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Endocrine Regulation of Adipose Tissue
Thermogenesis in the Fetal and Neonatal Sheep

by

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Thesis submitted to the University of Nottingham for the
degree of Doctor of Philosophy October 2001

Abstract

Leptin is produced primarily by white adipose tissue but also by brown adipose tissue (BAT) and the placenta. It has a major physiological role in the control of energy balance. BAT is critically important for the initiation of non-shivering thermogenesis in precocial newborns through the uncoupling protein, UCP1 unique to BAT. This is particularly important in neonatal lambs and infants in which levels of UCP1 peak at birth concomitant with a peak in plasma leptin and a surge in Cortisol. Other mitochondrial proteins may contribute to this effect e.g. VDAC which is located in the outer mitochondrial membrane.

The aim of this thesis was to:

1. investigate the role of the late gestation Cortisol surge on BAT development
2. investigate the effect of route of delivery on subsequent neonatal plasma leptin concentrations
3. assess the effect on of leptin administration to neonatal lambs on thermoregulation

Late gestation fetuses were infused with Cortisol or saline or underwent adrenalectomy or sham operation. BAT was sampled at 129 and 144 days of gestation, respectively. UCP1 abundance was significantly increased in the animals receiving Cortisol treatment compared to controls and was significantly reduced in adrenalectomised animals.

Plasma leptin was analysed from lambs delivered vaginally or near term by caesarean section (CS). Cord plasma leptin decreased significantly after birth, an adaptation that was delayed by CS delivery. Acute and chronic administration of leptin to neonatal lambs improved thermoregulation by preventing a decline in body temperature. Chronic leptin treatment over 7 days (100µg daily) promoted the loss of UCP1 mRNA and protein, but had no deleterious effects on body temperature

Polyclonal antibodies were developed against mitochondrial voltage-dependent anion channel (VDAC). The postnatal ontogeny of VDAC was found to be similar to that of UCP1 and cytochrome c, with abundance peaking around one day of age. VDAC was found in high abundance in organs with high metabolic requirements such as heart, muscle and BAT. These results suggest that VDAC is involved in ensuring BAT maintains a maximal rate of thermogenesis after birth.

In conclusion, I have shown for the first time that leptin has an important role in thermogenesis during the transition from fetal to neonatal life. This is attenuated in CS animals, possibly linked to a reduced sympathetic nervous system activity. The rapid loss of UCP1 mRNA, which occurs within the first few days of life, appears to be modulated by leptin, possibly stimulating the development of white adipose tissue and generation of body heat through mechanisms other than non-shivering thermogenesis in BAT. VDAC may be important during this period in ensuring adequate substrate delivery to BAT. Intact adrenal glands are also necessary for the increase in UCP1 abundance during late gestation, an effect mediated in part by Cortisol.

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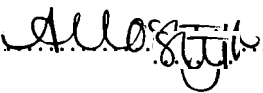
I would like to thank all the staff and students (past and present) in the Academic Divisions of Child Health and Obstetrics and Gynaecology at Queens Medical Centre, all of whom have made my time in the School of Human Development both fun and productive. Special thanks to Jennifer Dandrea (IT assistant!) and Helen Budge for conference fun here and abroad!

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Declaration

Work presented in this thesis was carried out during a University of Nottingham Research Scholarship in the Division of Child Health, School of Human Development, University of Nottingham between October 1998 and September 2001.

The thesis, and all publications arising, represents my own work, undertaken under the supervision of Dr Michael Symonds.

Signed: 

Alison Mostyn, February 2002

Introduction

1.1. Fetus to Neonate.....	1
1.2. Brown Adipose Tissue.....	1
1.2.1 BAT Morphology and Location.....	1
1.2.2 BAT Differentiation.....	5
1.2.3 BAT Development.....	9
1.2.4 Ovine Brown Adipose Tissue Regulation.....	14
1.3 Uncoupling Protein –1 (UCPI).....	16
1.3.1 Structure of UCPI.....	16
1.3.2 UCPI Mechanism of Action.....	20
1.3.3 Regulation of UCPI Activity.....	28
1.3.4 Assessment of UCPI Activity and Abundance.....	34
1.4 Other Mitochondrial Proteins.....	37
1.4.1 Uncoupling Protein 2 (UCP2).....	37
1.4.2 Uncoupling Protein 3 (UCP3).....	39
1.5 Leptin.....	41
1.5.1 Background.....	41
1.5.2 Leptin Gene and Protein Structure.....	42
1.5.3 Leptin Receptors.....	43
1.5.4 Physiological Regulation of Leptin.....	47
1.5.5 Leptin in the Fetus and Neonate.....	49
1.6 Summary and Aim of Thesis.....	52

Materials and Methods

2.1	Animals.....	53
2.1.1	<i>In vivo</i> measurements.....	54
2.1.1.a	Indirect calorimetry.....	54
2.1.1.b	Colonic Temperature.....	55
2.1.1.c	Breathing Frequency and Incidence of Shivering.....	55
2.1.2	Collection of Plasma Samples.....	55
2.1.3	Collection of Tissue Samples.....	56
2.2	Analysis of Plasma Samples.....	56
2.2.1	Glucose.....	56
2.2.2	NEFA.....	57
2.2.3	Leptin.....	58
2.2.4.	Prolactin.....	60
2.3	Analysis of Tissue Samples.....	61
2.3.1	Mitochondrial Preparation.....	62
2.3.2	Lowry Assay.....	62
2.3.4	Western Blotting.....	64
2.3.5	GDP Binding.....	71
2.3.6	RNA Preparation.....	72
2.3.7	Northern Blotting.....	75
2.3.8	Reverse Transcription and Polymerase Chain Reaction.....	81
2.3.9	Statistical Analysis.....	84

The Effect of Leptin Treatment on Thermoregulation of Neonatal Lambs

3.1 Introduction.....85

Part A Acute Leptin Administration

3.1.1 Hypothesis and Aim.....87

3.2 Methods.....87

3.2.1 Animals.....87

3.2.2 Experimental Design.....87

3.2.3 Laboratory Procedures.....89

3.2.4 Statistical Analysis.....89

3.3 Results.....90

3.3.1 Thermoregulation.....90

3.3.2 Plasma Metabolites and Hormones.....90

3.3.2 Lamb Body Weight and tissue Analysis.....103

Part B Long Term Leptin Administration

3.4 Introduction.....111

3.4.1 Hypothesis and Aim.....111

3.5 Methods.....111

3.5.1 Animals.....111

3.5.2 Experimental Design.....112

3.5.3 Laboratory Procedures.....112

3.5.4 Statistical Analysis.....113

3.6 Results.....113

3.6.1 Thermoregulation.....113

3.6.1 Plasma Metabolites and Hormones.....115

3.6.2 Lamb Body Weight and Tissue Analysis.....122

3.7 Discussion.....	135
3.7.1 Effect of Leptin Administration on Thermoregulation.....	136
3.7.2 Effect of Leptin Administration on Hormones and Metabolites...	140
3.7.2a Leptin.....	140
3.7.2b NEFA and Glucose.....	143
3.7.2c Prolactin.....	144
3.7.2d Body Weight and Tissue Analysis.....	144
3.8 Conclusion.....	148

The Effect of Route of Delivery on Plasma Leptin Concentrations in the Newborn Lamb

4.1 Introduction.....	149
4.1.1 Aim and Hypothesis.....	150
4.2 Methods.....	150
4.2.1 Experimental Design.....	150
4.2.2 Laboratory Procedures.....	151
4.2.3 Statistical Analysis.....	151
4.3 Results.....	151
4.3.1 Plasma Hormones.....	151
4.3.2 Lamb BAT and Body Weight.....	152
4.4 Discussion.....	159
4.4.1 Effect of Delivery on Plasma Leptin.....	159
4.5 Conclusion.....	163

Effect of Cortisol on Thermogenic Capacity of BAT in the Late Gestation

Fetal Lamb

5.1	Introduction.....	165
5.1.1	Aim and Hypothesis.....	165
5.2	Methods.....	166
5.2.1	Experimental Design.....	166
5.2.2	Laboratory Procedures.....	166
5.2.3	Statistical Analysis.....	167
5.3	Results.....	169
5.3.1	Plasma Hormones.....	169
5.3.2	Lamb Body Weight and BAT Analysis.....	173
5.4	Discussion.....	185
5.4.1	Effect of Cortisol Status on Plasma Hormones.....	185
5.4.2	Effect of Cortisol Status on Thermoregulation.....	186
5.5	Conclusion.....	190

Production of Polyclonal Antibodies Against a Specific Mitochondrial

Protein

6.1	Introduction.....	191
6.1.1	Aim.....	193
6.2	Methods.....	193
6.2.1	UCP2 Peptide Purification.....	193
6.2.2	Protein Concentration Determination.....	197
6.3.2	Protein Visualisation.....	198
6.3.2.a	Coomassie Blue Staining.....	198

6.2.3.b	Silver Staining	199
6.2.4	Immunisation of Rabbits	200
6.2.5	Antibody Screening	201
6.3	Results	205
6.3.1	Purification of UCP2	205
6.3.2	Antibody Selection	205
6.3.3	Sequencing and Confirmation	208
6.4	Discussion	211
6.5	Conclusion	213

Ontogeny of Ovine Brown and White Adipose Tissue Mitochondrial

Proteins and Tissue Distribution of VDAC

7.1	Introduction	214
7.1.1	Aim	215
7.2	Methods	215
7.2.1	Experimental Design	215
7.2.2	Laboratory Procedures	215
7.2.3	Statistical Analysis	216
7.3	Results	217
7.3.1	VDAC Ontogeny	217
7.3.2	UCP1 Ontogeny	217
7.3.3	Cytochrome <i>c</i> Ontogeny	217
7.3.4	UCP2 Tissue Distribution	218
7.3.5	VDAC Tissue Distribution	218
7.4	Discussion	232

7.4.1	BAT Ontogeny of VDAC, Cytochrome <i>c</i> and UCPI.....	232
7.7.3	Tissue Distribution of VDAC.....	234
7.7.4	UCP2 Tissue Distribution.....	235
Conclusion.....		236
Conclusion		
8.1	General Conclusions.....	238
8.1.1	Leptin and Neonatal Adipose Tissue Development.....	238
8.1.2	Cortisol and Adipose Tissue Development.....	240
8.1.3	Postnatal Ontogeny of Mitochondrial Proteins.....	240
8.2	Future Perspectives.....	242
	Appendix I.....	244
	Appendix II.....	247
	Appendix III.....	249
	Author Publication List.....	250
	Bibliography.....	254

CHAPTER 1

INTRODUCTION

1.1. Fetus to Neonate

The transition from fetus to neonate represents a major physiological, hormonal and environmental challenge. In order to survive, the fetus must be prepared to thermoregulate and feed independently shortly after birth. This requires a number of highly complex physical and hormonal interactions around the time of birth. Brown adipose tissue is activated around the time of birth by hormones such as Cortisol and triiodothyronine and also by the sympathetic nervous system. A recently discovered hormone, leptin, has been shown to be involved in thermoregulation in rodents, it is not known if leptin has such an effect in ovine neonates.

1.2. Brown Adipose Tissue

The transition from a maternally regulated thermal environment to one considerably colder (i.e. 39 °C *in utero* to 12 °C March/April air temperature, in the case of the sheep) requires the newborn to be able to rapidly produce heat in order not to become hypothermic. The neonatal lamb and human infant are able to produce large amounts of heat shortly after birth due to the presence of uncoupling protein 1 (UCPI) unique to the specialised adipose organ, brown adipose tissue (BAT).

1.2.1 BAT Morphology and Location

A brown fatty tissue was first observed in the marmot and noted by the 16th century physician and naturalist Conrad Gesner (Rothwell & Stock 1985). This tissue is now normally referred to as BAT and has been shown to be present in most mammals

studied to date (Rothwell & Stock 1985). BAT mitochondria may be distinguished from other cell types by presence of the unique uncoupling protein UCPI.

BAT is specialised for heat production due to recruitment of non-shivering thermogenesis (NST) and this is apparent from the properties, in addition to the presence of UCPI, that distinguishes BAT from the other adipose tissue type, white adipose tissue (WAT) (Figure 1.1). The other distinguishing features of BAT are outlined below:

- small cells (25-40 µm) with a central nucleus (Vernon 1986)
- multilocular triglyceride droplets (Alexander 1978)
- abundant mitochondria with dense parallel cristae richly endowed with respiratory chain enzymes (Cannon *et al.* 1977).
- high degree of vascularisation

WAT, on the other hand, contains a unilocular triglyceride droplet with few organelles that are pressed against the cell wall. Many of the prominent features of BAT relate to its function, for example the stores of triglycerides are necessary for combustion during non-shivering thermogenesis. Many mitochondria are required for the biochemical oxidation of non-esterified fatty acids (NEFA) released from triglycerides and the extensive vasculature supplies the high substrate and oxygen requirement necessary to support the high metabolic rate. The vasculature also constitutes a convective system for the capture and redistribution of heat. The dark

brown colour of BAT is derived from its rich blood supply and high cytochrome content. Both BAT and its blood vessels are innervated by sympathetic nerves resulting in a high tissue concentration of noradrenaline.

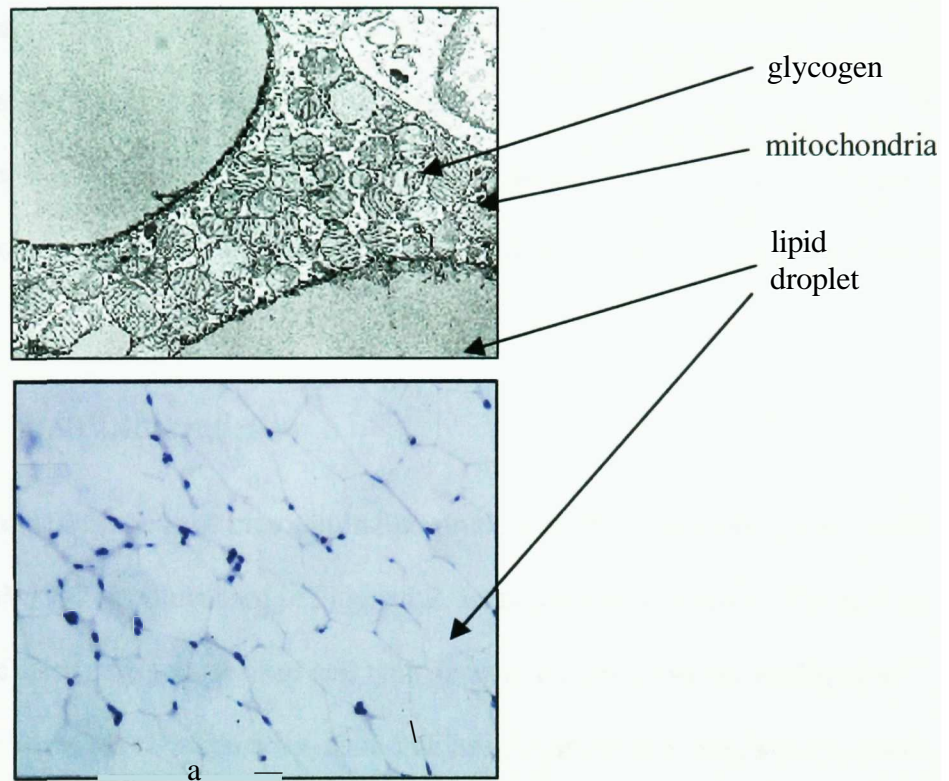


Figure 1.1

Microscopic images of brown (upper panel) and white (lower panel) adipose tissue. The BAT is an electron scanning microscope image (modified from Cinti 1999 (Cinti 1999)), WAT is a light microscopy image (courtesy of Victoria Wilson) 70X magnification.

In the lamb, the major depot of BAT (80 %) is found in the perirenal region (referred to as PAT throughout this thesis), with smaller amounts found in the pericardial area (Alexander 1981). This is in contrast to rodents and hibernating mammals, whose main depot is found in the inter-scapular region (Alexander 1981). Newborn infants have major BAT depots in the posterior cervical, axillary, supraciliac and perirenal regions, forming a "vest" like arrangement around the major organ systems (Merklin 1974).

1.2.2 BAT Differentiation

The adipocyte lineage is of mesodermal origin derived from embryonic stem cells (Dani *et al.* 1997) as illustrated in Figure 1.2. Increasing commitment of totipotent embryonic stem cells to a defined cell type gives rise to mesodermal multipotent stem cells, from which adipocytes, chondrocytes and myocytes originate (Ailhaud 1992; Gregoire *et al.* 1998). Direct evidence that brown adipocytes originate from the same mesodermal stem cells as white adipocytes comes from studies on C3H10T1/2 cells, that unlike pre-adipocyte cell lines, have not yet undergone commitment to any given lineage; i.e. they are a multipotent mesenchymal stem cell line. Studies have shown that treatment of these cells with insulin and thiazolidinediones (TDZ's) directed them towards differentiation into brown adipocytes i.e. they accumulated lipid, expressed UCPI and increased their mitochondrial mass (Paulik & Lenhard 1997). From a comparative study on primary cultured stromal vascular fractions of WAT and BAT, we know, that pre-adipocytes are already committed to either the white or brown adipocyte lineage as T3 evoked

differential metabolic and thermogenic effects between the adipose tissue types (Klaus *et al.* 1995). At some time point after commitment of the multipotent stem cell towards the adipose lineage, a further commitment occurs to either white or brown adipocyte lineage. However, at what point this occurs and what the underlying molecular mechanisms are is still completely unknown.

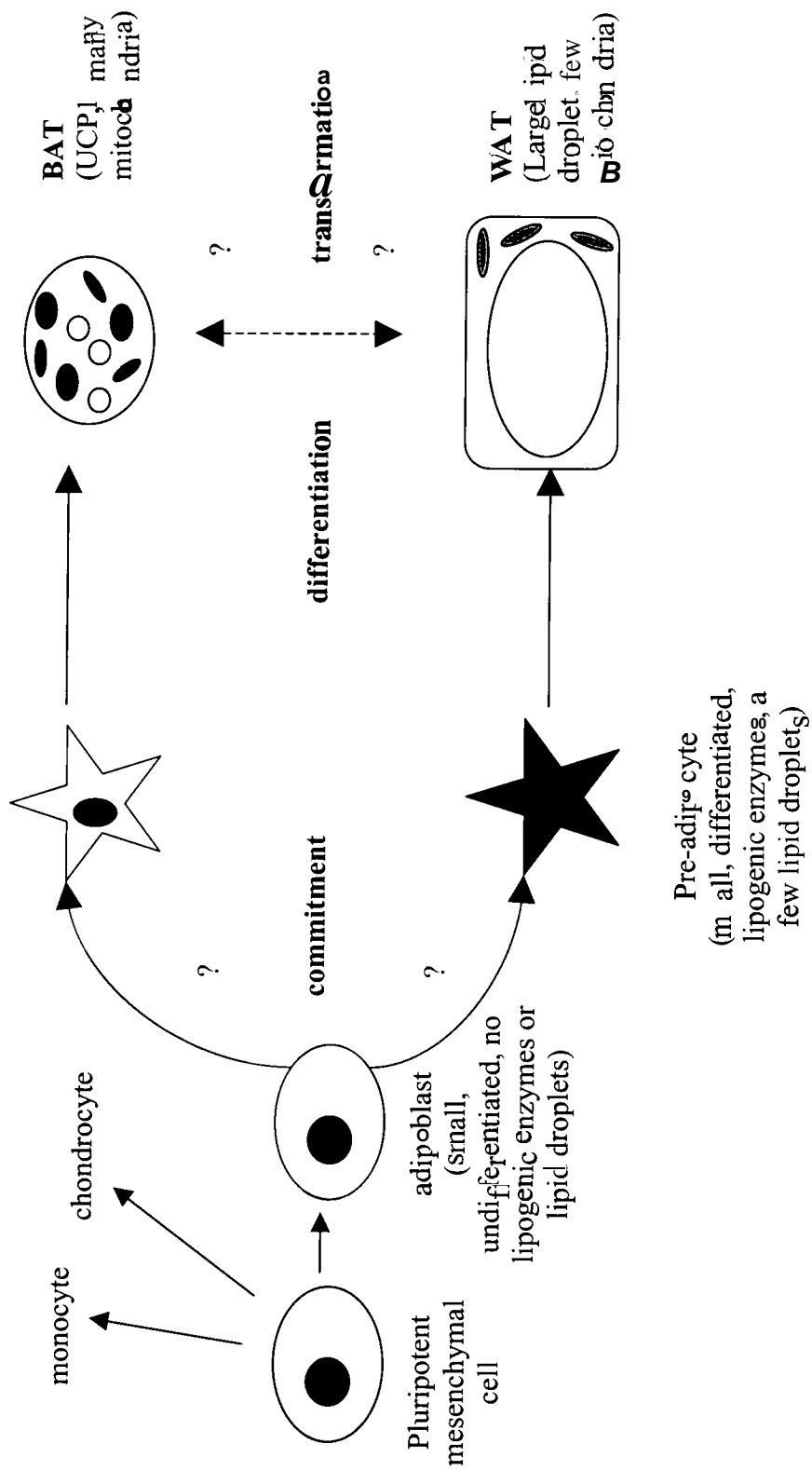


Figure 12

A summary of adipogenesis, modified from Wu *et al.* 1992, Ailhaud *et al.* 1992, Vermeulen 1986 and Gregoire *et al.* 1992. (Ailhaud 1992; Gregoire *et al.* 1992; Vermeulen 1986; Wu *et al.* 1992)

Factors inducing adipogenic conversion are similar for brown and white preadipocytes, whereas inducers of thermogenic function are only functional on brown preadipocytes. White and brown pre-adipocytes cultured in vitro can not be distinguished either by morphological, metabolic, or molecular criteria although, in cell culture, differentiated brown adipocytes can be recognised by the presence of UCP1 and a higher respiratory activity, i.e. increased mitochondrial content (Ailhaud 1992).

Therefore, although brown pre-adipocytes have a superficial resemblance to white pre-adipocytes, the brown adipocyte is not an intermediate in the development of the white adipocyte.

1.2.3 BAT Development

Ovine BAT development begins during fetal life and BAT is fully functional at birth.

The development of fetal BAT can be divided into three distinct phases:

- growth
- development
- maturation (Symonds *et al.* 1995)

Lobes of pre-adipocytes can be found in the perirenal and subcutaneous areas during the second trimester of gestation ~G70 (70 days gestation), by G90 there is a marked proliferation of adipocytes containing mitochondria and increasing lipid in the perirenal area (Alexander 1978; Vernon 1986) (Figure 1.3). Growth of the subcutaneous depots (i.e. WAT) commences around 2-3 weeks later (Alexander 1978) (Figure 1.3). Up to G120 PAT depots grow rapidly so that the amount per kg body weight increases, but after G120 until term growth of the depot continues at the same proportional rate as the fetus (Alexander 1978).

Lambs are a precocial species, born fully developed and able to thermoregulate independently minutes after birth and as such must have fully developed and functional BAT at birth. Lambs are normally born with 20 -30 g of perirenal adipose tissue which has the morphological and biochemical properties of BAT i.e. many mitochondria and multi-locular fat droplets (Alexander 1978; Cannon *et al.* 1977; Gemmel *et al.* 1972; Vernon 1986). However, lambs have little detectable subcutaneous adipose tissue (SAT) at birth (Figure 1.3). The amount of SAT in the

lamb fetus peaks around mid gestation then declines up to term as PAT increases. Any detectable SAT in the newborn lamb is BAT, not WAT as it contains UCP1 mRNA. It is possible that PAT tissue is laid down at the expense of SAT which is down-regulated towards term. This reinforces the importance of BAT in providing the newborn lamb with heat via NST.

During the first three weeks of postnatal age, adipose tissue in the perirenal region begins to adopt the characteristics and morphology of WAT and by one month of age is indistinguishable from the subcutaneous WAT depot, expressing no UCP1 (Casteilla 1989; Finn *et al.* 1998; Gemmel *et al.* 1972). Ovine and bovine species and humans do not express BAT, nor UCP1 again during adult life, although human patients with pheochromocytoma, a tumour inducing high plasma catecholamines, have been shown to have BAT depots (Lean *et al.* 1986a; Lean *et al.* 1986b; Ricquier *et al.* 1982b).

The mechanisms responsible for this alteration in BAT functional and structural characteristics are unknown, although it is possible that some sort of transformation from BAT to WAT occurs during the postnatal period. It has been suggested that programmed cell death, apoptosis, is responsible for the "remodelling" of BAT to WAT (Finn *et al.* 1998), although there is no direct evidence at present to substantiate this. Fetal and postnatal development of BAT are closely related to changes in P-adrenoceptor stimulation and subtype present in BAT. There are three subtypes of P-adrenoceptors, type three is the most recently discovered which is found predominantly in adipose tissue and thought to be involved in the regulation of

resting metabolic rate and lipolysis (Strosberg 1997). Mutation of the P3-adrenoceptor has been associated with predisposition to obesity and early onset of non-insulin dependent diabetes in Pima Indians (Clement *et al.* 1995; Walston *et al.* 1995). In the fetal calf P3-adrenoceptor mRNA appearance in BAT precedes that of UCPI and is maintained at a high level until around 3 months of postnatal age (Casteilla *et al.* 1994). However, by adulthood, β 3-adrenoceptors are undetectable in bovine adipose tissue (which has then transformed from brown to white). This is in contrast to rodent studies indicating a high expression of β 3-adrenoceptor mRNA in WAT and BAT of the adult rat (Muzzin *et al.* 1991). β 3-adrenoceptors act through a G-protein coupled receptor (Gs) on the surface of adipocytes to increase the intracellular activity of adenylyl cyclase, cAMP and subsequently lipolysis via hormone-sensitive lipoprotein lipase (Strosberg 1997). The developmental response of adenylyl cyclase to a p3-adrenoceptor agonist (i.e. BRL 37344, a synthetic β 3 specific agonist) is well correlated with β 3-adrenoceptor expression (Casteilla *et al.* 1994) and therefore BAT abundance. After birth the adenylyl cyclase response to BRL 37344 decreases rapidly (Casteilla *et al.* 1994), this could be due to receptor desensitisation as there is a peak in neurosympathetic stimulation during parturition (Padbury *et al.* 1981). Adrenergic stimulation is also known to regulate UCPI activity and this will be discussed in Chapter 1.3.3.

Small mammals do not share the same ontogeny of BAT as sheep. Mice and rats are an altricial species, immature at birth, they have no fur, their eyes are closed and they do not have fully developed BAT. In order to keep warm, rodents huddle in a nest

until BAT becomes fully functional around 2 weeks of age when they emerge from the nest.

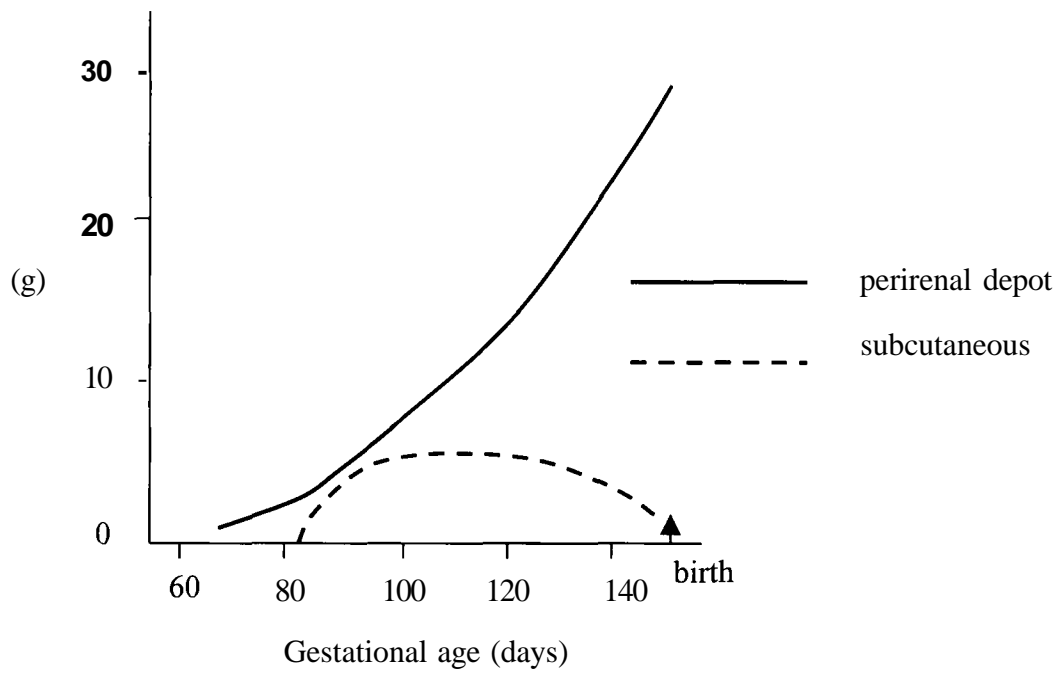


Figure 1.3

Adipose tissue growth in the fetal lamb. Modified from Alexander 1978 and Vemon 1986. (Alexander 1978; Vemon 1986)

1.2.4 Ovine Brown Adipose Tissue Regulation

Many factors influence BAT tissue development *in utero* and postnatally, including maternal nutrition (Alexander 1978; Budge *et al.* 2000; Symonds *et al.* 1998), fetal number (Alexander 1978), ambient temperature at birth (Clarke *et al.* 1994) and route of delivery (Chapter 4).

Maternal nutrition is known to influence lipid reserves in newborn lambs and BAT deposition (Alexander 1978; Budge *et al.* 2000; Symonds *et al.* 1998). Although, interestingly, increased nutrition does not necessarily equate to increased adipose tissue deposition. Alexander found that pregnant ewes fed a restricted diet of 10g/kg from 12 weeks gestation onwards (approximately 20 % less than the “*ad libitum*” ewes (Alexander 1978)) caused a reduction in BAT deposition (g per kg body) of the offspring. This has been confirmed by Symonds *et al* who found that under-feeding pregnant ewes in order to reduce fetal plasma glucose to 50 % of controls resulted in a significant reduction in fetal perirenal BAT (Symonds *et al.* 1998). In contrast, a study investigating the effects of maternal over-feeding during pregnancy (150 % versus 100 % in controls (Budge *et al.* 2000)) found that fetuses from well-fed ewes had less total perirenal BAT than control fed fetuses (Budge *et al.* 2000). However, BAT from the “well-fed” fetuses had an increased thermogenic potential than those from control fed mothers. These differential results suggest that there is an optimal energy requirement of BAT in the growing fetus, below this requirement, insufficient BAT is deposited and the neonate may not be able to effectively to thermoregulate at birth. Above this optimal energy requirement, BAT up-regulates

the mechanics of NST i.e. increased UCPI and GDP binding suggesting lipid is being oxidised. This may be a regulatory step by the fetus to stop over-deposition of BAT, the positive function of this is not apparent, but fits with studies of over-fed rodents (by a cafeteria diet) that demonstrate up-regulation of UCPI to maintain a steady body weight (Champigny & Ricquier 1990; Marchington *et al.* 1986; Rothwell & Stock 1984).

