± 1.1	21.5 ± 0.5	22.2 ± 0.7	21.4 ± 0.8	21.6 ± 0.4
	pCO_2 (mmHg)	51.3 ± 0.8	49.0 ± 1.5	51.9 ± 1.2	50.9 ± 1.0	49.1 ± 1.4
	pH	7.395 ± 0.005	7.404 ± 0.005	7.401 ± 0.006	7.403 ± 0.007	7.410 ± 0.007
	Hb (g/dl)	11.2 ± 0.2	10.5 ± 0.2	10.5 ± 0.3	10.7 ± 0.2	10.4 ± 0.2
	O_2 Saturation (%)	65.8 ± 3.1	65.4 ± 1.8	67.2 ± 2.1	65.1 ± 2.7	66.4 ± 3.0
Leptin infused	pO_2 (mmHg)	23.1 ± 0.5	23.1 ± 0.5	22.4 ± 1.2	22.6 ± 0.8	21.2 ± 0.5
	pCO_2 (mmHg)	49.3 ± 1.2	48.5 ± 1.1	49.0 ± 1.3	49.5 ± 1.1	49.6 ± 1.0
	pH	7.407 ± 0.006	7.406 ± 0.006	7.396 ± 0.005	7.405 ± 0.006	7.398 ± 0.004
	Hb (g/dl)	11.7 ± 0.2	11.1 ± 0.2	10.8 ± 0.3	10.8 ± 0.2	10.9 ± 0.3
	O_2 Saturation (%)	67.9 ± 2.0	67.5 ± 1.2	63.9 ± 3.7	65.4 ± 2.7	60.3 ± 2.0

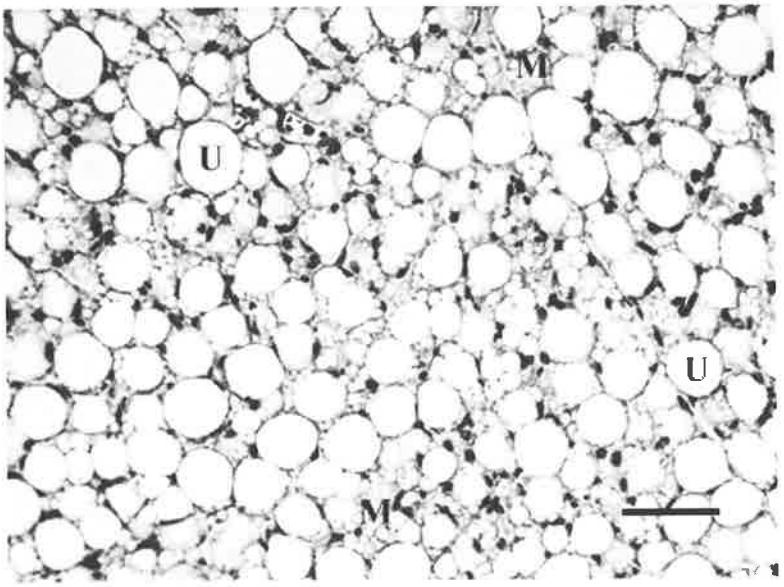
4.6.3 Plasma glucose and insulin concentrations

Fetal plasma glucose concentrations during the 4 d infusion period were not different between the saline (Basal: 1.85 ± 0.12 mmol/l, +96 h: 1.76 ± 0.12 mmol/l) and leptin infused groups (Basal: 1.52 ± 0.13 mmol/l, +96 h: 1.72 ± 0.14 mmol/l). Similarly plasma insulin concentrations were not different between the saline (Basal: 8.5 ± 0.8 μU/ml, +96 h: 9.8 ± 1.1 μU/ml) and leptin infused groups (Basal: 9.7 ± 2.0 μU/ml, +96 h: 9.2 ± 1.0 μU/ml) throughout the 4 d infusion period. On the first day of the infusion period, fetal glucose and insulin concentrations increased (P<0.05) during the 4-8 h period immediately after maternal feeding in both the saline infused (+8 h: glucose, 2.21 ± 0.21 mmol/l, insulin, 11.8 ± 0.9 μU/ml) and leptin infused (+8 h: glucose, 2.03 ± 0.11 mmol/l, insulin, 12.8 ± 1.8 μU/ml) groups and there was no difference between the groups in these responses to feeding.

4.6.4 Perirenal adipose tissue

There was no effect of leptin infusion on either the total mass (leptin infused, 20.2 ± 2.1 g; saline infused, 20.8 ± 1.7 g) or the relative mass (leptin infused, 4.16 ± 0.30 g/kg; saline infused, 4.43 ± 0.35 g/kg) of fetal PAT. The proportion of PAT comprised of unilocular tissue was lower (P<0.01) and the proportion comprised of multilocular tissue was higher (P<0.01), in the leptin infused group compared with the saline infused animals (Figure 4.2 and Figure 4.3). The relative mass of unilocular adipose tissue (g/kg) was also significantly lower (P<0.05) in the leptin infused group compared with the saline group (Figure 4.3).

A.



B.

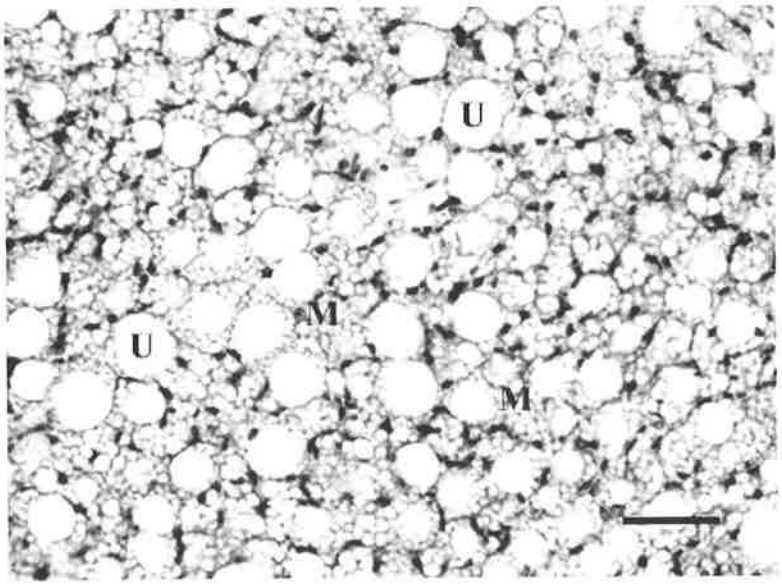


Figure 4.2 Photomicrographs of sections of perirenal adipose tissue from a saline infused (A) and a leptin infused (B) fetus.

Examples of tissue defined as unilocular (**U**) or multilocular (**M**) adipose tissue are indicated on the section. The horizontal dark bar represents 50 μm.

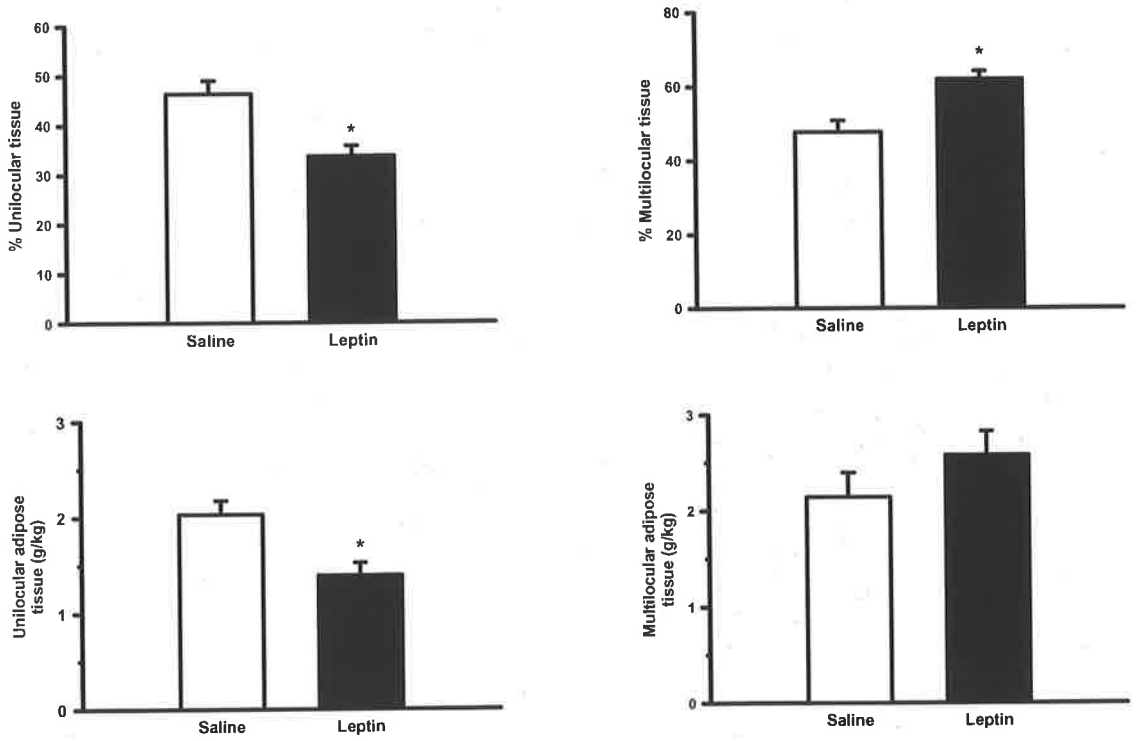


Figure 4.3 The proportion and relative mass of unilocular and multilocular tissue in fetal sheep infused either with saline or leptin.

The asterisks denote significant differences ($P<0.05$) between the saline and leptin treatment groups.

There was no difference in the density of the dominant lipid locules in the PAT from saline (687 ± 47 locules / mm^2) or leptin infused fetuses (703 ± 60 locules / mm^2). The median size of the dominant lipid locules present within fetal PAT tended to be smaller (P =0.06) in the leptin infused (323 ± 20 ìm^2) than in the saline infused group (376 ± 15 ^{2}) and the size distribution of the lipid locules in the fetal perirenal adipose depot was significantly different (P <0.05) in the 2 treatment groups (Figure 4.4). There were proportionately more lipid locules in the <200 μm^2 size range and proportionately fewer in the 200-600 μm^2 size range in the leptin infused group when compared with the saline infused fetuses (Figure 4.4).

4.6.5 Leptin mRNA expression in perirenal adipose tissue

Messenger RNA encoding leptin and β-actin were detected in total RNA extracted from fetal adipose tissue (Figure 4.5). The relative abundance of leptin mRNA in PAT was lower (P<0.001) in leptin infused (22.5 ± 3.7) compared with saline infused fetuses (54.1 ± 4.6) (Figure 4.5). There was also a direct correlation between the relative abundance of leptin mRNA and the proportion of unilocular tissue within the perirenal adipose depot (leptin mRNA : β-actin mRNA = 1.94 (proportion unilocular tissue) – 38.7; R=0.88, P <0.0001) when both treatment groups were combined (Figure 4.5).

4.6.6 Abundance of UCP-1 mRNA and UCP-1, Cytochrome *c* and VDAC protein in PAT

The relative abundance of UCP-1 mRNA was similar in the leptin infused (0.70 ± 0.13) and saline infused fetuses (0.88 ± 0.09) whereas UCP-1 protein content tended to be higher (P=0.06) in the leptin infused group (leptin infused, 74.8 ± 9.2 % reference: saline

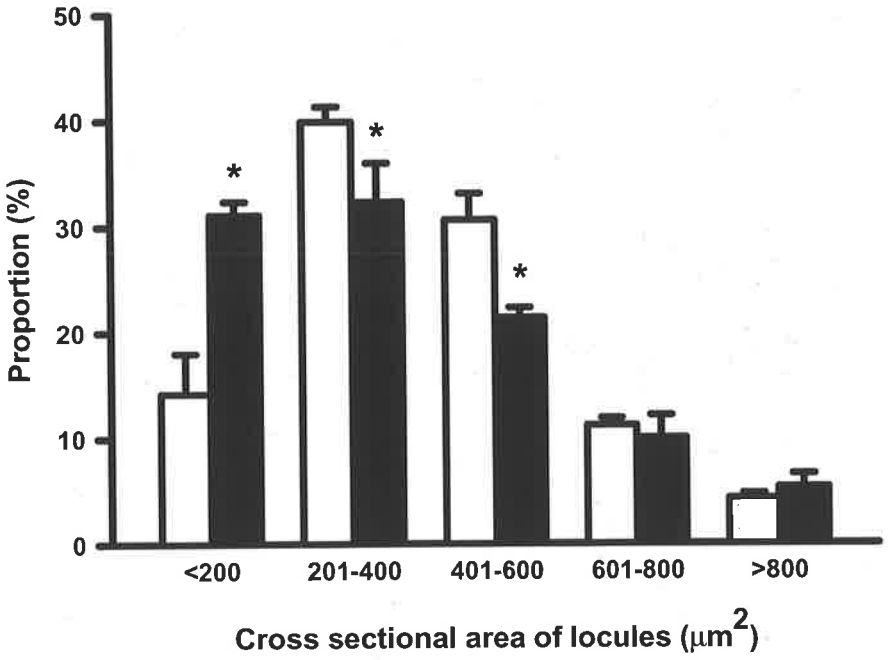


Figure 4.4 Proportion of dominant lipid locules in the perirenal adipose tissue of saline infused and leptin infused groups.

The proportion of the dominant lipid locules in the perirenal adipose tissue depots in each of the defined size ranges in the saline (open histograms) and leptin (closed histograms) infused fetuses. The asterisks denote significant differences ($P<0.05$) between the saline and leptin infused groups.

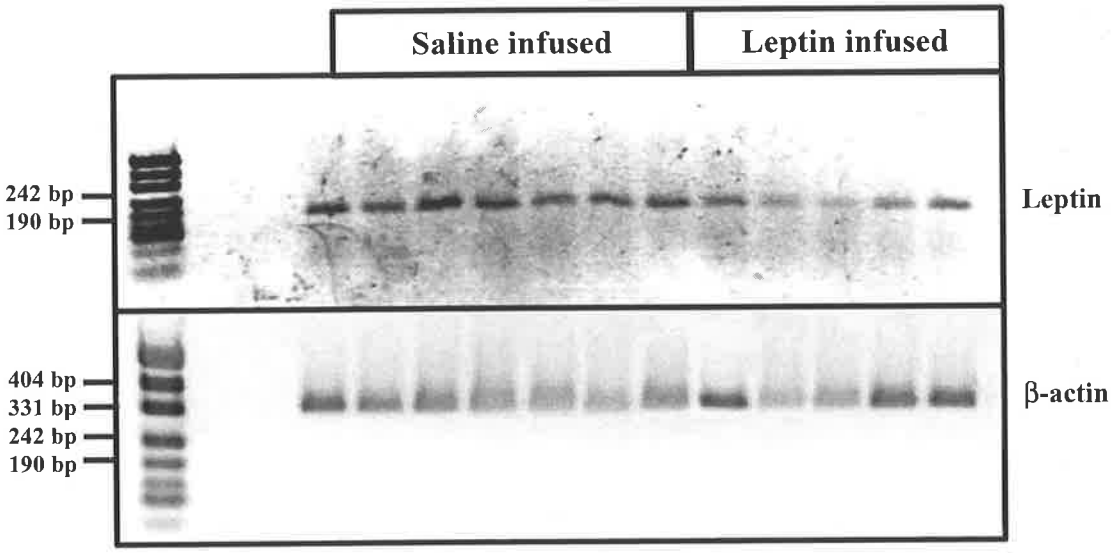
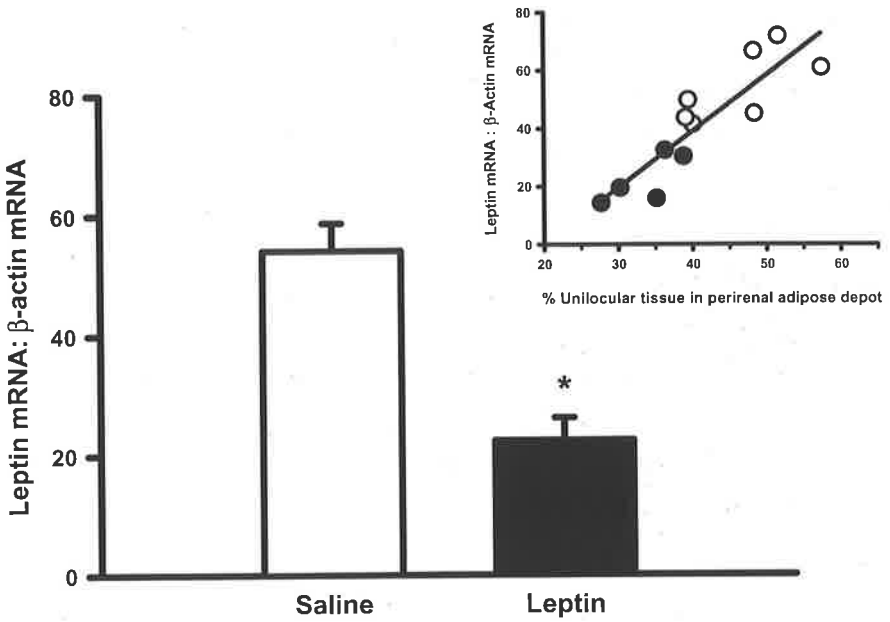


Figure 4.5 Leptin and β-actin expression in saline infused and leptin infused fetuses (A&B).

(A) Leptin (183 bp, upper panel) and β-actin (349 bp, lower panel) RT-PCR products were amplified from total RNA extracted from PAT of saline and leptin infused fetal sheep. Products were electrophoresed through an ethidium bromide stained agarose gel. Molecular markers were also electrophoresed in the same gel.



(B) The relative abundance of leptin mRNA in PAT from leptin infused and saline infused fetuses. The asterisks denote significant differences ($P<0.05$) between the saline and leptin treatment groups. (Inset) Relationship between the proportion of unilocular tissue and the relative abundance of leptin mRNA in the same adipose depot, (leptin mRNA : β-actin mRNA = 1.94 (proportion unilocular tissue) – 38.7; R=0.88, $P<0.0001$).

infused, 52.8 ± 1.1 % reference). The abundance of cytochrome *c* (saline infused, 86.6 ± 6.4 % reference; leptin infused, 81.3 ± 8.8 % reference) or VDAC protein (saline infused, 131.1 ± 8.9 % reference; leptin infused, 134.8 ± 12.8 % reference) in the PAT was not significantly different between the saline and leptin infused groups.

4.6.7 Fetal Growth

There was no effect of leptin infusion on fetal body weight (saline infused, 4.70 ± 0.23 kg; leptin infused, 4.83 ± 0.22 kg), the relative weight of any fetal organs or on any measures of fetal growth at 140 or 141 d gestation (Table 4.2).

4.7 DISCUSSION

In this Chapter, the infusion of leptin resulted in a significant shift in the size distribution of the lipid locules present within the fetal perirenal adipose tissue (PAT) with an increase in the proportion of smaller lipid locules. This was associated with an increase in the proportion of multilocular tissue and a decrease in the proportion and relative mass of unilocular tissue in the fetal PAT in the leptin infused fetuses. The relative abundance of leptin mRNA in PAT was also lower in leptin infused fetuses and there was a direct correlation between leptin mRNA expression and the proportion of unilocular tissue in the saline and leptin infused groups. These changes occurred in the absence of any effects of leptin on fetal plasma glucose or insulin concentrations or on fetal arterial blood gas status.

In the present study, intrafetal infusion of leptin at a dose rate of 0.48 mg/kg/d resulted in a 4-5 fold increase in circulating leptin levels. Fetuses were exposed to concentrations of

Table 4.2 Effect of a 4 d infusion of saline or leptin on fetal body weight, growth measurers and relative organ weights at 140 or 141 d gestation.

All values are expressed as mean ± SEM. Superscripts indicate significant differences between the mean values in the saline infused and leptin infused groups, ($P<0.05$).