Alexander has also demonstrated an effect of fetal number on adipose tissue deposition in the sheep, twins examined during late gestation were found to have significantly less perirenal BAT than singletons of the same gestational age (Alexander 1978). "Twinning" may also represent a nutritional effect, twin fetuses have lower plasma glucose than singletons of a similar age and are often thought of as "growth restricted" (Symonds *et al.* 2000).

The ambient environmental temperature at birth also represents a regulatory factor in BAT development. Clarke *et al* demonstrated that caesarean section delivery of lambs close to term into a cool ambient temperature (15 °C) caused a reduction of adipose tissue mass compared to lambs delivered by caesarean section into a warm ambient temperature (30 °C) (Clarke *et al.* 1994). However, this study only investigated caesarean section delivered animals, whom, as discussed in Chapter 4, display different adipose tissue abundance than vaginally delivered animals.

1.3 Uncoupling Protein -1 (UCP1)

The biochemical analysis of UCP1 began with the isolation of the intact peptide from BAT mitochondria in the 1980's (Lin & Klingenberg 1980; Lin & Klingenberg 1982). Prior to this, the thermoregulation of BAT was thought to be "peculiar" uncoupling of cellular respiration, related to a high ion conductance that could be suppressed by addition of purine nucleotides (Lin & Klingenberg 1980) or activated by the addition of fatty acids (Jezek *et al.* 1994). A nucleotide binding site on the inner mitochondrial membrane was also found around this time (Nicholls 1976), the number of these sites increased during cold exposure, suggesting a nucleotide binding, inner mitochondrial membrane protein was responsible for the uncoupling of cellular respiration. In view of this putative uncoupling function, the protein was "christened" uncoupling protein (UCP, as no family members had been discovered at this point). Other literature at the time coined the term "thermogenin" due to the heat producing effects of the BAT (Nedergaard & Cannon 1992).

In evolutionary terms, UCP1 has been proposed to be a late offspring from a group of H^+/OH^- substrate anion co-transporters (Klingenberg 1990). UCP1 is the only confirmed H^+ translocator of this type and bears considerable structural resemblance to the ADP/ADP and phosphate carriers (Klingenberg 1990).

1.3.1 Structure of UCP1

Mouse, rat and human UCP1 consists of 306 amino acids with a molecular weight of 32 - 33 kDa (Ricquier & Bouillaud 2000) although under physiological conditions

exists as a dimer (Lin & Klingenberg 1980; Lin & Klingenberg 1982) of 64 kDa.

UCPI belongs to the gene family of mitochondrial anion carrier proteins (MACP), which includes ADP/ATP and phosphate carriers.

UCPI is located on the inner mitochondrial membrane of BAT (Figure 1.4) where it acts as the only carrier known to transport H^+ alone. The exact tri-dimensional structure is unknown as it is difficult to obtain crystallization information on membrane proteins. Alternative strategies have included sequence analysis (Klingenberg 1990; Ricquier *et al.* 1982a), use of chemical and immunological probes (Gonzalez-Barroso *et al.* 1999; Miroux *et al.* 1992) and development of algorithms (Aquila *et al.* 1987). Based on the primary structure, a folding model of UCP 1 was derived which in its simplest form contains six transmembrane helices (Aquila *et al.* 1987). As a member of the MACP family, the structure can be divided into three similar repeat domains of about 100 residues, each containing two transmembrane helices this is called a "tripartite" or triad structure (Aquila *et al.* 1987; Klingenberg 1990). Both the N and C termini are located in the cytosol of the intermembrane space of the mitochondria (Figure 1.4). The three matrix loops are thought to contribute to the formation of the gating domain in UCPI and it has been proposed that they form a hydrophobic pocket that accommodates the purine moiety of the bound nucleotide. UCPI from hamster has been shown to contain 28 basic and 19 acidic charges resulting in excess of 9 positively charged residues, most of these are found in the hydrophilic matrix region. Positive or negative charges alternate at

the helix terminals on the cytosol or matrix side, resulting in "dipoles" which may form pairs by electrostatic attraction.

intermembrane space

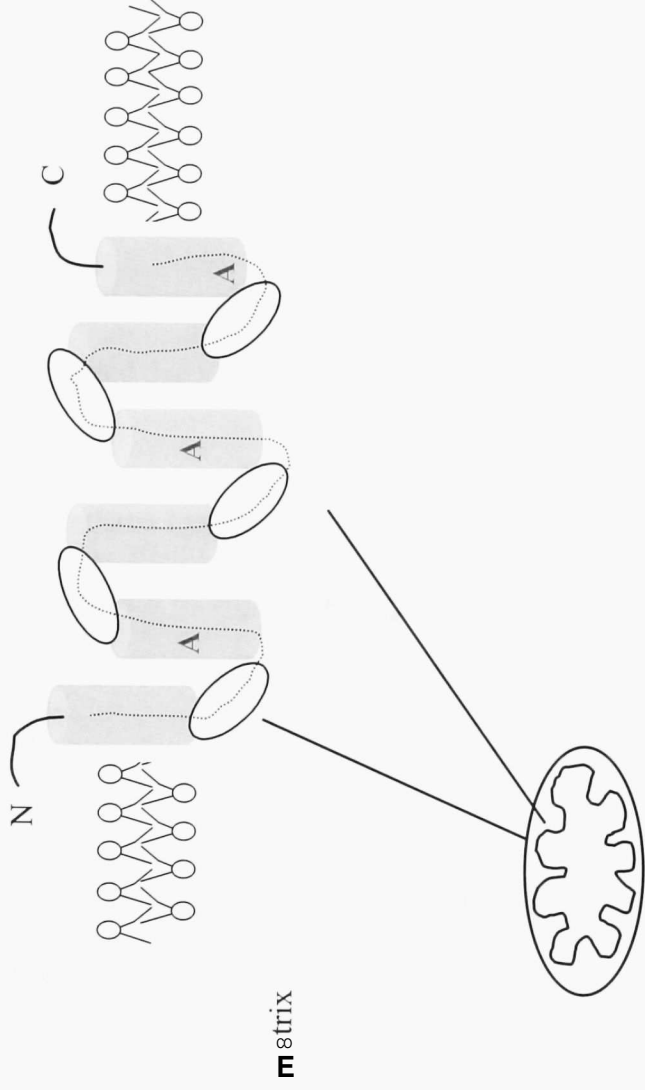


Figure 14

Diagram illustrating the structure of the inner mitochondrial membrane. Grey shaded areas represent the protein structure of UCP1. 'A's indicate the binding site for purine nucleotides. Modified from Jezek *et al.* 1998, Nedergaard and Klingenberg *et al.* 1999 (Jezek & Klingenberg *et al.* 1999; Nedergaard & Cannon 1992).

1.3.2 UCPI Mechanism of Action

The mechanism of UCPI is to uncouple normal cellular respiration. There are a number of theories at present regarding the exact mechanism of uncoupling; I will describe in this thesis the two main models.

Mammalian metabolic efficiency can be considered to be very close to zero i.e. what goes in, goes out. All energy supplied is essentially transformed into heat with a very small proportion used to perform work such as growth or for storage in, or extraction from the bodily reserves (Nedergaard *et al.* 2001). Metabolic respiratory control is highly regulated due to the following factors:

- ATP synthesis can only occur to replace de-phosphorylated ADP
- proton re-entry through ATP synthase is tightly coupled to ATP synthesis
- electron flow down the respiratory chain is tightly coupled to proton extmsion.
- proton extmsion and proton re-entry balance (Nicholls *et al.* 1986)

This idea is illustrated further in Figure 1.5. In the newborn sheep, stimulated BAT can contribute 30 % of the metabolic and 50 % upon arousal from hibernation in ground squirrels (Foster 1986). In order to produce these rates of thermogenesis observed *in vivo*, an alternative mechanism must be present in BAT to by-pass normal respiratory control.

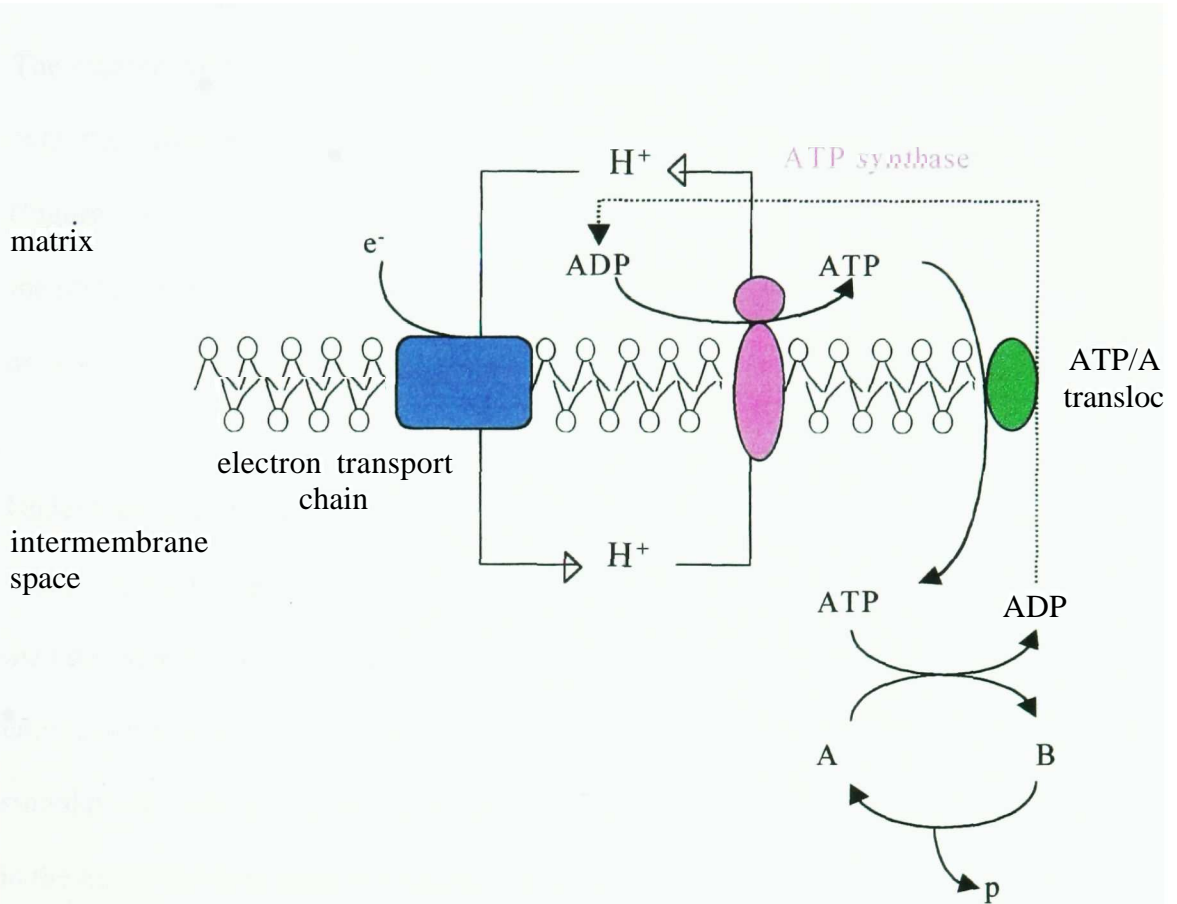


Figure 1.5

Example of conventional cellular respiration modified from Nicholls *et al* 1986 and Himms-Hagen 1990 (Hhms-Hagen 1990; Nicholls *et al.* 1986).

UCP1 is the by-pass mechanism employed by BAT to exploit cellular respiration. The presence of UCP1 in the inner mitochondrial membrane allows protons to re-enter the mitochondrial matrix via an alternative pathway, avoiding ATP synthase (Figure 1.6). The re-entry of protons into the mitochondrial matrix via UCP1 allows the proton motive force (or proton electrochemical gradient) energy to be dissipated as heat.

Under thermoneutral conditions, i.e. in the ovine fetus, UCP1 exists in a "masked" state; that is guanine di-phosphate (GDP) is bound to nucleotide binding sites on the outer side of UCP1 (Desautels *et al.* 1978; Nicholls 1976). This prevents the re-entry of protons into the matrix via UCP1. In the lamb, "unmasking" of UCP1 is stimulated by cold and increased sympathetic nervous activity - two factors critical in the transition from fetus to neonate. Other factors, such as diet, influence unmasking of UCP1 in rodent species (Peachy *et al.* 1988; Sudin & Cannon 1980). Acute stimulation of BAT causes rapid unmasking of available UCP1 before synthesis of new UCP1 occurs. Prolonged stimulation of BAT leads to increased UCP1 abundance and GDP binding in the mitochondria. Removal of the stimuli results in "re-masking" of active sites prior to any observed reduction in UCP1 abundance. It is thought that cold and sympathetic activity unmask UCP1 via stimulation of fatty acid liberation, fatty acids are known to activate H⁺ transport (Winkler & Klingenberg 1994).

Proton transport via UCP1 is inhibited by purine nucleotides (Desautels *et al.* 1978; Lin & Klingenberg 1982; Nicholls 1976). A nucleotide binding site is present on the outer surface of the inner mitochondrial membrane (Nicholls 1976). UCP1 has a strong preference for purine nucleotides and GDP and GTP bind with a higher affinity than ADP and ATP. It is thought that binding of GDP occurs in two stages, loose binding and tight binding (Huang & Klingenberg 1996; Huang *et al.* 1998). Loose binding occurs rapidly and there is a slow transition between loose and tight binding. Tight binding is highly correlated with the inhibition of H⁺ transport (Huang & Klingenberg 1996).

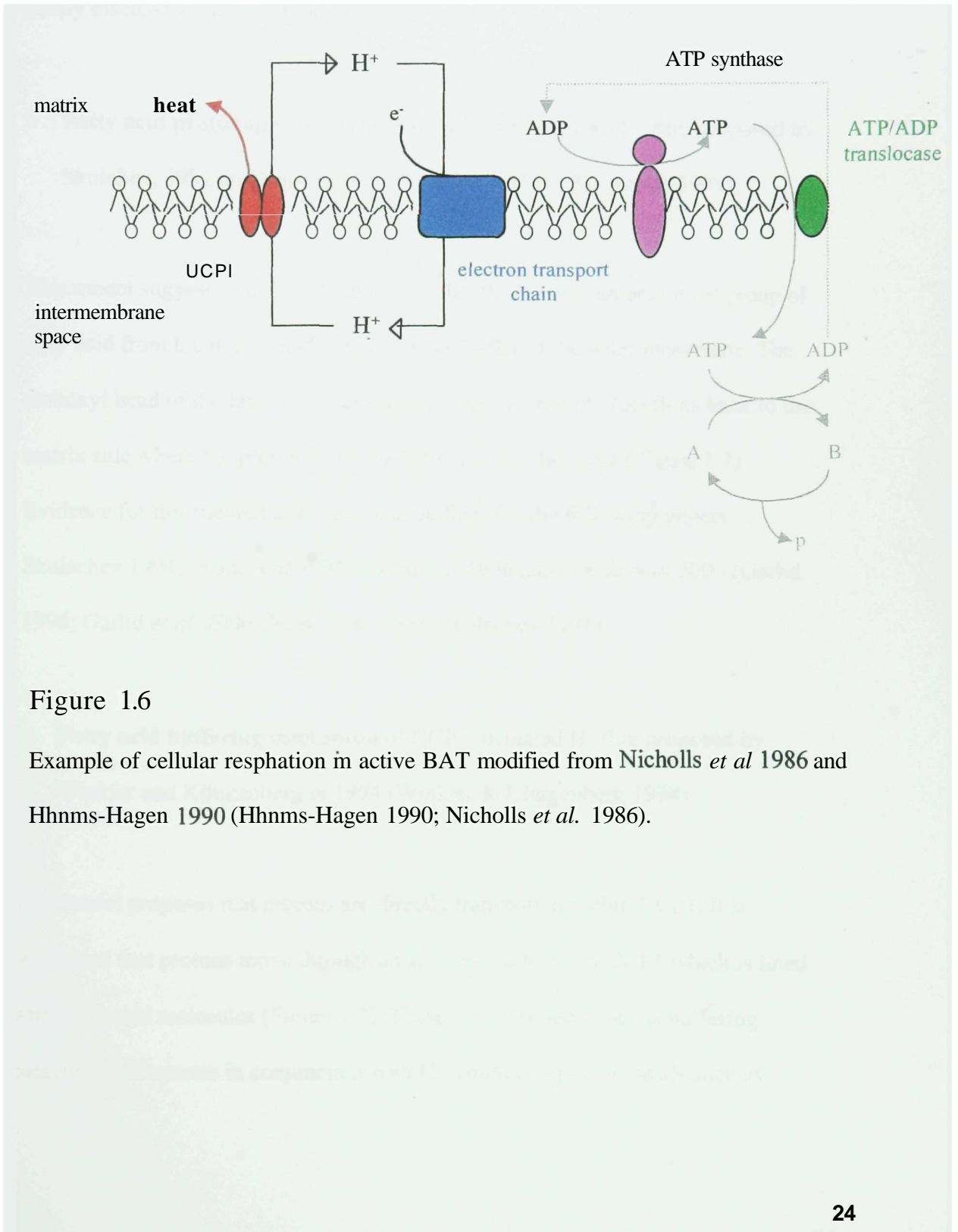


Figure 1.6

Example of cellular respiration in active BAT modified from Nicholls *et al* 1986 and Hhms-Hagen 1990 (Hhms-Hagen 1990; Nicholls *et al.* 1986).

There are two main models of how UCP1 mediates proton transport, these models were proposed in the early 1990's and are still hotly debated today. A full examination of these models is beyond the scope of this thesis and will only be briefly discussed. The two models are:

- **Fatty acid protonophore** mechanism of UCP1 mediated H^+ flux proposed by Skulachev, 1991 and Gariid *et al*, 1996 (Gariid 1996; Skulachev 1991).

This model suggests that UCP1 catalyses "flip-flop" of the anionic head group of fatty acid from the matrix leaflet to the outer leaflet of the inner membrane. The carboxyl head of the fatty acid picks up a proton and rapidly flip-flops back to the matrix side where the proton is released, completing the cycle (Figure 1.7).

Evidence for this method of H^+ flux can be found in the following papers: Skulachev 1991, Jezek *et al* 1994, Gariid *et al* 1996 and Gariid *et al* 2000 (Gariid 1996; Gariid *et al*. 2000; Jezek *et al*. 1994; Skulachev 1991).

- **Fatty acid buffering** mechanism of UCP1 mediated H^+ flux proposed by Winkler and Klingenberg in 1994 (Winkler & Klingenberg 1994).

This model proposes that protons are directly transported within UCP1. It is postulated that protons move through an aqueous pathway in UCP1 which is lined with fatty acid molecules (Figure 1.7). These are proposed to act as buffering cofactors that operate in conjunction with H^+ conducting amino acids such as

histidine. The fatty acid head group is proposed to oscillate from the aqueous phase into the protein channel and deliver a proton to an acceptor group which in turn delivers the proton to the matrix. UCPI in this model facilitates the return of the undissociated fatty acid to the cytosolic side for another H⁺ delivery cycle. Mutation of two histidine residues in UCPI has been shown to cause loss of H⁺ transport properties (Bienengraeber *et al.* 1998). Evidence for this model can be found in the following papers Winkler and Klingenberg 1994, Bienengraeber *et al.* 1998 and Klingenberg 1999 (Bienengraeber *et al.* 1998; Klingenberg 1999; Winkler & Klingenberg 1994).

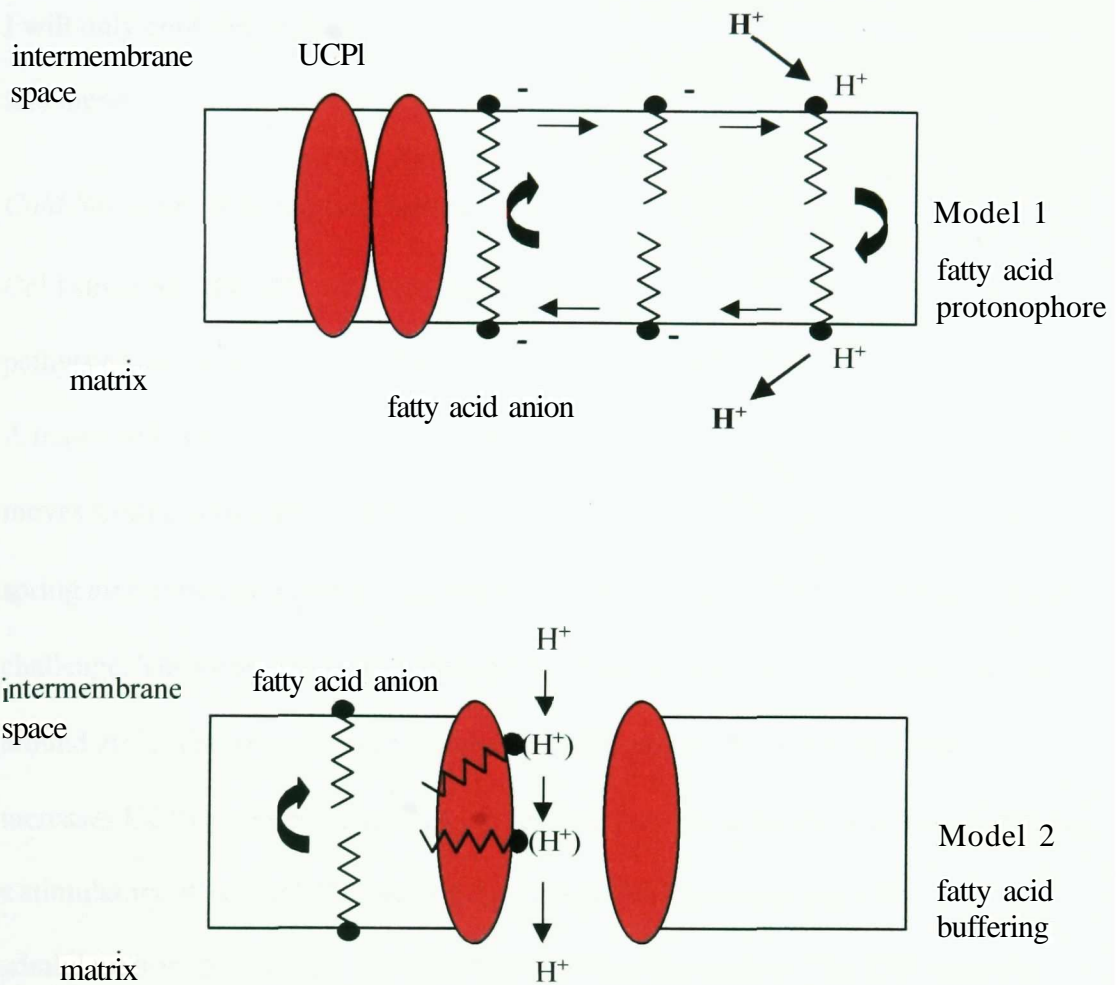


Figure 1.7

The two main models of H^+ flux mediated by UCPI. Model 1 modified from Skulachev 1991 and Gariid *et al* 1996 (Gariid 1996; Skulachev 1991); model 2 modified from Winkler and Klingenberg 1994 and Klingenberg 1999 (Klingenberg 1999; Winkler & Klingenberg 1994).

1.3.3 Regulation of UCPI Activity

The physiological activity of UCPI is known to be regulated by a number of factors, I will only consider those relevant to the period of transition from fetus to neonate in this thesis.

Cold Stress and Sympathetic Nervous System (SNS)

Cold stress and the SNS will be considered together as they act through the same pathway to activate UCPI.

A major activator of UCPI in the newborn lamb is cold exposure. At birth the lamb moves from a constantly maintained maternal temperature of 39 °C to that of the spring air temperature (approximately 12 °C), which represents a considerable cold challenge. The lamb therefore experiences a drop in environmental temperature of around 20 °C that stimulates an increased SNS output to BAT which in turn increases UCP 1 protein and mRNA expression. Noradrenaline (NA) is known to have a stimulatory effect on UCPI activity, evidence for this comes from NA administration (Mory *et al.* 1984) and sympathectomy studies. The SNS activates UCPI through a cascade of events culminating in increased lipolysis of BAT (Figure 1.8). Adrenergic nerves terminate at β 3-adrenoceptors on the cell surface of BAT, this stimulates the G protein Gs to activate adenylyl cyclase, thus increasing cyclic (c) AMP. cAMP acts to increase hormone sensitive lipase activity within BAT, liberating fatty acids from triglyceride stores (Figure 1.8). These fatty acids have a stimulatory effect on UCPI activity by "unmasking" the transport of protons. cAMP is also responsible for increased expression of the UCPI gene through a cascade of

cellular events involving protein kinase A, mitogen-activated kinase and genetic enhancers, although the exact mechanism is unknown (Cao *et al.* 2001).

Recent advances in molecular biology have enabled the production of UCPI ablated or “knockout” mice (Enerback *et al.* 1997). These mice have been found to be intolerant of cold exposure, UCPI ablated mice acclimatised at 24 °C and subsequently placed at 4 °C have been found to rapidly lose body temperature despite fully defending temperature through shivering. After around 200 minutes exposure to 4 °C these mice succumb to hypothermia, whereas wild type mice maintain body temperature through non-shivering thermogenesis. This finding confirms the presence of UCPI is essential for survival during cold exposure, at least in mice.

Sheep are unable to “recruit”UCPI in adult life during cold exposure as rodents can, but react to cold exposure at birth in the same way as rodents do in adulthood. As well as increasing UCPI activity, cold exposure and subsequent SNS activity also activate transcription of the UCPI gene (Bouillaud *et al.* 1984; Mory *et al.* 1984; Ricquier *et al.* 1986). There is evidence for the presence of a P-adrenergic response element in a 4.5-kilobase region upstream of the transcription start site of UCPI (Cassard-Doulier *et al.* 1998).

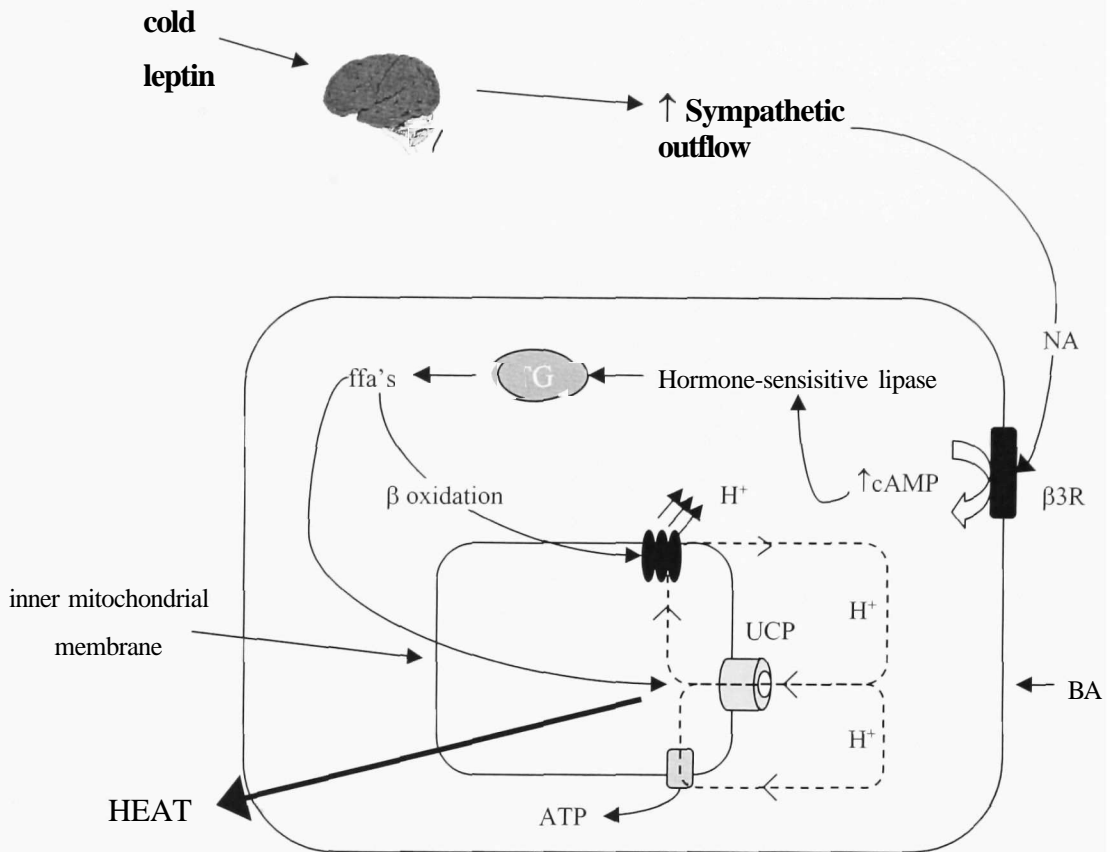


Figure 1.8

Effect of cold stress, leptin and SNS activation on UCP1 in BAT. Modified from Strosberg 1997 (Strosberg 1997). TG, triglyceride; ffa's, free fatty acids; $\beta 3R$, $\beta 3$ -adrenoceptor; NA, noradrenaline; ATP, adenosine triphosphate; BA, brown adipocyte.

The SNS is highly activated at the time of birth (Falconer & Lake 1982; Faxelius *et al.* 1983; Hagnevik *et al.* 1984; Lagercrantz & Bistoletti 1973), this is partly due to compression of the head during passage through the birth canal and the physical trauma of birth. As outlined above, the SNS has a stimulatory effect on UCP1 activity. Loss of the surge in SNS activity at birth, i.e. through caesarean section delivery results in a reduction in the stimulation of UCP1 and an attenuated ability to thermoregulate (Hagnevik *et al.* 1984; Irestedt *et al.* 1982) (Bird 1996; Clarke *et al.* 1997).

Thyroid hormones

Thyroid hormones are known to have a positive regulatory effect on UCP1 expression. In the neonate, as tri-iodothyronine (T3) levels increase rapidly with the onset of parturition and have been shown to be important in the initiation of thermoregulation in the lamb (Bird *et al.* 1998; Heasman *et al.* 2000; Schermer *et al.* 1996). T3 up-regulates and stabilises UCP1 expression (Guerra *et al.* 1996; Stein 1994) and abundance (Heasman *et al.* 2000) via responsive sequences on the UCP1 gene (Rabelo *et al.* 1995) (Cassard-Doulcier *et al.* 1998). T3 also acts to up-regulate the activity of mitochondrial carriers, proton leak and electron transport chain components (Goglia *et al.* 1999) these could all influence thermoregulation positively. The enzyme responsible for conversion of thyroxine (T4) to T3 and thus the increase in intracellular T3, is 5' deiodinase, this enzyme has been shown to be present in BAT (Trayhum *et al.* 1993) (Nedergaard & Cannon 1998). Abundance of 5' deiodinase in BAT increases markedly before birth, after which it spontaneously declines; the regulation of this decline is as yet unknown although it may be related

to the decline in sympathetic nervous system activity after birth (Nedergaard & Cannon 1998). The sympathetic nervous system is one of the factors that regulate 5' deiodinase activity in BAT. Noradrenaline can act through either β or α -adrenoceptors to increase cAMP and protein kinase c respectively to stimulate 5' deiodinase (Nedergaard & Cannon 1998). Regulation of UCP1 by T3 is further discussed in detail in Chapter 5.

Leptin

The hormone leptin is discussed in detail in Chapter 15 but its known role in thermogenesis will be summarised here. Studies in rodents have shown leptin to have a positive regulatory effect on thermoregulation (Pellemounter *et al.* 1995; Scarpace & Matheny 1998; Scarpace *et al.* 1997). Six days of leptin administration to leptin deficient *ob/ob* mice, who are hypothermic, stabilised their body temperature to values consistent with control wild type mice (Pellemounter *et al.* 1995). When this was repeated in normal rats, no increase in body temperature was observed, although there was an increase in whole body oxygen consumption. This increase was attributed to a significant increase in UCP1 mRNA expression (Scarpace *et al.* 1997). Induction of the UCP1 gene by leptin was found to be dependent on sympathetic innervation as no effect of leptin was observed in rats who had undergone denervation of interscapular BAT (Scarpace & Matheny 1998). This was consistent with the findings of Collins *et al.* 1996 (Collins 1996) who found that leptin administration increases noradrenaline turnover in BAT. Leptin is known to be a potent activator of the sympathetic nervous system, at least in rodents (Haynes *et al.* 1997b; Scarpace & Matheny 1998; Sivitz *et al.* 1999; Trayhum *et al.* 1999), so

can be included in Figure 1.8, inducing UCP1 via a similar mechanism as cold exposure. The action of leptin upon its receptors will be discussed in Chapter 1.5.3.

Glucocorticoids

Glucocorticoids are known to be important in the growth and development of the fetus (Bames *et al.* 1978; Fowden *et al.* 1998; Wu *et al.* 1978). There is limited evidence for a role of GCs in the regulation of thermogenesis. Dexamethasone treatment of pregnant ewes has been shown to improve thermogenic adaptation of prematurely delivered lambs (Clarke *et al.* 1998). It is unknown whether dexamethasone is acting on thermoregulation via UCP1 or another mechanism, although Cortisol is implicated in the maturation of the enzyme 5' deiodinase (Wu *et al.* 1978). This enzyme, as described above, increases plasma T3 by transformation of T4, this could increase UCP1 expression via an enhancer on the UCP1 gene (Guerra *et al.* 1996; Rabelo *et al.* 1995; Stein 1994). The role of glucocorticoids in the regulation of UCP1 is investigated further in Chapter 5.

Prolactin

The hormone prolactin is also proposed to have a role in the regulation of UCP1 and hence thermoregulation. The abundance of prolactin receptors in BAT increases coincidentally with UCP1, peaking around the time of birth. Administration of prolactin to pregnant mice has been found to increase the UCP1 abundance and promote lipid loss in the offspring of the treated rats (Budge *et al.* 2002; Yang *et al.* 2001). Administration of a molecular mimic of prolactin (a recombinant form of

endogenous phosphorylated prolactin [Chen, 1998 #270]) to newborn lambs has also been shown to improve thermoregulation (Pearce *et al.* 2001).

1.3.4 Assessment of UCPI Activity and Abundance

As this thesis focuses primarily on the activity of BAT and abundance of UCPI, it is important to comment on the interpretation of the biochemical methods used to assess BAT thermogenesis.

The major measurements made on BAT are

- gross assessments
- UCPI protein abundance
- UCPI mRNA
- GDP binding

Gross measurements made routinely on BAT are tissue weight and protein content.

Tissue weight gives an indication of the state of energy balance and the level of lipid stores, but does not give a measurement of thermogenic activity. According to Trayhum and Milner 1989 (Trayhum & Milner 1989) the "total protein content provides a crude index of active tissue mass", major changes of this measurement can be observed during initiation of thermogenesis. BAT depots contain other cell types such as endothelial cells and fibroblasts and any protein measurement made will include these, potentially overestimating total thermogenically active protein. The protein fraction used most commonly for thermogenic measurements is the

mitochondria, this is prepared by differential centrifugation as outlined in Chapter 2.3.1.a. This fraction is not purely mitochondria and will contain other organelles, however, this problem is overcome when investigating mitochondrial specific proteins. As mentioned above, the other cell types present will contribute to the mitochondria present, however, as the mitochondrial content of BAT is so high the proportion of non-BAT mitochondria is likely to be negligible (Trayhum & Milner 1989).

UCP1 protein abundance in our laboratory is analysed on western blots using a specific ovine UCP1 polyclonal antibody (Schermer *et al.* 1996). It is important to include negative and positive controls on initial western blots for UCP1 to confirm specificity of the antibody. The UCP1 antibody was produced from purified ovine UCP1 obtained by the procedure documented by Lin and Klingenberg in the 1980's (Lin & Klingenberg 1980; Lin & Klingenberg 1982; Schermer *et al.* 1996). This purification method is simple and produces a specific UCP1 peptide. The identification of UCP1 in an adipose tissue depot is regarded as definitive evidence that the tissue is BAT and not WAT (Trayhum & Milner 1989).

The UCP1 sequence is now available for sheep and many other species. In our laboratory we use an ovine oligonucleotide sequence in northern blotting to obtain a quantitative measurement of UCP1 mRNA (see Chapter 2.3.7 for more details). Any changes in UCP 1 mRNA do not provide quantitative evidence of changes in

thermogenesis, but indicate that the BAT is responding at a molecular level to the stimulus under test.

The GDP binding assay is the most widely used method of quantifying thermogenesis in BAT (Trayhum & Milner 1989). The assay uses the high affinity binding of GDP to UCP 1 to give a measurement of "active" sites available for proton transport activity [Nicholls, 1976 #498;Sudin, 1980 #566]. In our laboratory we employ a competitive assay to determine non-specific binding, this utilises a high, extra-physiological concentration of "cold" or un-radiolabeled GDP. Other purine nucleotides may be used in this assay, although ADP is translocated into mitochondria by the ADTP/ATP translocase thus requiring the addition of further chemicals to block the translocase.

When considered together, these biochemical analyses provide a detailed insight into the thermogenic capacity of the BAT studied:

- UCP1 protein measurements provide evidence for the presence and relative abundance of UCP1 hence the **thermogenic capacity**
- mRNA measurements provide information on the transcriptional state of UCP1
- GDP binding gives an estimate of the **potential thermogenic activity** of the tissue.

1.4 Other **Mitochondrial** Proteins

The search for other members of the uncoupling protein family began in the late 1990's. As UCP1 transports protons through the inner mitochondrial membrane of BAT, it was proposed that mitochondrial UCPs existed in tissues other than BAT and that they were responsible for the proton leak that accounts for a high percentage of total oxygen consumption in many tissues. Further evidence for the existence of other UCPs came from the observation that antibodies against UCP1 or UCP1 cDNAs were occasionally able to bind to protein or RNA from tissues other than BAT (Ricquier & Bouillaud 2000). Also, sequencing of the *S. cerevisiae* yeast genome revealed 34 proteins related to the mitochondrial carriers.

There is considerable disagreement as to whether the newly discovered UCP1 homologues actually uncouple respiration. A full examination of the evidence available on the newly discovered UCPs is beyond the capacity of this thesis and only a brief comment will be made on UCP2 and UCP3.

1.4.1 Uncoupling Protein 2 (UCP2)

UCP2 was isolated by Fleury *et al* in 1997 (Fleury *et al.* 1997) by comparing the cDNA of BAT UCP1 with a library of mouse skeletal muscle. UCP2 has 59 % sequence identity with UCP1 and has a much wider tissue expression. UCP2 exhibits several of the protein motifs common to the MACP and the amino acids required for nucleotide binding are conserved. UCP2 is expressed in skeletal muscle, kidney and the placenta. Interestingly, UCP2 has high expression in the spleen, thymus,

macrophages and leukocytes, suggesting a role in immunity (Arsenijevic *et al.* 2000; Fleury *et al.* 1997; Pecqueur *et al.* 2001). A role for UCP2 in the regulation of reactive oxygen species (ROS) production has also been proposed (Arsenijevic *et al.* 2000; Negre-Salvayre *et al.* 1997; Nicholls & Budd 2000). The chromosomal mapping of UCP2 is co-incident with quantitative trait loci for obesity and human insulin dependent diabetes locus-4 (Fleury *et al.* 1997; Pecqueur *et al.* 1999).

The regulation of UCP2 has been studied in many rodent tissues, however, most of these studies only present changes in UCP2 mRNA and use these as a indicator of altered thermogenesis. However, lack of protein analyses limit the conclusions that can be made (a full review of the regulation of UCP2 can be found in Fleury and Sanchis *et al.* 1999 (Fleury & Sanchis 1999)). Daniel Ricquier's group recently developed specific UCP2 antibodies that have been stringently tested in selected tissues from UCP2 knockout and wild type mice (Pecqueur *et al.* 2001). Results from these experiments indicate that UCP2 mRNA levels do not reflect UCP2 protein content in mitochondria. Despite the UCP2 protein content of lung and stomach mitochondria being 4 and 10 fold lower than that of spleen, the mRNA expression of UCP2 between these tissues was very similar (Pecqueur *et al.* 2001). Similar results were observed when the effects of fasting and administration of lipopolysaccharide were investigated, no change in UCP2 mRNA were found using northern blotting despite significant increases in UCP2 protein after both protocols (Pecqueur *et al.* 2001). The discrepancy between UCP2 protein and mRNA values has been attributed to an inhibitory open reading frame (ORF) in exon 2. Deletion of

this ORF in a UCP2 construct subsequently transfected in COS cells (a simian kidney epithelial cell line) resulted in UCP2 protein content 176 times that of spleen mitochondria (Pecqueur *et al.* 2001). Interestingly, all newly discovered UCPs have an upstream ORF (Pecqueur *et al.* 2001), indicating that caution must be made when interpreting UCP2 and UCP3 mRNA results.

1.4.2 Uncoupling Protein 3 (UCP3)

UCP3 was isolated in 1997 by Vidal-Puig *et al.* (Vidal-Puig 1997), Boss *et al.* (Boss 1997) and Gong *et al.* (Gong 1997) using the same method as Fleury *et al.* (Fleury *et al.* 1997). Human UCP3 was found to have 71 % sequence identity with human UCP2 and 57 % identity with UCP1, hydrophilicity plots indicate that these proteins are similar (Vidal-Puig 1997). UCP3 is mapped to the same quantitative trait loci as UCP2 (Gong 1997). UCP3 mRNA has been shown to be present in skeletal muscle, white and brown adipose tissue and to a lesser extent the heart (Gong 1997; Vidal-Puig 1997). UCP3 has been proposed to be regulated by thyroid hormones (Gong 1997; Gong *et al.* 2000), leptin (Gong 1997), noradrenaline (Gong 1997), fatty acids (Bmn *et al.* 1999; Hwang & Lane 1999) and fasting (Cadenas *et al.* 1999; Gong 1997), however, these studies relied upon mRNA expression and need verification with results from protein analysis. UCP3 knockout and over-expressing mice have been produced and provide a better insight into the function and regulation of UCP3. UCP3 knockout mice have been found not to be obese and have a phenotype similar to that of control mice, suggesting that a lack of UCP3 is not associated with obesity despite the genetic linkage studies (Gong *et al.* 2000; Vidal-Puig *et al.* 2000). When UCP3 over-expression was investigated, mice were found to have a 66-fold increase

in skeletal muscle UCP3 mRNA. This was associated with an increase in food intake, with no change in body weight (Clapham *et al.* 2000). The proton conductance in mitochondria from mice over expressing UCP3 was significantly increased (Clapham *et al.* 2000), suggesting a role for UCP3 in energy expenditure. However, Stuart *et al.* have suggested that a 66 fold increase in UCP3 mRNA and the corresponding (although unknown) increase in UCP3 protein could lead to alterations in the mitochondrial membrane integrity which could account for changes in proton leak (Stuart *et al.* 2001a).

There is intense debate regarding the functional role of UCP2 and 3 and whether they act as uncouplers of respiration. Much of the evidence for a role in uncoupling for UCP2 and 3 comes from yeast reconstitution studies which involve the insertion of UCP2 or 3 into the membrane of yeast and measurement of the membrane potential. As mentioned above, this can damage membrane integrity and provide false measurements of proton conductance (Stuart *et al.* 2001a), furthermore the very low levels of UCP2 in mammalian mitochondria have been shown not to uncouple yeast mitochondria (Stuart *et al.* 2001b). The information from mouse knock-out studies has been conflicting and no major conclusions have been drawn from them. There is little information regarding the role and regulation of UCP2 and 3 in the neonate, whether a true uncoupling function is active at this critical time is unknown. However, UCP2 and 3 have been shown to be present in the skeletal muscle and adipose tissue (white) of piglets, who do not have BAT at birth to provide heat via non-shivering thermogenesis (Damon *et al.* 2000).

1.5 Leptin

Many hormones and metabolites are critically important in ensuring a smooth transition from fetus to neonate. However, for purpose of this thesis I will only consider leptin, a recently discovered cytokine involved in energy regulation.

1.5.1 Background

Parabiosis (joining of animals by anastomosis of the skin, which allows cross-circulation) experiments in the 1950's provided evidence for a hormone acting as a satiety factor. In 1959 Hervey found that an appropriately placed hypothalamic lesion caused hyperphagia and obesity in rats which were members of a parabiotic pair. The un-operated partners of these rats however, became underweight –the immediate cause being that they ate less (Hervey 1959). These results were in accordance with the belief that adjustment of feeding is made in relation to the amount of stored fat in the body and that controlling centres in the hypothalamus are sensitive to the concentration of a "metabolite" in equilibrium with stored fat i.e. the "lipostatic theory" (Hervey 1969). It was not until 1994 that this satiety factor was identified with the cloning of the *ob* gene. The gene product was named "Leptin" from the Greek, Leptos, meaning thin (Halaas *et al.* 1995).

An obese strain of C57BL/6J mice named "*ob/ob*" was discovered in the Jackson laboratories in the 1950s. The *ob/ob* phenotype manifests as severe obesity, type II diabetes, decreased physical activity and hypothermia. A further mutation in C57BL/6J mice has been identified and termed *db* which is derived from "diabetic"

as the mice are obese and hyperglycaemic. A parabiosis experiment carried out between *ob/ob* and *db/db* mice found that the *ob/ob* partner reduced its food intake and lost weight, whereas the *db/db* mouse maintained a constant weight and food intake (Coleman 1973). This result led Coleman to believe that *ob/ob* mice fail to produce the signal which reduces food intake in response to body fat, and that the *db/db* mice produce this factor but their brains do not respond to it.

The *ob* gene was cloned in 1994 by the group of Friedman (Zhang *et al.* 1994). Two types of mutation in this gene have been observed in *ob/ob* mice that leads to abnormal production of the *ob* gene product leptin (Chua *et al.* 1996).

1.5.2 Leptin Gene and Protein Structure

WAT was initially thought to be the sole site of leptin production although recent evidence suggests that although WAT is the primary site of production, leptin may also be secreted from BAT and the placenta (Bi *et al.* 1997; Cinti *et al.* 1997; Dessolin 1997; Hassink *et al.* 1997; Hoggard *et al.* 1997a). The human *ob* gene consists of three exons and two introns, the promoter sequence contains a glucocorticoid response element and several cAMP response element binding sites (Isse *et al.* 1995). The coding region of the ovine leptin gene has 87 % homology to the corresponding human sequence (Dyer *et al.* 1997). The *ob* gene encodes a 4.5 kb adipose tissue mRNA with a highly conserved 166/167 amino acid reading frame and has no significant homology to any known peptide sequence. The signal peptide is 18 kDa and is cleaved to a mature 16 kDa protein. Leptin consists of 146 amino acids, is single stranded with a disulphide bond at the C terminus, this is thought to be

pivotal for leptin's stability and bioactivity. Analysis suggests that leptin is related in structure to the family of haemopoietic cytokines which includes interleukin-1 (IL-1) (Madej *et al.* 1995).

1.5.3 Leptin Receptors

In order to elicit a response, leptin must first interact with a high affinity binding protein. The leptin receptor (Ob-R) was cloned in 1995 by Tartaglia *et al* (Tartaglia *et al.* 1995). The *ob* receptor was found to be a single membrane-spanning receptor with considerable sequence homology to the glycoprotein 130 (gp130) subunit of the IL-6 receptor (Figure 1.9) which belongs to the cytokine class 1 family (Tartaglia *et al.* 1995). Parabiosis experiments suggested that *db/db* mice may be defective in the reception of the *ob* gene product. Subsequent genetic mapping of the gene encoding the Ob-R revealed its position to be within the same 300 kilobase interval as the *db* locus, suggesting the *db* gene is in fact the gene encoding the Ob-R (Lee *et al.* 1996; Tartaglia *et al.* 1995).

The leptin receptor is known to have at least six alternatively spliced forms, termed Ob-Rb, Ob-Ra, Ob-Rc, Ob-Rd and Ob-Re all with an identical extracellular domain (Figure 1.9). The long form of the receptor Ob-Rb has a complete intracellular portion complete with a box-2 sequence. This motif is required for the binding of Janus Kinase proteins (JAK) (Lee *et al.* 1996).

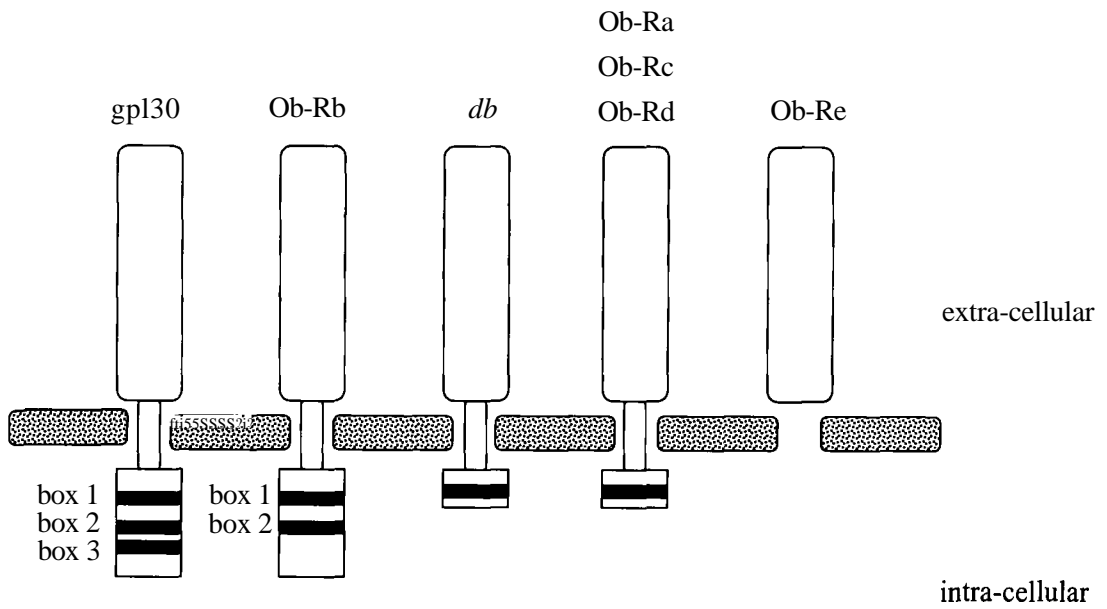


Figure 1.9

Comparison of the gp130 receptor and the 5 splice variants of the leptin receptor.

Modified from Miller and Bell 1996 (Miller & Bell 1996).

Ob-Rb is thought to act in a similar way to the gp130 receptor upon binding of an agonist (Baumann *et al.* 1996; Devos *et al.* 1997) i.e. the formation of dimers upon binding of leptin and binding of one of the JAKs to one of the intracellular "box" sequences (Bjorbeck *et al.* 1997; Devos *et al.* 1997). The JAK can then transduce signals through phosphorylation of tyrosine residues in the distal part of the receptor which activates binding of "signal transducers and activators of transcription" (STAT) - this can alter the transcription of key genes (Miller & Bell 1996). STAT-3 is one STAT known to be activated via Ob-Rb in the hypothalamus (Bjorbaek *et al.* 1997).

Ob-Rb is located primarily in the brain, but has been shown to be located at a lower abundance in several other tissues of the mouse including adipose tissue, ovaries, testis and placenta (Hoggard *et al.* 1997b; Mercer *et al.* 1996). In the brain, Ob-Rb is expressed at high levels in the supraoptic, arcuate, dorsomedial and ventromedial hypothalamic nuclei (Fei *et al.* 1997; Mercer *et al.* 1996; Udagawa *et al.* 2000). This distribution within the brain suggests Ob-Rb is involved in leptin's effects on body weight and energy regulation. There is evidence that leptin mediates its effects via the synthesis and release of a number of neuropeptides in specific brain areas. A number of these pathways have been identified and include neuropeptide Y, a potent stimulator of food intake (Kotz *et al.* 1998); α -melanocyte stimulating hormone, an inhibitor of food intake (Satoh *et al.* 1998) and corticotrophin releasing hormone a peptide with inhibitory effects on feeding, energy expenditure and body weight gain (Costa 1997).

A number of leptin's actions have been attributed to increased sympathetic activity (Figure 1.8), leptin has been shown to increase sympathetic nerve activity to BAT and the kidney in rats (Haynes *et al.* 1997 a; Haynes *et al.* 1997b). Leptin acts upon UCP1 expression in BAT via the sympathetic nervous system (SNS) as discussed in Chapter 1.3.3 (Figure 1.8). The increased peripheral SNS activity is thought to be mediated by activation of Ob-Rb in the ventromedial hypothalamus -sympathetic (or its neighbouring medial hypothalamus-sympathetic) nervous system. Leptin injection into this area increased glucose uptake in BAT through activation of the SNS, but had no effect on glucose uptake in WAT (Minokoshi *et al.* 1999). This increase in glucose was suppressed in after sympathetic denervation of BAT.

Outside the brain, Ob-Rb expression accounts for only small part of the total amount of leptin receptors. In the peripheral tissues the truncated forms Ob-Ra and Ob-Re are most abundant. Ob-Ra is expressed in many tissues, but mainly in kidney, lung and choroid plexus (Fei *et al.* 1997). The function of Ob-Ra is not known, but is thought to facilitate transport of leptin across the blood brain barrier (Boado *et al.* 1998). This is consistent with the pattern of high expression of Ob-Ra in brain microvessels and choroid plexus and low expression in the hypothalamus. There is some evidence that Ob-Ra has some signalling capabilities, although these *in vitro* results could not differentiate whether Ob-Ra homodimerised or formed heterodimers with Ob-Rb - or other cytokine receptors to induce signalling (Murakami *et al.* 1997; Yamashita *et al.* 1998).

The soluble form of the leptin receptor, Ob-Re is predicted to have no intracellular or transmembrane domains. It is proposed that Ob-Re is a soluble leptin binding protein (Huang *et al.* 2001). The level of Ob-R and leptin increase by up to 40 fold during the late stages of mouse pregnancy, suggesting that the soluble leptin receptor may modulate leptin action *in vivo* (Gavrilova *et al.* 1997). Over-expression of Ob-Re in rodents delays the clearance of leptin from the body, without affecting leptin expression (Huang *et al.* 2001).

1.5.4 Physiological Regulation of Leptin

Circulating levels and expression of leptin in the adult are regulated by a number of factors, these are summarised in Figure 1.10. Many of the factors responsible for the regulation of leptin are highly active during the transition from fetus to neonate, especially the SNS, cold and glucocorticoids. A full examination of the factors responsible for leptin regulation is not possible in this thesis, a detailed review can be found in Friedman and Halaas, 1998 (Friedman 1998) and Trayhum *et al* 1999 (Trayhum *et al.* 1999). However, it must be kept in mind that much of the information regarding leptin regulation comes from adult rodent studies and not large mammals.

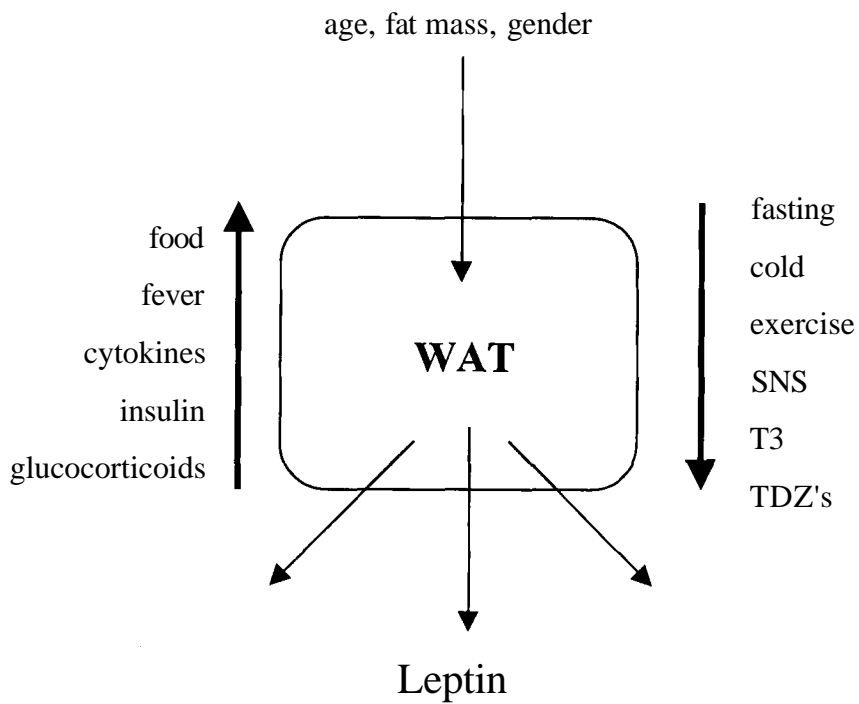


Figure 1.10

Summary of the physiological regulation of leptin. WAT, white adipose tissue; SNS, sympathetic nervous system; T3, triiodothyronine; TDZ's, thiazolidinediones.

1.5.5 Leptin in the Fetus and Neonate

Leptin mRNA is detectable in fetal adipose tissue of sheep by G90 gestation with levels increasing up to term (Yuen *et al.* 1999), which is ~147 days in the sheep. Leptin mRNA levels are closely correlated with fetal weight. The slope of the relationship between leptin mRNA abundance and fetal weight is steeper at G90 compared with G125-140 gestation (Yuen *et al.* 1999). This suggests that leptin mRNA is modulated by the increase in size and number of adipocytes which occurs during late gestation in the ovine fetus, but is also sensitive to the rapid increase in body weight at this period. The increase in leptin mRNA may also be attributed to the rise in circulating corticosteroids during late gestation as glucocorticoids have been shown to stimulate leptin expression both *in vitro* and *in vivo* (De Vos *et al.* 1995). Leptin receptor mRNA has been demonstrated in a variety of murine fetal tissues (Hoggard *et al.* 1997a; Hoggard *et al.* 2000) although the physiological relevance of these is as yet unknown.

It has been suggested that leptin may act as a growth factor in the fetus, directing growth and development via central or peripheral actions (Hassink *et al.* 1997; Stepan & Swick 1999; Udagawa *et al.* 2000). Many studies have been carried out measuring plasma leptin concentrations in newborn infants; however, the results from these are highly inconsistent, as shown in Table 1.1. These contrasting findings may be due to a number of confounding factors including geographical location, maternal and social differences along with the clinical status of the infant at the time of blood sampling as "well" infants are not routinely blood sampled. Also there are

known to be sensitivity problems with the RIA kit used in some of these studies (Linco Multi-Species), regarding the detection of low-range values and cross-reactivity with non-specific plasma proteins of the "multi-species" antibody (Delavaud *et al.* 2000; Imagawa *et al.* 1998).

Results from human clinical studies suggest that leptin values remain low during early gestation, are detectable by around G35 and increase towards term in response to the increased abundance of adipose tissue (Cinaz *et al.* 1999; Matsuda *et al.* 1999). Leptin levels peak around birth in the infant, then rapidly decline by day 3 of postnatal life (Hytinantti *et al.* 1999; Schubring *et al.* 1999). There is also evidence that leptin is expressed in rodent adipose tissue at birth (Dessolin 1997) and may play a role in energy regulation of the neonate (Yuan *et al.* 2000). These postnatal changes are likely to be in response to the dramatic alterations in energy balance experienced by the neonate during the transition to enteral feeding, as fasting has been shown to reduce circulating leptin levels (Ahima 1996). Initially there may be some delay before the mother's milk develops and during the first few days and colostrum has a low energy content. There is evidence that leptin levels are higher in female compared to male infants as leptin production is inhibited by testosterone (Figure 1.10) (Behre *et al.* 1997; Hassink *et al.* 1997). Recent work in our department has shown that ovine postnatal leptin levels do not follow this trend, leptin levels in the lamb decline after birth then increase to 8 days of age (Budge *et al.* 2001).

Country of study	Gestation	Cord leptin (ng/ml)	Neonatal leptin (ng/ml)	Notes	Reference
Finland	Term	9.7 ± 5.2		Leptin concentrations correlate with adiposity in female but not in male newborns	(Hytiroantti et al., 1999)
Sweden	Term	7.3 (median)		Cord leptin correlates with foetal tissue mass	(Mochini et al., 1998)
London	Term	5.9 ± 3.0		Fetal leptin levels increase towards term	Gentry et al., 1999)
Denmark	Term	3.6 ± 1.4	0.4 ± 0.3	Cord leptin relates with adiposity and levels decrease after birth	Schuhman et al., 1992)
Turkey	Term		2.22 ± 0.52	Placental leptin levels correlate with adiposity and are related to nutritional status	Cinaz et al., 1999)
USA	Term	4.1 ± 3.5		Cord placental levels are correlated with adiposity with no gender differences	(Shekhwast et al., 1998)
USA	Term	4.2 (median)		Cord placental levels are correlated with birth weight and are reduced in premature infants	Tsiquimi et al., 1999)
Japan	Term	19.6 ± 14.3	1.9 ± 1.1	Leptin concentrations are reduced after birth	(Matsuda et al., 1992)
Japan	Term	Artery: 9.8 ± 1.2 vein: 12.9 ± 1.8		Leptin levels are higher in umbilical veins than arteries. Leptin concentrations fall rapidly after birth	(Yura et al., 1998)

Table Summary compares findings from various studies investigating fetal and neonatal leptin concentrations in the human. (Mochini et al., 2001).