Treatments	Saline Infused	Leptin Infused	Significance, t-test
Fetal Weight (kg)	4.83 ± 0.22	4.70 ± 0.23	P=0.71
Crown Rump Length (cm)	55.8 ± 0.6	58.3 ± 1.7	P=0.26
Abdominal Circumference (cm)	36.9 ± 1.0	36.7 ± 1.5	P=0.95
Ponderal Index (cm^3)	2.42 ± 0.22	2.74 ± 0.09	P=0.27
Heart (g/kg)	7.45 ± 0.53	6.33 ± 0.28	P=0.11
Liver (g/kg)	27.3 ± 1.6	23.4 ± 1.1	P=0.07
Lung (g/kg)	33.0 ± 1.9	31.0 ± 1.5	P=0.53
Kidney (g/kg)	5.91 ± 0.32	5.84 ± 0.37	P=0.90
Spleen (g/kg)	1.73 ± 0.16	1.94 ± 0.34	P=0.56
Pancreas (g/kg)	0.70 ± 0.04	0.72 ± 0.06	P=0.86
Total Thyroid (g/kg)	0.27 ± 0.02	0.24 ± 0.03	P=0.09
Brain (g/kg)	12.8 ± 0.5	11.4 ± 0.9	P=0.18
Pituitary (g/kg)	0.030 ± 0.003	0.025 ± 0.002	P=0.24
Total Adrenal (g/kg)	0.10 ± 0.01	0.08 ± 0.01	P=0.41
Total Perirenal Adipose Tissue (g/kg)	4.43 ± 0.35	4.16 ± 0.30	P=0.57

circulating leptin similar to those measured in well-fed pregnant ewes where rapid maternal weight gain occurred (441). Whilst leptin infusion did not result in a significant change in the total or relative fetal fat mass, it did result in a decrease in the proportion of unilocular adipose tissue. This decrease appeared to be a consequence of the shift towards smaller lipid locules in the unilocular tissue and the associated increase in the proportion of the multilocular adipose tissue. The functional significance of this shift depends on the relative roles of the unilocular and multilocular components of fetal adipose tissue. In the adult, unilocular adipocytes are classified as white adipocytes and are the major sites of lipid storage and of leptin synthesis and secretion (466). In contrast, multilocular or brown adipocytes express the mitochondrial uncoupling protein, UCP-1 and participate in cold and dietary induced thermogenesis (467). Ultrastructural studies have demonstrated that in fetal sheep, both unilocular and multilocular adipocytes contain an abundance of mitochondria which is a characteristic feature of the thermogenic brown adipocyte (84) and whilst it is clear that leptin mRNA is expressed in fetal PAT (146, 443, 445), it is unknown whether leptin is expressed in either the unilocular or multilocular adipocytes. Circulating leptin concentrations are strongly correlated, however, with the relative mass of the unilocular, but not multilocular tissue, in the sheep fetus during late gestation and circulating leptin concentrations are also correlated with the relative abundance of leptin mRNA in fetal PAT (Chapter 3) (455). This suggests that adipose tissue may be a major site of leptin synthesis and secretion in the fetus (455). In the present study, leptin infusion decreased the relative proportion of unilocular fat and the relative abundance of leptin mRNA and there was also a strong correlation between the proportion of unilocular fat and leptin mRNA expression in fetal PAT. One possibility is that in fetal life, the unilocular adipocyte is a 'transitional' cell type that has the lipid storage and leptin synthetic characteristics typical of the white

adipocyte, in addition to the thermogenic characteristics of the brown adipocyte. Recent studies, however, suggest that during normal development, most white adipocytes are not derived from brown adipocytes and that there are distinct lineages of white and brown adipocytes (9). An alternative explanation is that the unilocular and multilocular cells are brown adipocytes, but that leptin gene expression in these cells is stimulated when the size of the dominant lipid locule exceeds and subsequently increases beyond a threshold value. This would explain the direct relationship between the relative abundance of leptin mRNA and the proportion of the PAT comprised of unilocular tissue in the current study as leptin infusion resulted in a shift in the size distribution of the lipid locules present in unilocular tissue. There is evidence from studies in a range of species including the human, rat and cow that circulating leptin concentrations are related to the size of the unilocular adipocytes i.e. large fat cells are associated with higher circulating leptin concentrations and a greater abundance of leptin mRNA (114, 125, 145). Independently of the cellular characteristics of the unilocular and multilocular adipose tissue, an increase in circulating leptin concentrations clearly results in a decrease in the lipid storage capacity of the unilocular adipose tissue and in the leptin synthetic capacity of PAT before birth.

Whilst leptin stimulated an increase in the multilocular component of PAT, this increase was not associated with an increase in UCP-1 mRNA expression in fetal PAT although UCP-1 protein levels tended to be higher (P=0.06) in the leptin infused group than controls. In contrast leptin infusion did not change the expression of VDAC, which is located on the outer mitochondrial membrane and cytochrome *c*, which is present in the mitochondrial space. Whilst these data suggest that leptin may act to increase the thermogenic potential of adipose tissue before birth, further studies are clearly required

to identify the localisation and expression of UCP-1 in fetal adipose tissue under a range of different physiological conditions. Interestingly, a recent study found that administration of leptin during the first 48 h after birth improved thermoregulation in newborn lambs and it was postulated that this may have been a result of enhanced lipolysis induced by leptin administration in PAT (337). After the first 48 h, however, chronic leptin administration promoted the loss of UCP-1 from PAT (337). The lack of a thermogenic effect of leptin at this age in the lamb may be related to the decrease in plasma catecholamines and in β-adrenoceptors in the perirenal adipose tissue which occurs in postnatal life (436).

In the adult rodent, central leptin administration decreases the mass of white adipose tissue and the gene expression of leptin within white adipocytes whilst increasing expression of the UCP-1 gene in brown adipocytes (468). Thus an increase in circulating leptin concentrations causes a shift from fat storage to fat mobilisation and oxidation. These effects occur independently of the leptin induced changes in food intake and are primarily a consequence of the actions of leptin at its functional receptors in the hypothalamus (13, 15, 109, 457). Recent studies using surgical (306), chemical (377) and transgenic (469) approaches have shown that in the adult, activation of the sympathetic nervous system is required for the effects of leptin on the gene expression of leptin and UCP-1 in white and brown adipose tissue respectively. In the sheep, sympathetic innervation in perirenal adipose tissue occurs at around 130 days gestation and there is an increase in the proliferation of sympathetic nerves within this tissue from 140 days gestation up to term (84). Furthermore prolonged infusion of the $\hat{a}_2$-adrenergic agonist, ritodrine into fetal sheep resulted in a decrease in the mass and lipid content of PAT and an increase in GDP binding to UCP-1 (322). One possibility is that leptin acts centrally

via leptin receptors located within the fetal hypothalamus to stimulate the sympathetic nervous system resulting in a decrease in the proportion of the unilocular adipose tissue and in the abundance of leptin mRNA and a concomitant increase in the proportion of the multilocular or thermogenic component of the PAT.

Whilst leptin may act centrally, it is also possible that leptin acts directly via leptin receptors on the unilocular and multilocular adipocytes to stimulate lipolysis and the shift in the distribution of the lipid locule sizes. Whilst it has been demonstrated that leptin can directly stimulate lipolysis in isolated adipocytes (304, 305, 470), a number of studies have failed to demonstrate local effects of leptin in adipocytes from a range of species including the human and sheep (471, 472).

In the present study, a 4-5 fold increase in fetal leptin concentrations had no effect on the basal plasma glucose and insulin concentrations or on the rise in fetal glucose and insulin concentrations after maternal feeding. Chronic leptin infusion in adult rats decreases plasma glucose, insulin and triglyceride concentrations and increases skeletal muscle glucose utilisation (446). Clearly further studies would be required to determine whether leptin acts to regulate glucose utilisation and energy consumption within specific fetal tissues.

In the present study I have found no evidence for an effect of increased circulating leptin concentrations on fetal body or organ growth after the 4 day infusion period. In the sheep, increases in leptin mRNA expression in fetal PAT and in circulating leptin concentrations occur during periods of rapid fetal growth late gestation (443, 446) and in the human, there are relationships between circulating leptin and measures of fetal size

or neonatal weight (393, 406, 413, 421, 423, 458). These relationships may be determined by the impact of the prenatal nutrient supply on fetal growth, adiposity and leptin synthesis and secretion, rather than reflect the actions of circulating leptin on prenatal growth.

In summary, this is the first demonstration that leptin can act to regulate the lipid storage characteristics, leptin synthetic capacity and potential thermogenic functions of fat before birth. These findings suggest that leptin may act as a signal of energy supply and have a 'lipostatic' role before birth. This role may be of particular importance when the fetus is exposed to an increase in a transplacental substrate supply such as occurs when maternal nutrient intake is increased (455) or in pregnancies complicated by maternal glucose intolerance and fetal hyperglycaemia (421). Future studies are required to determine the central and peripheral sites and mechanisms of action of leptin and the postnatal consequences of prenatal hyperleptinaemia.

Whilst exogenous leptin has been shown to stimulate changes within fetal adipose tissue, it remains to be determined whether leptin plays a role in other fetal regulatory systems. In the adult mammals, leptin can attenuate the hypothalamo-pituitary-adrenal (HPA) stress response through the suppression of ACTH and cortisol secretion. During late gestation the fetal HPA is activated during the prepartum period. It is therefore hypothesised, that administration of leptin may attenuate the activation of the fetal HPA axis through the suppression of ACTH and cortisol secretion leading to a prolonged gestation period in the sheep fetus.

CHAPTER 5

5. LEPTIN SUPPRESSES FETAL CORTISOL CONCENTRATIONS BUT DOES NOT ALTER THE TIMING OF PARTUTRITION

5.1 DECLARATION

During the course of my PhD candidature I conducted most of the experimental work presented in this chapter. Subsequently this work has been accepted for publication and the majority of the contents within this chapter can be found in the following periodical:

Yuen BSJ, Owens PC, Symonds ME, Keisler DH, McFarlane JR, Kauter KG and McMillen IC. Effects of leptin on fetal plasma ACTH and cortisol concentrations and the timing of parturition in the sheep. Biol Reprod. 2004 (Accepted for publication)

5.2 CONTRIBUTION OF COAUTHORS

Owens, PC

Co-supervised the experimental work contained within this publication, contributed to intellectual debates, assisted with data analyses and interpretation, and aided in the writing of the manuscript.

Signature.....Date 13 JAN 04

Symonds, ME

Assisted in the interpretation of the data, contributed to intellectual debates and evaluated the manuscript prior to submission.

Signature.....Date.....12/6/03.....

Keisler, DH

Generously provided recombinant ovine leptin and evaluated the manuscript prior to submission.

Signature.....Date..29 Jun 03.....

McFarlane, JR

Dr McFarlane generously agreed to assay maternal and fetal sheep plasma to determine circulating leptin concentrations. Contributed to intellectual debates, assisted with data interpretation and evaluated the manuscript prior to submission.

Signature.....Date..7/7/03.....

Kauter, KG

Ms Kauter was one of the first researchers to develop a leptin ELISA for use in the sheep. This assay system was used to measure the plasma leptin concentrations of the fetuses described in this study.

Signature.....Date.....10/7/03.....

McMillen, IC

Co-supervised the experimental work contained within this publication, contributed to intellectual debates, assisted with data analyses and interpretation, and aided in the writing of the manuscript.

Signature.....Date.....22/7/03.....

5.3 ABSTRACT

We have investigated whether leptin can suppress the prepartum activation of the fetal HPA axis and delay the timing of parturition in the sheep. Firstly, we investigated the effects of a 4 day intravascular infusion of recombinant ovine leptin (n=7) or saline (n=6) for on fetal plasma ACTH and cortisol concentrations starting from 136 days (d) gestation, i.e. at the onset of the prepartum activation of the fetal HPA axis. The effects of a continuous intrafetal infusion of leptin (n=7) or saline (n=5) from 144 d gestation on fetal plasma ACTH and cortisol concentrations and the timing of delivery were also determined in a separate study. There was an increase in fetal plasma ACTH ($P<0.01$) and cortisol ($P<0.001$) concentrations when saline was infused between 136/7 and 140/1 d gestation. There was no increase, however, in either plasma ACTH or cortisol concentrations when leptin was infused during this period of gestation. In saline infused fetuses, there was also a significant negative relationship between the plasma cortisol (y) and leptin (x) concentrations between 138 and 146 d gestation ($y = 81.4 - 7.7x$, $r=0.38$, $P<0.005$). When leptin was infused continuously from 144 d gestation until delivery, there was no effect of a 4-5 fold increase in circulating leptin on fetal ACTH concentrations during the week before delivery. In contrast, leptin infusion from 144 d gestation suppressed both fetal plasma cortisol concentrations between 90 and 42 h before delivery ($P<0.05$) and the ratio of fetal plasma cortisol : ACTH concentrations between 90 and 30 h before delivery ($P<0.05$). There was no difference, however, in the length of gestation between the saline and leptin infused groups (saline infused, 150.2 ± 0.5 d; leptin infused, 149.8 ± 1.0 d). This study provides evidence for an endocrine negative feedback loop between leptin and the HPA axis in fetal life which may play an important role in determining the fetal adrenal responsiveness to ACTH and other

trophic factors during intrauterine stress, parturition and the transition from intrauterine to extrauterine life.

5.4 INTRODUCTION

Leptin is a 16 kDa polypeptide hormone, which is principally synthesised and secreted by adipose tissue and which acts to regulate energy homeostasis and a range of neuroendocrine and reproductive functions (13, 15, 457). In the human infant, there is a positive relationship between cord blood concentrations of leptin at delivery and birth weight or neonatal adiposity (393, 406, 413). In animal species such as the sheep and pig, in which fat is deposited before birth, leptin is synthesised in fetal adipose tissue and is present in the fetal circulation throughout late gestation (146, 443, 445, 446, 455, 473, 474). In the sheep fetus, the expression of leptin mRNA in fetal adipose tissue is positively correlated with circulating leptin concentrations and there is also a positive relationship between fetal plasma leptin concentrations and the relative mass of lipid locules present within fetal adipose tissue (455, 474). We have recently shown that intravascular infusion of leptin in the sheep fetus during late gestation altered the lipid storage characteristics and suppressed leptin mRNA expression within fetal adipose tissue (475). Thus leptin may act as a circulating signal of fetal adiposity and have a 'lipostatic' role before birth.

Recent studies have indicated that there may be a functional interaction between circulating leptin and the fetal hypothalamic-pituitary-adrenal (HPA) axis in late gestation. It is well established in the sheep that the prepartum increase in circulating cortisol is required for the differentiation and maturation of key fetal organs such as the fetal lung, liver, kidney and brain and for the normal timing of parturition and the

successful transition to extrauterine life (298). Forhead and colleagues reported that in the sheep fetus, plasma cortisol and leptin concentrations increased in parallel and were positively related between 130 and 140 days gestation and that fetal adrenalectomy resulted in lower plasma leptin concentrations after 136 d (446). These findings are consistent with studies which have demonstrated that glucocorticoids stimulate both leptin gene expression and secretion from adult adipocytes *in vivo* and *in vitro* (187, 188, 191) and suggest that there is a positive relationship between the level of activation of the fetal HPA axis and leptin synthesis and/or secretion in late gestation. A separate study however, investigated the effects of intracerebroventricular (i.c.v.) infusion of leptin between 135 and 140 days gestation on the characteristics of plasma ACTH and cortisol pulses occurring during a 4 h sampling period on the first and last day of the infusion period (447). These authors found that i.c.v. leptin administration blunted the size of the increase which occurred in the amplitude and mean value of plasma ACTH and cortisol pulses between 135 and 140 days gestation (447). These data suggest that fetal leptin may inhibit ACTH and cortisol secretion during late gestation and are consistent with studies in the adult rat which have shown that administration of leptin can attenuate fasting or restraint induced stimulation of the HPA axis (153, 299). Given the potential role of leptin as a circulating signal of fetal adiposity in late gestation it is important to determine whether, as in the adult, a leptin mediated, endocrine negative feedback loop exists between adipose tissue and the HPA axis in the fetus during late gestation. In the present study we have determined whether intravascular administration of leptin can suppress the normal prepartum activation of the fetal HPA axis and thus delay the timing of parturition. Firstly, we measured the effects of a 4 day intrafetal infusion of leptin on fetal plasma ACTH and cortisol concentrations starting from 136 days gestation, i.e. at the onset of the prepartum activation of the fetal HPA axis.

Secondly, we infused leptin into fetal sheep from 144 days gestation until delivery and measured the effects of an increase in circulating leptin on the prepartum changes in fetal plasma ACTH and cortisol concentrations and on the timing of parturition.

5.5 MATERIALS AND METHODS

5.5.1 Animals and Surgery

These studies were approved by the University of Adelaide Animal Ethics Committee. Surgery was performed on dated pregnant Merino ewes (n=25) between 110 and 126 d gestation, as previously described (474, 476). Briefly, general anaesthesia was induced in ewes by an intravenous injection of sodium thiopentone (1.25 g i.v., Pentothal, Rhone Merieux, Pinkenba, Qld, Australia) and maintained by 2.5 - 4% halothane (Fluothane, ICI, Melbourne, Vic, Australia). Under aseptic conditions catheters were inserted into a maternal jugular vein, a fetal carotid artery and jugular vein, and the amniotic cavity. Catheters were filled with heparinised saline and the fetal vascular and amniotic catheters exteriorised through an incision made in the ewe's flank. Ewes and their fetuses received a 2 ml intramuscular injection of antibiotics (procaine penicillin 250 mg/ml; dihydrostreptomycin 250 mg/ml; procaine hydrochloride 20 mg/ml Penstrep Illium, Troy Laboratories, Smithfield, NSW, Australia). Animals were allowed to recover for at least 4 d after surgery before routine fetal arterial blood samples (3 ml) were collected every 2 - 3 d before the infusion studies commenced. Animals were housed in individual pens in rooms with a 12 h light/dark cycle, fed once daily at 1100 h and water was provided *ad libitum*.

5.5.2 Experimental Protocols

5.5.2.1 Leptin infusion from 136 or 137 d gestation

In thirteen pregnant ewes at 136/137 d gestation, fetal arterial blood samples (3 ml) were collected at -3 h, -2 h, -1 h and -30 min relative to the start of the infusion period at 1300 h. A bolus of either sterile saline (0.5 ml, n=7) or recombinant ovine leptin (0.25 mg in 0.5 ml sterile saline, n=6) (460) was infused into the fetal jugular vein, immediately followed by a continuous infusion (0.16 ml/h) of either sterile saline or leptin (0.48 mg/kg/d), respectively (475). Fetal arterial blood samples were collected at +2 min, +30 min, +1 h, +2 h, +4 h and +8 h on the first day of the infusion and at 0900 h, 1300 h and 1700 h on the second and third days and at 0900 h and 1300 h on the fourth day of the infusion. Blood samples were centrifuged at 1500 g for 10 min and plasma aliquots were separated and stored at -20°C. Fetal arterial blood samples (0.5 ml) were also collected daily to monitor fetal blood gases and pH (ABL 520 blood gas analyser, Radiometer, Copenhagen, Denmark).

After 96 h (at 140 or 141 d gestation), ewes were killed with an overdose of sodium pentobarbitone (Virbac Pty Ltd, Peakhurst, NSW, Australia) and fetuses delivered by hysterotomy, weighed and decapitated. It should be noted that these were the same fetueses described in Chapter 4 (475).

5.5.2.2 Leptin infusion from 144 d gestation

In 12 pregnant ewes at 144 d gestation, a bolus of saline (n=5) or recombinant ovine leptin (n=7, 0.5 mg/0.5 ml sterile saline) was infused into the fetal jugular vein immediately followed by a continuous infusion of saline or recombinant ovine leptin (1.0 mg/kg/d). On the first day of infusion, fetal arterial blood samples (3.5 ml), were

collected at -3 h, -2 h, -1 h and -30 min and at +2 min, +30 min, +1 h, +2 h, +4 h and +8 h relative to the start of the infusion at 1300 h. On the second, third and fourth days of the infusion, fetal blood samples were collected at 0900 h, 1300 h and 1700 h and on subsequent days, fetal blood samples were collected at 0900 h and 1700 h until either the ewe was in late labour (n=2) or the fetus delivered (n=10). Ewes were defined as being in late labour when the pressure of repeated intrauterine contractions were greater than 20 mmHg in amplitude. Intrauterine pressure was measured using a MacLab 1050 displacement transducer (ADInstruments, NSW, Australia) connected to the saline filled amniotic catheter (477). A MacLab data acquisition system was attached to the transducer and MacLab Chart software was used to analysis the intrauterine pressure recordings.

5.5.3 Leptin ELISA

A competitive ELISA specific for ovine leptin was used to measure plasma leptin concentrations in fetal sheep, as previously described (373, 474). Briefly, 6 ng recombinant bovine leptin was coated onto an ELISA plate by overnight incubation at 37°C. The plate was blocked with 200 µl of 5% skim milk in ELISA buffer for 1 h at 37 °C. Fetal plasma samples (100 µl) were added to wells containing a biotinylated chicken anti-recombinant bovine leptin in 100% Triton X 100, 0.5% SDS and 5% sodium deoxycholate (50 µl) and the plate was incubated overnight at 37°C. A biotinylated second antibody was added to the plate and incubated overnight at 37°C. The plate was then washed and incubated for 1 h with streptavidin conjugated to alkaline phosphatase (Amrad Biotech, Boronia, Vic, Australia) then developed with p-nitrophenylphosphate disodium salt hexahydrate. The sensitivity of the assay was 0.5 ng/ml and the inter assay and intra assay coefficients of variation were 11% and 9% respectively.