1.6 Summary and Aim of Thesis

The postulated peak, then rapid decline in plasma leptin concentrations after birth in human neonates is coincident with the height of UCPI activity. The factors that regulate UCPI and plasma leptin have a substantial "cross-over" with the factors important in the transition from fetus to neonate, such as the SNS, Cortisol and thyroid hormones. The aim of this thesis is to investigate the endocrine regulation of the development of neonatal brown adipose tissue thermogenesis, with special emphasis on leptin.

CHAPTER 2

MATERIALS AND METHODS

All operative and experimental procedures had the required Home Office approval and were carried out according to the regulations of the Animals (Scientific Procedures) Act, 1986.

Laboratory procedures were carried out in accordance with the Control of Substances Hazardous to Health (COSHH: SI No 1657 1988) regulations and following Good Laboratory Practice guidelines (University of Nottingham Safety Office).

Unless otherwise stated all chemicals and reagents were purchased from Sigma-Aldrich Company Ltd., and laboratory plastics and equipment from Scientific Laboratory Supplies. Addresses for all suppliers are located in Appendix I. Unless otherwise stated all aqueous solutions were made up in single distilled water (dH₂O).

2.1 Animals

All animals entered into these studies were housed at the University of Nottingham's Joint Animal Breeding Unit, Sutton Bonnington Campus, Sutton Bonnington with the exception of Chapters 4 and 5. Bluefaced Leicesters cross Swaledale ewes were mated with Texel rams and pregnancy was confirmed using real-time ultrasound echograph between 30 and 50 days gestation. Ewes were group housed at 6 weeks prior to their predicted lambing date and individually housed 1 week later. When individually housed post-partum ewes were fed daily at 08:30 hour with chopped

hay (~1 kg per day) and a barley based concentrate (2 X 500 g fed morning and evening), ewes had free access to water at all times.

2.1.1 *In vivo* measurements

All *in vivo* measurements of CO₂ production, shivering and colonic temperature made on neonatal lambs were recorded and analysed using the 'Cardas' sleep cardiorespirogram package and described in full in Chapters 2.1.1a, b and c.

2.LLa Indirect calorimetry

The rate of CO₂ production of lambs was measured using the technique of open-circuit indirect calorimetry (Symonds et al. 1986). Lambs were placed in an air-tight calorimetry chamber located within a larger temperature controlled room (Ilco Refrigeration), within which ambient temperature could be controlled to ± 1 °C. Air was drawn through the chamber by a vacuum pump (ECBI vacuum/compressor; BOC Edwards) at a rate of 50-70l/min, the exact flow rate was measured by an electronic flow meter (Percept PT4; SIP Analytical Ltd.). Air leaving the chamber was dehydrated as it passed through a silica gel column (4-7 mesh) and a continuous sample was analysed for carbon dioxide using an infra-red carbon dioxide analyser (ADC 439; Analytical Development Company Ltd.). The system was calibrated initially as the lamb was placed in the chamber then subsequently every 2 hours for the duration of the study, very little drift was observed during the studies.

Atmospheric air was used as the zero calibrator and compared to a mixture of gas of known CO₂ (0.191 % difference) concentration (Span Gas; Cryoservice Ltd.).

2.L1.b Colonic Temperature

Colonic temperature was measured using an electronic temperature probe (Type 3GID; Light Laboratories) inserted 8 cm into the rectum. The probe was secured to the base of the tail with a strip of sticking plaster.

2.1.1.c Breathing Frequency and Incidence of Shivering

Breathing frequency was measured by inductance plethysmography, using two respitrace bands, secured around the thorax and abdomen with sticking plaster. The bands consisted of teflon coated wires in a zig-zag arrangement. Resistance in the wire caused by expansion and contraction of the thorax during breathing movements generates a signal and hence the respiratory pattern. Interference on the inductance waveform was used to assess the incidence of shivering, and non-REM sleep was detected as a slow, regular pattern on the respiratory trace.

2.L2 Collection of Plasma Samples

All blood samples were taken through a polyvinyl catheter (internal diameter 1.1 mm, external diameter 1.6mm; Portex Ltd.) introduced into the jugular vein through a 14 gauge butterfly needle (Venoflux 247.21; Vygon) following shaving. This was carried out after local anaesthetic had been sprayed onto the area. Overnight, the catheter was filled with sterile saline (0.9 % (w/v) sodium chloride; Baxter Healthcare Ltd.) containing 250 units/ml of heparin, this concentration was reduced to 50 units/ml during sampling. Care was taken not to inject heparinised saline into the animal at any time. Approximately 5-10 ml of blood was sampled from each animal; prior to this a 2 ml volume of saline was taken and discarded,

ensuring that the sample was not contaminated. Blood samples were immediately placed on ice in tubes containing heparin (sodium salt) as an anticoagulant and stored at -20 °C following centrifugation at 800g at 4 °C for 15 minutes.

2.L3 Collection of Tissue Samples

All samples were taken as soon as possible after humane euthanasia of the animal (see individual studies for details) and snap frozen in liquid nitrogen. Samples were subsequently stored at -80 °C until analysis. All major organs were dissected out carefully and weighed prior to freezing with the exception of samples required for immunohistochemical analysis which were placed in 10 % formol saline (10 % (v/v) Formaldehyde in 0.9 % (v/v) sodium chloride).

2.2 Analysis of Plasma Samples

2.2.1 Glucose

Plasma glucose levels were determined using a quantitative enzymatic colourimetric assay, supplied in a kit (315-500 Sigma Aldrich Company Ltd.) based on the method of [Trinder, 1969 #602]

2.2.1.a Principle of the Assay

The enzyme Glucose Oxidase (GOD) catalyses the transformation of glucose and water in the presence of oxygen to gluconic acid and hydrogen peroxide. The hydrogen peroxide formed reacts with 4-aminoantipyrine and p-hydroxybenzene sulphate, which is catalysed by peroxidase, to form a quinoeimine dye with

maximal absorbance at 505nm . The intensity of the dye is directly proportional to the glucose concentration in the sample.

2.2.1.b Assay Procedure

A standard curve (0-16 mM) was prepared from a stock solution of 32 mM D- (+)-glucose diluted in dH₂O. Duplicate 10 µl aliquots of each standard and sample were transferred to a 96 well plate to which 200 µl Trinder reagent (0.5 mM 4-aminoantipyrine, 20 mM p-hydroxybenzene sulphonate, 15,000 U/L Glucose Oxidase, 10,000 U/L Horseradish Peroxidase in buffer at pH 7.0) was added. The plate was incubated on a shaker at room temperature for 15 minutes before measuring absorbance at 540 nm. (Anthos Reader 2001, Anthos Labtec Instruments) and plasma glucose concentration determined from regression analysis of the standard curve. The intra- and inter-assay coefficients of variation were 2.3 % (n=6) and 11.3 % (n=8), respectively.

2.2.2 NEFA

Plasma concentrations of NEFA were assessed using Waco Kh no. 994-75409 (Alpha Laboratories) as described by Symonds *et al* [Symonds, 1986 #2].

2.2.2.a Principle of the Assay

This assay utilises an *in vitro* enzymatic colourimetric method for the quantification of NEFA. NEFA in semm form thiol esters of coenzyme A (CoA) (known as acyl-CoA) when treated with acyl-CoA synthetase, in the presence of adenosine triphosphate (ATP), magnesium cations and CoA. Acyl-CoA is oxidised by acyl-

CoA oxidase to produce hydrogen peroxide. Peroxidase enzymes then catalyse the oxidative condensation of 3-methyl-N-ethyl-N-(4-hydroxyethylaniline) (MEHA) with 4-aminoantipyrine in the presence of hydrogen peroxide to form a purple coloured adduct with maximum absorption at 550 nm.

2.2.2.b Assay Procedure

A Standard curve (0 - 1.97 mM) was produced from the 1.0 mM oleic acid provided in the kit. Ten μl aliquots of standard or sample plasma were assayed in duplicate. Fifty μl of colour reagent A (acyl-coenzyme A Synthetase, ascorbate oxidase, coenzyme A, adenosine triphosphate and 4-aminoantipyrine) was incubated with the sample/standard at 37 °C for 10 minutes. 100 μl of colour reagent B was then added to each assay tube (acyl-coenzyme A oxidase, peroxidase, MEHA) for a further 10 minutes incubation at 37°C. Tube contents were equilibrated at room temperature for 10 minutes prior to measuring absorbance at 550 nm (reference wavelength 405 nm). Plasma NEFA concentrations were determined following linear regression analysis of the standard curve. The intra- and inter-assay coefficients of variation were 1.7 % (n=6) and 4.8 % (n=9), respectively.

2.2.3 Leptin

Plasma levels of leptin were analysed by Duane Keisler at the University of Missouri, using an ovine specific double-antibody, non-equilibrium radioimmunoassay according to the method of Delavaud [Delavaud, 2000 #405].

2.2.3.a Principle of the Assay

Leptin present in a sample/standard competes with a fixed amount of radiolabelled leptin for a limited number of binding sites provided by the addition of a polyclonal rabbit antibody raised against the purified leptin. Unbound radiolabelled leptin is separated from bound following the addition of a second antibody, raised in sheep directed against immuno- γ -globulin to which horse serum is added to equalise the protein content in all tubes. Precipitation of the antibody-antigen complexes was accomplished by the addition of polyethylene glycol followed by centrifugation. The radioactivity in the pellet is measured with the disintegrations per minute (dpm) being inversely proportional to the concentration of leptin in the initial sample or standard.

2.2.3.b Assay Procedure

A standard curve (0.0833 - 4.0 ng) was prepared from 1 $\mu\text{g}/\text{ml}$ leptin. Fifty μl aliquots of standards and one hundred μl plasma aliquots were assayed in triplicate. Standards and samples were incubated for 24 hours at 4 °C with 50 μl of 1:1200 working dilution of leptin antiserum (diluted in buffer containing 1:100 normal rabbit serum) to achieve a total volume of 400 μl in incubation buffer. One hundred μl ^{125}I -ovine leptin (20,000 c.p.m.) was then added to each tube and incubation continued for a further 20 hours at 4 °C. The final dilution of leptin antisera was 1:15000. Bound and free ligands were separated by the addition of 100 μl specific anti-rabbit ram plasma diluted either 1:5 in horse serum for standard curves or 1:5 in incubation buffer for unknown plasma samples, this equalised the protein content of

all tubes. Antibody-antigen complexes were precipitated by the addition of 2 ml 4.4% polyethylene glycol and centrifugation at 3000g at 4 °C for 25 minutes. Unbound ¹²⁵I-ovine leptin was removed by aspiration of the supernatant and the radioactivity of the remaining pellet was counted with a Cobra II gamma counter (Packard Inc., Downers Grove, Australia). The intra- and inter-assay coefficients of variation were 4 % and 11 %, respectively [Delavaud, 2000 #405]. The lower limit of sensitivity was 0.83ng/ml for a 100 µl sample [Delavaud, 2000 #405].

2.2.4. Prolactin

Plasma levels of prolactin were analysed by Helen Budge using a specific double-antibody, non-equilibrium radioimmunoassay according to the method of McMillen *et al* 1987 [McMillen, 1987 #630].

2.2.4.a Principle of the Assay

The prolactin assay relied on the same methodology to that of the leptin assay (section 2.2.3) with the exception that the plasma was incubated with a specific anti-sera raised against prolactin in sheep and the second antibody was goat anti-rabbit serum.

2.2.4.b Assay Procedure

A standard curve (0.078 - 10.0ng/100 µl) was prepared from 50ng/ml prolactin (a gift from National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)). One hundred µl aliquots of standards or 5 µl sample were made up to 250 µl with assay buffer (0.015 M sodium chloride, 0.015 M sodium azide, 2 %

(w/v) BSA made up to volume with 0.5 M phosphate buffer (pH 7.4)) and were assayed in duplicate. Standards and samples were incubated for 18 hours at 4 °C with 50 µl of 1:21,000 working dilution of prolactin antisem (diluted in assay buffer containing 2 % (v/v) normal rabbit sem) and 100 1 ¹²⁵ I-ovine prolactin (10,000 c.p.m./100 l). Bound and free ligands were separated by the addition of 100 µl specific anti-rabbit goat plasma (diluted 1:8) and 100 µl of normal rabbit sem to equalise the protein content of all tubes followed by an 18 hour incubation at 4 °C. Antibody-antigen complexes were precipitated by centrifugation at 4000g at 4 °C for 30 minutes. Unbound ¹²⁵ I-ovine prolactin was removed by aspiration of the supernatant and the radioactivity of the remaining pellet was counted with a 1260 Multigamma II gamma counter (LKB, Pharmacia Diagnostics). The intra- and inter-assay coefficients of variation were 4.9 % (n=4) and 9.0 % (n=6), respectively. The sensitivity of the assay (defined by the dose required to produce a 10% displacement) was 0.1 ng/tube [Houghton, 1997 #685].

2.3 Analysis of Tissue Samples

Preparation and sorting of tissue samples prior to analysis was carried out on dry ice so no significant thawing of the samples occurred. For each analysis, the required amount of frozen tissue was cut from the sample and the remainder returned to the freezer.

2.3.1 Mitochondrial Preparation

Mitochondria from various tissues were prepared according to the method of Symonds *et al* (1992)[Symonds, 1992 #603] for analysis of thermogenic potential i.e. GDP binding.

2.3.1.a Mitochondria Preparation Method

Approximately 1 g of perirenal adipose tissue was thawed on ice in 10 ml tris [hydroxymethyl] amino-methane (tris) - sucrose homogenisation buffer (10 mM tris, 250 mM sucrose and 1 mM ethylenediaminetetraacetic acid (EDTA) pH7.4) and homogenised using an Ultra Turrax homogeniser for 2 x 30 seconds. The homogenate was transferred to a glass homogenisation tube and further homogenised with 10 strokes in a Potter-Elvehjem homogeniser. The homogenate was made up to 20 ml with homogenising buffer and an aliquot of each sample was collected for total protein concentration analysis.

The remaining homogenate was centrifuged at 800g for 10 minutes at 4 °C and the supernatant passed through 2 layers of surgical gauze in order to remove lipid. The supernatant was then centrifuged at 10,000 rpm for 30 minutes at 4 °C. The resulting mitochondrial pellet was resuspended a total volume of around 1ml homogenisation buffer and stored at -20 °C until further analysis.

2.3.2 Lowry Assay

Protein concentration of mitochondria and whole cell homogenates were assessed according to the method of Lowry, Rosenbrough, Farr & Randall (1951)[Lowry,

1951 #604] taking into account the modifications of Dulley & Grieve [Dulley, 1975 #623] using Folin reagent.

2.3.2.a Principle of the Assay

The Lowry method relies on two different reactions. The first is the formation of a copper ion complex with amide bonds, forming reduced copper in alkaline solutions. This is called a "Biuret" chromophore. The second is the reduction of Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate) by tyrosine and tryptophan residues. The reduced Folin-Ciocalteu reagent is blue and thus detectable with a spectrophotometer in the range of 500-750 nm. The Biuret reaction itself is not very sensitive. Using the Folin-Ciocalteu reagent to detect reduced copper makes the assay nearly 100 times more sensitive than the Biuret reaction alone. The Lowry assay is relatively sensitive, but susceptible to many interfering compounds, particularly detergents therefore the BCA assay (see section 2.3.3 for details) was employed for analysis of samples containing these. The standard curve is linear in the 1 to 100 μg protein region.

2.3.2.b Assay Procedure

Mitochondrial suspensions and cell homogenates were diluted 1 in 50 and 20 respectively in dH_2O to ensure concentrations fell within the linear range. A standard curve (0 – 100 μg) was prepared using bovine serum albumin (BSA) in dH_2O . Solution A (0.2 % (w/v) sodium dodecyl sulphate (SDS), 2 % (w/v) sodium carbonate in 0.1 M sodium hydroxide) and B (1 % (w/v) potassium sodium tartrate,

0.5% (w/v) copper sulphate pentahydrate) were prepared and a ratio of 100A:2B was mixed on the day of assay. One ml of A:B mix was added to 200 μ l sample/standards in duplicate which were incubated at room temperature for 10 minutes. One hundred μ l of Folin's reagent (1:1 with dH₂O) was added to each tube and the contents were mixed well. After 45 minutes incubation at room temperature the absorbance at 620 nm (reference wavelength 405nm) was measured. Protein concentrations were determined following linear regression analysis of the standard curve and corrected for the initial dilution. The intra- and inter-assay coefficients of variation were 6.0 % and 6.8 %, respectively.

2.3.3 Western Blotting

The abundance of selected proteins was measured using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Bumette (1981) [Bumette, 1981 #620]. All western blots were performed in duplicate.

2.3.3.a Principle of the Assay

Sodium dodecyl sulphate (SDS) is an anionic detergent that denatures proteins by "wrapping around" the polypeptide backbone, SDS binds to proteins specifically in a mass ratio of 1.4 g SDS : 1 g protein. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length - ie: the denatured polypeptides become "rods" of negative charge with equal charge or charge densities per unit length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with 2-

mercaptoethanol or dithiothreitol (DTT). The SDS-protein complexes are then electrophoresed through a polyacrylamide gel, with proteins moving towards the anode at a rate inversely related to their size, due to the sieving effect of the gel. The pore size of the acrylamide gel is dependent on the concentration of acrylamide and cross-linking bisacrylamide used in the gel preparation. Therefore, in SDS-polyacrylamide gel electrophoresis (PAGE) migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight. Proteins separated by SDS-PAGE can be transferred to nitrocellulose membrane for immunoblotting analysis. For this procedure, an electric current is applied to the gel so that the separated proteins transfer through the gel and onto the membrane in the same pattern as they separated on the SDS-PAGE. Areas of the membrane that do not contain blotted protein from the gel, can be non-specifically "blocked" so that antibody will not bind to them, causing a false positive result. To detect a specific antigen blotted on the membrane, for example UCPI, a primary antibody is added at an appropriate dilution and incubated with the membrane. If there are any antibodies present which are directed against one or more of the blotted antigens, those antibodies will bind to the protein(s) while other antibodies will be washed away at the end of the incubation. In order to detect the antibodies which have bound, anti-immunoglobulin antibodies coupled to a reporter group such as the enzyme horseradish peroxidase (HRP) are added; this anti-Ig-enzyme is commonly called a "second antibody". Finally, after excess second antibody is washed free of the blot, a substrate is added which emits light upon reaction with the conjugate, resulting in a visible band where the primary antibody bound to the protein.

2.3.3.b Assay Procedure

Gel Preparation

Polyacrylamide gels were cast using alumina and glass plates and teflon spacers and combs supplied with a dual gel caster (SE 245; Hoefer Pharmacia Biotech Inc.).

Unless otherwise stated, all gels were 12 % acrylamide. The resolving gel (10 ml resolving gel: 3.3 ml dH₂O, 4.0 ml Acrylamide-bisacrylamide solution (37:5:1), 2.5 ml 1.5M Tris (pH 8.8), 100 µl 10 % (w/v) SDS, 100 µl 10% (w/v) Ammonium persulphate and 4 µl N,N,N',N'-tetramethylethylenediamine (TEMED)) was poured between an alumina and glass plate at a thickness of 0.75 mm to a level 1.5 cm below the position of the wells. One ml of water saturated butanol was placed on top of the resolving gel to ensure a uniform surface and polymerisation of the gel. After 45 minutes the butanol was decanted and the gel surface rinsed with dH₂O. Stacking gel (10 ml stacking gel: 6.8 ml dH₂O, 1.7 ml Acrylamide-bisacrylamide solution (37:5:1), 1.25 ml 1.0 M Tris (pH 6.8), 100 µl 10% (w/v) SDS, 100 µl 10% (w/v) ammonium persulphate and 10 µl TEMED) was poured on top of the resolving gel and a 15 well comb inserted between the glass and alumina plates to create wells. After polymerisation the comb was removed and the gels were transferred to a Mighty Small™ II (SE 250; Hoefer Pharmacia Biotech Inc.) vertical slab electrophoresis unit. The wells, upper and lower chambers were filled with 1 X tris-glycine electrophoresis buffer (25 mM Tris, 250mM Glycine and 0.1 % (w/v) SDS).

Sample preparation and Electrophoresis

Mitochondrial suspensions of known protein concentration were diluted in tris-sucrose homogenisation buffer to 4.2 mg/ml in a final volume of 20 μ l, this concentration equates to 10 g protein in each well. Fifty μ l of protein dissociation buffer (10 % (v/v) glycerol, 2 % (w/v) SDS and 5 % (v/v) -mercaptoethanol made up to volume in 50 mM tris (pH 6.8)) and 14 μ l glycerol-bromophenol blue (16 % (v/v) glycerol and 0.001 % (w/v) bromophenol blue in dH₂O) was added to each sample which were incubated at 100 °C for 4 minutes. Ten μ l of sample, reference or molecular weight marker was loaded into each lane, empty lanes were filled with 10 μ l glycerol-bromophenol blue, and electrophoresed at 40 mA per gel for approximately 45 minutes, or the dye front was sufficiently near the bottom of the gel.

Electroblotting

Proteins from the polyacrylamide gel were transferred to a solid support (Hybond™ - C Super, Amersham Life Science) using electroblotting (EF100B Electroblotter; Cambridge Electrophoresis Ltd). The gel and membrane were sandwiched between 2 layers of blotting paper soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20 % (v/v) methanol, 0.037 % (w/v) SDS made up to volume with dH₂O) and supported by 2 scotch-Brite pads as demonstrated in Figure 2.1. This "sandwich" was placed in a cassette and inserted into the electroblotter that was filled with transfer buffer. A cooling bath was connected to the electroblotter to ensure that the proteins were not denatured during the high voltage transfer process. The proteins were then blotted for 1 hour at 80 mA.

(+) POSITIVE ELECTRODE (ANODE)
 ***** Plastic cassette
 ||||| Scotch-Brite pads
 2 sheets blotting paper
 _____Nitrocellulose membrane
 _____Polyacrylamide gel
 2 sheets blotting paper
 ||||| Scotch-Brite pads
 ***** Plastic cassette
 (-) NEGATIVE ELECTRODE (CATHODE)

Figure 2.1

Diagram outlining the westem blot procedure.

Following electroblotting, the proteins were reversibly visualised using Ponceau S stain (2 % (w/v) ponceau S, 30 % (w/v) trichloroacetic acid, 30 % (w/v) sulphosalicylic acid in dH₂O, this stock solution was diluted 1 in 10 with dH₂O prior to use) which allowed for the position of molecular weight markers to be noted and verification of even blotting. Ponceau S dye was removed by rinsing the membrane in Tween-tris buffered saline (TTBS, 0.2 % (v/v) Tween 20 in 20 mM tris, 500 mM NaCl in dH₂O, pH 7.5).

Immunodetection

Nitrocellulose membrane were incubated overnight at 4 °C in blocking buffer (10 % (w/v) dried milk powder (Marvel; Premier Brands UK Ltd.) in TTBS) in order to block any remaining binding sites on the membrane. The blocking solution was discarded and the membrane rinsed twice in TTBS before incubation at room temperature for 1 hour with an appropriate dilution of primary antibody (see appendix III for details) or normal rabbit serum in order to establish non-specific binding (TTBS containing primary antibody and 3 % (w/v) dried milk powder). The membrane was then washed for 3 x 10 minutes with TTBS before addition of an appropriate dilution of secondary antibody (TTBS containing 3 % (w/v) dried milk powder and HRP conjugated swine anti-rabbit immunoglobulins (PO 217; Dako Ltd.) unless otherwise indicated) and incubated for 1 hour at room temperature. The secondary antibody was discarded and the membrane was washed for 4 x 15 minutes in TTBS. Finally the membrane was washed, then soaked in tris buffered saline (TBS) for 30 and 45 minutes respectively.

Enhanced Chemiluminescence

The membrane was incubated in ECL western blotting reagents (1:1 of reagents A and B, Amersham Life Science) for exactly 1 minute before exposure in a CCD camera (Fugi; Luminescent Image Analyser LAS-1000). Exposure time depended on the nature of the tissue type and the antibody used, but was normally between 1 and 15 minutes.

Densitometric analysis was carried out on the bands visualised using Aida software (Aida version 2.0; raytest Isotopenmeßgeräte GmbH), in order to calculate the relative abundance of the protein studied. Protein values were expressed as a percentage of a reference sample present on all gels. Approximate size of the visualised band was calculated using regression analysis of the molecular weight markers.

2.3.4 GDP Binding

GDP binding was carried out according to the method of Nicholls 1976 [Nicholls, 1976 #498] as described by Symonds *et al* [Symonds, 1992 #603]. Mitochondrial GDP-binding is the most widely used *in vitro* measurement of thermogenesis in BAT [Trayhum, 1989 #501], taking advantage of the high affinity of UCPI for purine nucleotides [Nicholls, 1976 #498]. Although other purine nucleotides may be used, GDP is almost always selected, primarily as it shows one of the highest affinities for UCPI [Lin, 1982 #333] and is not translocated into the inner mitochondrial space such as is ADP.

2.3.4.a Principle of the Assay

Mitochondria are incubated with a fixed amount of radiolabelled GDP ($[^3\text{H}]\text{-GDP}$) and one of two concentrations of unlabelled or "cold" GDP. One concentration of GDP (2 pM) represents the normal substrate range and the other (200 pM) is past the point of substrate saturation. This high concentration of cold GDP is included to determine the extent of non-specific binding. The bound GDP is separated from the free by centrifugation leaving the bound $[^3\text{H}]\text{-GDP}$ to be assessed by counting β radiation.

2.3.4.b Assay Procedure

Mitochondrial suspensions (see section 2.3.1) were diluted to approximately 1 mg/ml in tris-sucrose homogenisation buffer. One hundred μl of diluted mitochondria was added to reaction tubes containing 850 μl incubation buffer

(100mM sucrose, 20mM N-fris (hydroxymethyl) methyl-2-aminoethane sulphonic acid (potassium salt) (TES), 1mM EDTA, 10 mM choline chloride, 0.8 % (w/v) BSA (essential fatty acid free), 5 pM rotenone, 79 pM [3H]-GDP (10 Ci/mmol, Amersham International PLC) and either 50 μ l 2 μ M cold GDP (in triplicate) or 50 μ l 200 μ M cold GDP in incubation buffer.

GDP binding was assessed over a 7 minute incubation at room temperature with continuous shaking. The reaction was stopped by centrifugation for 3 minutes at 10,000 rpm. The supernatant was discarded and the pellet dissolved by addition of 100 μ l 0.75 M sodium hydroxide with a further incubation at 55 °C for 15 minutes. A 50 μ l volume was taken from the dissolved pellet solution and mixed with 5 ml Ecoscint A (National Diagnostics and [3H] was counted using a Packard Tri-card 460 CD scintillation counter (Packard Instruments). The specific binding was then calculated following correction for non-specific binding i.e. measured using 200nM GDP.

2.3.56 RNA Preparation

RNA was prepared from BAT using the TRI REAGENT™ (Sigma Aldrich Company Ltd.) which is an improved version of the single-step RNA isolation developed by Chomczynski and Sacchi [Chomczynski, 1987 #624].

2.3.5.a Principle of the assay

The TRI REAGENT™ is a reagent for use in the simultaneous isolation of RNA, DNA and protein. The procedure is an improvement of the single step method of total RNA isolation developed by Chomczynski and Sacchi [Chomczynski, 1987

#624]. TRI REAGENT™ is a mixture of guanidine thiocyanate and phenol in a mono-phase solution that effectively dissolves RNA upon homogenisation of the tissue; addition of chloroform separates the homogenate into three phases, the aqueous one containing the RNA. Separation of the phases and further alcohol precipitation yields intact RNA with little or no contaminating DNA or protein.

2.3.5.b Assay Procedure

RNA Precautions

RNA is more susceptible to degradation than DNA, due to the ability of the 2' hydroxyl groups adjacent to the phosphodiester linkages in RNA to act as intramolecular nucleophiles in both base- and enzyme-catalyzed hydrolysis.

Therefore, it is essential when working with RNA that special precautions are made, such as wearing gloves and keeping samples on ice, in order to reduce degradation of RNA. Diethyl Pyrocarbonate (DEPC) is used to treat water (DEPC-H₂O) and solutions used to prepare RNA. DEPC derivitizes histidine residues and is therefore an effective method to inactivate nucleases including RNase. DEPC has a half-life of approximately 30 minutes in water, and at a DEPC concentration of 0.1 %, solutions autoclaved for 15 minutes/litre can be assumed to be DEPC-free.

RNA Preparation

Approximately 100 mg of perirenal adipose tissue was homogenised in TRI REAGENT™ (1 ml/100 mg tissue). The homogeniser was rinsed between each sample tip in a beaker of DEPC-H₂O so not to cross-contaminate samples.

Perirenal adipose tissue (PAT) contains high levels of fat that can contaminate the RNA, PAT samples were homogenised at this point at 12,000g, 4 °C for 10 minutes. The fatty material on the surface of the aqueous phase was discarded and the supernatant transferred to a fresh tube. Samples were incubated at room temperature for 5 minutes to ensure total dissociation of nucleoprotein complexes before addition of 200 μ l chloroform. Samples were vortexed and incubated at room temperature for a further 15 minutes.

The resulting mixture was centrifuged at 12,000g, 4 °C for 15 minutes in order to separate the 3 phases: a red organic phase, an interphase and a colourless upper aqueous phase which contains RNA, this phase was transferred to a fresh tube to which 500 μ l isopropanol was added. Samples were incubated at room temperature for 10 minutes prior to centrifugation at 12,000g, 4 °C for 10 minutes. The supernatant was removed and the RNA pellet washed in 1 ml of 75 % (v/v) ethanol prior to centrifugation at 7,500g, 4 °C for 5 minutes. Again the supernatant was discarded and the pellet was dried in air for 5-10 minutes.

The resulting pellet was dissolved in an appropriate volume of DEPC-H₂O (usually ~20 μ l) and stored at -80 °C until further analysis.

Determination of RNA concentration

RNA can be accurately quantified by measuring its absorbance in a spectrophotometer [Wilkinson, 1995 #627]. The optical density (OD) of RNA is measured at its maximum wavelength of 260 nm. One OD unit is equivalent to

40 µg/ml of RNA. RNA samples were diluted 1:500 in DEPC-H₂O for concentration determination and placed in a quartz cuvette. Absorbance was measured at 260 and 280 nm in a spectrophotometer (UV 1101 Biotech Photometer) for determination of RNA concentration as well as DNA concentration, this gives an indication of the degree of contamination of genomic DNA. RNA concentration was calculated using the following equation:

$$\text{RNA concentration } (\mu\text{g}/\mu\text{l}) = (\text{OD}_{260} \times 40 \times 500) / 1000$$

A 260OD/280OD ratio between 1.6 and 1.8 was accepted as uncontaminated.

2.3.6 Northern Blotting

Northern blotting was carried out according to the method of Alwine *et al* 1977 [Alwine, 1977 #718] on PAT RNA in order to determine the expression of UCPI.

2.3.6.a Principle of Assay

Northern blotting is the term that refers to the transfer of RNA from a gel to a filter. An RNA sample is subjected to formaldehyde gel electrophoresis and transferred to a filter so that the separation achieved on the gel is maintained on the solid support. Formaldehyde is used to prevent RNA forming secondary structures. Signals obtained after subsequent hybridisation with a suitable probe can be compared to control samples to give information about the abundance (signal intensity) or size (distance of migration) of RNA transcripts [Dyson, 1995 #628].

2.3.6.b Assay Procedure

Gel preparation

A 1.4 % (w/v) formaldehyde gel (1.4 g agarose, 83 ml 1 X 3-(N-Morpholino) Propanesulphonic Acid (MOPS) heated until the agarose dissolved, cooled to 60 °C then 17 ml 37 % formaldehyde was added) was prepared and poured into a gel caster containing a comb which formed wells. The gel was left to set in a fume hood. When set, the comb was removed and the gel placed in a gel running compartment filled with 1 X MOPS buffer.

Sample Preparation and Gel Electrophoresis

Thirty pg of RNA was aliquoted into a fresh tube and made up to 4.8 l with DEPC-H₂O. To each sample 10 µl formamide, 2 µl 10 X MOPS and 3.2 µl formaldehyde was added and the samples were incubated at 65 C for 5 minutes. Samples were chilled on ice for 5 minutes and 2 µl of gel loading buffer (50 % (v/v) glycerol, 0.1 mg/ml bromophenol blue made up to volume with DEPC-H₂O) and 0.1 µl of ethidium bromide (EtBr) was added.

The whole volume of each samples, along with reference and base pair markers, were gently pipetted into the wells of the formaldehyde gel. The gel running compartment was attached to a power pack and the gel was run at 80 V for approximately 3 hours or until the dye front was sufficiently near the bottom of the

gel. The gel was visualised under UV illumination to ensure equal loading and that no degradation had occurred.

Blotting

RNA from the formaldehyde gels was transferred to a solid support (+vely charged membrane details) using capillary blotting. Gels were washed in DEPC-H₂O for 30 minutes prior to blotting to remove formaldehyde. A plastic stand was placed in a tray containing 150 ml 10 X standard saline citrate (SSC) (dilute 20 X SSC; 3 M sodium chloride and 0.3 M sodium citrate, make up to volume with DEPC-H₂O, pH 7.0). A glass plate was placed over the stand and 3 pre-soaked sheets of filter paper were draped over it forming a wick. The gel was inverted and placed on the filter paper. A sheet of nylon membrane (positively charged nylon membrane; Roche Molecular Biochemicals) was cut to the size of the gel and soaked in 10 X SSC prior to being placed on top of the gel so that its edges ran parallel with the top of the gel. Bubbles between the gel and membrane were removed with gently rolling of a sterile glass rod across the membrane. Three sheets of filter paper were soaked in 10 X SSC and placed on top of the membrane followed by a 6 cm stack of paper towels and a glass plate. The whole "sandwich" was held in place with a 500 g weight on top of the glass plate. Enough 10 X SSC was added to the tray to give a depth of 1 cm. The blotting process was left to proceed over night. When blotting was complete, the stack was dismantled and the membrane placed on filter paper. The membrane was cross-linked at maximum intensity (UV Stratalinker 1800, Stratagene) on both sides and stored at room temperature sealed in an air-tight bag until further analysis.

Probe Labelling

Anti-sense oligonucleotide probes for UCP1 and 18S (see below) were identified and produced in-house.

UCP1: 5'GAT CCC GGA CTT TGG CGG TGT CCA GCG GGA AGG TGA TG 3'

18S: 5'CTA GAC GCC TGC TGC CTT CCT TGG ATG TGG TAG CCG T 3'

Oligonucleotides were end-labelled using a DIG-oligonucleotide 3'-end labelling kit (Roche Molecular Biochemicals) which adds digoxigenin-ddUTP conjugates to the 3' end of an oligonucleotide. Five μl of a selected oligonucleotide was incubated at 37 °C for 15 minutes with 4 μl tailing buffer, 4 μl cobalt chloride solution, 1 μl DIG-ddUTP solution and 1 μl (50 units) terminal transferase (all Cat. No. 1362 372; Roche Molecular Biochemicals). The tailing reaction was then stopped by the addition of 2 μl glycogen solution (1 μl glycogen in 200 μl 0.2 M EDTA (pH 8.0) Cat. No. 1362 372; Roche Molecular Biochemicals). The labelled oligonucleotide was then precipitated with 2.5 μl 4 M lithium chloride and 75 μl pre-chilled absolute ethanol. After a 30 minute incubation at -80 °C the sample was centrifuged at 12,000 g for 5 minutes at 4 °C, the supernatant was discarded and the pellet washed with 50 μl pre-chilled 70 % (v/v) ethanol. The pellet was air dried, dissolved in an appropriate volume of DEPC-H₂O (10 μl is acceptable) and stored at -20 °C until use.

Chemiluminescent Detection

Detection of UCP1 and 18S on northern blots was carried out using the DIG (Roche Molecular Biochemicals) non-isotopic detection system. The DIG system is based on the steroid hapten digoxigenin that occurs in certain digitalis plants. The digoxigenin molecule may be coupled as a hapten to a suitable carrier molecule to produce high affinity antibodies, e.g. in sheep. Since digoxigenin occurs exclusively in digitalis plants there are no endogenous background problems with these antibodies as in the case of other haptens, such as biotin. Digoxigenin can also be coupled to nucleotides such as dUTP or UTP and incorporated into nucleic acids using generally available polymerases like Klenow polymerase, Taq polymerase, or RNA polymerases. The probes thus generated can be used in northern blotting and detected with anti-digoxigenin conjugates and the resulting signals can be detected by chemiluminescence.

The RNA membranes were washed for 5 minutes in 2 X SSC prior to a 3 hour pre-hybridisation in hybridisation buffer (50 % deionised formamide, 5 X SSC, 0.1 % (w/v) n-lauroylsarcosine, 0.02 % (w/v) SDS and 2 % blocking reagent (Roche Molecular Biochemicals) made up to volume with DEPC-H₂O) at 42 °C in a hybridisation oven (Hybaid micro 4, Hybaid). Membranes were then incubated overnight at 42 °C in hybridisation buffer containing an appropriate dilution of probe (10 µl end-labelled probe in 20 ml hybridisation buffer). The following day, membranes were washed twice for 5 minutes in wash 1 (2 X SSC, 10 % SDS made up to volume with DEPC-H₂O) at room temperature, and twice for 15 minutes in

wash 2 (0.1 X SSC, 0.1 % SDS made up to volume with DEPC-H₂O) at 48 °C. Membranes were then washed in maleic acid wash buffer (0.3 % tween-20 in maleic acid buffer (maleic acid buffer; 0.1 M maleic acid, 0.15 M sodium chloride, (pH 7.5) pH adjusted by addition of solid sodium hydroxide and made up to volume with DEPC-H₂O and autoclaved) for 5 minutes at room temperature. Membranes were then incubated in blocking buffer (10 % blocking reagent (Roche Molecular Biochemicals) made up to volume with maleic acid buffer) for 1 hour at room temperature and then in antibody solution (Anti-Digoxigenin -AP fab fragments (Roche Molecular Biochemicals) diluted 1:10,000 in blocking buffer) for 1 hour at room temperature. Membranes were then washed twice at room temperature in maleic acid wash buffer for 15 minutes and equilibrated for 2 minutes in detection buffer (0.1 M tris hydrochloride and 0.1 M sodium chloride made up to volume with DEPC-H₂O; pH 9.5) before excess wash buffer was drained from the membrane onto blotting paper. Membranes were placed on a sheet of saran wrap and incubated with approximately 3 ml of Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-yl)phenyl phosphate (CSPD) (Roche Molecular Biochemicals), then covered with another sheet of saran wrap a glass plate. After a 10 minute incubation at room temperature, excess CSPD was "squeezed" from the saran wrap using a tissue and the whole sandwich was heat - sealed around the edges to keep the membrane moist. After a 30 minute at 37 °C, chemiluminescence was detected using a CCD camera (Fugi; Luminescent Image Analyser LAS-1000).

Densitometric analysis was carried out on the bands visualised using Aida software (Aida version 2.0; raytest Isotopenmeßgeräte GmbH), in order to calculate the relative expression of the mRNA studied. UCPI mRNA expression was expressed as a percentage of 18S rRNA. Approximate size of the visualised band was calculated using regression analysis of the base pair markers.

2.3.7 Reverse Transcription and Polymerase Chain Reaction

Reverse Transcription and Polymerase Chain Reaction (RT-PCR) is a sensitive method for the detection and analysis of rare mRNA transcripts such as leptin. RNA cannot serve as a template for PCR, so it must first be reverse transcribed into cDNA (e.g. with reverse transcriptase from Moloney murine leukemia virus (M-MuLV)). Powell et al. first described a combined technique (now commonly known as RT-PCR) in which reverse transcription (RT) is coupled with PCR amplification of the resulting cDNA.

2.3.7.a Principles of the Assay

Reverse transcription is the process by which RNA is converted into DNA, catalysed by the enzyme reverse transcriptase. M-MLV Reverse Transcriptase is used to extend a random hexamer hybridized to an RNA sample containing the message of interest. The cDNA produced by reverse transcription is then utilised by PCR. The PCR reaction uses two oligonucleotide primers that hybridize to opposite strands and flank the target DNA sequence that is to be amplified. The elongation of the primers is catalyzed by a heat-stable DNA polymerase (Taq DNA Polymerase). A repetitive series of cycles involving template denaturation, primer annealing, and

extension of the annealed primers by the polymerase results in exponential accumulation of a specific DNA fragment. The ends of the fragment are defined by the 5' ends of the primers. Because the primer extension products synthesised in a given cycle can serve as a template in the next cycle, the number of target DNA copies approximately doubles every cycle; thus, 20 cycles of PCR yield about a million copies of the target DNA.

2.3.7.b Assay Procedure

Reverse Transcription

Reverse transcription was carried out on RNA produced from PAT (section 2.3.6). 1 μl of 1 $\mu\text{g}/\mu\text{l}$ RNA was incubated at 70 °C for 5 minutes with 0.5 μl hexamer mix (Roche Molecular Biochemicals) and 9.5 μl DEPC-H₂O then placed on ice. Then 8.5 μl of master mix (4 μl 5 x RT buffer (Promega), 2 μl 10 mM DNTPs (10 mM of each; dATP, dCTP, dGTP and dTTP), 0.5 μl RNase inhibitors (Roche Molecular Biochemicals) and 2 μl DEPC-H₂O) was added to each sample and incubated at room temperature for 5 minutes. Reverse transcriptase (M-MLV, Promega) (0.5 μl) was then added to each tube and incubated at 25 °C for 10 minutes (Progene PCR block, Techne), 42 °C for 1 hour and 70 °C for 10 minutes. Reverse transcriptase products (cDNA) were stored at -20 °C until further use.

Polymerase Chain Reaction (PCR)

Primers for ovine leptin were taken from Yuen et al [Yuen, 1999 #398] and produced in house. Primers for 18 were obtained from Ambion (Quantum RNA 18 S internal standards).

PCR was carried out on the cDNA produced in the reverse transcription step. A PCR master mix was prepared (11.25 μ l dH₂O, 2 μ l 10 X PCR buffer (Promega), 1 μ l dNTPs, 1.5 μ l leptin sense primer, 1.5 μ l leptin anti-sense primer and 1 μ l 18S primer/competimer mix (Ambion) per template) and 18.5 μ l was added to 1 μ l of template and 0.75 μ l Taq Polymerase (Promega) was added last.

The samples were placed in the PCR block and cycled through the following:

1.	94 °C	15 minutes	} 30 cycles
2.	94 °C	0.5 minutes	
3.	60 °C	0.5 minutes	
4.	72 °C	1.0 minutes	
5.	72 °C	7.0 minutes	
6.	4 °C	indefinitely	

The PCR products were stored at -20 °C until gel electrophoresis.

Gel Electrophoresis

Agarose gel electrophoresis was carried out in order to assess the size and abundance of the PCR product. A 2 % (w/v) tris-acetate (TAE) agarose gel (2 % (w/v) agarose made up to volume with 1 X TAE (0.04 M tris-acetate, 0.001 M EDTA made up to volume with dH₂O, pH 8.0) was prepared and poured into a gel caster containing a comb which formed wells. The gel was left to set in a fume hood.

When set, the comb was removed and the gel placed in a gel running compartment filled with 1 X TAE buffer.

Two μl of gel loading buffer (50 % (v/v) glycerol, 0.1 mg/ml bromophenol blue made up to volume with DEPC-H₂O) and 0.1 μl of ethidium bromide (EtBr) was added to 10 μl of PCR product, reference or base pair marker (DNA 100 base pair ladder, Promega). Samples were loaded into individual wells and the running compartment attached to a power pack. PCR gels were electrophoresed for approximately 2 hours, or until the dye face was 1 cm from the end of the gel.

Gels were visualised under UV illumination using a CCD camera (Fugi; Luminescent Image Analyser LAS-1000). Densitometric analysis was carried out as section 2.3.7.b. Leptin expression was expressed as a percentage of 18S rRNA.

Approximate size of the visualised bands was calculated using regression analysis of the base pair markers.

2.3.8 Statistical Analysis

The statistical tests applied to results are described in the methods section of the relevant chapter. Analyses were performed using the SPSS for Windows package (Release 9.0.0; SPSS Inc., 1989-1999).

CHAPTER 3

THE EFFECT OF LEPTIN TREATMENT ON THERMOREGULATION OF NEONATAL LAMBS

3.1 Introduction

Survival of the newborn lamb is primarily dependent on its ability to maintain a stable body temperature in response to the transition from a warm uterine, to cold external environment. Precocial newborns, such as the lamb and human infant, must have sufficient reserves of BAT for non-shivering thermogenesis at birth in order to avoid hypothermia. In the newborn lamb, non-shivering thermogenesis constitutes ~40 % of the maximal response to cold (Gemmell *et al.* 1972).

BAT produces heat through the unique uncoupling protein UCP1 (see Chapter 1) which as its name suggests uncouples the respiratory chain in the inner mitochondrial membrane, dissipating the energy liberated as heat. BAT is particularly well vascularised and the heat is conveyed to other parts of the body by blood vessels. The activation of non-shivering thermogenesis at birth is regulated by a number of factors (see Chapter 1.2.1 for details), one of which is an increase in sympathetic activity mediated by the cold challenge.

Many hormones, including triiodothyronine and Cortisol peak around the time of birth in the lamb in order to co-ordinate the transition from fetus to neonate.

Non-shivering thermogenesis is one of the mechanisms modulated by the hormone surge at the time of birth. Leptin has been shown to peak around the time of birth in humans (Hyttinanti *et al.* 1999; Matsuda *et al.* 1997; Matsuda *et al.* 1999; Schubring *et al.* 1999), although it is not known if this is the case for sheep. Leptin administration to rodents has been shown to increase body temperature via increased sympathetic nervous system activity and UCP1

expression (Pelleymounter *et al.* 1995; Scarpace & Matheny 1998; Scarpace *et al.* 1997). These studies were carried out on adult rodents, some of whom were *ob/ob* mice that do not express nor secrete leptin so it was not known if a similar thermogenic effect would be observed in a precocial species. A mechanism that would enhance non-shivering thermogenesis in the newborn lamb would be highly beneficial, as many lambs die every year from hypothermia. The aim of these studies therefore was to investigate the effect of leptin administration to neonatal lambs.

Part A Acute Leptin Administration

3.1.1 Hypothesis and Aim

The effect of acute leptin administration to neonatal lambs on thermoregulation and adipose tissue function was investigated. It was hypothesised that acute leptin treatment of day old lambs would increase body temperature via increased UCP1 expression.

3.2 Methods

3.2.1 Animals

Eight pairs of triplet lambs, born normally at term to Bluefaced Leicester cross Swaledale ewes were entered into the study. Each lamb remained with their ewe until 16 ± 2.5 hours after birth and all lambs entered into the study obtained adequate amounts of colostrum, this was monitored by experienced farm staff. Supplementary colostrum was provided for lambs that were thought not to have received enough.

3.2.2 Experimental Design

Pairs of lambs were selected on the basis of matched body weight ($\pm 10\%$) and were placed in a constant temperature room of 15 ± 1 °C. The third lamb remained with its ewe. A jugular vein catheter was inserted into each lamb as (described in chapter 2.1.2.) to enable vehicle or leptin treatment and blood sampling

Each lamb was subsequently placed in an indirect calorimeter maintained at

14 °C. Continuous measurements of colonic temperature and breathing pattern using inductance plethysmography were made and sleep state was determined from these respiratory pattern measurements (Symonds *et al.* 1989a). The occurrence of interference on the respiratory patterns was also used to assess the incidence of shivering whilst in non-rapid eye movement sleep. Carbon dioxide production was measured continuously using indirect open-circuit calorimetry. The mean values presented represent values obtained during periods of non-rapid eye movement sleep. This is in order to minimise variations due to animal movement, and were recorded using two identical indirect-calorimetry systems based on that described by (Symonds *et al.* 1989b), with the modification that airflow was measured using a differential flow indicator (see chapter 2.1.1 for details)

One lamb from each pair was randomly selected to receive recombinant ovine leptin (a gift from Professor Duane Keisler, University of Missouri, USA). Once placed in the calorimetry box an initial 5 ml blood sample was taken and designated sample 1. This was followed by a 1 hour acclimatisation period in the calorimeter box after which both lambs were injected intravenously with 1 ml vehicle (sterile water). Approximately 40 - 60 minutes after the injection, while the lamb was still sleeping a 5 ml blood sample was taken (sample 2). One lamb was then treated with 10 pg of recombinant ovine leptin in 1 ml sterile water while its sibling received water alone. Blood samples were again taken 40 - 60 minutes after treatment as it was not possible to blood sample both lambs at the same time. This procedure was then repeated twice with the modification that treated lambs were injected with 100 pg leptin. Between 70 - 90 minutes after

the final injection each lamb was humanely euthanased by intravenous administration of barbiturate (100 mg kg⁻¹ pentobarbital sodium: Euthatal: RMB Animal Health, UK). Perirenal adipose tissue was rapidly removed and stored as described in chapter 2.1.3.

3.2.3 Laboratory Procedures

Mitochondria and RNA were prepared from PAT as described in chapters 2.3.1 and 2.3.6 in order to assess UCPI abundance, expression and activity (GDP binding) and VDAC abundance. Plasma concentrations of glucose, NEFA, leptin and prolactin were carried out as described in chapter 2.2.

3.2.4 Statistical Analysis

Statistical analysis with respect to significant differences ($p < 0.05$) between values obtained from treated and untreated lambs was carried out using Mann-Whitney U test or repeated measures GLM. The repeated measures GLM considered together the effect of time, treatment and gender. Correlations were investigated using Spearman's Rho test.

3.3 Results

3.3.1 Thermoregulation

In vehicle, but not leptin treated lambs, body temperature declined throughout the study (Figure 3.1). As a consequence, the overall change in colonic temperature was significantly different between vehicle treated lambs ($P=0.018$) compared to their leptin treated siblings (Figure 3.2). All lambs shivered during the study period, but after the second dose of 100 μg leptin, the treated lambs shivered for half as much time as controls (Figure 3.3). Carbon dioxide production was not influenced by leptin treatment (Table 3.1).

3.3.2 Plasma Metabolites and Hormones

At the start of the study leptin concentrations were similar between groups, although female lambs had significantly higher levels than males (Figure 3.4). Leptin treatment caused a dose dependent increase in plasma leptin treatment that was not observed in vehicle treated animals, irrespective of the animals gender (Figure 3.5). The increase in plasma leptin concentration after the final 100 μg treatment was significantly greater than both pre-treatment and control animal values.

Plasma glucose concentrations were found to be similar at the start of the study period (Figure 3.6) and exhibited a decline in both leptin and vehicle treated groups. There were no differences attributable to leptin treatment at any time point.

Plasma NEFA concentrations were similar between treatment groups at the start of the study (Figure 3.7). Plasma NEFA concentrations in the vehicle treated group declined significantly through out the study period, a trend that was not observed in the leptin treated animals. This resulted in vehicle treated animals having significantly lower plasma NEFA concentrations than their leptin treated siblings after the first 100 pg leptin treatment, by the end of the study NEFA concentrations had equalised between groups. Prior to any treatment, at hour 0, NEFA and leptin concentrations were positively correlated (Figure 3.8) ($R^2=0.522$, $P=0.017$) no other correlation between NEFA and leptin levels was observed.

Prolactin levels were similar between groups at the start of the study and remained so throughout the study (Figure 3.9). There was no effect of time or treatment on prolactin levels. A weak negative correlation was observed between leptin and prolactin plasma concentrations in the leptin group after the initial 100 pg dose of leptin ($R^2=0.417$, $P=0.052$) (Figure 3.10).

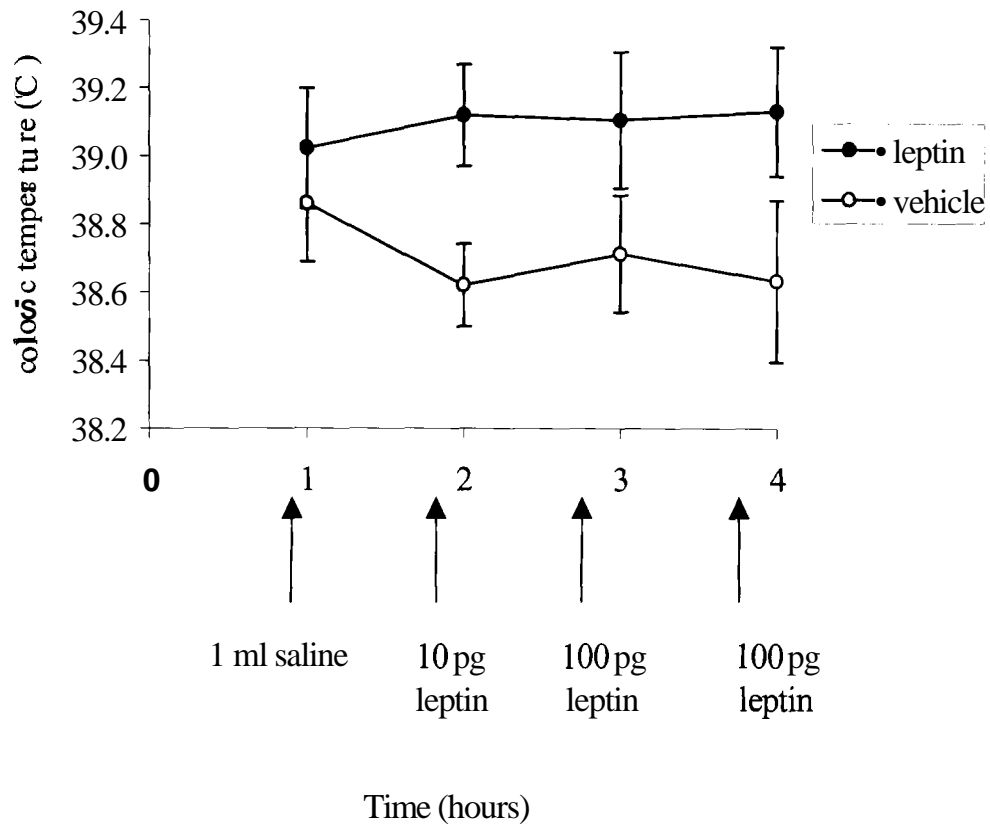


Figure 3.1

Effect of leptin administration on colonic temperature (°C) in neonatal lambs.

Values are means \pm SEM.

(• leptin, n = 8; O vehicle, n = 8)

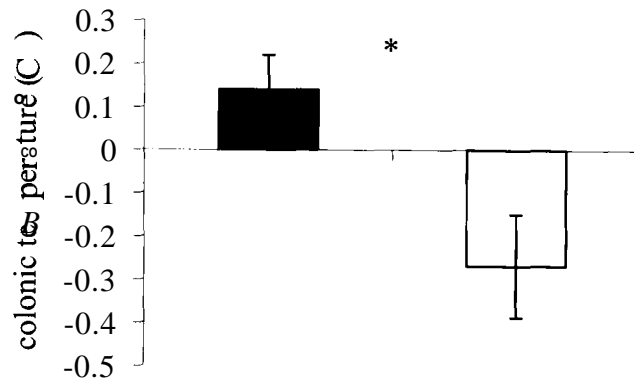


Figure 3.2

Effect of leptin administration on overall change in colonic temperature in neonatal lambs.

Values are means \pm SEM.

(• Leptin n = 8; □ Vehicle, n = 8)

* indicates $P < 0.05$ for treatment effect.

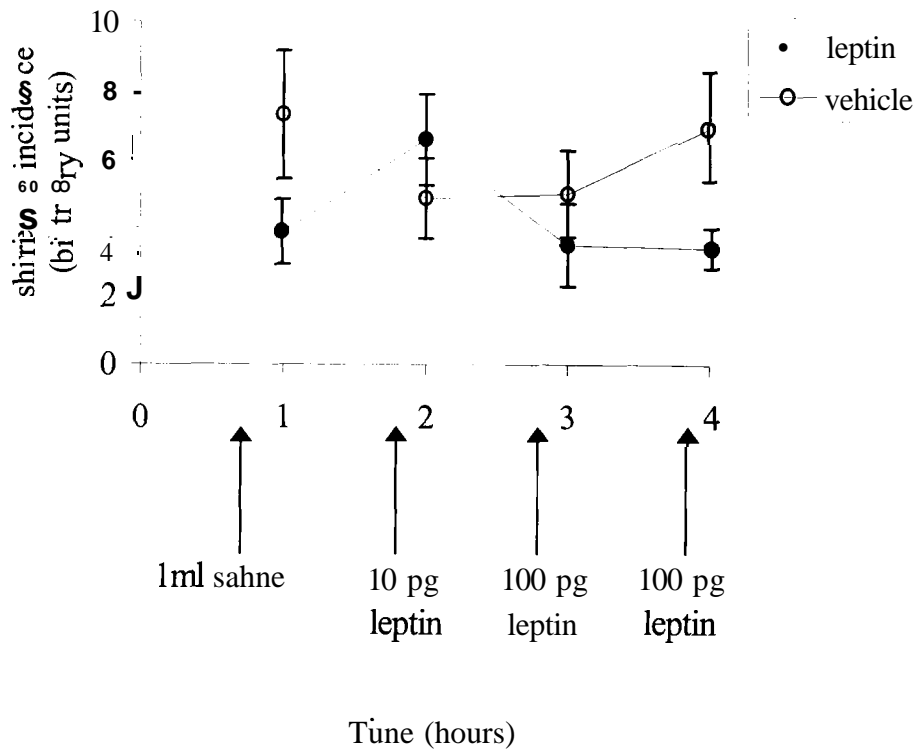


Figure 3.3

Effect of leptin administration on shivering in neonatal lambs.

Values are means \pm SEM.

(• leptin, n = 8; O vehicle, n = 8)

Table 3.1

The effect of leptin administration on CO₂ production in neonatal lambs maintained at a constant ambient temperature (14°C).

Leptin n=8, Vehicle n=8.

	Vehicle	Leptin
Time after start of study (hours)	CO ₂ production (ml min ⁻¹ kg ⁻¹) Mean ± SEM	CO ₂ production (ml min ⁻¹ kg ⁻¹) Mean ± SEM
0	15.16±1.75	14.66 ± 2.65
1	15.88 ± 1.63	13.79 ± 1.90
2	15.18 ±1.66	14.68 ± 1.70
3	14.68 ± 1.54	15.45 ± 1.76
4	14.53 ± 1.30	13.57±2.33

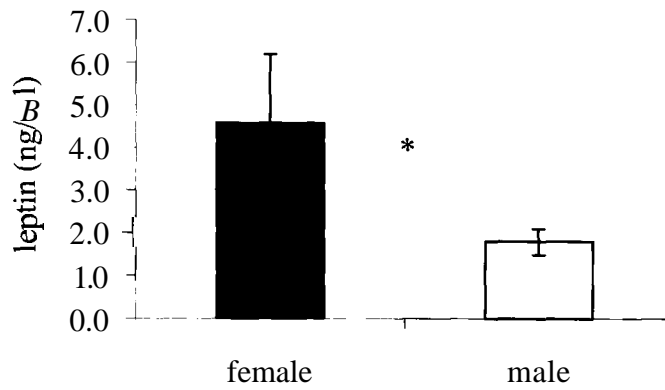


Figure 3.4

Effect of gender on neonatal plasma leptin concentrations in neonatal lambs prior to any experimental intervention.

Values are means \pm SEM.

(• Female, n = 5; □ Male, n = 7)

* indicates $P < 0.05$ for gender effect.

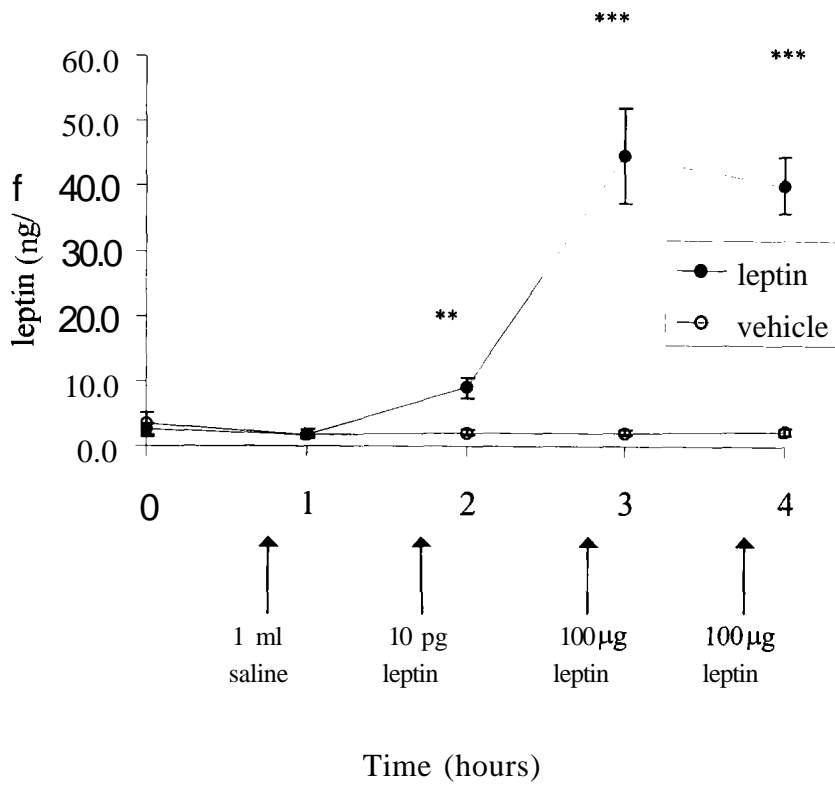


Figure 3.5

Effect of leptin administration on plasma leptin concentrations in neonatal lambs.