5.5.4 ACTH radioimmunoassay

ACTH concentrations in fetal sheep plasma were measured by radioimmunoassay (DiaSorin, Stillwater, Minnesota, USA), previously validated for fetal sheep plasma (478). The cross-reactivity of the rabbit anti-ACTH antisera was <0.01% with α-MSH, β-Endorphin, β-Lipotropin, parathyroid hormone, Vasopressin and growth hormone. Briefly, rabbit anti-ACTH serum (50 µl) was added to each sample (50 µl) and incubated overnight at 4°C. Radiolabelled ^{125}I-ACTH (50 µl) was added to each tube and incubated overnight at 4°C. Rabbit serum (200 µl), pre-precipitated with goat anti-rabbit serum and polyethylene glycol, was added to samples that were then centrifuged. The inter assay coefficient of variation was 11.5% and the intra assay coefficient of variation was 5.2%.

5.5.5 Cortisol radioimmunoassay

Cortisol was extracted from fetal plasma using dichloromethane as previously described (479). The efficiency of recovery of radiolabelled ^{125}I-cortisol from fetal plasma using this extraction procedure was >90%. The cross-reactivity of the rabbit anti-cortisol antisera was <1% with pregnenolone, aldosterone, progesterone and oestradiol. Fetal cortisol concentrations were then measured using a Amersham Radioimmunoassay kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA). Briefly, 100 nM of hydrocortisone (Sigma Chemical Co., St.Louis MO, USA) was serially diluted in buffer (0.1 mol/l Tris-HCl, pH 7.4, 0.5% BSA, 0.1% sodium azide) to generate a standard curve. Plasma extracts (100 µl) were incubated with rabbit anti-cortisol antisera (100 µl) overnight at 4°C. Radiolabelled ^{125}I-cortisol (100 µl) was then added to the samples that

were then incubated overnight at 4°C. The inter- and intra-assay coefficients of variation were 10% and 5% respectively.

5.6 STATISTICAL ANALYSES

Data are presented as the mean ± SEM. Fetal hormone data were logarithmically transformed where required to reduce heterogeneity of variance. Analyses of Variance (ANOVA) with repeated measures were performed using the Statistical Package for Social Sciences (SPSSX, Chicago, IL, USA) on a VAX mainframe computer. The Duncan's new multiple range test was used post hoc to identify significant differences between mean values.

5.6.1 Leptin infusion from 136/137 d gestation

Mean values for fetal arterial pO_2, pCO_2 and pH were calculated using fetal blood gases and pH values obtained between +0.5 and +96 h in the saline infused and leptin infused groups. A Student's unpaired t-test was performed to determine whether fetal blood gases and pH values were different between the treatment groups.

A mean value for the basal plasma concentration of leptin, ACTH or cortisol was calculated for each fetus as the average of the 5 values during the pre-infusion period (i.e. for those samples collected at -3 h, -2 h, -1 h, -0.5 h and 0 h). The change in fetal hormones during the infusion period was calculated by subtraction of the mean basal value obtained during the pre-infusion period, from hormone values at each time point during the infusion period. The effects of leptin infusion on fetal hormone concentrations were analysed using a two way ANOVA with treatment (saline infused vs leptin infused)

and the length of time relative to the start of the infusion as the specified factors. Where there was a significant interaction between the effects of treatment and the length of time of infusion on plasma hormone concentrations, the data were split based on the interaction and reanalysed.

5.6.2 Leptin infusion from 144 d gestation

Linear regression analysis was used to assess potential relationships between the plasma leptin and either ACTH or cortisol concentrations in samples collected between 125 to 137 d i.e. prior to the onset of the prepartum increase in circulating cortisol and between 138 to 146 d gestation i.e. after the onset of the prepartum increase in cortisol in fetuses which were infused with saline from 144 d gestation.

The effects of leptin on fetal hormone concentrations during the first 20 h of the infusion period were determined by a two way ANOVA with treatment (saline infused vs leptin infused) and time relative to the start of the infusion as the specified factors.

As the length of gestation varied between animals (147-153 d), the hormonal data from each animal were expressed relative to the known or estimated time of delivery. Intrauterine pressure traces from animals that delivered were analysed to establish the relationship between frequency of contractions with an amplitude of >20 mmHg and time before birth. For the 2 fetuses that were killed during late labour, the intrauterine pressure traces were analysed to determine the frequency of contractions (>20 mmHg) during labour and the time of delivery was then estimated. Hormone data were then grouped into 12 h time blocks in relation to the actual or estimated time of delivery: 6-18 h, 18-30 h, 30-42 h, 42-54 h, 54-66 h, 66-78 h, 78-90 h, 90-102 h and 102-114 h before

delivery. The effects of leptin infusion on fetal leptin, ACTH and cortisol concentrations were determined by a two way ANOVA using treatment (saline infused vs leptin infused) and time before birth as the specified factors. A Student's unpaired t-test was performed to determine whether the length of gestation was different between saline and leptin infused fetuses.

5.7 RESULTS

5.7.1 Leptin infusion from 136/137 d gestation

5.7.1.1 Plasma leptin concentrations and fetal blood gas status

During the pre-infusion period, there was no difference in the plasma leptin concentrations between fetuses assigned to the saline (3.0 ± 0.9 ng/ml) or leptin (4.4 ± 1.0 ng/ml) infusion groups. Plasma leptin concentrations increased (P<0.001) during the leptin infusion period (+24 h: 22.9 ± 3.9 ng/ml, +92-96 h, 20.1 ± 1.5 ng/ml) but not during the saline infusion period. There was no significant difference in mean fetal arterial blood gas and pH values between the saline and leptin infused groups during the infusion period (pO_2 - saline infused, 21.7 ± 0.6 mmHg; leptin infused, 22.3 ± 0.5 mmHg, pCO_2 - saline infused, 50.4 ± 1.2 mmHg; leptin infused, 49.1 ± 0.8 mmHg, pH - saline infused, 7.404 ± 0.006; leptin infused, 7.401 ± 0.005).

5.7.1.2 Fetal plasma ACTH and cortisol concentrations

Plasma ACTH concentrations during the pre-infusion period were not different between fetuses assigned to the saline (28.5 ± 3.5 pg/ml) or leptin (26.9 ± 1.6 pg/ml) infusion groups. During the infusion period, there was a significant interaction between the effects of treatment and time on ACTH concentrations expressed relative to those in the

preinfusion period ($P<0.05$; Figure 5.1). In the saline infused group, the change in plasma ACTH concentrations relative to baseline values was greater at 96 h (13.7 ± 7.8 pg/ml, $P<0.01$) than at between 2 h and 44 h after the start of the infusion (-6.8 ± 3.4 pg/ml). In contrast, in fetuses infused with leptin, there was no change in plasma ACTH concentrations during the 96 h infusion period (+96 h: -4.1 ± 2.0 pg/ml).

There was no difference during the preinfusion period in the plasma cortisol concentrations between the fetuses assigned to the saline (14.4 ± 2.5 nmol/l) and leptin (13.8 ± 6.2 nmol/l) infusion groups. During the infusion period, there was a significant interaction between the effects of treatment and time on cortisol concentrations relative to basal levels ($P<0.02$; Figure 5.1). In the saline infused group, the change in plasma cortisol concentrations was greater at 96 h (54.1 ± 7.5 nmol/l; $P<0.001$) when compared with between 3 h before and 24 h after the start of the infusion (14.9 ± 2.9 nmol/l). In fetuses infused with leptin, there was no change, however, in plasma cortisol concentrations throughout the infusion period (+96 h: 7.0 ± 3.9 nmol/l; Figure 5.1).

The ratios of plasma cortisol : plasma ACTH concentrations during the pre-infusion (saline infused: 0.61 ± 0.15, leptin infused: 0.56 ± 0.21) and infusion periods were not different between the two treatment groups (Figure 5.1). The ratio of plasma cortisol : ACTH concentrations were significantly higher ($P<0.001$) in both treatment groups, however, at +96 h (saline infused: 1.66 ± 0.23, leptin infused: 1.12 ± 0.25), when compared to the period from 3 h before until 52 h after the start of the saline (0.82 ± 0.26) or leptin (0.63 ± 0.22) infusion (Figure 5.1).

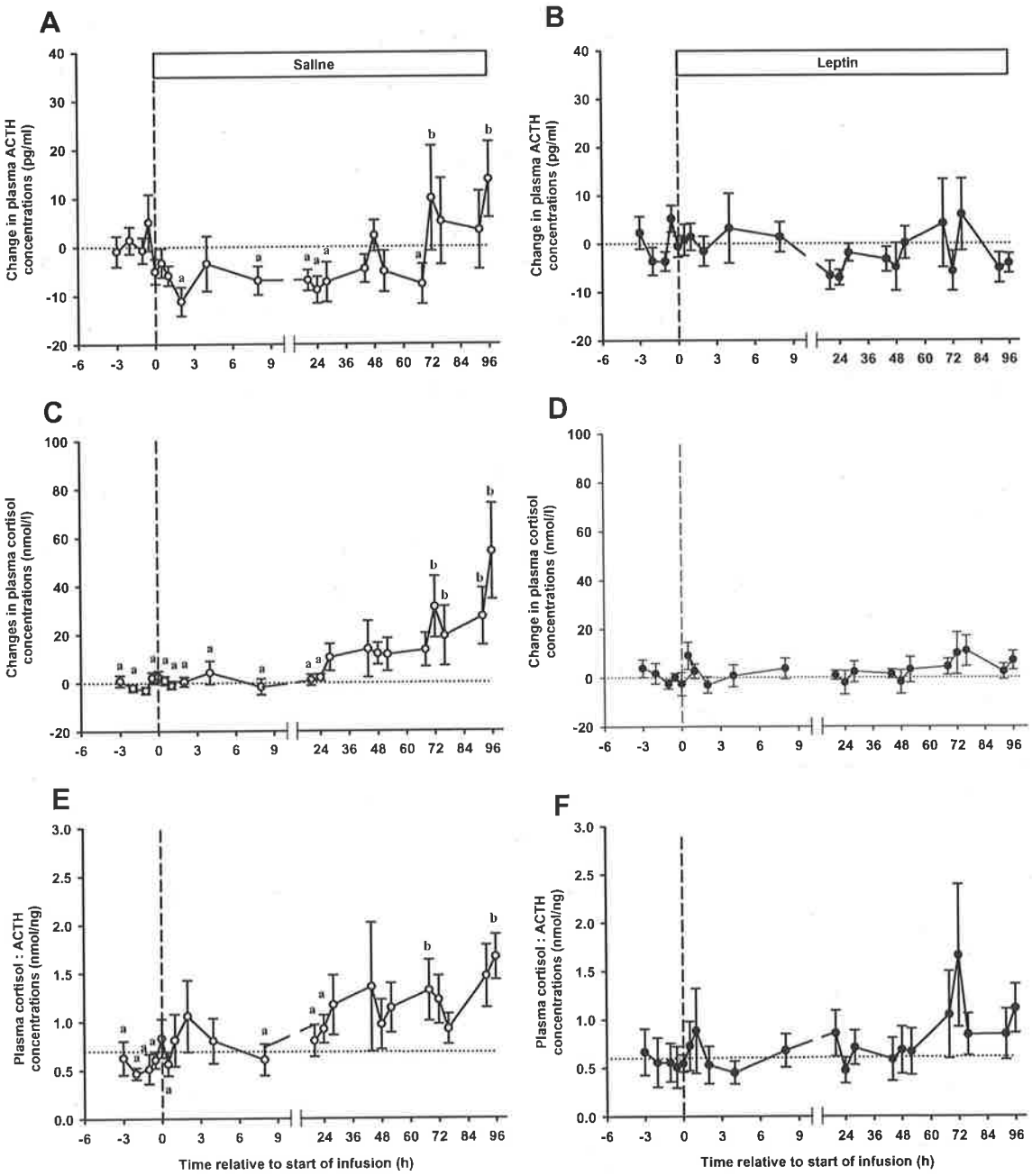


Figure 5.1 Plasma ACTH, cortisol and the ratio of plasma cortisol : ACTH concentrations in fetuses infused with saline (open; A,C,E) or leptin (closed; B,D,F) for 96 h from 136/137 d gestation.

Different alphabetic superscripts denote mean values which are significantly different ($P<0.05$) from each other within a treatment group.

5.7.2 Fetal plasma leptin, cortisol and ACTH concentrations in saline infused fetuses between 125 and 150 d gestation

There was a significant increase in plasma cortisol concentrations between 125 and 150 d gestation in saline infused fetuses (Figure 5.2). There was no change, however, in plasma leptin concentrations in these fetuses during this period (Figure 5.2). Whilst there was no relationship between fetal plasma cortisol (y) and leptin (x) concentrations at 125 - 137 d gestation, there was a significant negative relationship between the plasma concentrations of these two hormones at 138-146 d gestation ($y = 81.4 - 7.7x$, $R=0.38$, $P<0.005$) (Figure 5.3). In contrast, there was no significant relationship between fetal plasma ACTH and cortisol concentrations during this period.

5.7.3 Leptin infusion from 144 d gestation

5.7.3.1 Plasma leptin, ACTH and cortisol concentrations during the first 20 h of the saline and leptin infusion

During the pre-infusion period, there was no difference in the plasma leptin concentrations between fetuses assigned to the saline (2.8 ± 0.9 ng/ml) or leptin (3.5 ± 0.8 ng/ml) infusion groups. During the first 20 h of infusion, plasma leptin concentrations increased significantly in the leptin infused (+20 h: 18.3 ± 2.1 ng/ml) and not in the saline infused fetuses (+20 h: 2.0 ± 0.7 ng/ml) (Figure 5.4).

During the preinfusion period, there was no difference in either fetal plasma cortisol or ACTH concentrations between fetuses assigned to the saline (62.8 ± 18.6 nmol/l and 41.5 ± 5.1 pg/ml respectively) and leptin infusion groups (81.4 ± 22.2 nmol/l and 42.0 ± 6.4 pg/ml respectively). At 20 h after the start of the infusion there was no difference in

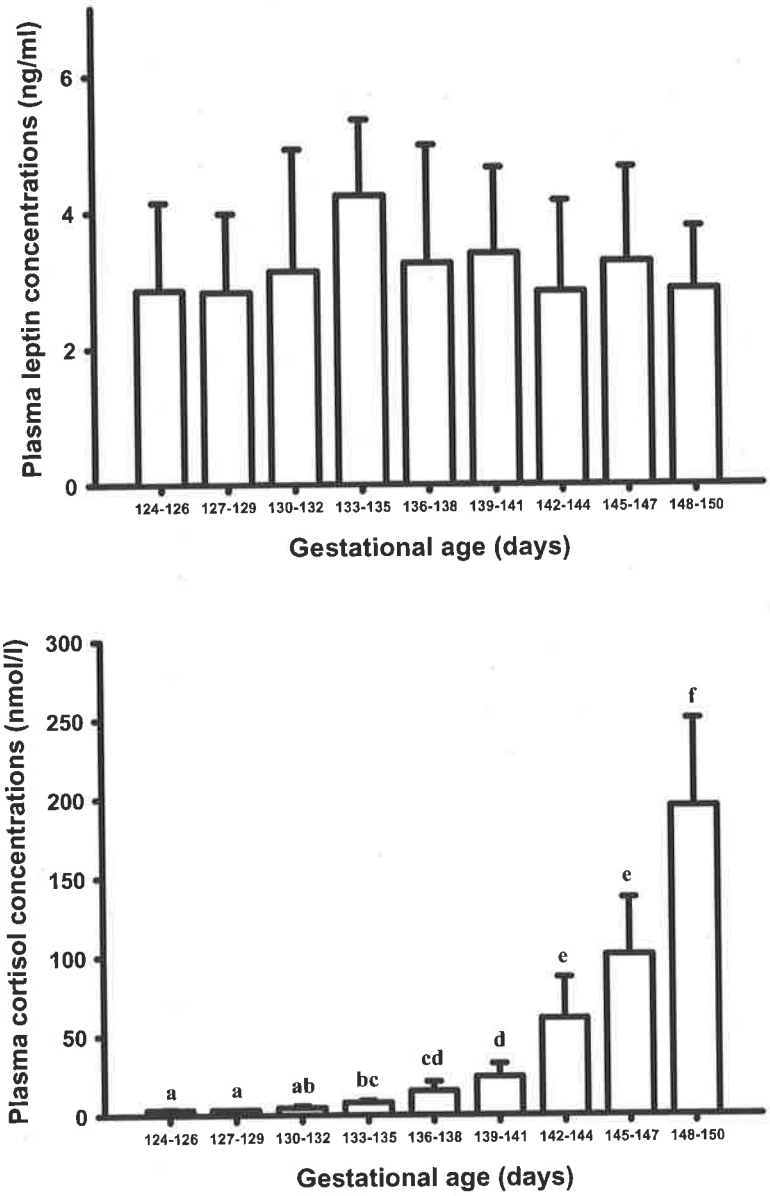


Figure 5.2 Plasma cortisol and leptin concentrations between 125 and 150 d gestation in fetal sheep assigned to the saline infused group.

Different alphabetic superscripts denote mean hormone values which are significantly different ($P<0.05$) from each other during late gestation.

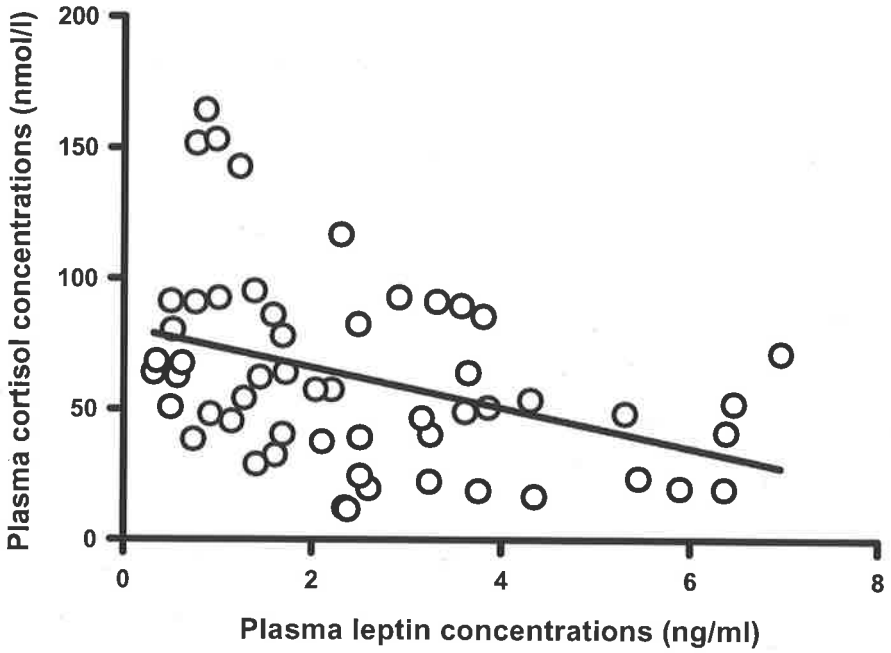


Figure 5.3 The relationship between plasma cortisol and leptin concentrations in saline infused fetuses between 138 and 146 d gestation.

The relationship is described by the equation, y=81.4 – 7.7x (R=0.38, $P<0.05$).

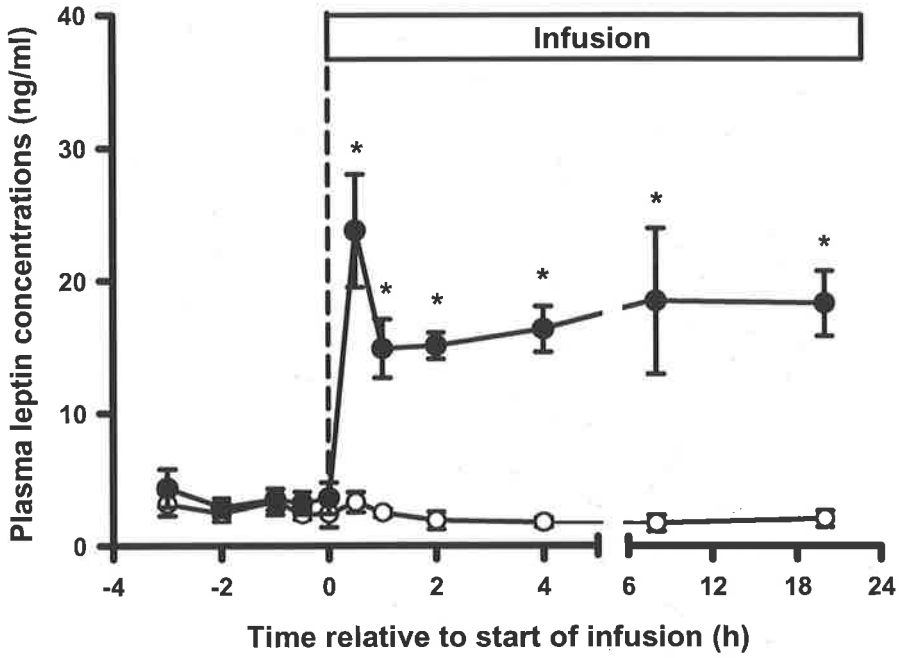


Figure 5.4 Plasma leptin concentrations in saline infused (open circles) and leptin infused (closed circles) fetuses during the first 20 h of the infusion period starting at 144 d gestation.

either plasma cortisol (saline infused, 61.4 ± 5.1 nmol/l; leptin infused, 81.4 ± 22.2 nmol/l) or ACTH (saline infused, 41.0 ± 4.9 pg/ml; leptin infused, 34.1 ± 5.0 pg/ml) concentrations between the saline and leptin infused groups.

5.7.3.2 *Effects of leptin on the timing of delivery and on plasma ACTH and cortisol concentrations preceding delivery*

There was no difference in the length of gestation between the saline and leptin infused groups (saline infused, 150.2 ± 0.5 d; leptin infused, 149.8 ± 1.0 d).

Circulating leptin concentrations were higher in leptin infused than saline infused fetuses from 114 h before and up to delivery (leptin infused: 16.3 ± 2.9 ng/ml; saline infused, 2.5 ± 0.7 ng/ml; $P<0.001$). There was no significant change with time in plasma leptin concentrations in either the leptin or saline infused fetuses during this period (Figure 5.5).

There was no difference in plasma ACTH concentrations between the saline infused (70.8 ± 46.8 pg/ml) and leptin infused (69.7 ± 22.6 pg/ml) fetuses during the period 114-6 h before delivery. In both the saline and leptin infused groups, plasma ACTH concentrations were significantly higher ($P<0.001$) during the period from 18 to 6 h before delivery when compared with either 90-78 h or 114-102 h before delivery (Figure 5.6).

There was a significant interaction ($P<0.05$) between the effects of leptin infusion and time before delivery on fetal plasma cortisol concentrations (Figure 5.6). Between 90 and 42 h before delivery, circulating cortisol concentrations were significantly higher in the

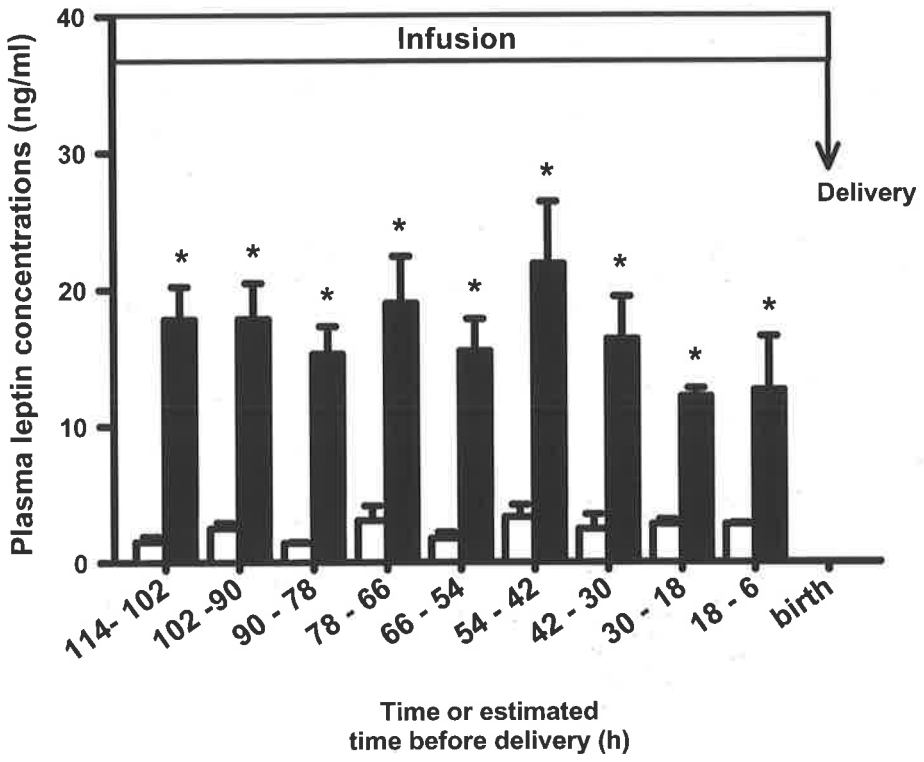


Figure 5.5 Plasma leptin concentrations in saline infused (open bars) and leptin infused (closed bars) fetuses during the period from 114 h until 6 h before birth.

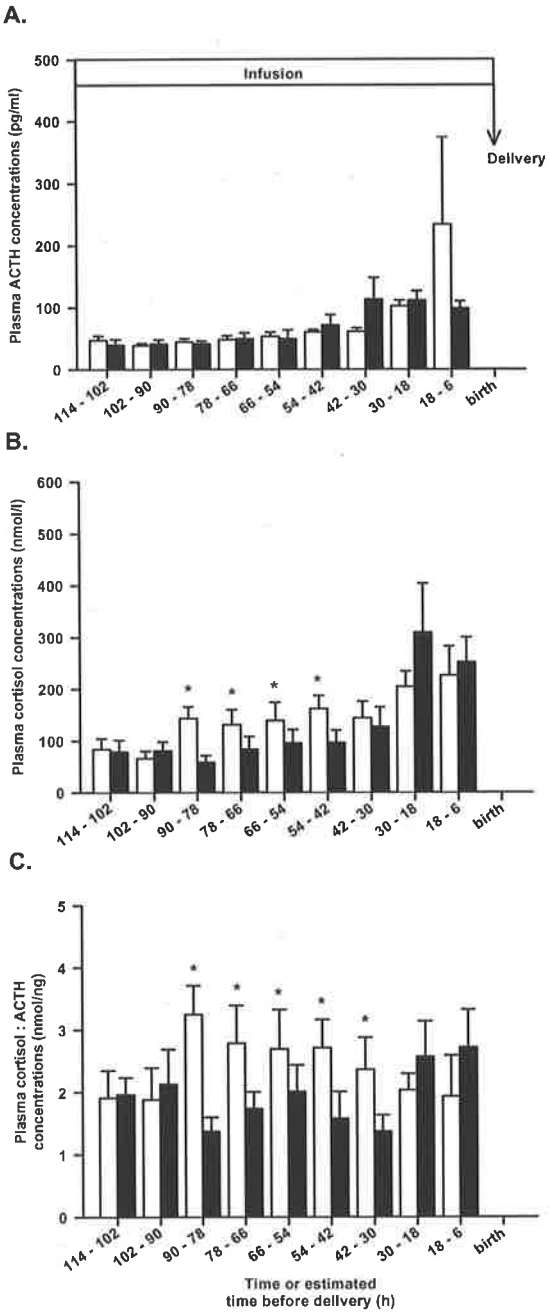


Figure 5.6 Plasma ACTH (A), cortisol (B) and the ratio of plasma cortisol : ACTH (C) concentrations in saline infused (open) and leptin infused (closed) fetuses from 114 h before delivery.

Asterisks denote significant differences ($P<0.05$) between mean values in the saline and leptin infused groups.

saline infused fetuses (90-42 h: saline infused, 142.5 ± 27.6 nmol/l; leptin infused, 84.3 ± 22.7 nmol/l; $P<0.05$) (Figure 5.6). During the period 42 to 6 h before delivery, however, there was no difference in plasma cortisol concentrations between the saline infused (188.0 ± 42.4 nmol/l) and leptin infused groups (244.5 ± 69.3 nmol/l). Plasma cortisol concentrations ($P<0.001$) were highest in both leptin and saline infused groups from between 30 and 6 h before delivery (Figure 5.6).

There was a significant interaction between the effects of treatment and the time relative to delivery on the ratio of plasma cortisol : ACTH concentrations ($P<0.005$) (Figure 6C). There was no significant difference between the saline and leptin infused fetuses in the ratio of plasma cortisol : ACTH concentrations between 114 and 90 h before delivery (saline infused: 1.89 ± 0.45, leptin infused: 2.04 ± 0.42). The plasma cortisol : ACTH ratios were lower, however, in the leptin infused fetuses between 90 and 30 h before delivery (saline infused, 2.78 ± 0.53; leptin infused, 1.62 ± 0.34) (Figure 6C).

5.8 DISCUSSION

We have demonstrated that infusion of leptin into fetal sheep, resulting in a 4-5 fold increase in circulating leptin concentrations suppressed the normal increase in fetal cortisol concentrations at the onset of the prepartum activation of the fetal HPA between 136 and 140 days gestation. Furthermore, intrafetal infusion of leptin from 144 days gestation until delivery also suppressed fetal plasma cortisol concentrations for an extended period from 90 to 42 h before delivery. Whilst plasma cortisol concentrations were reduced by around 40% during this period in the leptin infused group, there was no difference in the timing of parturition between leptin and saline infused fetuses.

In a previous study, Howe and colleagues (447) infused leptin via the lateral cerebral ventricle in fetal sheep between 135 and 140 days gestation and measured plasma ACTH and cortisol concentrations during a 4 h sampling period at 135 days and at 140 days gestation. They found that the increases in the mean value and amplitude of the pulses in plasma ACTH and cortisol concentrations between 135 and 140 days gestation were less in the leptin infused compared to the vehicle infused fetuses (447).

In the present study, there was an increase in fetal plasma ACTH and cortisol concentrations when saline was infused for a 96 h period between 136 and 141 days gestation as expected. There was no increase, however, in either plasma ACTH or cortisol concentrations when leptin was infused during this gestational age range. During this period, the inhibitory effect of leptin on the prepartum increase in fetal cortisol concentrations appeared to be relatively greater than its impact on fetal plasma ACTH concentrations. Furthermore when leptin was infused continuously from 144 days gestation, there was no effect on fetal ACTH concentrations during the week before delivery. In marked contrast, leptin infusion from 144 days gestation suppressed fetal plasma cortisol concentrations and the ratio of fetal plasma cortisol : ACTH concentrations for an extended period from 90 h until around 42 to 30 h before delivery. The suppression of fetal plasma cortisol concentrations and the decrease in the ratio of plasma cortisol : ACTH concentrations was not maintained, however, during the last 30 h before delivery despite continued infusion of leptin. Plasma cortisol concentrations were similar in both the leptin and saline infused fetuses on the day before delivery and there was no difference between these two groups in the timing of delivery which occurred between 148 and 153 d gestation. In summary, evidence from the current study

suggests that an increase in circulating leptin concentrations in the sheep fetus during late gestation can blunt, but not block, the prepartum activation of the HPA axis and delivery. Whilst there may be a transient impact of leptin on fetal plasma ACTH concentrations during the early phase of activation of the fetal pituitary-adrenal axis in late gestation, the predominant action of leptin appears to be to suppress the normal prepartum increase in circulating cortisol and adrenal responsiveness to ACTH.

In adult sheep it has been demonstrated that i.c.v. infusion of leptin suppressed food intake and resulted in a decrease in the expression of the mRNA for the orexigenic peptide, Neuropeptide Y (NPY) in the hypothalamic arcuate nucleus (480). This is consistent with the localisation of the long form of the leptin receptor in around 60% of NPY containing cells in the sheep hypothalamus (481). There is also evidence in the sheep that hypothalamic NPY can regulate the synthesis and secretion of the ACTH secretagogues, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) (294). NPY is present within the arcuate nucleus of the fetal sheep hypothalamus during late gestation (482) and it is possible that leptin acts centrally via leptin receptors located within the fetal hypothalamus to suppress NPY, CRH and/or AVP secretion and hence result in a decrease in fetal plasma ACTH concentrations in late gestation. It appears from the present study, however, that any inhibitory effect of an increase in circulating leptin concentrations on fetal ACTH secretion is not maintained during the week before delivery. The sustained increase in circulating leptin concentrations may induce resistance to the central actions of leptin as this has been proposed to underlie reduced sensitivity to peripherally administered leptin in genetically wild type mice, primates and lambs (483-485). There is evidence that high circulating leptin concentrations may induce a decrease in the transport or access of leptin to the brain (486-488). Whilst this is

possible, it should be noted that we found no evidence that leptin infusion at 144 days resulted in an initial decrease in fetal ACTH concentrations during the first day of the infusion period. An alternative explanation is that the hypothalamic mechanisms that stimulate fetal pituitary ACTH synthesis and secretion during the prepartum period are not suppressed by an increase in peripheral leptin concentrations. In the present study, we have demonstrated, however, that leptin infusion blunted the increase in fetal cortisol at the onset of the prepartum stimulation of the fetal HPA axis between 136 and 141 days gestation and resulted in a suppression of fetal cortisol during the week before delivery. A range of studies have reported that that the long form of the leptin receptor is expressed in human, rat and mouse adrenal and that leptin acts directly to inhibit ACTH stimulated glucocorticoid secretion by the bovine (300), human and rat adrenal gland (208). Leptin acts to decrease the expression of the steroidogenic enzymes, cytochrome P450 C21-hydroxylase, side chain cleavage and C17 α hydroxylase in the bovine adrenal and it has recently been reported that leptin reduces the ACTH stimulation of steroidogenic acute regulatory protein (StAR) expression in the rat adrenal (301, 489). It has been proposed that in the adult, a leptin mediated endocrine feedback loop exists between adipose tissue and the HPA axis as glucocorticoids can stimulate leptin expression and secretion from the adipocyte (187, 188), whereas rising circulating leptin concentrations can directly down regulate adrenal cortisol synthesis and secretion. The current study provides evidence that leptin can act directly at the fetal adrenal and raises the novel possibility that a leptin mediated endocrine feedback loop between fetal adipose tissue and the HPA axis is present in late gestation.

In the present study, intrafetal leptin infusion resulted in circulating leptin concentrations of around 15-20 ng/ml. Whilst these concentrations are similar to those measured in

well-fed pregnant ewes where rapid maternal weight gain has occurred (441), they are significantly higher than those measured by us or others in the fetal sheep of well nourished ewes in late gestation (146, 446, 455, 474). Significant fetal hyperleptinaemia has been reported in human pregnancies complicated by maternal glucose intolerance and fetal hyperglycaemia (56-58) and in these pregnancies it is possible that the increase in fetal leptin concentrations may regulate adrenal responsiveness to ACTH and other stimulatory hormones. What is currently unclear is the extent of the endocrine interaction between fetal adipose tissue and the HPA axis in normal pregnancy. In the sheep fetus, circulating leptin concentrations are positively correlated with the relative mass of lipid stored in dominant cellular lipid locules within the fetal perirenal adipose tissue (455) and leptin is therefore an endocrine signal of the lipid storage capacity of this tissue. Forhead and colleagues (446) have reported that plasma cortisol and leptin concentrations increase in parallel during late gestation and are positively related between 130 and 140 days in the sheep fetus. Furthermore they reported that fetal adrenalectomy resulted in lower plasma leptin concentrations in fetal sheep after 136 days (446). In contrast, two recent studies found that plasma leptin concentrations did not increase between 116 and 140-145 days gestation in fetal sheep of either well nourished or undernourished ewes (455, 474). Furthermore cortisol infusion or adrenalectomy did not alter leptin mRNA levels in perirenal adipose tissue in the late gestation sheep fetus (490). It is unclear therefore to what extent cortisol exerts a stimulatory effect on fetal leptin synthesis and secretion during late gestation. In the present study we found that in saline infused fetuses, there was no change in fetal plasma leptin concentrations during the last 3 weeks of gestation and that there was no relationship between plasma cortisol and leptin concentrations between 125 and 137 days gestation. There was, however, a negative relationship between circulating cortisol and leptin during the week before

delivery such that around 14% of the variation in plasma cortisol in the saline infused group was explained by the variation in fetal leptin concentrations. Thus whilst the initiation of the prepartum increase in fetal plasma cortisol does not appear to be related to any concomitant fall in circulating leptin, leptin may act to inhibit the output of cortisol from the fetal adrenal during the week before delivery.

In summary we have demonstrated that an increase in circulating leptin concentrations in fetal sheep suppressed the normal increase in fetal cortisol concentrations at the onset of the prepartum activation of the fetal HPA between 136 and 140 days gestation. Furthermore intrafetal infusion of leptin from 144 days gestation until delivery also suppressed fetal plasma cortisol concentrations for an extended period from between 90 - 42 h before delivery. Whilst plasma cortisol concentrations were reduced by around 40% in leptin infused fetuses, there was no difference in the timing of parturition between the leptin and saline infused groups. Whilst fetal plasma leptin concentrations do not fall before the prepartum increase in fetal cortisol, there is a negative relationship between circulating cortisol and leptin concentrations in the week before delivery. This study therefore provides evidence that a leptin mediated, endocrine negative feedback loop between fetal adipose tissue and the HPA axis may be present before birth and play an important role in determining the fetal adrenal responsiveness to ACTH and other trophic factors during intrauterine stress, parturition and the transition from intrauterine to extrauterine life.

CHAPTER 6

6. GENERAL DISCUSSION

The work described in this thesis investigates the regulation and actions of leptin before birth in sheep and includes some of the first reported studies of this hormone in a mammalian fetus. At the time these studies commenced, it was known that leptin protein was present in umbilical cord blood samples in newborn human infants which suggested that leptin might be an important regulatory hormone before birth in mammals. Adipocytes that contain visible lipid locules were reported to be present in perirenal adipose tissue from as early as the middle of gestation in the sheep fetus and it is possible to work with the chronically catheterised sheep fetus during late gestation (84). Therefore the sheep appeared to be a valid model in which to investigate the developmental physiology of leptin in mammals. To undertake this work, a method was developed and validated to measure ovine leptin mRNA by RT-PCR. The concurrent establishment of an immunoassay for ovine leptin protein in sheep plasma by external Australian collaborators and the generous donation of biologically active recombinant ovine leptin by an American collaborator permitted expansion of the research investigation.

In Chapter 2, leptin mRNA expression in adipose tissue was discovered well before the time of birth, from at least 90 days gestation in the sheep fetus. This is less than two-thirds of normal gestation in the sheep. This study also revealed that the abundance of leptin mRNA in adipose tissue progressively increases during the last third of fetal development in this species. The original observation that the abundance of leptin mRNA in adipose tissue of the sheep fetus is positively correlated with the body weight of the fetus (Chapter 2), provided indirect evidence that fetal production of leptin may be

controlled by factors that regulate growth and development of the fetus (443). Subsequently, other researchers showed that the expression of leptin mRNA could be detected in this tissue from as early as 75 days gestation in the sheep fetus (445).

More recently, leptin protein has been detected in the circulation of fetal sheep from as early as the first quarter (40 days) of gestation (146). As this is before the appearance of fetal adipose tissue it indicates that leptin protein may be derived from other feto-placental tissues (146). Indeed, leptin mRNA has been detected in other organs of the sheep fetus, including the brain and liver, from as early as 40 days gestation (146). Unlike the placenta of other mammals (humans (130), rats (131), mice (127)), which produces as much as 15% of the leptin protein detected in the fetal circulating (403-405), the ovine placenta does not express leptin mRNA (445)(Yuen, unpublished data), although, leptin protein has been detected in the sheep placenta (127).

It is thought that leptin is able to cross cell membranes, e.g. blood brain barrier (234-242), however, the exact mechanisms by which leptin is able to cross the cell membranes remains to be determined. Some have proposed that Ob-Ra - the short form of the leptin receptor, which is expressed in the mammalian placenta (214, 227, 228), may be involved in the transport of leptin (238, 242). Studies by Smith and Waddell (214, 407), demonstrated when iodinated leptin was infused into the maternal circulation of rats, the radiolabelled leptin could later be detected in the fetal circulation. It suggests that, at least in the rat, leptin is able to cross the placenta. This may explain how leptin protein is detected in the circulation of fetal sheep as early as 40 days gestation. I have reported that a positive relationship exists between maternal leptin and fetal leptin concentrations (Chapter 3), and this is certainly consistent with matero-fetal leptin transport and could

explain how detectable concentrations of leptin are present in the fetal circulation of sheep as early as 40 days gestation (84).

By 130 days gestation, leptin mRNA expression in the brain and liver of the sheep fetus is almost undetectable. In late gestation it appears that fetal adipose tissue is the major contributor to circulating leptin levels in the fetal sheep. Gemmell *et al* (84) examined the ultrastructure of perirenal and subcutaneous adipose tissue in sheep fetus, and observed that both depots contained adipose tissue, which contained lipid locules. I demonstrated that leptin mRNA expression in adipose tissue increases between 90 and 144 days gestation in the sheep fetus (Chapter 2) and reported that the abundance of leptin mRNA in the perirenal adipose tissue of fetal sheep between 144 and 147 days gestation was positively correlated with circulating leptin concentrations in the fetus (Chapter 3). Furthermore, it has been demonstrated that the abundance of leptin mRNA in fetal adipose tissue was positively correlated with the proportion of adipose tissue comprised of lipid locules which were greater than 75 μm^2 (Chapter 4) and the mass of this unilocular proportion of fetal adipose tissue was positively correlated with circulating leptin concentrations in the sheep fetus (455). Together these findings suggest that in the fetus, like in the adult, leptin expression and secretion occurs from cells that are predominantly unilocular, i.e. contain a dominant lipid locule. Moreover, it may be that in the fetus, as in the adult, factors that alter adipocyte or lipid locule size, such as glucose, insulin, cortisol and IGF-I, may also regulate circulating leptin concentrations (6, 7, 491, 492).