Values are means \pm SEM.

(• leptin, n = 7; O vehicle, n = 5)

** indicates $P < 0.001$ for treatment effect

*** indicates $P < 0.0001$ for treatment effect

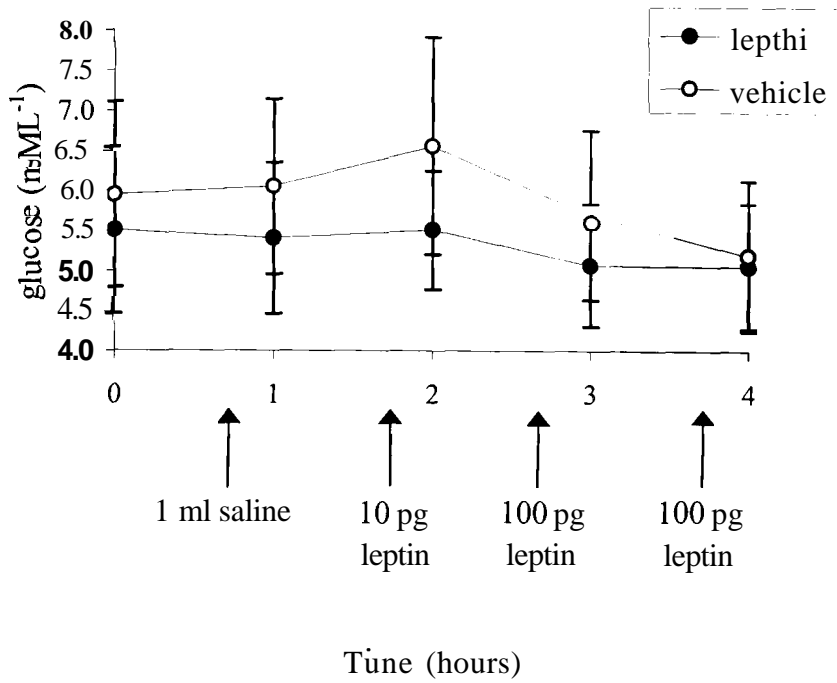


Figure 3.6

Effect of leptin administration on plasma glucose concentrations in neonatal lambs.

Values are means \pm SEM.

(• leptin, n = 7; O vehicle, n = 5)

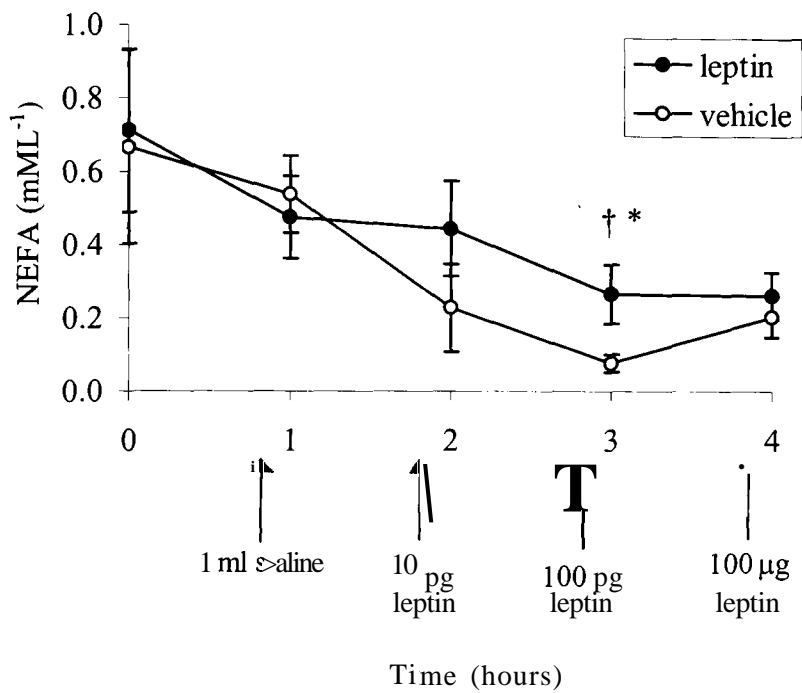


Figure 3.7

Effect of leptin administration on plasma NEFA concentrations in neonatal lambs.

Values are means \pm SEM.

(• leptin, n = 7; O vehicle, n = 5)

* indicates $P < 0.05$ for treatment effect

t indicates $P < 0.05$ for time effect

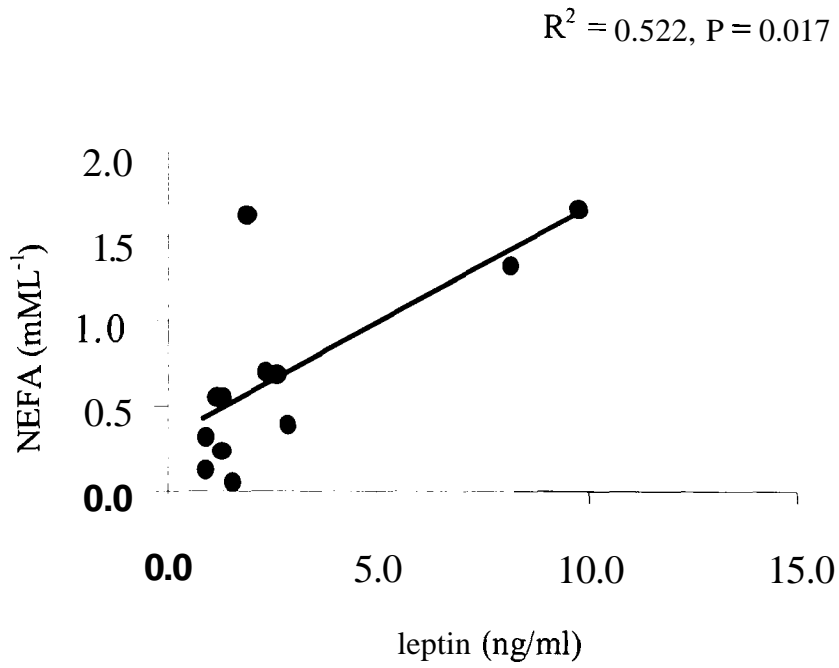


Figure 3.8

Relationship between leptin and NEFA in neonatal lambs prior to any experimental intervention.

(● hour 0, n = 12)

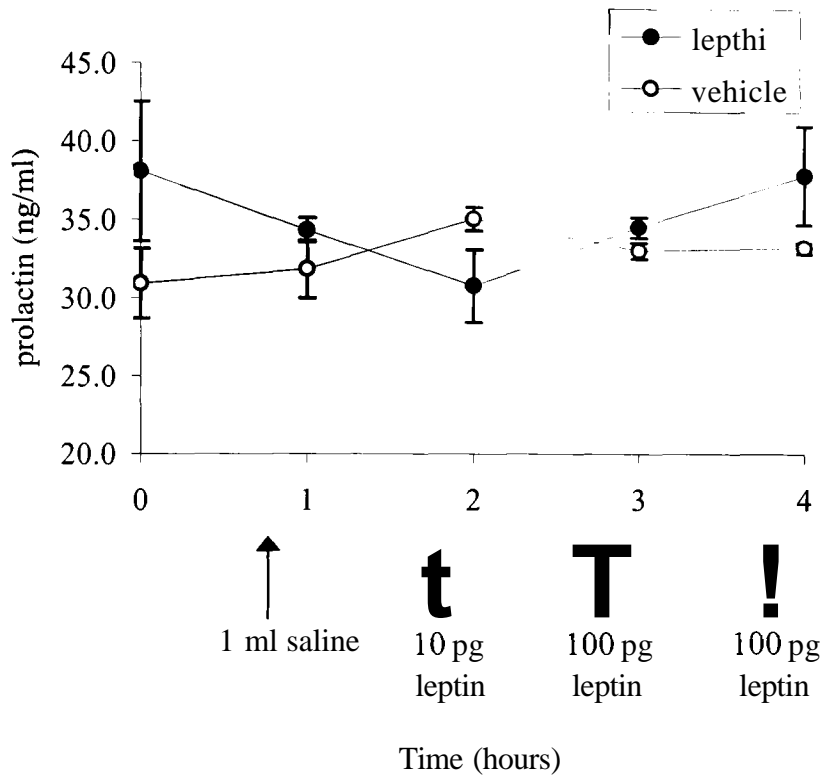


Figure 3.9

Effect of leptin administration on plasma prolactin concentrations in neonatal lambs.

Values are means \pm SEM.

(• leptin, n = 7; O vehicle, n = 5)

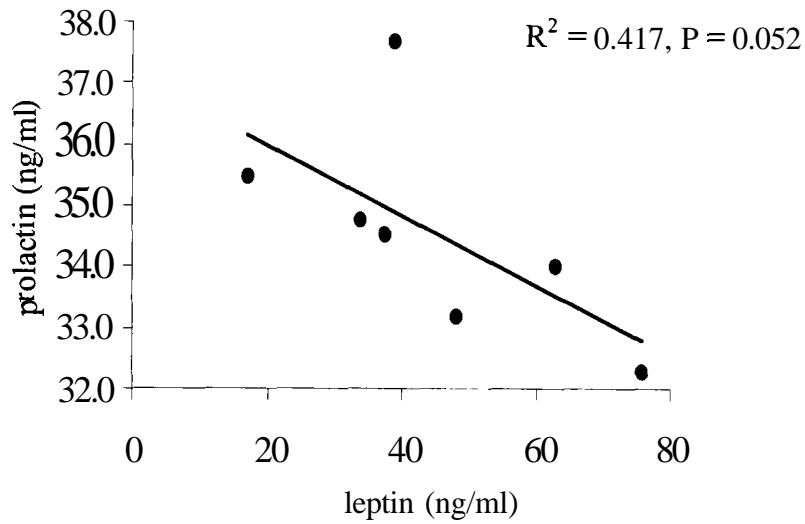


Figure 3.10

Relationship between leptin and prolactin in neonatal lambs treated with 10, then 100 μ g leptin.

(• hour 4, n = 7)

3.3.2 Lamb Body Weight and tissue Analysis

There was no effect of leptin administration on body weight, PAT weight or any of the organs studied (Table 3.2).

The potential thermogenic activity, as assessed by GDP binding, and abundance of UCPI was similar between groups (Table 3.3 and Figure 3.11). UCPI expression as measured by Northern blotting was found to be lower in leptin treated group (Figure 3.12). Although this did not reach statistical significance, six out of the eight pairs of lambs studied expressed less UCPI mRNA following leptin administration.

Leptin mRNA abundance was assessed using RT-PCR and expressed as a percentage of 18S and an internal reference sample (Figure 3.13). There was a trend for the leptin treated animals to have lower leptin mRNA expression than vehicle treated siblings, but this was not statistically significant. Leptin mRNA was found to be positively related to BAT weight at the end of the study in the vehicle group ($R^2=0.496$, $P=0.037$) (Figure 3.14), although it was only possible to study 5 animals.

VDAC protein abundance in BAT was assessed using western blotting with a specific ovine antibody. VDAC abundance was similar between groups at 1 day of age (Figure 3.15).

Table 3.2

Body and organ weights of lambs following leptin administration.

Leptin n=8 Vehicle n=8

	Vehicle	Leptin
Body weight (kg)	3.93 ±0.24	3.98 ±0.29
PAT weight (g)	15.56 ±1.69	16.23 ±1.24
Pericardial AT (g)	3.37 ±0.42	3.19 ±0.27
Brain (g)	43.37 ±0.77	42.94 ± 0.97
Heart (g)	32.83 ±1.87	32.24 ±2.57
Liver (g)	87.04 ±6.12	80.09 ±7.39
Lung (g)	79.33 ±4.21	76.28 ±5.29
Mean Thyroid (g)	0.47 ± 0.03	0.46 ± 0.04
Mean Adrenal (g)	0.46 ± 0.04	0.47 ± 0.03
Mean Kidney (g)	11.33 ±0.53	11.79 ±0.62

Table 3.3

UCPI protein abundance and potential activity following leptin administration.

Leptin, n=8; Vehicle, n=8

	Vehicle	Leptin
UCPI abundance (% of reference)	138.0 \pm 5.2	150.5 \pm 6.6
GDP Binding (pM/mg MP)	102.3 \pm 8.3	104.8 \pm 9.9

MP, mitochondrial protein.

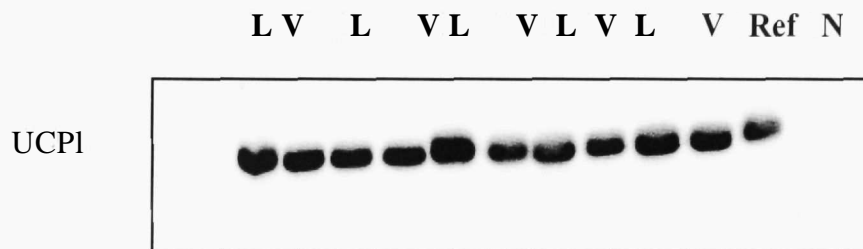
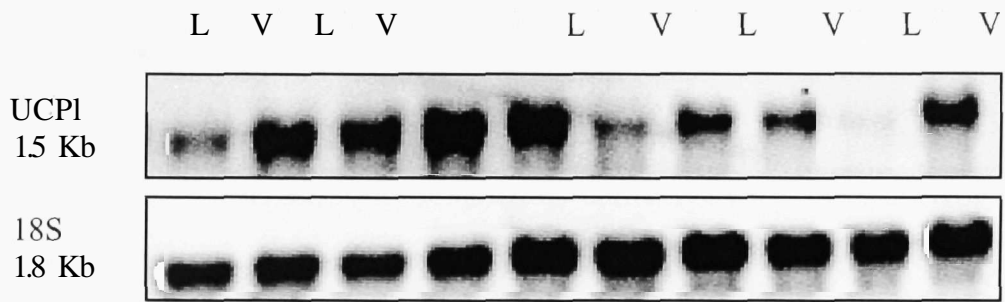


Figure 3.11

Representative image of a Western blot for UCP1 (L = leptin, V = vehicle) 10 pg MP each lane.

MP, mitochondrial protein; Ref, reference sample (4 hour old lamb PAT mitochondria); N, negative control (liver mitochondria).

a)



b)

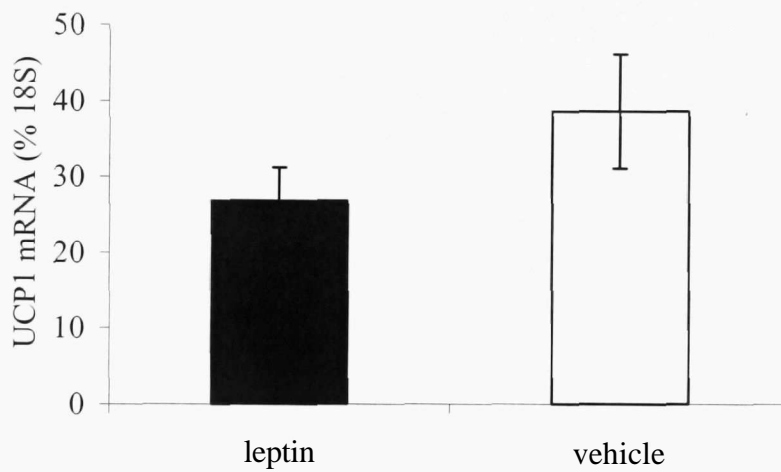


Figure 3.12

a) Representative image of a Northern blot probed for UCPI and 18S (L = leptin, V = vehicle) 10 μ g RNA each lane.

b) Effect of leptin administration on UCP 1 mRNA expression

Values are means \pm SEM.

(• Leptin, n = 8; DVehicle, n = 8)

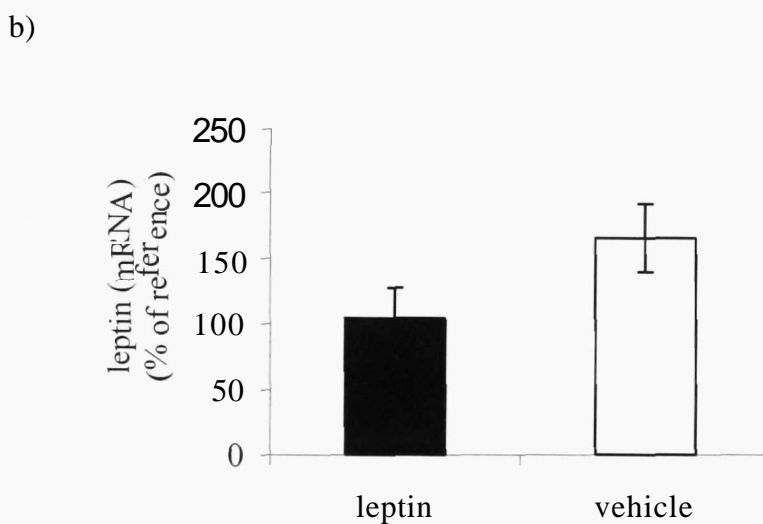
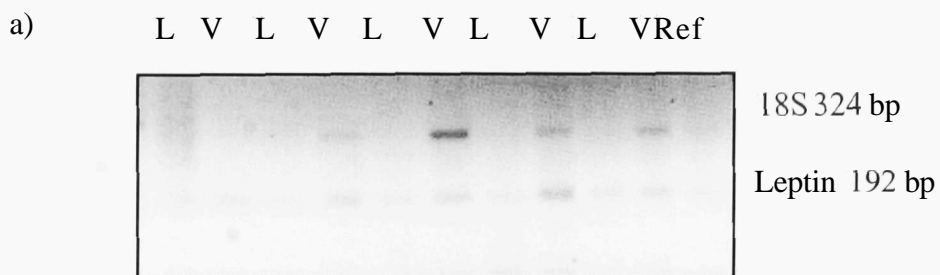


Figure 3.13

a) Representative image of a DNA gel containing leptin and 18S PCR products from BAT

(L = leptin, V = vehicle)

b) Effect of leptin administration on leptin mRNA expression

Values are means \pm SEM.

(• Leptin, n = 5; DVehicle, n = 5)

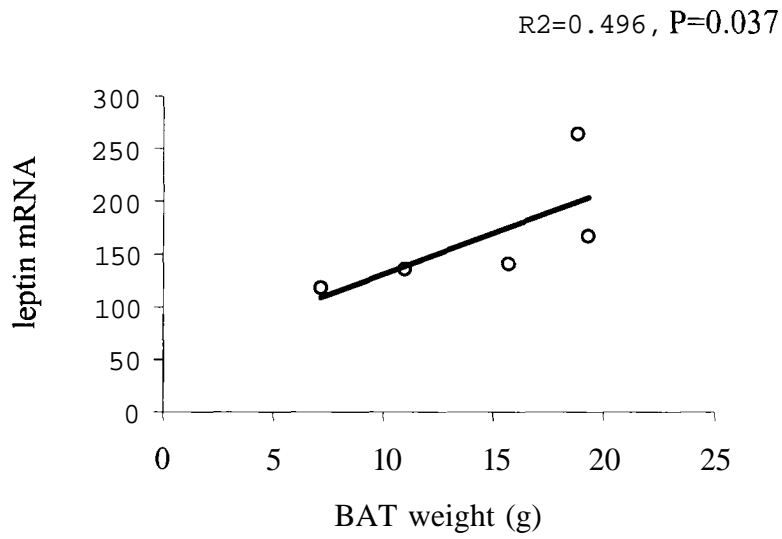


Figure 3.14

Relationship between leptin mRNA and BAT weight in vehicle treated neonatal lambs.

(O hour 4, n = 5)

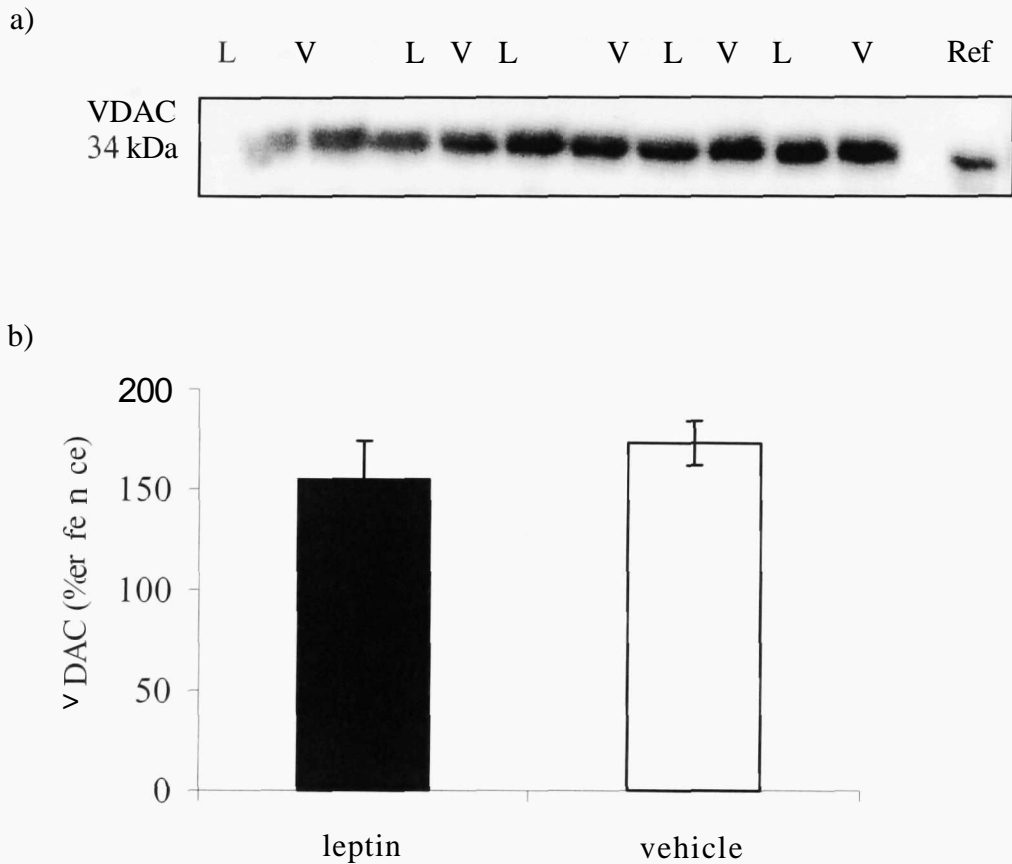


Figure 3.15

a) Representative image of a Western blot for VDAC in BAT (L = leptin, V = vehicle) 10 pg MP each lane.

b) Effect of leptin administration on BAT VDAC protein abundance

Values are means \pm SEM.

(• Leptin, n = 5; □ Vehicle, n = 5)

MP, mitochondrial protein; Ref, reference sample (7 day old lamb skeletal muscle mitochondria).

3.4 Introduction

This study was carried out to investigate further the results from acute leptin administration to neonatal lambs. To determine whether the effects of leptin administration to day old lambs were transient due to the complex milieu of hormones present at birth, a longer-term, "chronic", administration of leptin to postnatal lambs was carried out.

3.4.1 Hypothesis and Aim

The effect of chronic leptin administration to neonatal lambs on thermoregulation and adipose tissue function has been investigated. It was hypothesised that long-term leptin treatment of postnatal lambs would maintain body temperature through a mechanism other than increased UCP1 expression.

3.5 Methods

3.5.1 Animals

Nine pairs of female triplet lambs, born normally at term to Bluefaced Leicester cross Swaledale ewes were entered into the study. Lambs remained with their ewe throughout the study in individual pens. All lambs entered into the study obtained adequate amounts of colostrum.

3.5.2 Experimental Design

Pairs of lambs were selected on the basis of matched body weight ($\pm 10\%$) and gender, only female lambs were entered into this study to remove any effect of gender on plasma leptin concentrations. The third lamb remained in the pen with its ewe and siblings. A jugular vein catheter was inserted into each lamb as (described in chapter 2.1.2.) to enable vehicle or leptin treatment and blood sampling

One lamb from each pair was selected to receive recombinant ovine leptin (a gift from Professor Duane Keisler, University of Missouri, USA). Lambs were entered into the study on day one of life and treated daily for six days at 9.30 am with either 100 pg leptin, or vehicle (sterile water). Colonic temperature was measured daily prior to treatment using an electronic thermometer, as was body weight of all lambs. Blood samples were taken daily before treatment. On day seven each lamb was humanely euthanased by intravenous administration of barbiturate (100 mg kg⁻¹ pentobarbital sodium: Euthatal: RMB Animal Health, UK). Perirenal adipose tissue was rapidly removed and stored as described in chapter 2.1.3.

3.5.3 Laboratory Procedures

Mitochondria and RNA were prepared from PAT as described in chapters 2.3.1 and 2.3.6 in order to assess UCP1 abundance, expression and activity (GDP binding) and VDAC and cytochrome c abundance. Plasma concentrations of glucose, NEFA, leptin and prolactin were carried out as described in chapter 2.2.

3.5.4 Statistical Analysis

Statistical analysis with respect to significant differences ($p < 0.05$) between values obtained from treated and untreated lamb pairs was carried out using the Mann Whitney U test or repeated measures GLM followed with post hoc Mann-Whitney U tests. Correlations were investigated using Spearman's Rho test.

3.6 Results

3.6.1 Thermoregulation

Colonic temperatures were similar between groups on all days throughout the study, however, leptin treated lambs were better able to maintain body temperature between days 1 and 2 when the vehicle treated group exhibited a significant decline in colonic temperature ($P=0.011$) (Figure 3.16).

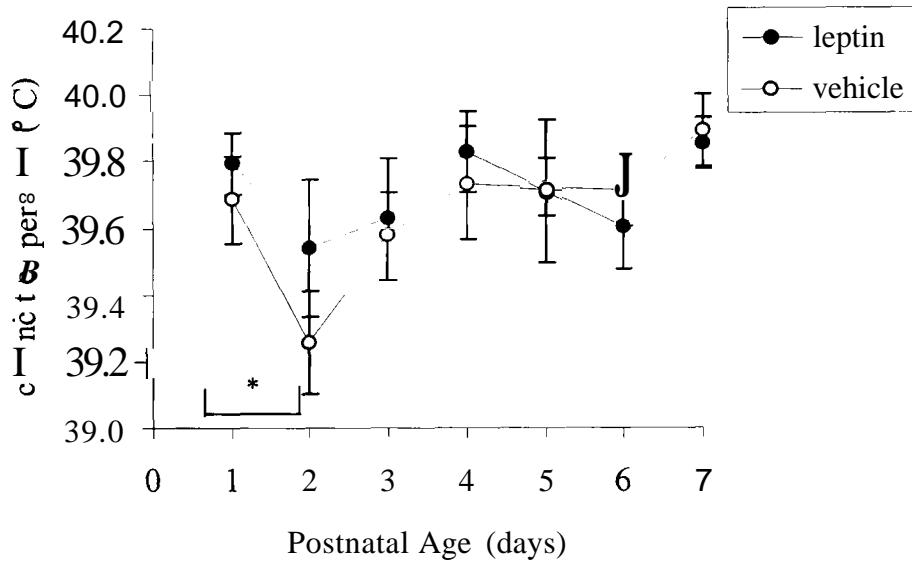


Figure 3.16

Effect of leptin administration on temperature in postnatal lambs.

Values are means \pm SEM.

(• leptin, n = 9; O vehicle, n = 9)

* indicates $P < 0.05$ for time effect

3.6.1 Plasma Metabolites and Hormones

Plasma leptin concentrations were significantly higher overall in treated lambs, although, on day three of postnatal age there was no difference between the groups (figure 3.17).

This study also gave us the opportunity to study the natural postnatal ontogeny of leptin in the lamb (Figure 3.18). Plasma leptin levels were similar between days one, two and three of postnatal age, then significantly increased to a plateau at days five and six. Plasma leptin concentrations were positively correlated with colonic temperature at postnatal day 1 prior to any intervention ($R^2=0.145$, $P=0.018$).

Plasma glucose concentrations were found to be similar on day one of postnatal age (Figure 3.19). There were no differences attributable to leptin treatment at any time point.

Plasma NEFA concentrations were similar between treatment groups on day one of postnatal age (Figure 3.20). Plasma NEFA concentrations in the vehicle treated group declined significantly between day one and day two of postnatal age ($P=0.028$), a similar trend was also observed in the leptin treated animals. This resulted in vehicle treated animals having significantly lower plasma NEFA concentrations than their leptin treated siblings on the third day of life, however, NEFA concentrations subsequently equalised between groups. Prior to experimental intervention on day one of age there was a significant positive relationship between plasma NEFA levels and colonic temperature ($R^2=0.24$,

P=0.035). In the leptin treated group, there was a weak negative correlation between plasma NEFA and leptin concentrations on day 4 of postnatal age ($R^2=0.631$, $P=0.042$).

Plasma prolactin concentrations were similar between treatment groups at the start of the study (Figure 3.21). Plasma prolactin concentrations in the leptin treated group declined between day 1 and day 2 of postnatal age, although this did not reach statistical significance ($P=0.066$). This trend was not observed in their vehicle treated siblings. Prolactin concentrations remained similar throughout the study until the sixth postnatal day when the vehicle treated group had significantly higher levels ($P=0.016$). Plasma prolactin levels were found to be negatively related to plasma leptin on day 1 prior to experimental intervention ($R^2=0.13$, $P=0.023$) and also to plasma NEFA concentrations on day 2 of postnatal age in the leptin treated group ($R^2=0.62$, $P=0.019$). This was the day when leptin treated animals maintained body temperature better than their vehicle treated siblings.