The positive relationship between maternal and fetal leptin concentrations and the possibility that maternally derived leptin protein is transported into the fetal circulation is

intriguing, however, this conclusion is inconsistent with the positive relationship found between leptin synthesis and circulating leptin concentrations in the sheep fetus during late gestation. One possible explanation is that fetal cortisol concentrations regulate the transplacental passage of leptin and during late gestation, as circulating cortisol concentrations increase in the sheep fetus, the transplacental passage of leptin decreases. Smith and Waddell (407) showed that in pregnant rats, maternal dexamethasone treatment, which is known to increase fetal dexamethasone concentrations, caused a decrease (77%) in the placental transfer of leptin. Conversely, maternal metyrapone treatment, which is known to decrease maternal and fetal corticosterone concentrations, increased (55%) the placental transfer of leptin (407). Thus it can be concluded that cortisol is an important determinant of matero-fetal leptin transport. It is well known that the fetal HPA axis becomes increasingly active during late gestation and fetal cortisol concentrations increase from ~135 days gestation. Hence one possibility is that fetal cortisol concentrations decreases the transplacental passage of leptin resulting in a greater contribution of the fetal adipose tissue to circulating leptin concentrations in the sheep fetus.

Although studies by Yuen (Chapter 3), Muhlhausler (455) and colleagues showed that moderate changes in maternal food intake do not significantly affect leptin expression and/or circulating leptin levels in fetal sheep, it is unclear whether changes in maternal nutrition during pregnancy will effect postnatal leptin concentrations. There is evidence to suggest that, in some species, a suboptimal intrauterine environment causes the development of obesity and alters circulating leptin concentrations in progeny, however, in these studies no measurements of circulating leptin were taken at or before birth. Vickers and colleagues (453, 493) showed that decreased maternal food intake during

pregnancy resulted in rats that were lighter and shorter in length at birth, however no measurements of plasma leptin concentrations were made at this time. These offspring exhibited catch-up growth during early life and by young adulthood, the rat offspring were obese, hyperphagic and had elevated circulating leptin concentrations, suggesting that these animals had become leptin resistant. Vickers (494) showed that these effects could be attenuated with the administration of IGF-I during young adulthood. Certain findings from the Vicker's rodent model mimics observations in adult humans who were small at birth. Low birthweight newborns that experience catch up growth during childhood are found to develop adult obesity (59). Whilst circulating leptin concentrations were not measured in these subjects (59), it is well established that obese humans have high circulating leptin concentrations compared to lean individuals and is thought to be a consequence of these individuals being leptin resistant (15, 117, 133, 379). Therefore, a suboptimal environment during pregnancy followed by certain postnatal events can lead to the development of adult obesity, elevated circulating leptin concentrations and leptin resistance in offspring. Ekert (454) showed that in pigs, however, that a moderate feed restriction during pregnancy decreased the abundance of leptin mRNA in adipose tissue and concentrations of leptin protein in blood in adolescent offspring. These differences maybe related to differences in the development of adipose tissue and the leptin axis in these species (492, 495).

In Chapters 4 and 5, I examined the effects of intravascular infusion of leptin into fetal sheep on adipose tissue and on the HPA axis in the sheep fetus. Leptin treatment altered the structural and functional characteristic of fetal adipose tissue (Chapter 4) by increasing the proportion of adipose tissue with a multilocular appearance and reducing the proportion of the tissue that was unilocular. Leptin expression was decreased, UCP-1

protein abundance tended to increase and the abundance of other mitochondrial proteins were not altered in the adipose tissue of fetuses infused with leptin. These experiments show that leptin can down regulate its own expression and can potentially stimulate thermogenesis in fetal adipose tissue.

In Chapter 5, the effects of leptin on the fetal pituitary-adrenal axis were described. I demonstrated that during the early phase of prepartum activation of the fetal HPA axis, continuous administration of leptin appeared to suppress both ACTH secretion by the pituitary gland and cortisol secretion by the fetal adrenal gland. When leptin was continuously administered later in gestation, cortisol concentrations in fetal blood were reduced for an extended period although leptin was unable to prevent the prepartum surge in cortisol that occurred during the 2 days before delivery. Interestingly around 14% of the variation in fetal cortisol concentrations was explained by variation in leptin concentrations in the late gestation sheep fetus. These data suggest that in the fetus, as in the adult (299, 496, 497), a leptin-mediated feedback loop may exist between adipose tissue and the pituitary and/or adrenal glands in the sheep fetus.

In conclusion, the studies presented in this thesis demonstrate that leptin is expressed in fetal adipose tissue, and that leptin expression and circulating leptin concentrations are not altered in response to moderate changes in maternal food intake during pregnancy. Furthermore, intravenous administration of leptin to fetal sheep causes structural and functional changes in fetal adipose tissue resulting in a greater proportion of the adipose tissue having a multilocular appearance, which may have increased thermogenic activity. In the fetal sheep, leptin treatment also suppresses the typical prepartum surge in cortisol concentrations, although the effectiveness of leptin to attenuate rising cortisol

concentrations was dependent on time relative to birth. These effects are not mediated by changes in ACTH concentrations and therefore implicate a direct action of leptin on the fetal adrenal gland.

CHAPTER 7

7. REFERENCES

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CHAPTER 8

8. APPENDIX: PUBLICATIONS ARISING FROM THIS THESIS

The following papers have been published in journals of international standing:

Yuen B.S., McMillen I.C., Symonds M.E., Owens P.C. 1999 Abundance of leptin mRNA in fetal adipose tissue is related to fetal body weight. Journal of Endocrinology. 163:R11-R14.

Yuen B.S., Owens P.C., McFarlane J.R., Symonds M.E., Edwards L.J., Kauter K.G., McMillen I.C. 2002 Circulating leptin concentrations are positively related to leptin messenger RNA expression in the adipose tissue of fetal sheep in the pregnant ewe fed at or below maintenance energy requirements during late gestation. Biology of Reproduction 67:911-916.

Yuen B.S., Owens P.C., Muhlhausler B.S., Roberts C.T., Symonds M.E., Keisler D.H., McFarlane J.R., Kauter K., Evens Y., McMillen I.C. 2003 Leptin alters the structural and functional characteristics of adipose tissue before birth. The FASEB Journal 10.1096/fj.02-0756fje.

The follow paper have been accepted for publication:

Yuen B.S., Owens P.C., Symonds M.E., Keisler D.H., McFarlane J.R., Kauter K.G., McMillen I.C. 2004 Effects of leptin on fetal plasma ACTH and cortisol concentrations and the timing of parturition in the sheep. Biology of Reproduction.

Yuen, B.S., McMillen, I.C., Symonds, M.E., and Owens, P.C., (1999) Abundance of leptin mRNA in fetal adipose tissue is related to fetal body weight.
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Circulating Leptin Concentrations Are Positively Related to Leptin Messenger RNA Expression in the Adipose Tissue of Fetal Sheep in the Pregnant Ewe Fed at or Below Maintenance Energy Requirements During Late Gestation[1]

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ABSTRACT

We have investigated the effects of maternal undernutrition during late gestation on maternal and fetal plasma concentrations of leptin and on leptin gene expression in fetal perirenal adipose tissue. Pregnant ewes were randomly assigned at 115 days of gestation (term = 147 ± 3 days [mean ± SEM]) to either a control group (n = 13) or an undernourished group (n = 16) that received ~50% of the control diet until 144–147 days of gestation. Maternal plasma glucose, but not leptin, concentrations were lower in the undernourished ewes. A significant correlation was found, however, between mean maternal plasma leptin (y) and glucose (x) concentrations ($y = 2.9x - 2.4$; $r = 0.51$, $P < 0.02$) when the control and undernourished groups were combined. Fetal plasma glucose and insulin, but not fetal leptin, concentrations were lower in the undernourished ewes, and no correlation was found between mean fetal leptin concentrations and either mean fetal glucose or insulin concentrations. A positive relationship, however, was found between mean fetal (y) and maternal (x) plasma leptin concentrations ($y = 0.18x + 0.45$; $r = 0.66$, $P < 0.003$). No significant difference was found in the relative abundance of leptin mRNA in fetal perirenal fat between the undernourished (0.60 ± 0.09, n = 10) and control (0.70 ± 0.08, n = 10) groups. Fetal plasma concentrations of leptin (y) and leptin mRNA levels (x) in perirenal adipose tissue were significantly correlated ($y = 1.5x \pm 0.3$; $r = 0.69$, $P < 0.05$). In summary, the capacity of leptin to act as a signal of moderate maternal undernutrition may be limited before birth in the sheep.

insulin, leptin, pregnancy

INTRODUCTION

Leptin is a 16-kDa protein hormone that is principally synthesized and secreted by adipocytes and that suppresses appetite and increases energy expenditure in the adult [1]. During adult life, plasma concentrations of leptin and the abundance of leptin mRNA in adipose tissue correlate positively with body weight and adiposity, and these concentrations are altered by long-term changes in dietary intake in the rodent, human, and sheep [2–7]. A positive relationship also exists between leptin expression in fetal adipose tissue and fetal weight in the sheep [8], and leptin concentrations in umbilical cord blood correlate positively with birth weight in the human [9, 10]. Because fetal growth rate and body weight at birth are positively affected by nutrition during pregnancy, we hypothesized that the synthesis and secretion of leptin may be regulated by the fetal nutrient supply. In the present study, we therefore investigated the effects of maternal undernutrition during late gestation in the sheep on maternal and fetal plasma concentrations of leptin and on leptin gene expression in fetal adipose tissue. We also investigated the relationship between the abundance of leptin mRNA in this fetal tissue and circulating fetal glucose, insulin, and leptin concentrations.

MATERIALS AND METHODS

Animals and Surgery

All procedures were approved by the Adelaide University Animal Ethics Committee. Surgery was performed on 29 pregnant Border-Leicester Merino cross-bred ewes under aseptic conditions between 109 and 113 days of gestation (term = 147 ± 3 days [mean ± SEM]) with general anesthesia induced by sodium thiopentone (1.25 g i.v.; Pentothal; Rhone Merieux, Pinkenba, Qld, Australia) and maintained with 2.5–4% (v/v) halothane (Fluothane; ICI, Melbourne, Vic, Australia) in oxygen. Vascular catheters were implanted in a maternal jugular vein, a fetal carotid artery and jugular vein, and the amniotic cavity as previously described [11]. Catheters were filled with heparinized saline, and the fetal catheters were exteriorized through an incision made in the ewes' flank. During surgery, ewes and fetuses received a 2-ml i.m. injection of antibiotics (procaine penicillin [250 mg/ml], dihydrostreptomycin [250 mg/ml], and procaine hydrochloride [20 mg/ml]; Penstrep Illium; Troy Laboratories, Smithfield, NSW, Australia). Ewes were housed in individual pens in rooms with a 12L:12D photoperiod and fed once daily at 1100 h with water provided ad libitum. Animals were allowed to recover from surgery for at least 4 days before collection of fetal and maternal blood samples commenced.

Feeding Regime

Pregnant ewes were randomly assigned at 115 days either to a control group weighing 56.7 ± 1.9 kg (n = 13) that received 19.8 ± 0.2 g/kg of lucerne and 3.0 ± 0.1 g/kg of oats per day or to an undernourished group weighing 53.5 ± 2.3 kg (n = 16) that received 10.3 ± 0.1 g/kg of lucerne and 1.6 ± 0.1 g/kg of oats per day. Maternal food allocation was increased in both the control and undernourished groups (lucerne by 15%, oats by 10%) every 10 days until postmortem at 144–147 days of pregnancy [11].

Blood Sampling Protocol

Maternal venous (5 ml) and fetal arterial (3.5 ml) blood samples were collected between 0800 and 1100 h, before the ewes were fed, three times each week between 116 and 140 days of gestation. Blood samples were centrifuged at 1500 × *g* for 10 min, and plasma was separated into aliquots and stored at −20°C for subsequent glucose and hormone assay. At times during the 25-day protocol, blood samples could not be collected due to technical problems (primarily related to blocked vascular catheters). The

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number of maternal and fetal blood samples that were available for glucose, insulin, and leptin determination are detailed in the subsequent assay sections. Fetal arterial blood (0.5 ml) samples were also collected for the measurement of arterial blood gas status (ABL 520 blood gas analyzer; Radiometer, Copenhagen, Denmark).

Tissue Collection

Ewes were killed between 144 and 147 days of pregnancy with a lethal overdose of sodium pentobarbitone (Virbac Pty Ltd.; Peakhurst, NSW, Australia). Fetuses were delivered by hysterotomy, weighed, and killed by decapitation (control group, 12 singletons and 2 twins; undernutrition group, 15 singletons and 1 twin). Fetal perirenal adipose tissue was collected and weighed, and a sample was frozen in liquid nitrogen and stored at −80°C.

Glucose Assay

Plasma glucose concentrations were determined in 234 maternal plasma samples (control group, 90 samples, n = 8 sheep; undernutrition group, 144 samples, n = 13 sheep) and 348 fetal plasma samples (control group, 160 samples, n = 13 sheep; undernutrition group, 188 samples, n = 16 sheep) by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase and measuring the formation of NADH spectrophotometrically at 340 nm (COBAS MIRA automated analysis system; Roche Diagnostic, Basel, Switzerland) [11]. The intra- and interassay coefficients of variation were both <5%.

Insulin Radioimmunoassay

Fetal plasma insulin concentrations were measured in 196 samples (control group, 88 samples, n = 12 sheep; undernutrition, 108 samples, n = 13 sheep) using a commercial kit (Phadaseph radioimmunoassay kit; Pharmacia & Upjohn, Uppsala, Sweden). The detection range of the assay was 1.5–240 μU ml^{-1}. Guinea pig anti-insulin antisera and [^{125}I]human insulin (100 μl) were added to plasma samples (100 μl), which were then incubated for 2 h at room temperature before the addition of 2 ml of sheep anti-guinea pig immunoglobulin G. Samples were allowed to stand at room temperature for a further 30 min before being centrifuged at 1500 × *g* for 10 min as described previously [11]. The inter- and intraassay coefficients of variation were <10%.

Leptin Assay

Plasma leptin concentrations were determined in 119 maternal plasma samples (control group, 50 samples, n = 10 sheep; undernutrition group, 69 samples, n = 15 sheep) and 99 fetal plasma samples (control group, 44 samples, n = 9 sheep; undernutrition group, 55 samples, n = 12 sheep) using a competitive ELISA previously validated for sheep plasma [12]. The ELISA plate was coated with 6 ng of recombinant bovine leptin in 50 μl of 0.1 M bicarbonate buffer (pH 9.0) overnight at 37°C. The plate was blocked with 200 μl of 5% skim milk in ELISA buffer for 1 h at 37°C. Samples (100 μl) were assayed in duplicate and added to wells containing 50 μl chicken antirecombinant bovine leptin antisera in 100% Triton-X 100, 0.5% SDS, and 5% sodium deoxycholate, and the plate was incubated overnight at 37°C. Strepavidin conjugated to alkaline phosphatase (Amrad Biotech; Boronia, Vic, Australia) was added, and after incubation for 1 h, the plate was developed with *p*-nitrophenylphosphate disodium salt hexahydrate. The sensitivity of the assay was 0.25 ng/ml, and the inter- and intraassay coefficients of variation were 15.7% and 11%, respectively.

Leptin Reverse Transcription-Polymerase Chain Reaction

Perirenal adipose tissue was collected from 20 (control group, n = 10; undernourished group, n = 10) of the 29 fetal sheep, and total RNA was extracted as previously described [8]. Briefly, approximately 100 mg of fetal adipose tissue were homogenized with 1 ml of Sigma Trireagent (Sigma Chemical Co., St. Louis, MO) and allowed to stand at room temperature for 5 min. This was then mixed with 1-bromo-3-chloro-propane (100 μl), left standing at room temperature for 10 min, and then centrifuged at 4°C at 3500 × *g* for 10 min. An aliquot of the aqueous layer (500 μl) was recovered and mixed with isopropanol (500 μl). The RNA was precipitated by centrifugation at 3500 × *g* for 5 min at 4°C. The pellet was washed in 70% ethanol and allowed to air dry. The RNA pellet was then dissolved in sterile water (20 μl), and 1 μl of the solution was diluted in sterile water (500 μl) for determination of the spectrophotometric absorbance at 260 and 280 nm. The ratio of nucleic acid to protein was >1.6, and the RNA yield was 0.44 ± 0.02 μg/mg adipose tissue. Integrity of RNA preparations was evaluated by agarose gel electrophoresis, followed by ethidium bromide staining and identification of ribosomal RNA.

Ovine leptin and β-actin cDNA were amplified by reverse transcription-polymerase chain reaction (RT-PCR) as previously described [8]. Briefly, cDNA was obtained by RT of 2 μg of total RNA with random hexamer oligonucleotides (GeneWorks, Adelaide, SA, Australia) and Super-Script RNase H$^-$ (Gibco BRL, Gaithersburg, MD). A fragment of ovine leptin cDNA was amplified through 26 cycles of 60 sec at 94°C, 15 sec at 53°C, and 60 sec at 72°C (Hybaid PCR Express, Teddington, U.K.) from 5 μl of RT product using Taq DNA polymerase (Biotech International, Bently, WA, Australia) according to the manufacturer's instructions with 5′-GACATCTCACACACGCAG-3′ and 5′-GAGGTTCTCCAGGTCATT-3′ (GeneWorks) as primers. This produced a double-stranded fragment of ovine leptin of 183 base pairs (bp) whose sequence was confirmed. A fragment of ovine β-actin cDNA was similarly amplified separately by PCR of the same RT product used for amplification of leptin cDNA with 5′-TGGATGGTGGGTATATGGGTC-3′ and 5′-TAGATGGGCACAGTGTGGGT-3′, and the identity of the 349-bp product was confirmed by sequencing. Both cDNA products from RT-PCR (8 μl) were electrophoresed through a 2.0% (w/v) agarose gel, stained with ethidium bromide, visualized by ultraviolet transillumination, photographed using a digital camera, and quantified using 1D Image Analysis Software Electrophoresis Documentation and Analysis System 120 (Kodak dS Digital Science, Rochester, NY).

Statistical Analysis

Data are presented as the mean ± SEM. The effects of maternal nutrition on fetal body weight, total perirenal fat mass, mean gestational arterial PO_2, and the relative abundance of leptin mRNA (ratio of leptin mRNA to β-actin mRNA) in fetal perirenal adipose tissue were determined using unpaired Student *t*-test. The effects of maternal nutrition on maternal plasma glucose and leptin concentrations were determined by multifactorial ANOVA with repeated measures using feeding group (control vs. undernutrition) and gestational age (in 5-day blocks) as the specified factors. Similarly, the effects of maternal nutrition and gestation on fetal plasma glucose, insulin, and leptin concentrations were also determined using multifactorial ANOVA with repeated measures. Data were transformed when required to reduce heterogeneity of variance and overcome nonnormal distributions. The Duncan new multiple-range test was used after ANOVA to identify significant differences between mean values. Linear regression analysis was used to assess the relationship between the mean plasma leptin and the mean plasma glucose concentrations measured in each ewe and fetus from 116 to 140 days of pregnancy. Similarly, linear regression analysis was also used to assess relationships between mean plasma leptin and mean plasma glucose or insulin concentrations measured in each fetus from 116 to 140 days of pregnancy. Relationships between mean fetal plasma leptin concentrations and fetal body weight, fetal fat mass, and maternal plasma leptin concentrations were similarly determined. A probability of 5% ($P < 0.05$) was taken as the level of significance in all analyses.

RESULTS

Fetal Outcome

The mean fetal arterial PO_2 throughout late gestation was not different between the control (21.9 ± 0.5 mm Hg) and undernourished (23.4 ± 0.6 mm Hg) groups. No difference was found in fetal body weights (control, 5.02 ± 0.12 kg; undernourished, 4.70 ± 0.16 kg) or relative fat mass (control, 3.89 ± 0.15 g/kg; undernourished, 4.13 ± 0.29 g/kg) between the two groups.