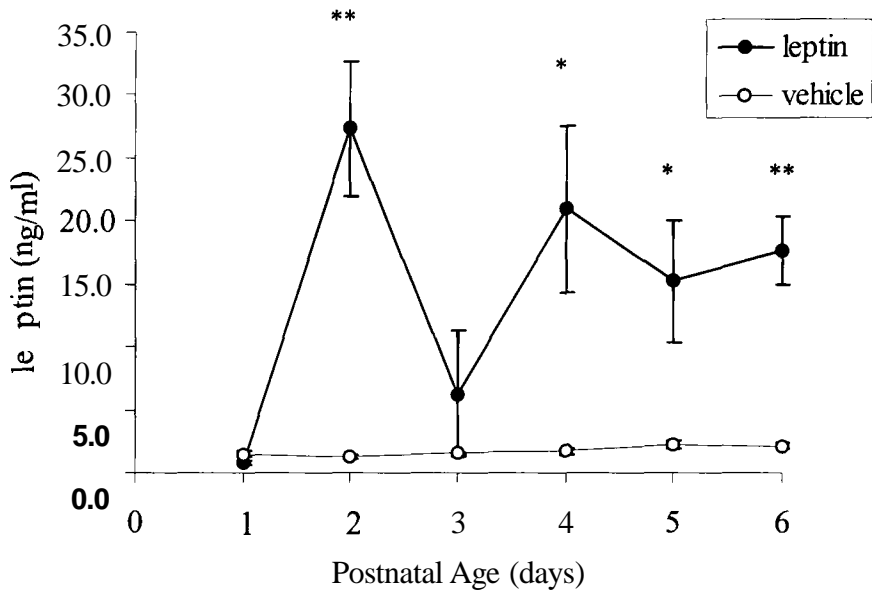


Figure 3.17

Effect of leptin administration on plasma leptin concentrations in postnatal lambs.

Values are means \pm SEM.

(• leptin, n = 9; O vehicle, n = 7)

* indicates $P < 0.05$ for treatment effect

** indicates $P < 0.01$ for treatment effect

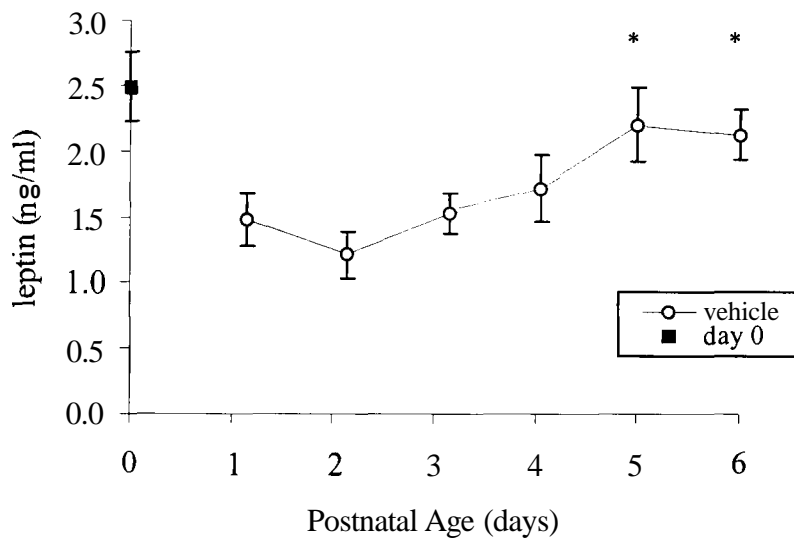


Figure 3.18

Ontogeny of plasma leptin in vehicle treated postnatal lambs

Values are means \pm SEM.

(O vehicle, n = 7)

* indicates $P < 0.05$ for age effect

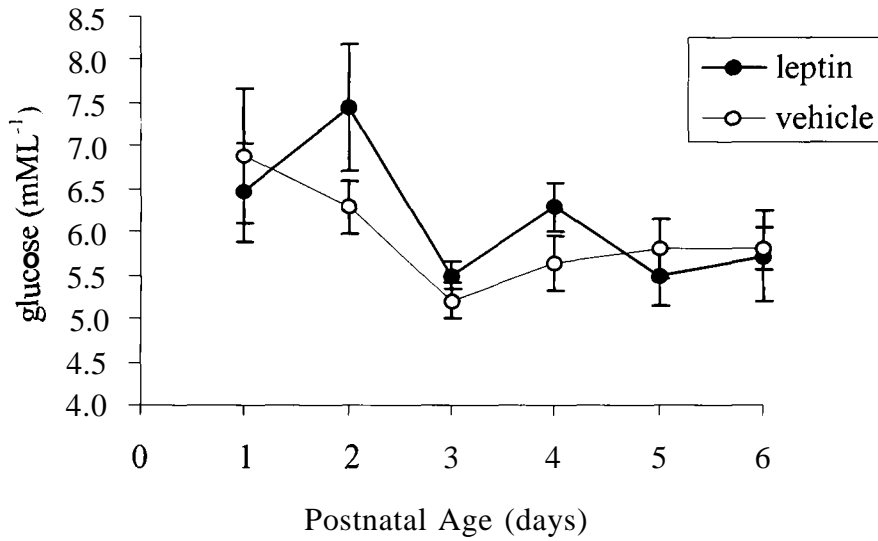


Figure 3.19

Effect of leptin administration on plasma glucose concentrations in postnatal lambs.

Values are means \pm SEM.

(• leptin, n = 9; O vehicle, n = 7)

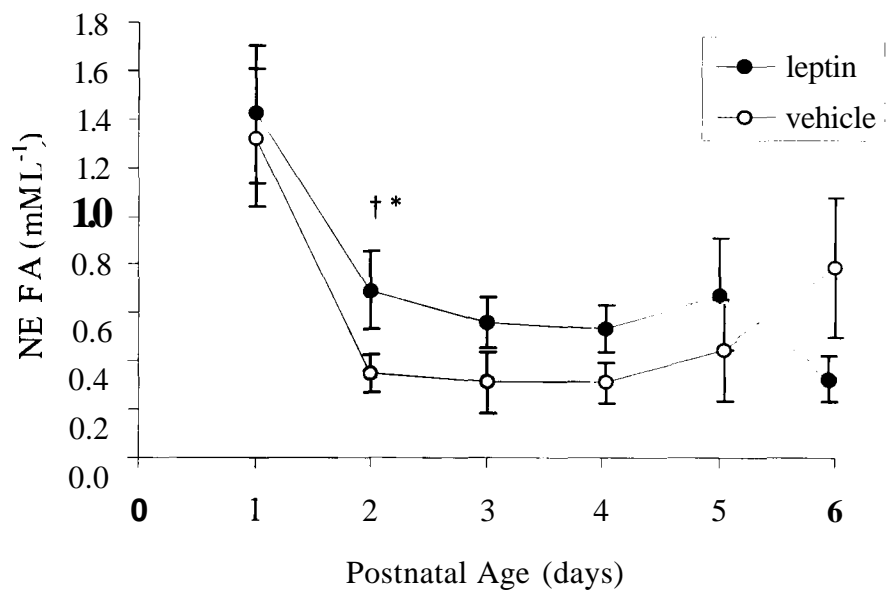


Figure 3.20

Effect of leptin administration on plasma NEFA concentrations in postnatal lambs.

Values are means \pm SEM.

(• leptin, n = 9; O vehicle, n = 7)

* indicates $P < 0.05$ for treatment effect

t indicates $P < 0.05$ for time effect

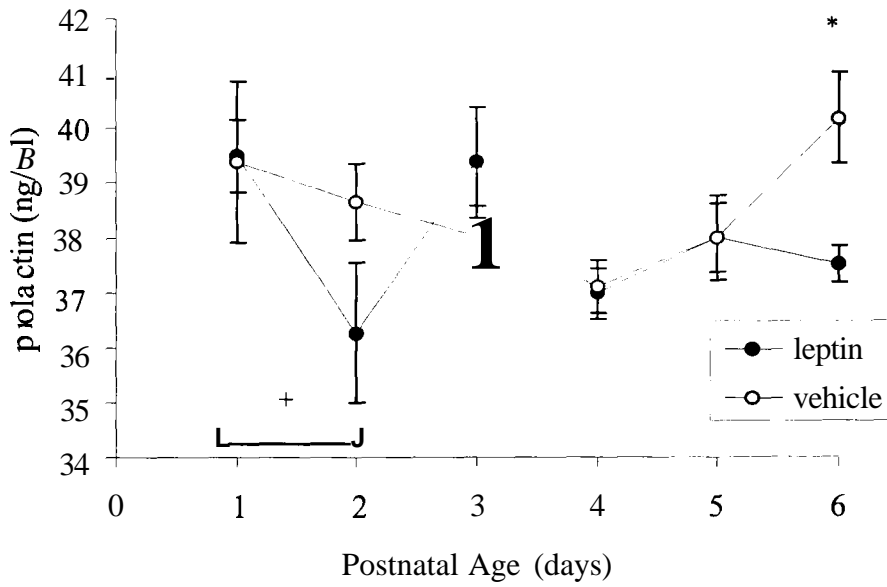


Figure 3.21

Effect of leptin administration on plasma prolactin concentrations in postnatal lambs.

Values are means \pm SEM.

(• leptin, n = 9; O vehicle, n = 7)

* indicates $P < 0.05$ for treatment effect

+ indicates $P = 0.06$ for time effect

3.6.2 Lamb Body Weight and Tissue Analysis

There was no affect of leptin administration on PAT weight or any of the organs studied (Table 3.4). Lambs from the leptin administration group had slightly heavier hearts than their vehicle treated siblings although this was not significant ($P=0.063$) and the effect disappeared when heart weight was expressed as a ratio of body weight.

Leptin treatment had no effect on body weight, growth rate or weight gain and all lambs displayed normal linear growth over the seven day study period (Figure 3.22) with an average daily weight gain of $0.20 \text{ kg} \pm 0.03$, leptin treated and $0.21 \text{ kg} \pm 0.02$, vehicle treated.

Table 3.4

Body and organ weights of lambs on day 8 of postnatal age.

Leptin (n=9), Vehicle (n=9)

	Vehicle	Leptin
Body weight (kg)	5.89 ±0.44	5.62 ±0.36
PAT weight (g)	27.19 ±2.40	28.66 ±4.16
Pericardial AT (g)	5.53 ±0.53	6.30 ±0.31
Brain (g)	46.76 ±1.06	48.54 ±1.43
Heart (g)	41.89 ±1.38	46.37 ± 3.44
Liver (g)	156.93 ±9.88	165.79 ±12.42
Lung (g)	129.16 ±13.32	136.98 ±9.34
Mean Thyroid (g)	0.43 ± 0.06	0.52 ±0.05
Mean Adrenal (g)	0.60 ± 0.03	0.61 ±0.03
Mean Kidney (g)	16.82 ±0.98	18.51 ±1.16

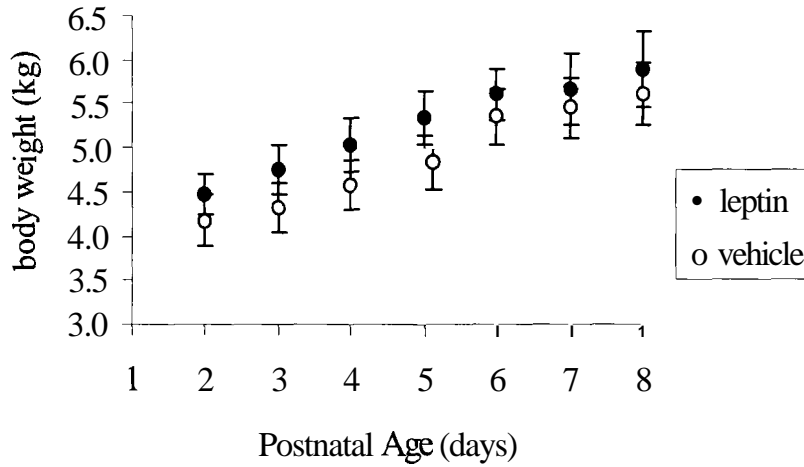


Figure 3.22

Effect of leptin administration on lamb body weight.

Values are means \pm SEM.

(• leptin, n = 9; O vehicle, n = 9)

The potential thermogenic activity, as assessed by GDP binding was similar between groups (Figure 3.23). UCP1 abundance as measured by western blotting was lower in the leptin treated group (Figure 3.24). UCP1 mRNA expression as measured by Northern blotting was found to be significantly lower in leptin treated group ($P=0.028$) (Figure 3.25). Leptin mRNA expression was found to be similar between groups at day 7 of postnatal age (Figure 3.26) although values of the control group were significantly lower than those observed at 1 day of age ($P=0.002$) (Figure 3.27). Unlike the vehicle treated one day old lambs, BAT weights were not correlated with leptin mRNA levels (Figure 3.14), but were weakly positively related to plasma leptin concentrations ($R^2=0.26$, $P=0.086$) although this was not statistically significant.

VDAC abundance in BAT and skeletal muscle was assessed by western blotting with a specific ovine antibody and was found to be similar between groups (Figures 3.28 and 3.29). Cytochrome *c* abundance in BAT was also measured using western blotting with a purchased multi-species antibody (Santa Cruz sc-7159). Leptin treated lambs had a higher mitochondrial abundance of cytochrome *c* compared to their vehicle treated siblings, however, this did not reach statistical significance (Figure 3.30).

Associations between UCP1 mRNA and protein, colonic temperature and GDP binding were investigated using Spearman's Rho test, the results are shown in Table 3.5.

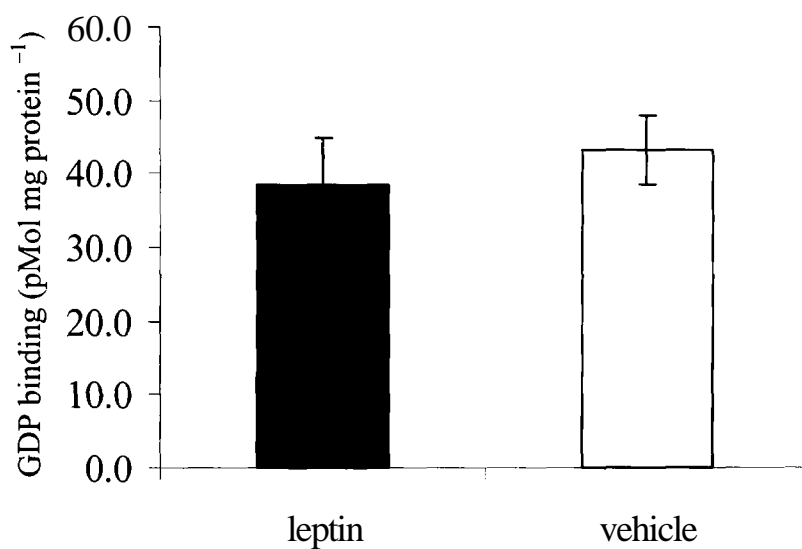


Figure 3.23

Effect of leptin administration on GDP binding in BAT from neonatal lambs.

Values are means \pm SEM.

(• Leptin n = 9; DVehicle, n = 9)

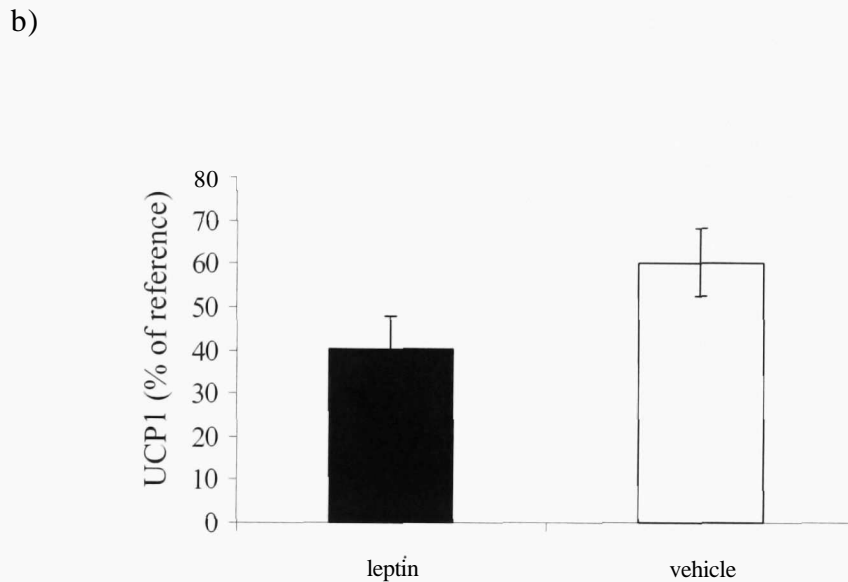
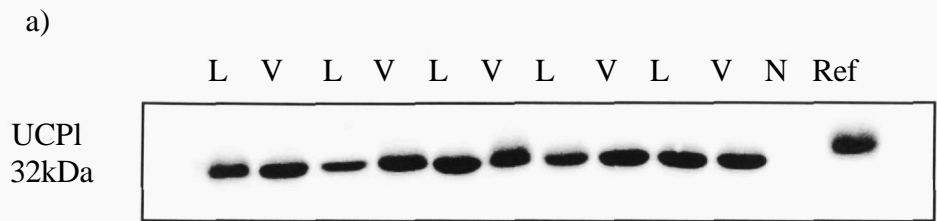


Figure 3.24

a) Representative image of a Western blot for UCPI (L = leptin, V = vehicle)

10 pg MP each lane.

b) Effect of leptin administration on UCPI protein abundance

Values are means \pm SEM.

(• Leptin, n = 9; □ Vehicle, n = 9)

MP, mitochondrial protein; Ref, reference sample (4 hour old lamb PAT

mitochondria); N, negative control (liver mitochondria).

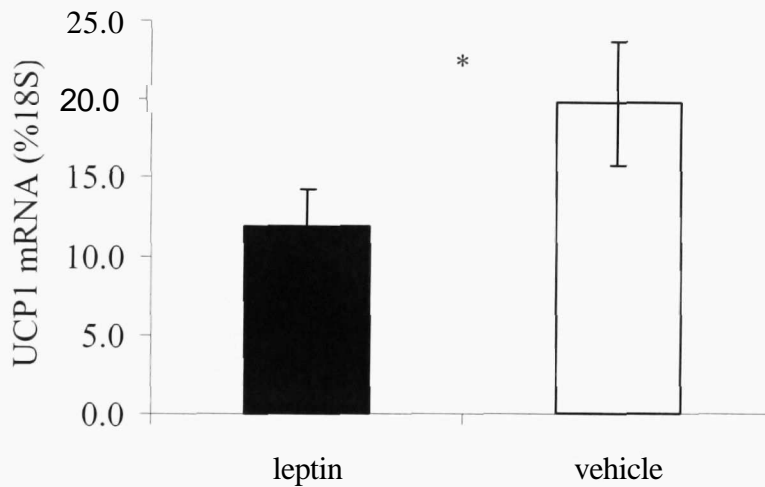
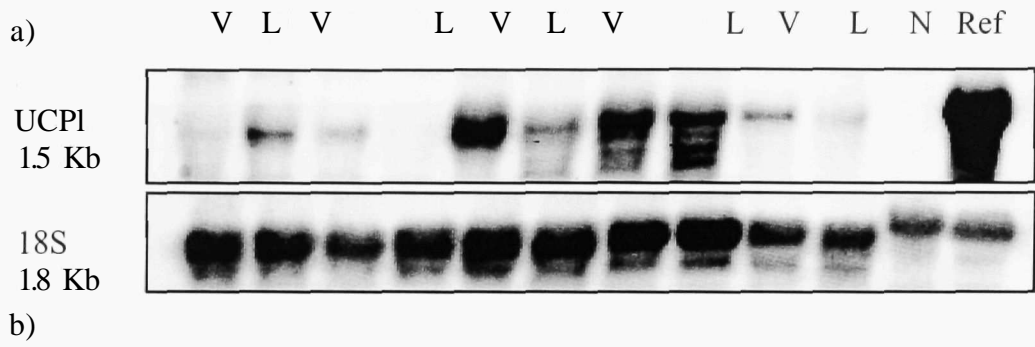


Figure 3.25

a) Representative image of a Northern blot probed for UCP1 and 18S (L = leptin, V = vehicle) 30 pg RNA each lane.

b) Effect of leptin administration on UCP 1 mRNA expression

Values are means \pm SEM.

(• Leptin n = 9; DVehicle, n = 9)

Ref, reference sample (4 hour old lamb PAT mRNA); N, negative control (liver mRNA).

* indicates $P < 0.05$ for treatment effect

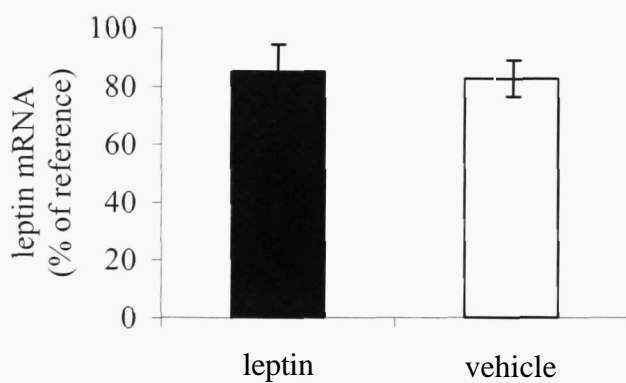


Figure 3.26

a) Representative image of a DNA gel containing leptin and 18S PCR products from BAT

(L = leptin, V = vehicle)

b) Effect of leptin administration on leptin mRNA expression

Values are means \pm SEM.

(• Leptin n = 5; DVehicle, n = 5)

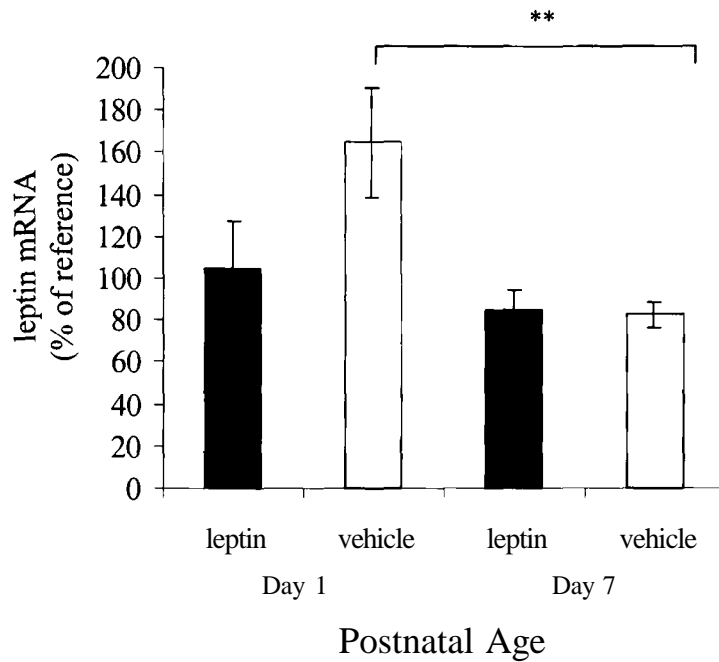


Figure 3.27

b) Effect of leptin administration on leptin mRNA expression

Values are means \pm SEM.

(Day 1 • Leptin n = 5; DVehicle, n = 5; Day 7 • Leptin n = 8; DVehicle, n = 8)

** indicates $P < 0.01$ for time effect

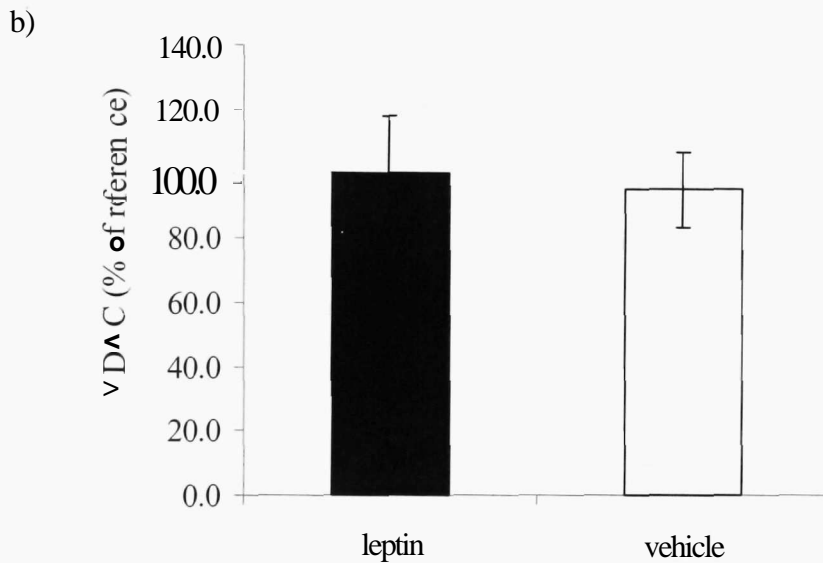


Figure 3.28

a) Representative image of a Western blot for VDAC in BAT (L = leptin, V = vehicle) 10 μ g MP each lane.

b) Effect of leptin administration on BAT VDAC protein abundance

Values are means \pm SEM.

(• Leptin, n = 5; □ Vehicle, n = 5)

MP, mitochondrial protein; Ref, reference sample (7 day old lamb skeletal muscle mitochondria).

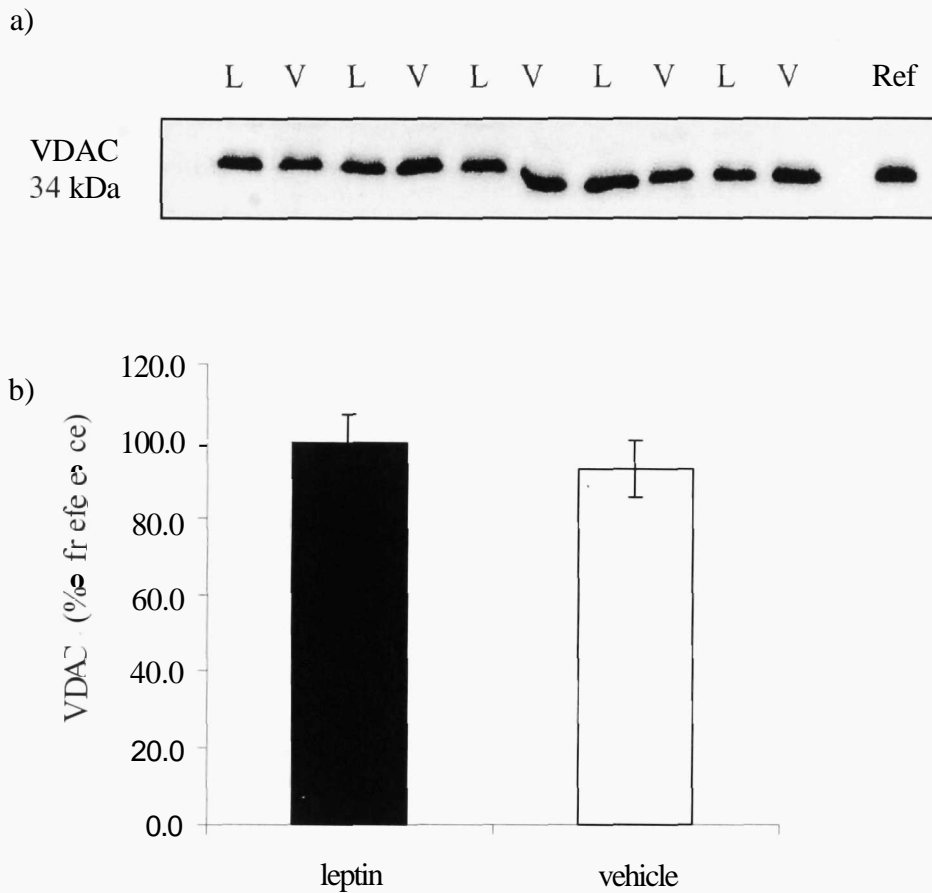


Figure 3.29

a) Representative image of a Western blot for VDAC in skeletal muscle (L = leptin, V = vehicle) 10 μ g MP each lane.

b) Effect of leptin administration on skeletal muscle VDAC protein abundance

Values are means \pm SEM.

(• Leptin, n = 5; DVehicle, n = 5)

MP, mitochondrial protein; Ref, reference sample (7 day old lamb skeletal muscle mitochondria).

a)



b)

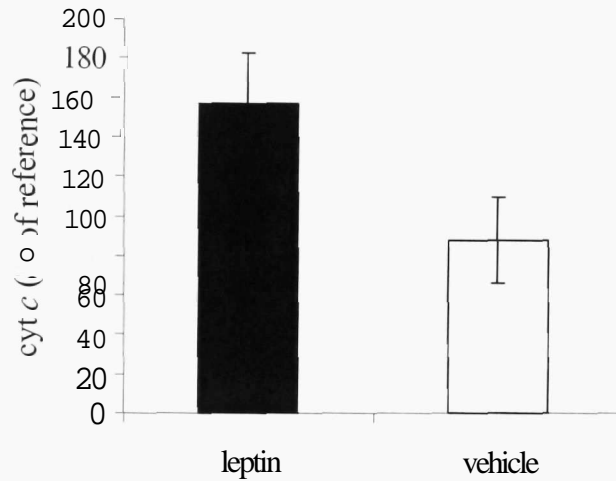


Figure 3.30

a) Representative image of a Western blot for cytochrome *c* in BAT (L = leptin, V = vehicle) 10 μ g MP each lane.

b) Effect of leptin administration on BAT cytochrome *c* protein abundance

Values are means \pm SEM.

(• Leptin, n = 4; □ Vehicle, n = 4)

MP, mitochondrial protein

Table 3.5

Associations between measured thermogenic parameters in the acute and long-term leptin treatment studies.

temp, temperature at time of tissue sampling; vs, versus; NS, non significant result.

	Day 1		Day 7	
	Vehicle	Leptin	Vehicle	Leptin
UCP1 mRNA vs UCP1 protein	R ² = 0.009 NS	R ² = 0.27 P = 0.051 *	R ² = 0.27 NS	R ² = 0.67 P = 0.021 *
temp vs UCP1 mRNA	R ² = 0.24 NS	R ² = 0.45 P = 0.036*	R ² = 0.18 NS	R ² = 0.53 P = 0.006 **
temp vs UCP1 protein	R ² = 0.057 NS	R ² = 0.48 P = 0.012*	NS	NS
GDP vs UCP1 mRNA	NS	NS	R ² = 0.01 NS	R ² = 0.78 P < 0.001 ***
GDP vs UCP1 protein	NS	NS	R ² = 0.09 NS	R ² = 0.55 P = 0.004 **

3.7 Discussion

Previous studies in rodents have reported that leptin treatment can increase body temperature through enhanced UCPI activity (Scarpace *et al.* 1997) (Pelleymounter *et al.* 1995), therefore reducing the requirement for shivering thermogenesis. However, since one of these studies were carried out in adult *ob/ob* mice, who are deficient in leptin and the other studied adults, these are obviously not good models for the neonatal period.

Plasma leptin concentrations have been shown to fall dramatically after birth in the newborn human infant (Cetin *et al.* 2000; Geary *et al.* 1999; Matsuda *et al.* 1999; Schubring *et al.* 1999) and remain significantly lower than cord values for up to six days postnatally (Matsuda *et al.* 1999). However, it has recently been demonstrated that ovine leptin concentrations do not follow this trend at birth. Ovine plasma leptin concentrations decline during the immediate six hours after birth, then exhibit a concomitant increase in plasma leptin and leptin mRNA over the first seven days of neonatal life (Bispham *et al.* 2001; Budge *et al.* 2001).