Maternal Plasma Glucose and Leptin Concentrations

Maternal plasma concentrations of glucose were significantly lower ($F = 5.13$, $P < 0.05$) in undernourished ewes throughout late pregnancy (Fig. 1A). Plasma glucose concentrations were also lower ($F = 4.88$, $P < 0.002$) in both control and undernourished ewes after 120 days when compared with earlier in pregnancy (Fig. 1A). No significant effect of undernutrition, however, was found on maternal

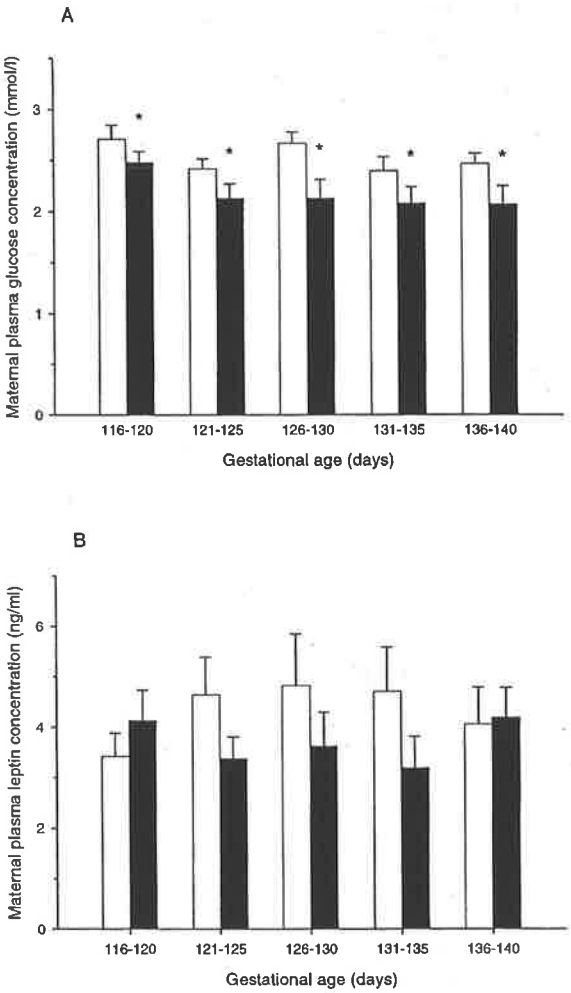

FIG. 1. Maternal plasma glucose (A) and leptin concentrations (B) in control (open histograms) and undernourished (dark histograms) ewes between 116 and 140 days of gestation. Asterisks denote a significant effect of undernutrition on maternal glucose concentrations throughout late gestation.

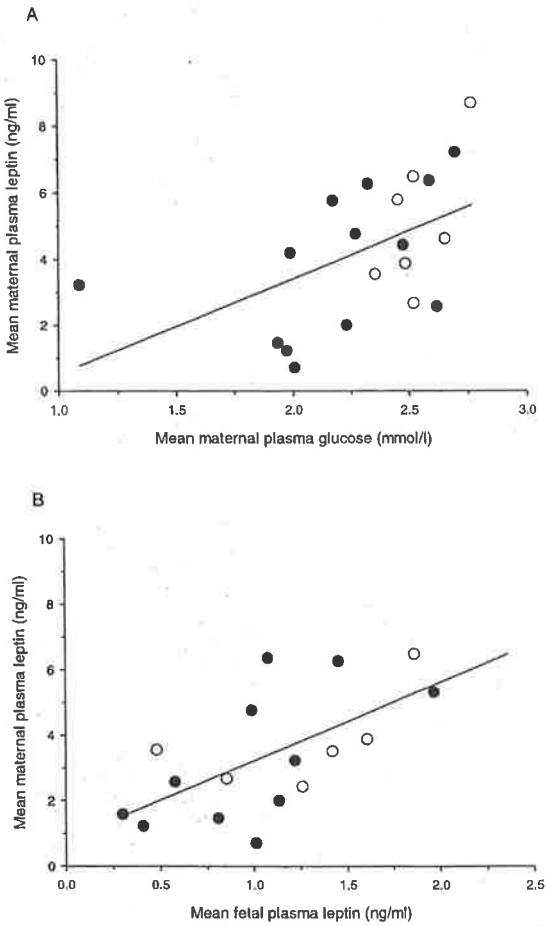

FIG. 2. Relationship between maternal plasma leptin and glucose concentrations (A) or between fetal and maternal plasma leptin concentrations (B) in undernourished (closed symbols) and control (open symbols) groups.

plasma concentrations of leptin, and no significant change in maternal leptin concentrations was found between 116 and 140 days of pregnancy in either the control or the undernourished ewes (Fig. 1B). Mean maternal plasma leptin (y) and glucose (x) concentrations were not correlated within each separate feeding group; however, they were significantly correlated when data from the control and undernourished groups were combined ($y = 2.9x - 2.4$; $r = 0.51$, $P < 0.02$, $n = 20$) (Fig. 2A). No relationship was observed between the mean maternal plasma concentrations of leptin and either maternal body weight at 110–115 days of gestation or fetal body weight at 144–147 days of gestation.

Fetal Plasma Glucose, Insulin, and Leptin Concentrations

Fetal plasma concentrations of glucose ($F = 10.13$, $P < 0.005$) and insulin ($F = 6.64$, $P < 0.02$) were significantly lower in the undernourished group (Fig. 3, A and B). Fetal plasma concentrations of insulin were lowest ($P < 0.003$) between 131 and 135 days compared with other gestational periods. No significant effect of maternal undernutrition on fetal leptin concentrations was found, however, and no significant change in fetal plasma leptin concentrations was observed between 116 and 140 days of gestation in either the undernourished or the control group (Fig. 3C). Also, no difference was observed between plasma leptin concentrations in male and female fetuses.

No significant correlation was found between mean fetal plasma concentrations of leptin and either glucose or insulin when data from the undernourished and control groups were combined. Mean fetal (y) and maternal (x) plasma leptin concentrations were significantly correlated ($y = 0.18x + 0.45$; $r = 0.66$, $P < 0.003$, $n = 17$) (Fig. 2B). The mean fetal plasma leptin concentrations between 116 and 140 days of gestation were not correlated with either fetal body weight or with absolute or relative fetal fat mass at 144–147 days of gestation.

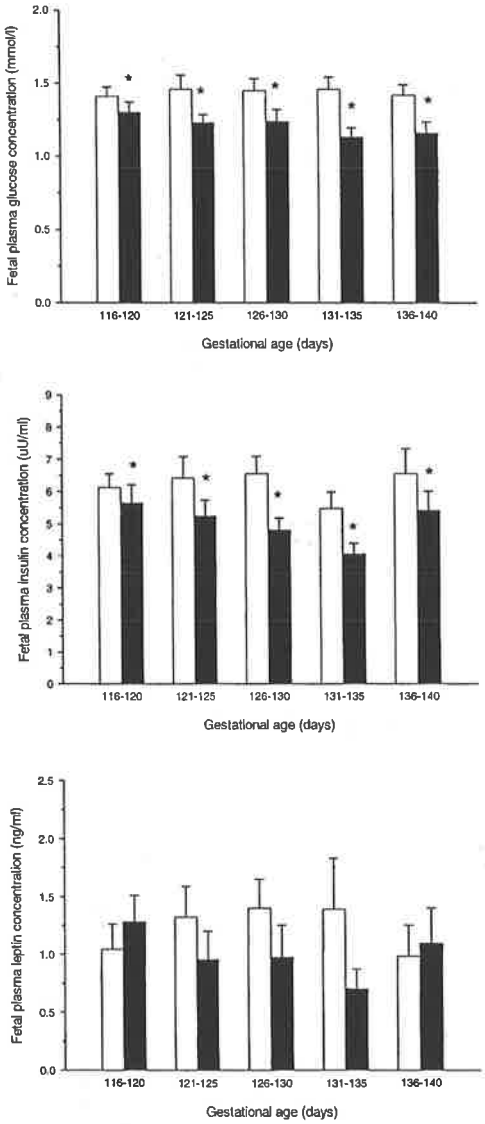

FIG. 3. Fetal plasma glucose (A), insulin (B), and leptin concentrations (B) in control (open histograms) and undernourished (dark histograms) groups between 116 and 140 days of gestation. Asterisks denote a significant effect of undernutrition on fetal glucose and insulin concentrations throughout late gestation.

Leptin mRNA Expression in Fetal Perirenal Adipose Tissue

No significant difference was found in the relative abundance of leptin mRNA in fetal perirenal fat between the undernourished (0.60 ± 0.09, n = 10) and control (0.70 ± 0.08, n = 10) groups. The mean fetal plasma concentrations of leptin (y) and the relative abundance of leptin mRNA (x) in perirenal adipose tissue were significantly correlated ($y = 1.5x + 0.3$; $r = 0.69$, $P < 0.05$, n = 9) (Fig. 4). Leptin mRNA expression in fetal adipose tissue was not related to either fetal weight ($P = 0.09$), fetal perirenal fat mass, mean fetal glucose, or insulin concentrations.

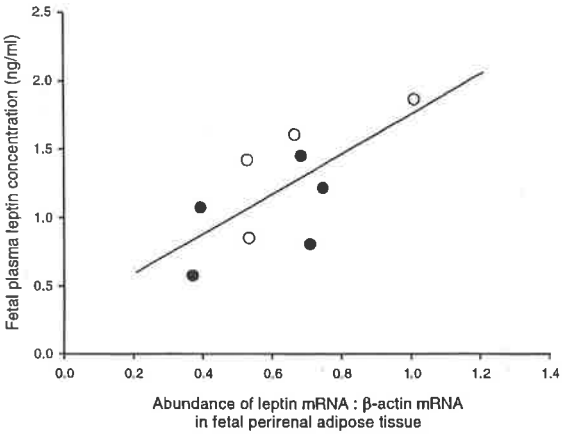

FIG. 4. Relationship between fetal plasma leptin concentrations and relative abundance of leptin mRNA in fetal perirenal adipose tissue in undernourished (closed symbols) and control (open symbols) groups.

DISCUSSION

In the present study, a positive relationship was found between mean plasma concentrations of leptin and glucose during the last 30 days of pregnancy when data from the control and well-fed ewes were combined. Thomas et al. [13] recently reported that when the dietary intake of adolescent pregnant ewes was increased from moderate to high or reduced from high to moderate at Day 50 of pregnancy, circulating maternal leptin concentrations changed within 48 h of the alteration in maternal diet. They suggested that this was likely to be a "direct" nutritional effect. These authors also found that at some 50–90 days after the change in diet, circulating leptin concentrations were correlated with indices of body composition in the pregnant ewe. They were unable, however, to distinguish whether dietary intake or changed body composition due to the nutritional treatments was the primary factor influencing circulating leptin concentrations. In the present study, differences in maternal body composition may also explain some of the variation in maternal leptin concentrations.

We have also found that maternal plasma leptin concentrations varied between 3 and 10 ng/ml throughout late pregnancy and that no significant change occurred in circulating leptin concentrations between 115 days of pregnancy and term in either control or undernourished adult ewes. These circulating leptin concentrations are similar to those reported by Thomas et al. [13] in moderately fed, adolescent pregnant ewes from 50 days of pregnancy until term. Those authors also found no significant change in plasma leptin concentrations throughout late pregnancy. The lack of a change in plasma leptin concentrations toward the end of pregnancy in the sheep is in contrast to the increase in plasma leptin concentrations that occurs during late pregnancy in the human [14], rat [15], and mouse [16]. Adipose tissue is the main source of circulating leptin in all species, but to what extent other tissues, such as the placenta, are also a source of leptin in the maternal circulation during late pregnancy is unclear. Leptin gene expression is relatively high in the human placenta [17] and is also detectable in the rodent placenta [18]. Species-specific differences in the relative level of placental leptin expression may account for differences in the effect of preg-

nancy on the maternal plasma concentrations of leptin between sheep and other species. The sheep placenta also expresses the leptin receptor gene [13], and it is therefore possible that maternal leptin may interact with leptin receptors within the placenta to impact on fetal growth and development. Overfeeding the adolescent ewe throughout pregnancy increases maternal growth at the expense of the placenta, leading to growth restriction of the fetus [19]. In a cohort of overfed and normally fed adolescent pregnant ewes, a negative association was found between maternal plasma leptin concentrations and birth weight, placental weight, and number of placentomes [13]. In the present study in the mature ewe, however, we found no significant relationship between maternal plasma leptin concentrations and fetal body weight. Clearly, further work is required to define the relative roles that maternal leptin and nutrients play in placental and fetal growth and development at different stages of reproductive maturity.

Plasma concentrations of leptin in the fetus (<0.3–3 ng/ml) were substantially lower than those in the pregnant ewe, and no effect of either maternal undernutrition or gestational age was found on circulating fetal leptin concentrations between 116 and 140 days of gestation. In a previous study [8], we reported that the abundance of leptin mRNA in fetal adipose tissue increased between 125 and 144 days of gestation. It may be that leptin concentrations increase in the fetal circulation after 140 days of gestation. We also found a positive relationship between fetal and maternal plasma concentrations of leptin during late gestation. One possible explanation is that maternal body composition or fatness either at the beginning or during pregnancy determines the leptin synthetic and secretory capacity of both maternal and fetal adipose tissue or the amount of fetal adipose tissue deposited during late gestation. A positive relationship was found between circulating fetal leptin and the relative abundance of leptin mRNA in fetal adipose tissue; however, no relationship was found with either maternal or fetal leptin concentrations or with the absolute or relative fetal fat mass. Thus, any impact of maternal body composition on circulating fetal leptin concentrations is presumably expressed through the leptin synthetic and secretory capacity of the fetal adipose tissue. An alternative explanation for the close correlation between maternal and fetal plasma leptin concentrations is that the placental leptin receptor may mediate the uptake of leptin from the maternal into the fetal circulation. This would be similar to the postulated mode of action for the short isoform of the leptin receptor in the choroid plexus epithelium to transport leptin from plasma into the cerebrospinal fluid [20].

In the present study, maternal feed availability was reduced by 50% below maintenance for 29–32 days, and this was associated with an ~16% fall in maternal glucose concentrations and a 20% fall in fetal plasma glucose and insulin concentrations. No significant effect, however, of this level of maternal undernutrition was found on the fetal plasma concentrations of leptin or on the relative abundance of leptin mRNA in the perirenal adipose tissue. It has recently been reported that continuous infusion of insulin into pregnant ewes for up to 34 days resulted in fetal hypoglycemia and hypoinsulinemia and reduced fetal body weight, but that no change occurred in the expression of leptin mRNA in fetal perirenal fat [21]. Those authors also reported, however, that if the period of continuous insulin infusion was prolonged beyond 36 days (36–76 days), then fetal glucose and insulin concentrations were reduced by 30–50% and leptin mRNA expression was suppressed in fetal perirenal fat [21]. Together, these studies indicate that the synthesis and secretion of leptin in the sheep fetus is resistant to the changes in fetal glucose and insulin concentrations associated with moderate maternal undernutrition. Fetal leptin synthesis is suppressed, however, in the presence of profound fetal hypoglycemia or hypoinsulinemia, which may occur as a consequence of either pharmacological induction of maternal hypoglycemia or severe maternal undernutrition.

In the human, strong positive associations exist between umbilical cord blood leptin concentrations at delivery and infant body weight at birth as well as with other anthropometric markers of fetal growth, including estimates of fetal fat mass [9, 10, 22–25]. We have also previously reported [8] that the abundance of leptin mRNA in fetal adipose tissue was positively correlated with fetal body weight in a cohort of fetuses at an earlier gestational age than those used in the present study. In the present study, however, whereas the relationship between leptin mRNA expression in fetal adipose tissue and fetal weight tended to be positive ($P = 0.09$), no relationship was found between circulating leptin concentrations and either fetal weight or relative fat mass. These differences between the sheep and the human may be explained, in part, by the different patterns of fat deposition that occur in these species during fetal life. In the sheep fetus, fat is deposited at ~0.8 g/kg fetal body weight per day, the proportion of body fat at term is ~0.3–2.0%, and the major fat depot is the perirenal adipose tissue, which is comprised predominantly of brown fat cells [26]. Whether leptin is expressed uniformly in all perirenal adipocytes in the sheep fetus before birth is unknown. In contrast, in the human fetus, fat is deposited at a higher rate (~3.5 g/kg fetal body weight per day), the proportion of body fat at term is ~16%, and subcutaneous fat depots are comprised predominantly of white fat cells [27]. Despite these differences between sheep and human fetuses in the rate of fat deposition, the leptin synthetic capacity of fat stores, and the effect of undernutrition on leptin concentrations during late gestation, it is interesting that perturbations of the intrauterine environment may program the development of postnatal obesity in these and other species. Restricted fetal nutrient supply programs alterations in adiposity or leptin synthesis beyond the postnatal period in the human [28], sheep [29], rat [30], and pig [31]. Further work is required to identify those periods during intrauterine life when changes in the long-term development of the adipocyte and the leptin signaling system are initiated and to clarify the relative importance of maternal body composition and the level of fetal nutrition in the mechanistic pathway that underlies the association between poor intrauterine growth and postnatal obesity.

In summary, we have reported, to our knowledge for the first time, the effect of maternal undernutrition during late pregnancy on maternal and fetal plasma concentrations of leptin and on leptin gene expression in fetal adipose tissue in the sheep. We have found that maternal plasma concentrations of leptin and glucose are positively correlated across the range of circulating glucose concentrations present in well-fed and undernourished pregnant ewes. Interestingly, we have found a positive relationship between the fetal and maternal plasma concentrations of leptin during late gestation, suggesting that maternal body composition during early pregnancy may determine the leptin synthetic and secretory capacity of maternal and fetal adipose tissue. We have found no effect, however, of maternal undernutri-

tion on circulating leptin concentrations or on the abundance of leptin mRNA in adipose tissue in the sheep fetus. The capacity of leptin to act as a signal of moderate maternal undernutrition may, therefore, be limited in this species before birth.

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Effects of leptin on fetal plasma ACTH and cortisol concentrations and the timing of parturition in the sheep

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Abstract

We have investigated whether leptin can suppress the prepartum activation of the fetal HPA axis and delay the timing of parturition in the sheep. Firstly, we investigated the effects of a 4 day intravascular infusion of recombinant ovine leptin (n=7) or saline (n=6) on fetal plasma ACTH and cortisol concentrations, starting from 136 days (d) gestation, *ie* at the onset of the prepartum activation of the fetal HPA axis. The effects of a continuous intrafetal infusion of leptin (n=7) or saline (n=5) from 144 d gestation on fetal plasma ACTH and cortisol concentrations and the timing of delivery were also determined in a separate study. There was an increase in fetal plasma ACTH ($P<0.01$) and cortisol ($P<0.001$) concentrations when saline was infused between 136/7 and 140/1 d gestation. Plasma ACTH and cortisol concentrations did not rise, however, when leptin was infused during this period of gestation. When leptin was infused after 144 d gestation, there was no effect of a 4-5 fold increase in circulating leptin on fetal ACTH concentrations. In contrast, leptin infusion from 144 d gestation suppressed ($P<0.05$). fetal plasma cortisol concentrations by around 40% between 90 and 42 h before delivery. There was no difference, however, in the length of gestation between the saline and leptin infused groups (saline infused, 150.2 ± 0.5 d; leptin infused, 149.8 ± 1.0 d). In saline infused fetuses, there was a significant negative relationship between the plasma concentrations of cortisol (y) and leptin (x) between 138 and 146 d gestation ($y = 81.4 - 7.7x$, $r=0.38$, $P<0.005$). This study provides evidence for an endocrine negative feedback loop between leptin and the HPA axis in fetal life.

48 **Introduction**

49 Leptin is a 16 kDa polypeptide hormone, which is principally synthesised and
50 secreted by adipose tissue and which acts to regulate energy homeostasis and a range
51 of neuroendocrine and reproductive functions [1-3]. In the human infant, there is a
52 positive relationship between cord blood concentrations of leptin at delivery and
53 either birth weight or neonatal adiposity [4-6]. In animal species such as the sheep and
54 pig, in which fat is deposited before birth, leptin is synthesised in fetal adipose tissue
55 and is present in the fetal circulation throughout late gestation [7-13]. In the sheep
56 fetus, the expression of leptin mRNA in fetal adipose tissue is positively correlated
57 with circulating leptin concentrations and there is also a positive relationship between
58 fetal plasma leptin concentrations and the relative mass of lipid locules present within
59 fetal adipose tissue [11,13]. We have recently shown that intravascular infusion of
60 leptin in the sheep fetus during late gestation altered the lipid storage characteristics
61 and suppressed leptin mRNA expression within fetal adipose tissue [14]. Thus leptin
62 may act as a circulating signal of fetal adiposity and have a 'lipostatic' role before
63 birth.
64
65 It is well established in the sheep that the prepartum increase in circulating cortisol is
66 required for the differentiation and maturation of key fetal organs such as the fetal
67 lung, liver, kidney and brain and for the normal timing of parturition and the
68 successful transition to extrauterine life [16]. Forhead and colleagues reported that in
69 the sheep fetus, plasma cortisol and leptin concentrations increased in parallel and
70 were positively related between 130 and 140 d gestation and that fetal adrenalectomy
71 resulted in lower plasma leptin concentrations after 136 d [10]. These findings are
72 consistent with studies which have demonstrated that glucocorticoids stimulate both

leptin gene expression and secretion from adult adipocytes *in vivo* and *in vitro* [17-20] and suggest that there is a positive relationship between the level of activation of the fetal HPA axis and leptin synthesis and/or secretion in late gestation. A separate study however, investigated the effects of intracerebroventricular (icv) infusion of leptin between 135 and 140 d gestation on the characteristics of plasma ACTH and cortisol pulses occurring during a 4 h sampling period on the first and last day of the infusion period [15]. These authors found that icv leptin administration blunted the size of the increase which occurred in the amplitude and mean value of plasma ACTH and cortisol pulses between 135 and 140 d gestation [15]. These data are consistent with studies in the adult rat which have shown that administration of leptin can attenuate fasting or restraint induced stimulation of the HPA axis [21-22].

Given the conflicting nature of the previous studies, we have directly determined whether leptin can act to suppress the normal prepartum activation of the fetal HPA axis and delay the timing of parturition. Firstly, we measured the effects of a 4 d intrafetal infusion of leptin on fetal plasma ACTH and cortisol concentrations starting from 136 d gestation, ie at the onset of the prepartum activation of the fetal HPA axis. Secondly, we infused leptin into fetal sheep from 144 d gestation until delivery and measured the effects of an increase in circulating leptin on the prepartum changes in fetal plasma ACTH and cortisol concentrations and on the timing of parturition.

Materials And Methods

Animals and Surgery

These studies were approved by the University of Adelaide Animal Ethics Committee. Dated pregnant Merino ewes (n=25) were housed in individual pens in

98 rooms with a 12 h light/dark cycle and fed once daily (~10-12 MJ/kg metabolisable
99 energy) with a diet consisting of lucerne chaff (85% dry matter) and concentrated
100 pellets containing: straw, cereal, hay, clover, barley, oats, lupins, almond shells, oat
101 husks and limestone (90% dry matter; Johnsons and Sons, Kapunda, SA, Australia) at
102 1100 h with water provided ad libitum. Surgery was performed between 110 and 126
103 d gestation, as previously described [13,23]. Briefly, general anaesthesia was induced
104 in ewes by an intravenous injection of sodium thiopentone (1.25 g iv, Pentothal,
105 Rhone Merieux, Pinkenba, Qld, Australia) and maintained by 2.5 - 4% halothane
106 (Fluothane, ICI, Melbourne, Vic, Australia). Under aseptic conditions catheters were
107 inserted into a maternal jugular vein, a fetal carotid artery and jugular vein, and the
108 amniotic cavity. Catheters were filled with heparinised saline and the fetal vascular
109 and amniotic catheters exteriorised through an incision made in the ewe's flank. Ewes
110 and their fetuses received a 2 ml intramuscular injection of antibiotics (procaine
111 penicillin 250 mg/ml; dihydrostreptomycin 250 mg/ml; procaine hydrochloride 20
112 mg/ml Penstrep Illium, Troy Laboratories, Smithfield, NSW, Australia). Animals
113 were allowed to recover for at least 4 d after surgery before routine fetal arterial blood
114 samples (3 ml) were collected every 2 - 3 d before the infusion studies commenced.
115
116 ***Experimental Protocols***
117 *Leptin infusion from 136 or 137 d gestation*
118 In thirteen pregnant ewes at 136/137 d gestation, fetal arterial blood samples (3 ml)
119 were collected at -3 h, -2 h, -1 h and -30 min relative to the start of the infusion period
120 at 1300 h. A bolus of either sterile saline (0.5 ml, n=7) or recombinant ovine leptin
121 (0.25 mg in 0.5 ml sterile saline, n=6; provided by Professor Duane Keisler,
122 Department of Animal Sciences, University of Missouri, Columbia, MO, USA) was

123 infused into the fetal jugular vein, immediately followed by a continuous infusion
124 (0.16 ml/h) of either sterile saline or leptin (0.48 mg/kg/d) [24], respectively. Fetal
125 arterial blood samples were collected at +2 min, +30 min, +1 h, +2 h, +4 h and +8 h
126 on the first day of the infusion and at 0900 h, 1300 h and 1700 h on the second and
127 third days and at 0900 h and 1300 h on the fourth day of the infusion. Blood samples
128 were centrifuged at 1500 g for 10 min and plasma aliquots were separated and stored
129 at -20°C. Fetal arterial blood samples (0.5 ml) were also collected daily to monitor
130 fetal blood gases and pH (ABL 520 blood gas analyser, Radiometer, Copenhagen,
131 Denmark). After 96 h (at 140 or 141 d gestation), ewes were killed with an overdose
132 of sodium pentobarbitone (Virbac Pty Ltd, Peakhurst, NSW, Australia) and fetuses
133 (saline infused group: 5 singletons and 2 twins; leptin infused group: 3 singletons and
134 3 twins) delivered by hysterotomy, weighed and decapitated.
135
136 *Leptin infusion from 144 d gestation*
137 In 12 pregnant ewes at 144 d gestation, a bolus of saline (n=5) or recombinant ovine
138 leptin (n=7, 0.5 mg/0.5 ml sterile saline) was infused into the fetal jugular vein
139 immediately followed by a continuous infusion of saline or recombinant ovine leptin
140 (1.0 mg/kg/d). On the first day of infusion, fetal arterial blood samples (3.5 ml), were
141 collected at -3 h, -2 h, -1 h and -30 min and at +2 min, +30 min, +1 h, +2 h, +4 h and
142 +8 h relative to the start of the infusion at 1300 h. On the second, third and fourth
143 days of the infusion, fetal blood samples were collected at 0900 h, 1300 h and 1700 h
144 and on subsequent days, fetal blood samples were collected at 0900 h and 1700 h until
145 either the ewe was in late labour (n=2) or the fetus delivered (n=10). There were 4
146 singletons and 1 twin lamb (3 male, 2 female) in the saline infused group and 7
147 singletons (4 male, 2 female, 1 unknown) in the leptin infused group. Ewes were

148 defined as being in late labour when the pressure of repeated intrauterine contractions
149 were greater than 20 mmHg in amplitude. Intrauterine pressure was measured using a
150 MacLab 1050 displacement transducer (ADInstruments, NSW, Australia) connected
151 to the saline filled amniotic catheter [24]. A MacLab data acquisition system was
152 attached to the transducer and MacLab Chart software was used to analysis the
153 intrauterine pressure recordings.
154
155 ***Leptin ELISA***
156 A competitive ELISA specific for ovine leptin was used to measure plasma leptin
157 concentrations in fetal sheep, as previously described [13,25]. Briefly, 6 ng
158 recombinant bovine leptin was coated onto an ELISA plate by overnight incubation at
159 37°C. The plate was blocked with 200 µl of 5% skim milk in ELISA buffer for 1 h at
160 37 °C. Fetal plasma samples (100 µl) were added to wells containing a biotinylated
161 chicken anti-recombinant bovine leptin in 100% Triton X 100, 0.5% SDS and 5%
162 sodium deoxycholate (50 µl) and the plate was incubated overnight at 37°C. A
163 biotinylated second antibody was added to the plate and incubated overnight at 37°C.
164 The plate was then washed and incubated for 1 h with streptavidin conjugated to
165 alkaline phosphatase (Amrad Biotech, Boronia, Vic, Australia) then developed with p-
166 nitrophenylphosphate disodium salt hexahydrate. The sensitivity of the assay was 0.5
167 ng/ml and the inter assay and intra assay coefficients of variation were 11% and 9%
168 respectively.
169
170 ***ACTH radioimmunoassay***
171 ACTH concentrations in fetal sheep plasma were measured by radioimmunoassay
172 (DiaSorin, Stillwater, Minnesota, USA), previously validated for fetal sheep plasma

[26]. The cross-reactivity of the rabbit anti-ACTH antisera was <0.01% with α-MSH, β-endorphin, β-lipotropin, parathyroid hormone, vasopressin and growth hormone. Briefly, rabbit anti-ACTH serum (50 µl) was added to each sample (50 µl) and incubated overnight at 4°C. Radiolabelled [I^{125}]-ACTH (50 µl) was added to each tube and incubated overnight at 4°C. Rabbit serum (200 µl), pre-precipitated with goat anti-rabbit serum and polyethylene glycol, was added to samples that were then centrifuged. The inter assay coefficient of variation was 11.5% and the intra assay coefficient of variation was 5.2%.

Cortisol radioimmunoassay

Cortisol was extracted from fetal plasma using dichloromethane as previously described [27]. The efficiency of recovery of radiolabelled [I^{125}]-cortisol from fetal plasma using this extraction procedure was >90%. The cross-reactivity of the rabbit anti-cortisol antisera was <1% with pregnenolone, aldosterone, progesterone and oestradiol. Fetal cortisol concentrations were then measured using a Amersham Radioimmunoassay kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA). Briefly, 100 nM of hydrocortisone (Sigma Chemical Co., St.Louis MO, USA) was serially diluted in buffer (0.1 mol/l Tris-HCl, pH 7.4, 0.5% BSA, 0.1% sodium azide) to generate a standard curve. Plasma extracts (100 µl) were incubated with rabbit anti-cortisol antisera (100 µl) overnight at 4°C. Radiolabelled [I^{125}]-cortisol (100 µl) was then added to the samples that were then incubated overnight at 4°C. The inter- and intra-assay coefficients of variation were 10% and 5% respectively.

196 ***Statistical analyses***

197 Data are presented as the mean ± standard error of the mean (SEM). Fetal hormone
198 data were logarithmically transformed where required to reduce heterogeneity of
199 variance. Analyses of Variance (ANOVA) with repeated measures were performed
200 using the Statistical Package for Social Sciences (SPSSX, Chicago, IL, USA) on a
201 VAX mainframe computer. The Duncan's new multiple range test was used post hoc
202 to identify significant differences between mean values.
203
204 *Leptin infusion from 136/137 d gestation*
205 Mean values for fetal arterial pO_2, pCO_2 and pH between 0 and +96 h were calculated
206 and a Student's unpaired t-test was performed to determine whether fetal blood gases
207 and pH values were different between the saline and leptin infused groups.
208
209 A mean value for the basal plasma concentration of leptin, ACTH or cortisol was
210 calculated for each fetus as the average of the 5 values during the pre-infusion period.
211 The change in fetal hormones during the infusion period was then calculated by
212 subtraction of the mean preinfusion hormonal value at each time point and the effects
213 of leptin infusion on fetal hormone concentrations were analysed using a two way
214 ANOVA with treatment and the length of time relative to the start of the infusion as
215 the specified factors.
216
217 *Leptin infusion from 144 d gestation*
218 The effects of leptin on fetal hormone concentrations during the first 20 h of the
219 infusion period were determined by a two way ANOVA with treatment and time
220 relative to the start of the infusion as the specified factors.As the length of gestation

varied between animals (147-153 d), the hormonal data from each animal were expressed relative to the known or estimated time of delivery. Intrauterine pressure traces from animals that delivered were analysed to establish the relationship between frequency of contractions with an amplitude of >20 mmHg and time before birth. For the 2 fetuses that were killed during late labour, the intrauterine pressure traces were analysed to determine the frequency of contractions (>20 mmHg) during labour and the time of delivery was then estimated. Hormone data were then grouped into 12h time blocks in relation to the actual or estimated time of delivery. In the leptin infused group, 94 plasma samples were assayed for ACTH, 93 samples were assayed for cortisol and 92 samples were assayed for leptin. In the saline infused group, 67 plasma samples were assayed for ACTH, 68 samples were assayed for cortisol and 38 samples were assayed for leptin. The effects of leptin infusion on fetal leptin, ACTH and cortisol concentrations were determined by a two way ANOVA using treatment and time before birth as the specified factors.

Linear regression analysis was used to assess relationships between the plasma leptin and either ACTH or cortisol concentrations in samples collected between 125 to 137 d *ie* prior to the onset of the prepartum increase in circulating cortisol and between 138 to 146 d gestation *ie* after the onset of the prepartum increase in cortisol in fetuses which were infused with saline from 144 d gestation.

Results

243 ***Leptin infusion from 136/137 d gestation***

244 *Plasma leptin concentrations and fetal blood gas status*

245 Plasma leptin concentrations increased ($P<0.001$) during the leptin infusion period
246 (+24 h: 22.9 ± 3.9 ng/ml, +92-96 h, 20.1 ± 1.5 ng/ml) but not during the saline
247 infusion period (+24h: 1.9 ± 0.7 ng/ml, +92-96h, 4.1 ± 1.6 ng/ml). There was no
248 significant difference in mean fetal arterial blood gas and pH values between the
249 saline and leptin infused groups during the infusion period (pO_2 - saline infused, 21.7
250 ± 0.6 mmHg; leptin infused, 22.3 ± 0.5 mmHg, pCO_2 - saline infused, 50.4 ± 1.2
251 mmHg; leptin infused, 49.1 ± 0.8 mmHg, pH - saline infused, 7.404 ± 0.006; leptin
252 infused, 7.401 ± 0.005).

253

254 *Fetal plasma ACTH and cortisol concentrations*

255 During the infusion period, there was a significant interaction between the effects of
256 treatment and time on ACTH concentrations expressed relative to those in the
257 preinfusion period ($P<0.05$; Figure 1A and B). In the saline infused group, the change
258 in plasma ACTH concentrations relative to baseline values was greater at 96 h (13.7 ±
259 7.8 pg/ml, $P<0.01$) than at between 2 h and 44h after the start of the infusion (-6.8 ±
260 3.4 pg/ml). In contrast, in fetuses infused with leptin, there was no change in plasma
261 ACTH concentrations during the 96 h infusion period (+96 h: -4.1 ± 2.0 pg/ml). There
262 was a significant interaction between the effects of treatment and time on cortisol
263 concentrations relative to basal levels ($P<0.02$; Figure 1C and D). In the saline
264 infused group, the change in plasma cortisol concentrations was greater at 96 h (54.1
265 ± 7.5 nmol/l; $P<0.001$) when compared with between 3 h before and 24 h after the
266 start of the infusion (14.9 ± 2.9 nmol/l). In fetuses infused with leptin, there was no
267 change, however, in plasma cortisol concentrations throughout the infusion period

(+96 h: 7.0 ± 3.9 nmol/l). The ratios of plasma cortisol : ACTH concentrations were significantly higher (P<0.001) in both treatment groups at +96 h (saline infused: 1.66 ± 0.23 nmol/ng, leptin infused: 1.12 ± 0.25 nmol/ng), when compared to the period from 3 h before until 52 h after the start of the saline (0.82 ± 0.26 nmol/ng) or leptin (0.63 ± 0.22 nmol/ng) infusion (Figure 1E and F).

Fetal plasma leptin, cortisol and ACTH concentrations in saline infused fetuses between 125 and 150 d gestation

There was a significant increase in plasma cortisol concentrations between 125 and 150 d gestation in saline infused fetuses. There was no change, however, in plasma leptin concentrations in these fetuses during this period (Figure 2). Whilst there was no relationship between fetal plasma cortisol (y) and leptin (x) concentrations at 125 - 137 d gestation, there was a significant negative relationship between the plasma concentrations of these two hormones at 138 - 146 d gestation (y = 81.4 - 7.7x, n=84, r=0.38, P<0.005) (Figure 3).

Leptin Infusion from 144 d gestation

Plasma leptin, ACTH and cortisol concentrations during the first 20 h of the infusion period

During the first 20 h of infusion, plasma leptin concentrations increased significantly (P<0.001) in the leptin infused (+20 h: 18.3 ± 2.1 ng/ml and not in the saline infused fetuses (+20 h: 2.0 ± 0.7 ng/ml). At 20 h after the start of the infusion there was no difference in either plasma cortisol (saline infused, 61.4 ± 5.1 nmol/l; leptin infused, 81.4 ± 22.2 nmol/l) or ACTH (saline infused, 41.0 ± 4.9 pg/ml; leptin infused, 34.1 ± 5.0 pg/ml) concentrations between the saline and leptin infused groups.

293

294 *Effects of leptin on the timing of delivery and on plasma ACTH and cortisol*
295 *concentrations preceding delivery*

296 There was no difference in the length of gestation (saline infused, 150.2 ± 0.5 d; leptin
297 infused, 149.8 ± 1.0 d) or birth weight (saline infused 4.9 ± 0.3 kg; leptin infused 5.2
298 ± 0.1 kg) between the saline and leptin infused groups.

299

300 Circulating leptin concentrations were higher in leptin infused than saline infused
301 fetuses from 114 h before and up to delivery (leptin infused: 16.3 ± 2.9 ng/ml; saline
302 infused, 2.5 ± 0.7 ng/ml; $P<0.001$) (Figure 4).

303

304 There was no significant difference in mean fetal arterial blood gas and pH values
305 between the saline and leptin infused groups during the infusion period (pO_2 - saline
306 infused, 20.4 ± 1.3 mmHg; leptin infused, 20.1 ± 1.2 mmHg, O_2 saturation- saline
307 infused, 52.8 ± 2.8 mmHg; leptin infused, 52.3 ± 5.4 mmHg, pH - saline infused,
308 7.396 ± 0.008; leptin infused, 7.372 ± 0.032).

309

310 There was no difference in plasma ACTH concentrations between the saline infused
311 (70.8 ± 46.8 pg/ml) and leptin infused (69.7 ± 22.6 pg/ml) fetuses during the period
312 114 – 6 h before delivery. In both the saline and leptin infused groups, plasma ACTH
313 concentrations were significantly higher ($P<0.001$) during the period from 18 to 6 h
314 before delivery when compared with either 90 - 78 h or 114 - 102 h before delivery
315 (Figure 5A).

316

317 There was a significant interaction ($P<0.05$) between the effects of leptin infusion and
318 time before delivery on fetal plasma cortisol concentrations. Between 90 and 42 h
319 before delivery, circulating cortisol concentrations were significantly higher in the
320 saline infused fetuses compared to the leptin infused fetuses (90 - 42 h: saline
321 infused, 142.5 ± 27.6 nmol/l; leptin infused, 84.3 ± 22.7 nmol/l; $P<0.05$; Figure 5B).
322 During the period 42 to 6h before delivery, however, there was no difference in
323 plasma cortisol concentrations between the saline infused (188.0 ± 42.4 nmol/l) and
324 leptin infused groups (244.5 ± 69.3 nmol/l). Plasma cortisol concentrations ($P<0.001$)
325 were highest in both leptin and saline infused groups from between 30 and 6 h before
326 delivery (Figure 5B).
327
328 There was a significant interaction between the effects of treatment and the time
329 relative to delivery on the ratio of plasma cortisol : ACTH concentrations ($P<0.005$)
330 (Figure 5C). There was no significant difference between the saline and leptin infused
331 fetuses in the ratio of plasma cortisol : ACTH concentrations between 114 and 90 h
332 before delivery (saline infused: 1.89 ± 0.45, leptin infused: 2.04 ± 0.42). The plasma
333 cortisol : ACTH ratios were lower, however, in the leptin infused fetuses between 90
334 and 30 h before delivery (saline infused, 2.78 ± 0.53; leptin infused, 1.62 ± 0.34)
335 (Figure 5C).
336
337 **Discussion**
338 We have demonstrated that infusion of leptin into fetal sheep, resulting in a 4-5 fold
339 increase in circulating leptin concentrations suppressed the normal increase in fetal
340 cortisol concentrations at the onset of the prepartum activation of the fetal HPA
341 between 136 and 140 days gestation. Furthermore, intrafetal infusion of leptin from

144 days gestation until delivery also suppressed fetal plasma cortisol concentrations for an extended period from 90 to 42 h before delivery. Whilst plasma cortisol concentrations were reduced by around 40% during this period in the leptin infused group, there was no difference in the timing of parturition between leptin and saline infused fetuses.

In the present study, there was an increase in fetal plasma ACTH and cortisol concentrations when saline was infused for a 96 h period between 136 and 141 days gestation as expected. There was no increase, however, in either plasma ACTH or cortisol concentrations when leptin was infused during this gestational age range. In a previous study, Howe and colleagues [15] infused leptin via the lateral cerebral ventricle in fetal sheep between 135 and 140 days gestation and measured plasma ACTH and cortisol concentrations during a 4 h sampling period at 135 days and at 140 days gestation. They found that the increases in the mean value and amplitude of the pulses in plasma ACTH and cortisol concentrations between 135 and 140 days gestation were less in the leptin infused compared to the vehicle infused fetuses.

In the present study when leptin was infused continuously from 144 days gestation, there was no effect on fetal ACTH concentrations during the week before delivery. In marked contrast, leptin infusion from 144 days gestation suppressed fetal plasma cortisol concentrations and the ratio of fetal plasma cortisol : ACTH concentrations for an extended period from 90 h until around 42 to 30 h before delivery. The suppression of fetal plasma cortisol concentrations and the decrease in the ratio of plasma cortisol : ACTH concentrations was not maintained, however, during the last 30 h before delivery despite continued infusion of leptin. Plasma cortisol

concentrations were similar in both the leptin and saline infused fetuses on the day before delivery and there was no difference between these two groups in the timing of delivery. In summary, evidence from the current study suggests that an increase in circulating leptin concentrations in the fetus during late gestation can blunt the prepartum activation of the HPA axis but not block or delay the timing of delivery. Whilst there may be a transient impact of leptin on fetal plasma ACTH concentrations during the early phase of activation of the fetal pituitary-adrenal axis in late gestation, the predominant action of leptin appears to be to suppress the normal prepartum increase in circulating cortisol and adrenal responsiveness to ACTH.

In adult sheep it has been demonstrated that icv infusion of leptin suppressed food intake and resulted in a decrease in the expression of the mRNA for the orexigenic peptide, Neuropeptide Y (NPY) in the hypothalamic arcuate nucleus [28]. This is consistent with the localisation of the long form of the leptin receptor in around 60% of NPY containing cells in the sheep hypothalamus [29]. There is also evidence in the sheep that hypothalamic NPY can regulate the synthesis and secretion of the ACTH secretagogues, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) [30]. NPY is present within the arcuate nucleus of the fetal sheep hypothalamus during late gestation [31] and it is possible that leptin acts centrally via leptin receptors located within the fetal hypothalamus to suppress NPY, CRH and/or AVP secretion and hence result in a decrease in fetal plasma ACTH concentrations in late gestation. It appears from the present study, however, that any inhibitory effect of an increase in circulating leptin concentrations on fetal ACTH secretion is not maintained during the week before delivery. The sustained increase in circulating leptin concentrations may induce resistance to the central actions of leptin as this has

392 been proposed to underly reduced sensitivity to peripherally administered leptin in
393 genetically wild type mice, primates and lambs [32-34]. There is evidence that high
394 circulating leptin concentrations may induce a decrease in the transport or access of
395 leptin to the brain [35-37]. Whilst this is possible, it should be noted that we found no
396 evidence that leptin infusion at 144 days resulted in an initial decrease in fetal ACTH
397 concentrations during the first day of the infusion period. An alternative explanation is
398 that the hypothalamic mechanisms which stimulate fetal pituitary ACTH synthesis
399 and secretion during the prepartum period are not suppressed by an increase in
400 peripheral leptin concentrations.
401
402 A range of studies have reported that that the long form of the leptin receptor is
403 expressed in human, rat and mouse adrenal and that leptin acts directly to inhibit
404 ACTH stimulated glucocorticoid secretion by the bovine [17], human and rat adrenal
405 gland [38]. Leptin acts to decrease the expression of the steroidogenic enzymes,
406 cytochrome P450 C21-hydroxylase, side chain cleavage and C17 α hydroxylase in the
407 bovine adrenal and it has recently been reported that leptin reduces the ACTH
408 stimulation of steroidogenic acute regulatory protein (StAR) expression in the rat
409 adrenal [39-40]. It has been proposed that in the adult, a leptin mediated feedback
410 loop exists between adipose tissue and the HPA axis, as glucocorticoids can stimulate
411 leptin expression and secretion from the adipocyte [18,20] whereas rising circulating
412 leptin concentrations can directly down regulate adrenal cortisol synthesis and
413 secretion. Thus it is possible that leptin acts directly at the fetal adrenal and that there
414 is a similar endocrine feedback loop between fetal adipose tissue and the HPA axis in
415 late gestation.
416

In the present study, intrafetal leptin infusion resulted in circulating leptin concentrations of around 15-20 ng/ml. Whilst these concentrations are similar to those measured in well-fed pregnant ewes where rapid maternal weight gain has occurred [41], they are significantly higher than those measured by us or others in the fetal sheep of well nourished ewes in late gestation [9,11,13,42]. It has been shown, however, that fetal plasma leptin concentrations are increased up to 9 fold higher in human pregnancies which are complicated by maternal glucose intolerance and fetal hyperglycaemia when compared with fetuses in normal pregnancies [43-45] and in these pregnancies it is possible that such an increase in fetal leptin concentrations may regulate adrenal responsiveness to ACTH and other stimulatory hormones. What is currently unclear is the extent of the endocrine interaction between fetal adipose tissue and the HPA axis in normal pregnancy. In the sheep fetus, circulating leptin concentrations are positively correlated with the relative mass of lipid stored in dominant cellular lipid locules within the fetal perirenal adipose tissue [11] and leptin is therefore an endocrine signal of the lipid storage capacity of this tissue. Forhead and colleagues [10] have reported that plasma cortisol and leptin concentrations increase in parallel during late gestation and are positively related between 130 and 140 days in the sheep fetus. Furthermore they reported that fetal adrenalectomy resulted in lower plasma leptin concentrations in fetal sheep after 136 days [10]. Cortisol infusion or fetal adrenalectomy, however, did not alter leptin mRNA levels in perirenal adipose tissue in the late gestation sheep fetus [46]. In the present study we found that in saline infused fetuses, there was no change in fetal plasma leptin concentrations during the last 3 weeks of gestation and there was also no relationship between plasma cortisol and leptin concentrations between 125 and 137 days gestation. The difference between studies in the extent to which plasma cortisol and

leptin are related between 130 and 140 days gestation may be related to the differences in circulating fetal leptin concentrations between the sheep breeds used in the studies. The fetus of the Welsh mountain ewe appears relatively hypoleptinaemic [10] when compared with the fetus of the Merino ewe used in the current and previous studies [11,13].

One further potential source of circulating leptin in the fetus is the placenta. Whilst the placenta has been proposed as a possible source of fetal leptin in the hman, baboon and rat [47-50], the levels of leptin mRNA present in the sheep placenta are negligible [9,41]. It should be noted, however, that the leptin receptor is expressed in the sheep placenta [41], that there is evidence for transplacental transfer of leptin in the rat [51] and that maternal and fetal plasma leptin concentrations are correlated during late gestation in the sheep [13]. Whether there is a major contribution of maternal leptin to circulating leptin in the fetus and the extent to which this may vary across different breeds of sheep has yet to be determined.

In the present study, there was a negative relationship between circulating cortisol and leptin in the fetus in the week before delivery such that around 14% of the variation in plasma cortisol in the saline infused group was explained by the variation in fetal leptin concentrations. Thus whilst the initiation of the prepartum increase in fetal plasma cortisol does not appear to be related to any concomitant fall in circulating leptin, leptin may act to inhibit the output of cortisol from the fetal adrenal during the week before delivery.

In summary we have demonstrated that an increase in circulating leptin concentrations in fetal sheep suppressed the normal increase in fetal cortisol concentrations at the onset of the prepartum activation of the fetal HPA between 136 and 140 days gestation. Furthermore intrafetal infusion of leptin from 144 days gestation until delivery also suppressed fetal plasma cortisol concentrations for an extended period from between 90 - 42 h before delivery, although there was no difference in the timing of parturition between the leptin and saline infused groups. This study provides evidence therefore that fetal hyperleptinaemia, which is present in pregnancies complicated by gestational diabetes, may act to limit the fetal adrenal responsiveness to ACTH and other trophic factors during the transition from intrauterine to extrauterine life.

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484

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FIGURE LEGENDS

Figure 1. Plasma ACTH, cortisol and the ratio of plasma cortisol:ACTH concentrations in fetuses infused with saline (open circles; A,C,E) or leptin (closed circles; B,D,F) for 96 h from 136/137 d gestation. Different alphabetic superscripts denote mean values which are significantly different ($P<0.05$) from each other within a treatment group.

Figure 2. Plasma cortisol and leptin concentrations between 125 and 150 d gestation in fetal sheep which were infused with saline from 144 d gestation. Different alphabetic superscripts denote mean hormone values which are significantly different ($P<0.05$) from each other during late gestation.

Figure 3. The relationship between plasma cortisol and leptin concentrations between 138 and 146d gestation in those fetal sheep which were infused with saline from 144d gestation. The relationship is described by the equation, $y=81.4 - 7.7x$ ($r=0.38$, $P<0.05$).

Figure 4. Plasma leptin concentrations in saline infused (open bars) and leptin infused (closed bars) fetuses during the period from 114 h until 6 h before birth.

Figure 5. Plasma ACTH (A), cortisol (B) and the ratio of plasma cortisol : ACTH (C) concentrations in saline infused (open bars) and leptin infused (closed bars) fetuses from 114h before delivery. Asterisks denote significant differences ($P<0.05$) between mean values in the saline and leptin infused groups.

Figure 1.

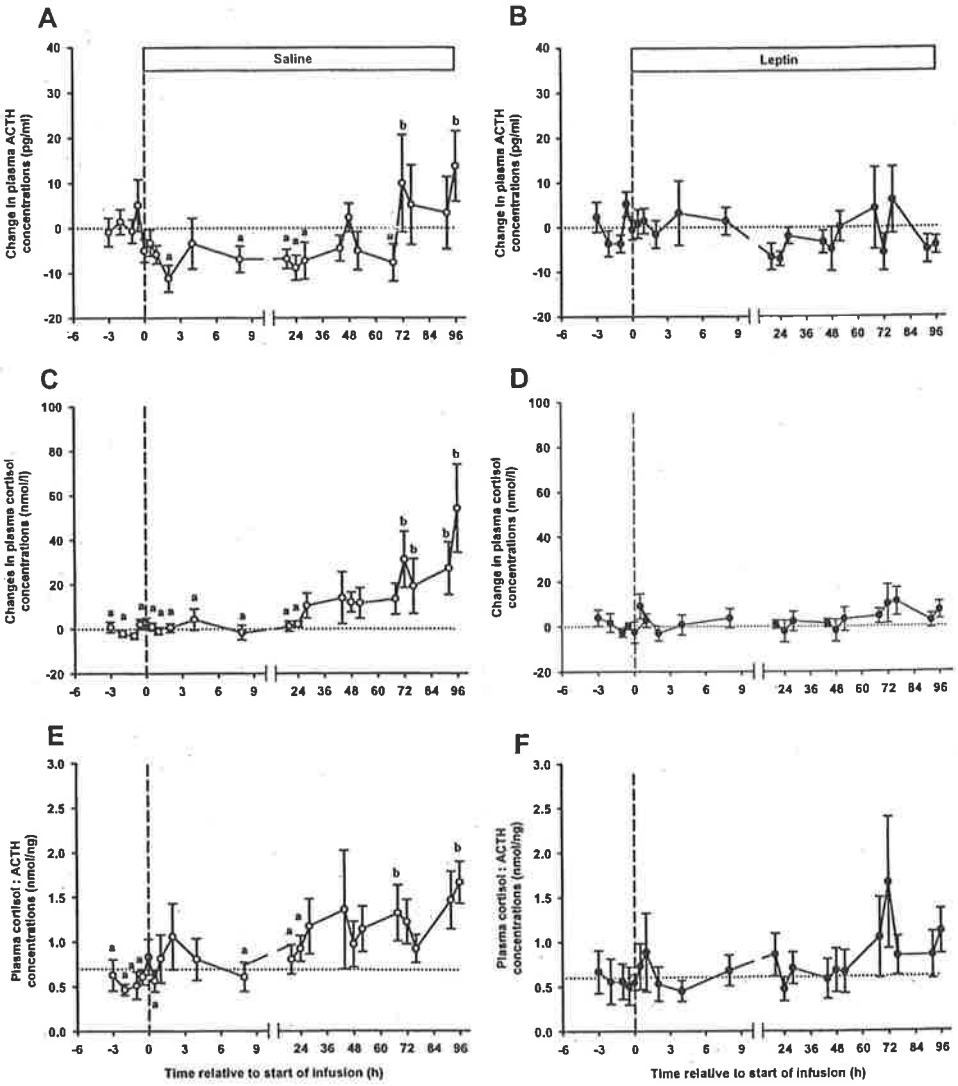

A
Saline
B
Leptin
C
D
E
F
Change in plasma ACTH concentrations (pg/ml)
Changes in plasma cortisol concentrations (nmol/l)
Change in plasma cortisol concentrations (nmol/l)
Plasma cortisol : ACTH concentrations (nmol/ng)
Time relative to start of infusion (h)

Figure 2.

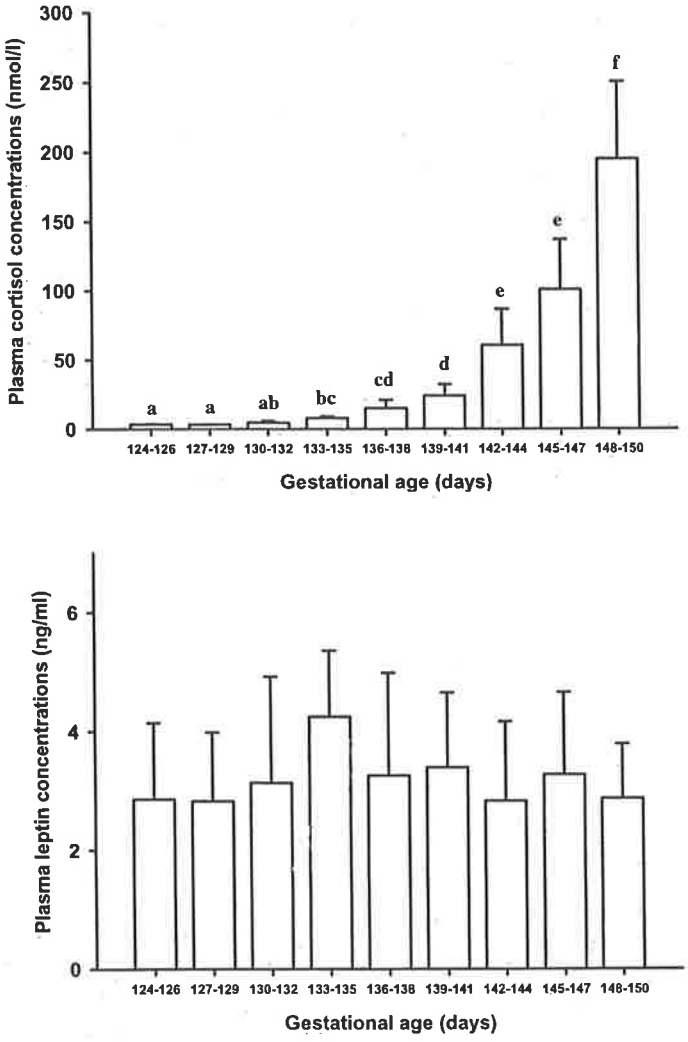

Plasma cortisol concentrations (nmol/l)
300
250
200
150
100
50
0
a
a
ab
bc
cd
d
e
e
f
124-126
127-129
130-132
133-135
136-138
139-141
142-144
145-147
148-150
Gestational age (days)
Plasma leptin concentrations (ng/ml)
6
4
2
0
124-126
127-129
130-132
133-135
136-138
139-141
142-144
145-147
148-150
Gestational age (days)

Figure 3.

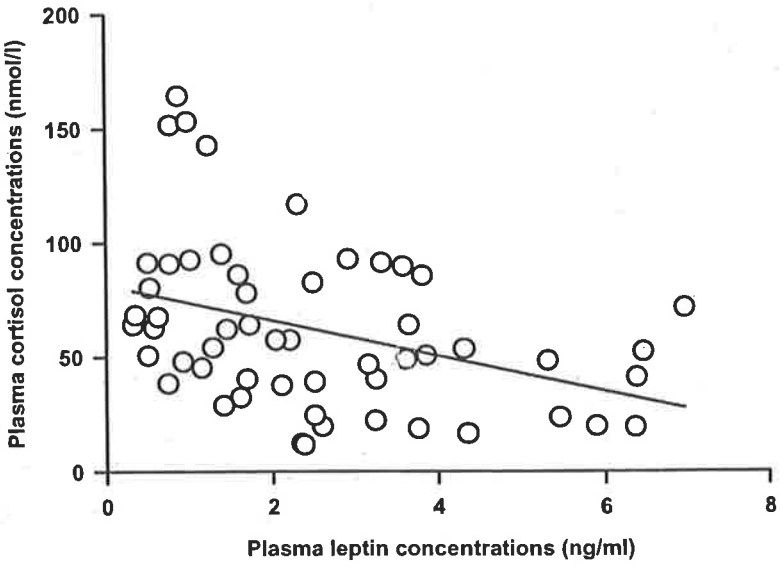

Plasma cortisol concentrations (nmol/l)
200
150
100
50
0
0
2
4
6
8
Plasma leptin concentrations (ng/ml)

Figure 4.

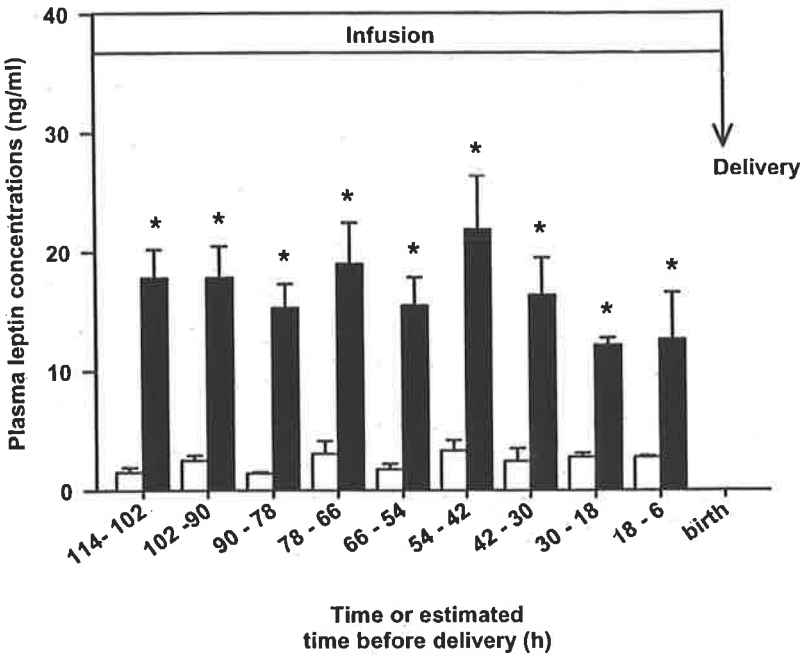
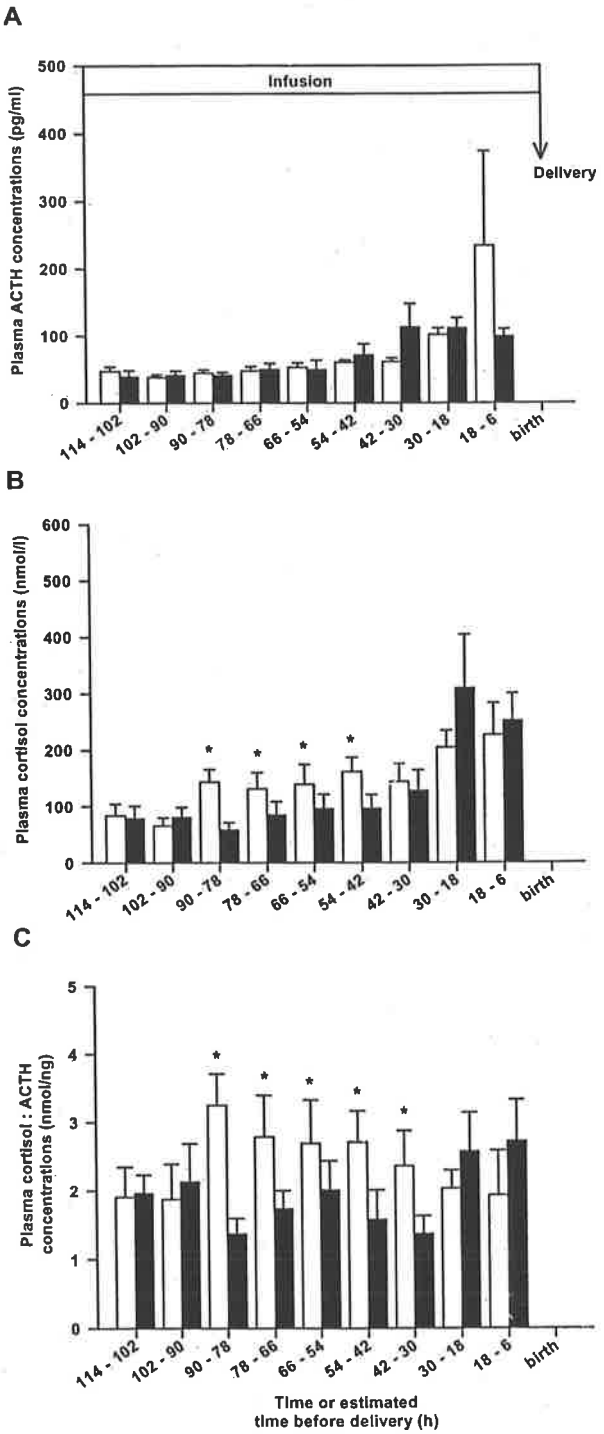

Infusion
Delivery
Plasma leptin concentrations (ng/ml)
40
30
20
10
0
114- 102
102 -90
90 - 78
78 - 66
66 - 54
54 - 42
42 - 30
30 - 18
18 - 6
birth
Time or estimated
time before delivery (h)

Figure 5.


A
Infusion
Delivery
Plasma ACTH concentrations (pg/ml)
500
400
300
200
100
0
114 - 102
102 - 90
90 - 78
78 - 66
66 - 54
54 - 42
42 - 30
30 - 18
18 - 6
birth
B
Plasma cortisol concentrations (nmol/l)
600
500
400
300
200
100
0
C
Plasma cortisol : ACTH concentrations (nmol/ng)
5
4
3
2
1
0
Time or estimated time before delivery (h)