I hypothesised that leptin treatment of neonatal lambs would enhance thermoregulation through increased activity of UCPI, resulting in a reduced reliance on shivering thermogenesis. The principal finding of the present study was that leptin treatment maintained colonic temperature of neonatal lambs, despite a reduction in UCPI protein abundance and mRNA expression which was not observed in vehicle treated siblings. These apparently contradictory findings suggest that regulation of thermoregulation by leptin in is considerably

different between precocial and altricial species and between adults and neonates.

3.7.1 Effect of Leptin Administration on Thermoregulation

Leptin was found to maintain colonic temperature on day one of postnatal age in the acute administration study, in contrast, untreated siblings lost body temperature. As a result, the control lambs had an average colonic temperature that was 40% lower than their leptin treated siblings at the end of the study. In support of the hypothesis, plasma leptin was positively correlated with colonic temperature prior to intervention in the long-term study. All the animals studied in the acute experiment shivered during the experiment, but the leptin treated group shivered for half as much time as the controls. The reduction in shivering time suggested that leptin treated lambs were maintaining their colonic temperature via non-shivering thermogenesis or increased thermal efficiency with reduced heat loss. However, UCP1 and GDP activity were similar between groups. Surprisingly, UCP1 expression was reduced in the acute leptin treated group although this was not significant. After the first dose of 100 pg leptin plasma NEFA concentrations are significantly higher in the treated lambs suggesting increased unmasking of GDP binding sites, it was not possible analyse this however as no tissue samples were taken at this time point.

When leptin administration over day two to eight of postnatal age was investigated, a similar, initial, maintenance of colonic temperature was observed. Between day two and three of postnatal age the vehicle treated group exhibited a significant decline in colonic temperature that was not observed in leptin treated

siblings. However, after 6 days of leptin treatment there was no difference in GDP binding, but both UCP 1 abundance and expression were reduced in the leptin treated group. Significant associations between thermogenic parameters (e.g. UCP1 mRNA and protein, GDP and colonic temperature) were found only in the leptin treated animals from both the acute and long-term leptin treatment studies (Table 3.5). These results were dependent on postnatal age, but not gender. These results suggest that leptin treatment alters the relationships between these thermogenic factors.

These results are in contradiction to rodent studies (both short and long-term leptin treatment) that have reported enhanced body temperature due to increased UCP1 after leptin administration and may be explained partially by a study reporting that leptin activates the proinflammatory cytokine interleukin-1 (IL-1) (Luheshi *et al.* 1999). Leptin has been proposed to induce IL-1 β expression in the hypothalamus to a magnitude similar to that of a pyrogenic dose of bacterial lipopolysaccharide. This mechanism would increase body temperature transiently, with no requirement for increased UCPI.

Alternatively, leptin may be directly activating one of the homologues of UCPI, UCP2 or UCP3. Although there has been no direct evidence that leptin up-regulates either of these proteins during the neonatal period, it has been established that leptin treatment of rodents up-regulates BAT and skeletal muscle UCP3 and 2 (Cusin *et al.* 1998, Scarpace, 1998 #156, Scarpace, 1998 #227). UCP3 has been shown to increase postnatally in skeletal muscle of rodents (Bmn

et al. 1999; Carmona *et al.* 1998) and to be present in newborn piglet muscle (Damon *et al.* 2000). Interestingly, IL-1 β and TNF- α have been shown to upregulate UCP2 in liver, muscle and adipose tissue of adult mice (Faggioni *et al.* 1998) although there is some debate whether UCP2 protein is present in liver and muscle (Pecqueur *et al.* 2001).

The elucidation of the role of these UCP homologues has been slow due to the lack of specific antibodies, and the ability of these proteins to mediate adaptive non-shivering thermogenesis has been thrown into doubt after the production of UCP1 ablated mice (Enerback *et al.* 1997, Nedergaard, 2001 #640). These mice were found to become hypothermic during cold exposure, despite high expression of UCP2. These mice were also unable to initiate non-shivering thermogenesis in response to noradrenaline administration. However, recent work has shown no UCP2 protein to be present in BAT from UCP1 KO mice. However, these studies have only investigated the thermoregulatory capacity of UCP1 deficient adults. The adult rodents used were progeny of the original UCP1 knock out mice (Enerback *et al.* 1997) but no mention is made of their thermoregulatory ability as neonates, with no UCP1 to produce heat after the birth the young mice must be initiating other mechanisms of heat production. Also, many publications demonstrating an effect of leptin on the UCP homologues only provide evidence of mRNA changes, which are not correlated with a functional response, for example, little is known about the translational efficiency of UCP2 or 3. Also, changes in mRNA levels do not confirm translation of the corresponding protein nor import and insertion of the protein

into the mitochondrial membrane. However, work in our laboratory on UCP1 suggests that these unusual transcriptional effects are limited to UCP2 as UCP1 mRNA is closely correlated with UCP1 protein when the protein half-life is taken into consideration.

Leptin is known to be a potent stimulator of the sympathetic nervous system (Haynes *et al.* 1997b; Scarpace & Matheny 1998; Sivitz *et al.* 1999; Trayhum *et al.* 1999). Sympathetic activity mediates the increase in free fatty acids and "switching - on" of UCP1 during adaptive thermogenesis (Chapter 1.3.2). Leptin administration may also be increasing the noradrenaline turnover in BAT (Collins 1996) along with increasing sympathetic outflow, thus liberating more fatty acids from triglycerides. In both the acute and long - term ovine studies of leptin administration, plasma NEFA levels declined more slowly in the leptin treated lambs (Figures 3.7 and 3.18) over the study period. These increased NEFA levels may have caused increased activation of UCP1 through unmasking, leading to a higher body temperature. As the differences in plasma NEFA concentrations occurred prior to tissue sampling (postnatal hour 3 and day 3) and equalised between groups by the end of the studies, it is possible that significant differences in GDP binding at the time of maintained body temperature may have been missed.

A more recently discovered effect of the sympathetic nervous system is to promote angiogenesis through the transient induction of vascular endothelial growth factor (VEGF) in BAT (Fredriksson *et al.* 2000). Leptin has previously been shown to have an angiogenic effect when administered to endothelial cells

and rodent comea in culture (Sierra-Honigmann *et al.* 1998). It is possible that noradrenaline is the physiological inducer of VEGF expression during periods of cold exposure and it is thought that a transient elevation of VEGF is sufficient to support angiogenesis during BAT recruitment (Fredriksson *et al.* 2000). In the present studies, leptins positive regulation of sympathetic outflow to BAT and noradrenaline turnover in BAT may be improving thermoregulation through increased blood flow to the tissue. This could be investigated further through quantitative analysis of VEGF in BAT from the present studies.

3.7.2 Effect of Leptin Administration on Hormones and Metabolites

3.7.2a Leptin

Leptin administration in both the acute and long-term studies increased plasma leptin concentrations significantly. Plasma leptin concentrations in treated animals from the acute study were higher than those of the long-term study. This was probably due to plasma leptin levels being measured the day after administration in the long-term study. The "chronic" leptin administration study provided the opportunity to look at the natural ontogeny of plasma leptin over the first week of life, something that has not previously been undertaken in sheep. Results from human clinical studies suggest that leptin values remain low during early gestation and increase towards term in response to the increased abundance of adipose tissue (Cinaz *et al.* 1999; Matsuda *et al.* 1999). Leptin concentrations peak around the time of birth in the infant then rapidly decline to day 3 of postnatal life (Hytinantti *et al.* 1999; Schubring *et al.* 1999) and remain low up to at least 17 days of age (Matsuda *et al.* 1999). However, there is no published data

demonstrating tissue mRNA levels of leptin in newborns. The results of the present study suggest that ovine plasma leptin levels decrease after postnatal day one, then gradually rise and plateau at the end of the first postnatal week despite no concomitant rise in leptin mRNA, in fact a decrease was observed.

A possible explanation for the differential mRNA and plasma concentrations of leptin is that the newborn lamb obtains leptin from ovine milk, this would increase plasma concentrations without the need for increased mRNA expression. Human, porcine and ovine breast milk have been shown to contain maternally derived leptin that is transferred to the infants' blood stream (Estienne *et al.* 2000; Houseknecht *et al.* 1997), providing leptin at a time when the digestive tract of the newborn can absorb whole proteins. At birth, only 66 % of UK babies are breastfed, this figure falls rapidly to 56 % at one week (data from The Office for National Statistics Infant Feeding Survey 1995 report) this is low compared to the number of lambs that suckle. This may influence plasma leptin concentrations and could be an important source of leptin not available to 34% of British infants and a possible explanation for the difference in human and ovine postnatal ontogeny and the discrepancy between mRNA and plasma values.

Although BAT stores of triglycerides are depleted by non-shivering thermogenesis in the first few days after birth in the lamb, there is still a significant accumulation (43 % increase in perirenal adipose tissue weight, Tables 3.2 and 3.4) of this depot in the first week of life. Leptin has been shown to be produced in BAT of newborn rats (Cancello *et al.* 1998; Dessolin 1997)

therefore the increased abundance of this tissue has been suggested to represent one of the sources of leptin over the neonatal period. However, in the study's presented we found no significant correlation between BAT weight and plasma leptin after one week of life, suggesting that plasma concentrations of leptin are not associated with concomitant changes in adipose tissue deposition. Human infants on the other hand, experience weight loss during the week after birth, then slowly increase their body weight (approximately 1kg in the first month) suggesting that the production of endogenous leptin may be reduced and circulating levels remain low.

Gender related differences in leptin have been well documented, as have the regulatory role of the sex hormones in leptin production (Behre *et al.* 1997; Mannucci *et al.* 1998; Tome *et al.* 1997). Results from the acute leptin administration study confirmed the link between gender and plasma leptin concentrations, with female lambs exhibiting significantly higher plasma leptin levels than their male siblings, however, the ability of the lambs to respond to leptin treatment was unaffected by gender and no further effect of gender was observed in any parameter measured. The role of gender in leptin regulation was not examined further as all animals in the long-term study were female.

A negative relationship between leptin and prolactin has not been reported previously, although there is limited evidence that prolactin stimulates leptin secretion in BAT in rats (Gualillo *et al.* 1999). It is possible that leptin acts to down-regulate prolactin through an unknown negative feedback mechanism.

3.7.2b NEFA and Glucose

Both long and short - term leptin administration was found to transiently attenuate the decline in plasma NEFA concentrations observed in untreated lambs (Figures 3.7 and 3.17) and to be positively correlated with body temperature in the long term study. Leptin has been shown to increase the rate of lipolysis in WAT *in vitro* and *in vivo*, (Shimabukuro *et al.* 1997; Siegrist-Kaiser *et al.* 1997) leading to a reduction in triglyceride stores and preventing accumulation of triglycerides in non-adipose tissues such as the liver (Unger *et al.* 1999). However, the documented increase in rate of lipolysis in WAT was not associated with an increase in plasma NEFA, possibly due to increased intracellular oxidation (Shimabukuro *et al.* 1997). Leptin has also been shown to increase lipoprotein lipase mRNA in cultured BAT (Siegrist-Kaiser *et al.* 1997) which would liberate more NEFA. This is one way in which leptin may be modulating UCPI without the requirement of increased protein abundance. These observations, however, are from adult studies. The situation during the neonatal period is likely to be different and the transient changes in NEFA in the present studies may reflect a physiological mechanism "resisting" the breakdown of essential adipose tissue stores.

Plasma glucose levels remained similar between groups in both the short and long-term study, this is in agreement with studies carried out in lean mice that have shown leptin treatment to have no effect on plasma glucose, insulin or glucagon. Leptin has been proposed to increase glucose uptake in BAT and muscle and to reduce hepatic glycogen stores associated with increased glucose production (Nonogaki 2000). Further analysis could be carried out to

investigate the hepatic and skeletal muscle glycogen content of neonatal lambs after leptin administration.

3.7.2c Prolactin

In the long-term study, there was a non-significant decline in prolactin plasma concentrations in the leptin group between day 1 and 2 of postnatal age that was not observed in their vehicle treated siblings. This decrease in prolactin was not associated with any other factor although leptin was found to negatively regulate plasma prolactin concentrations prior to intervention as discussed in 3.7.2a .

Plasma prolactin concentration was found to be negatively related to plasma NEFA on day 2 of age in the leptin treated group. This was at a time when the treated group were maintaining their colonic temperature better than vehicle treated siblings, suggesting that a low plasma prolactin level is conducive for effective thermoregulation. This is however in contrast to previous studies documenting a positive thermogenic effect of maternal prolactin treatment on neonatal rodents (Budge *et al.* 2002) and prolactin treatment of neonatal lambs (Pearce *et al.* 2001). At the end of the first postnatal week, plasma prolactin levels were significantly reduced in the leptin treated lambs, again this was unassociated with leptin or any other factor investigated.

3.7.2d Body Weight and Tissue Analysis

There was no effect of leptin administration on body weight, PAT weight or any other organ investigated in either the acute or long-term studies despite many rodent studies demonstrating an anorectic effect of leptin (De Vos *et al.* 1995; Halaas *et al.* 1995; Pelleymounter *et al.* 1995). Lamb growth in the long-term

study was also similar between treatment groups, these results were in accordance with a rodent study which found that leptin had no anorectic effect on neonatal mice prior to two weeks of age (Mistry *et al.* 1999). Neonatal lambs must maximise their milk intake and maintain high metabolic rates in order to survive, so this differential effect of leptin administration between adults and neonates is not surprising.

When BAT thermogenesis was investigated, GDP binding was found to be similar between both groups of animal in the acute and long-term studies. GDP binding was reduced at 8 compared to 1 days of age. This is in accordance with previous ovine studies (Clarke *et al.* 1997b) demonstrating a decline in BAT thermogenesis with increasing age. Surprisingly, UCPI mRNA expression was reduced with leptin treatment, but UCP 1 protein abundance was similar between groups in the acute study. Although the reduction in UCPI mRNA was not significant, six out of the eight leptin treated lambs had lower UCPI mRNA when compared to their vehicle treated siblings. This result was in contradiction compared to previous published work demonstrating an increase in UCPI with leptin treatment (Pelleymounter *et al.* 1995; Scarpace & Matheny 1998; Scarpace *et al.* 1997). The half-life of UCPI protein is approximately five days (Nedergaard *et al.* 2001), so no changes related to reduced mRNA would be expected in the acute study. It was predicted that seven days of leptin treatment would allow any protein changes to become apparent, however, only a non-significant reduction in protein for UCP1 was observed in the long-term study. Increasing postnatal age is associated with loss of transcription of the UCPI gene

and by one month of age UCPI is undetectable in PAT of lambs (Clarke *et al.* 1997b).

The loss of BAT UCPI is an indication of the changing role of adipose tissue with age. BAT, and therefore UCP1 is not expressed in adult sheep, there is no requirement for recruitable thermogenesis as they have deposited subcutaneous WAT and developed fleece. The loss of UCPI in the leptin treated lambs suggests that leptin is acting to promote or "signal" the transition from BAT to WAT. Leptin is known to modulate adipose tissue mass in rodents via lipolysis and deletion of adipocytes through apoptosis (Qian *et al.* 1998) although the signalling pathway mediating this process is unknown. So it is possible that leptin treatment of neonatal lambs is activating apoptosis of BAT thus reducing the number of adipocytes present, apoptosis was suggested as a possible mechanism for the transition of BAT to WAT in 1998 by Finn *et al.* (Finn *et al.* 1998). However, as there was no difference in adipose tissue weight between treatment groups, microscopy would have to be carried out to verify cell size and the lipid content of individual adipocytes. Assays of apoptosis, such as TUNEL staining and DNA laddering are required to determine the apoptotic state of the tissue.

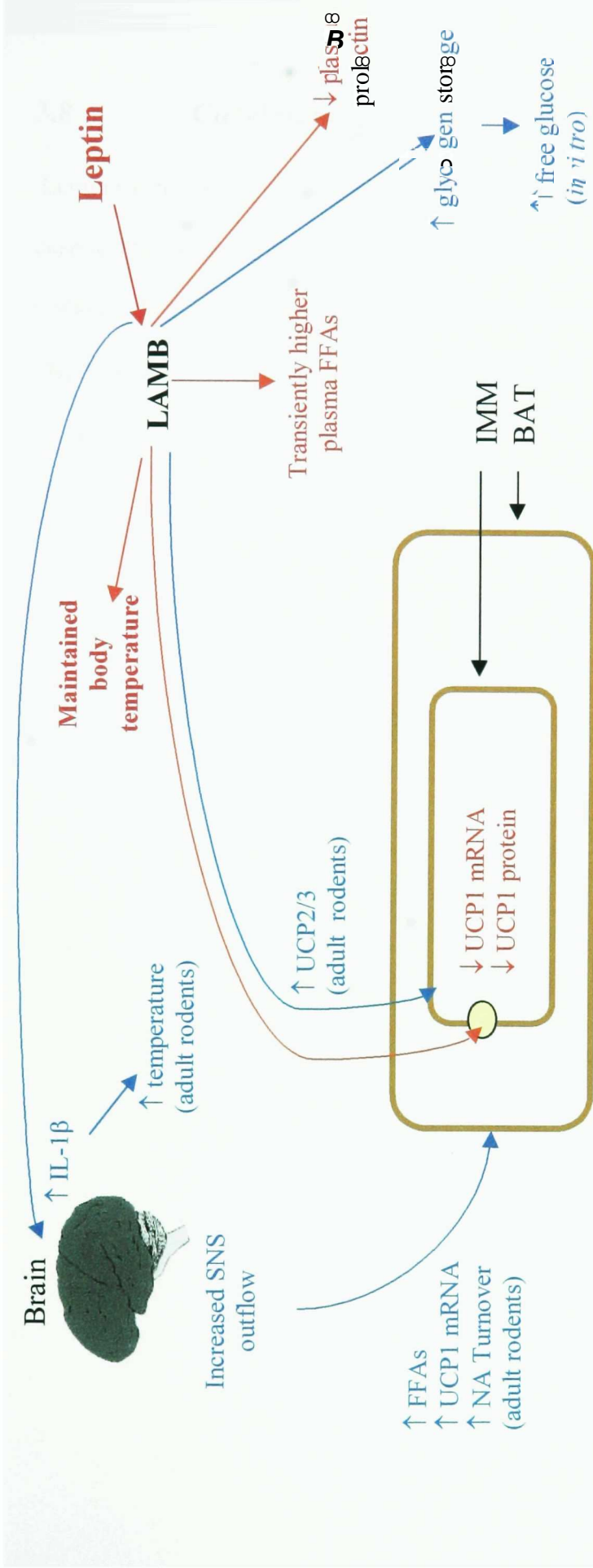


Figure 3.26

Diagrams showing the major findings from the two leptin studies.

Red lines and text indicate experimental findings from the present studies; blue lines and text published data

IMM, inner mitochondrial membrane; SNS, sympathetic nervous system; BAT, brown adipose tissue

3.8 Conclusion

Leptin treatment of neonatal lambs causes maintenance of body temperature despite a reduction in UCP1 protein and mRNA expression. This change occurs without effect on lamb or organ growth and development. Leptin, in this way may be acting through mechanisms other than UCP1 to maintain temperature.

CHAPTER 4

THE EFFECT OF ROUTE OF DELIVERY ON PLASMA LEPTIN CONCENTRATIONS IN THE NEWBORN LAMB

4.1 Introduction

Leptin mRNA is detectable in fetal adipose tissue of sheep by around G90 (Yuen *et al.* 1999). Abundance of leptin then increases up to term and is closely correlated with fetal weight (Yuen *et al.* 1999). This suggests that the production of leptin mRNA is modulated by the increase in size and number of adipocytes which occurs during late gestation in the ovine fetus (Alexander 1978; Lonqvist *et al.* 1997). The rise in leptin could also be due to increasing corticosteroids during late gestation as these have been shown to stimulate leptin expression both *in vitro* and *in vivo* (De Vos *et al.* 1995).

Many studies have measured plasma leptin concentrations in newborn infants; however, the findings from these studies have been highly inconsistent (see Chapter 1). Clinical human studies suggest that plasma leptin remains low throughout gestation, are first detectable by 35 weeks of gestation, and increase towards term in response to the increased abundance of adipose tissue (Cinaz *et al.* 1999; Matsuda *et al.* 1999). Leptin levels have been shown to peak around the time of birth in the infant, then rapidly decline by day 3 of postnatal age (Hytinanti *et al.* 1999; Schubring *et al.* 1999). These postnatal changes are likely to occur in response to the dramatic alterations in energy balance and the surge in endocrine and sympathetic activity experienced at birth. The sympathetic nervous system, Cortisol and thyroid hormones are known to regulate leptin expression (De Vos *et al.* 1995; Flier *et al.* 2000; Mostyn *et al.* 1998); all of which are critically important at parturition and for the initiation of breathing and thermoregulation at birth (Symonds 1995).

The route of delivery of a fetus influences the postnatal changes outlined above (Clarke *et al.* 1997c). Caesarean section delivery is known to reduce neonatal plasma concentrations of Cortisol and thyroid hormones by reducing the normal stress experienced by the infant during the process of parturition. This results in a neonate with significantly reduced ability to thermoregulate after birth (Clarke *et al.* 1997c). However, the effect of the route of delivery on plasma leptin concentrations is unknown.

4.1.1 Aim and Hypothesis

The aim of this study was to investigate the effect of route of birth, adipose tissue mass and body weight on plasma leptin in neonatal lambs. It was hypothesised that caesarean section delivery would alter leptin secretion.

4.2 Methods

4.2.1 Experimental Design

All animal work was carried as described by Clarke *et al.* (Clarke *et al.* 1997c) prior to the development of the plasma leptin assay. Full experimental procedures may be found in Clarke *et al.* 1997 (Clarke *et al.* 1997c). Briefly, twenty Bluefaced Leicester cross Swaledale ewes of recorded mating date and confirmed as bearing twins were entered into the study. Ten sets of twins were delivered by caesarean section at G146 into warm or cool ambient temperatures. An umbilical cord blood sample was taken immediately before cord clamping. A jugular vein catheter was then inserted into each lamb and blood samples were

taken hourly for the first 5 hours after birth. A similar protocol was performed on the remaining lambs that were all born normally at term (G147 in this breed), with the modification that it was not possible to obtain umbilical cord samples. There was no effect of delivery temperature on plasma leptin in either caesarean section or vaginally delivered lambs, so only mean results with respect to route of delivery are shown. Body weights and perirenal adipose tissue mass were measured post euthanasia, 6 hours after birth.

4.2.2 Laboratory Procedures

Plasma leptin was analysed by Professor Duane Keisler using a radioimmunoassay as described in Chapter 2.2.3.

4.2.3 Statistical Analysis

Statistical analysis with respect to significant differences ($p < 0.05$) between values obtained from the different treatment groups was carried out using Mann-Whitney U tests. The effect of postnatal age was assessed by the Wilcoxon test.

4.3 Results

4.3.1 Plasma Hormones

Plasma leptin concentrations were significantly higher in fetal than postnatal samples ($P=0.009$) (Figure 4.1). When route of delivery was considered, cord plasma leptin levels were not significantly higher than the first venous sample taken from caesarean section delivered neonates (Figure 4.1 and 4.2). However, cord values were significantly higher than the first sample taken from vaginally

delivered lambs ($P=0.001$) (Figure 4.1 and 4.2) and this leptin concentration was significantly lower than that of the caesarean section delivered lambs ($P=0.002$) (Figure 4.1). Figure 4.2 shows the ontogeny of leptin in caesarean section and vaginally delivered lambs over the first 5 hours of life. Plasma leptin concentrations in caesarean section delivered lambs were significantly lower than cord values by 2 ($P=0.038$), 3 ($P=0.031$) and 4 ($P=0.008$) hours of life by which time leptin concentrations were similar between delivery groups. Irrespective of delivery group, there was no effect of gender on neonatal leptin.

4.3.2 Lamb BAT and Body Weight

Lambs delivered by caesarean section had significantly more fat per kg than their vaginally delivered counterparts ($P=0.001$) (figure 4.3), however, vaginally delivered lambs were significantly heavier ($P<0.001$) (Figure 4.4).

Plasma leptin was positively correlated with body weight in the vaginally delivered group only ($R^2=0.22$, $P=0.05$) (Figure 4.5). No correlation between body weight and leptin was found in the caesarean section delivered group, although male animals exhibited a strong relationship between leptin and fat per kg body weight ($R^2=0.85$, $P=0.004$) (Figure 4.6).

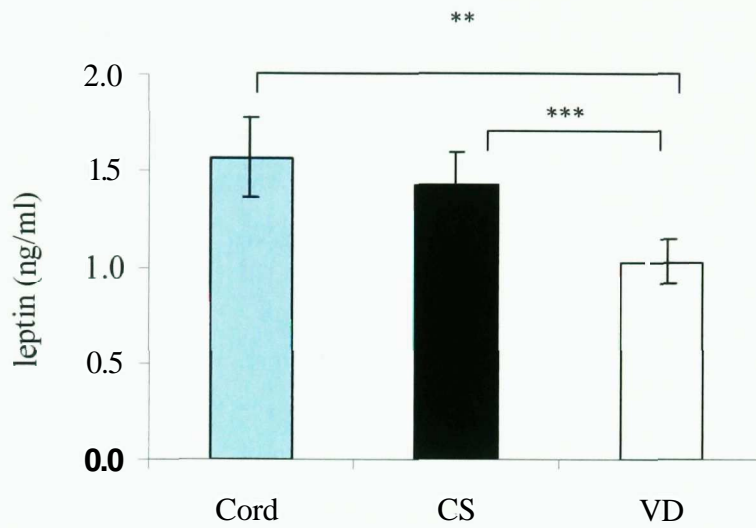


Figure 4.1

Influence of route of delivery on plasma leptin concentrations.

Values are means \pm SEM (cord n=19, caesarean delivery (CD) n=19, vaginal delivery (VD) n=20).

*** indicates $P < 0.001$ for delivery effect

** indicates $P < 0.01$ for age effect

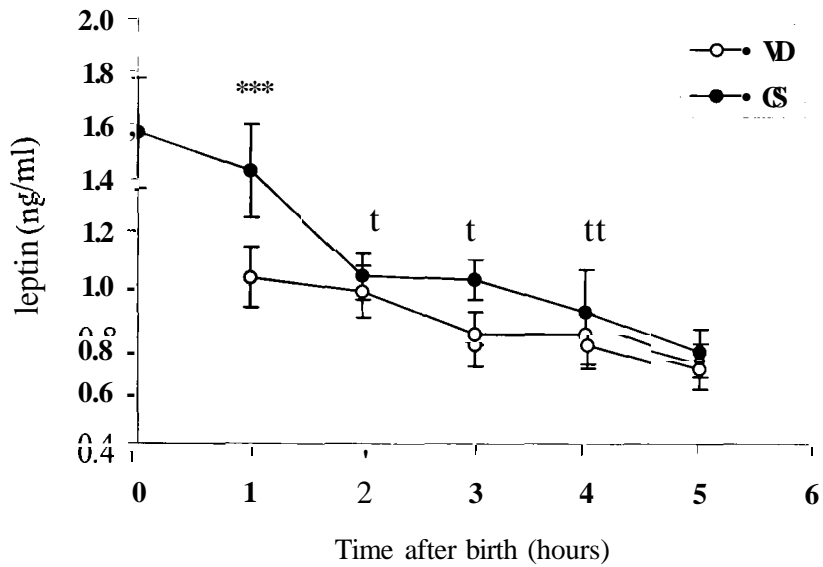


Figure 4.2

Influence of route of delivery on plasma leptin concentrations.

Values are means \pm SEM (cord n=19, caesarean delivery (CD) n=19, vaginal delivery (VD) n=20).

*** indicates $P < 0.001$ for delivery effect

t indicates $P < 0.05$ for age effect (when compared to cord values)

tt indicates $P < 0.01$ for age effect (when compared to cord values)

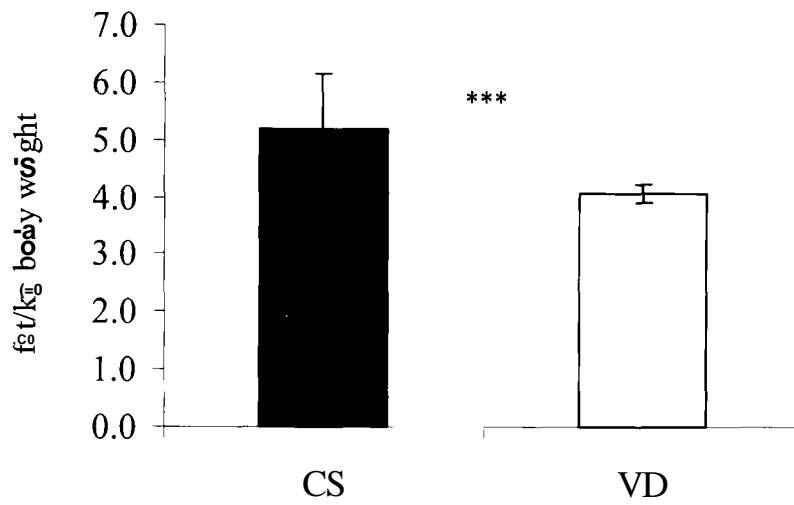


Figure 4.3

Influence of route of delivery on fat per kg body weight in neonatal lambs.

Values are means \pm SEM (caesarean delivery (CD) n=20, vaginal delivery (VD) n=22).

*** indicates $P < 0.001$ for delivery effect

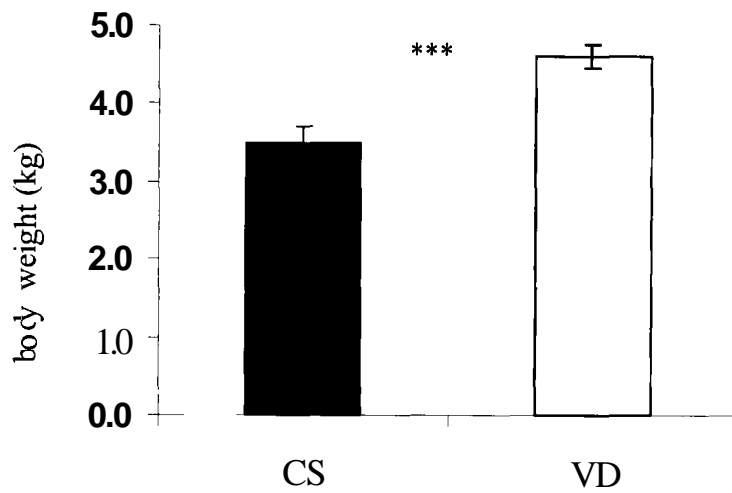


Figure 4.4

Influence of route of delivery on body weight in neonatal lambs.

Values are means \pm SEM (caesarean delivery (CD) n=20, vaginal delivery (VD) n=22).

*** indicates $P < 0.001$ for delivery effect

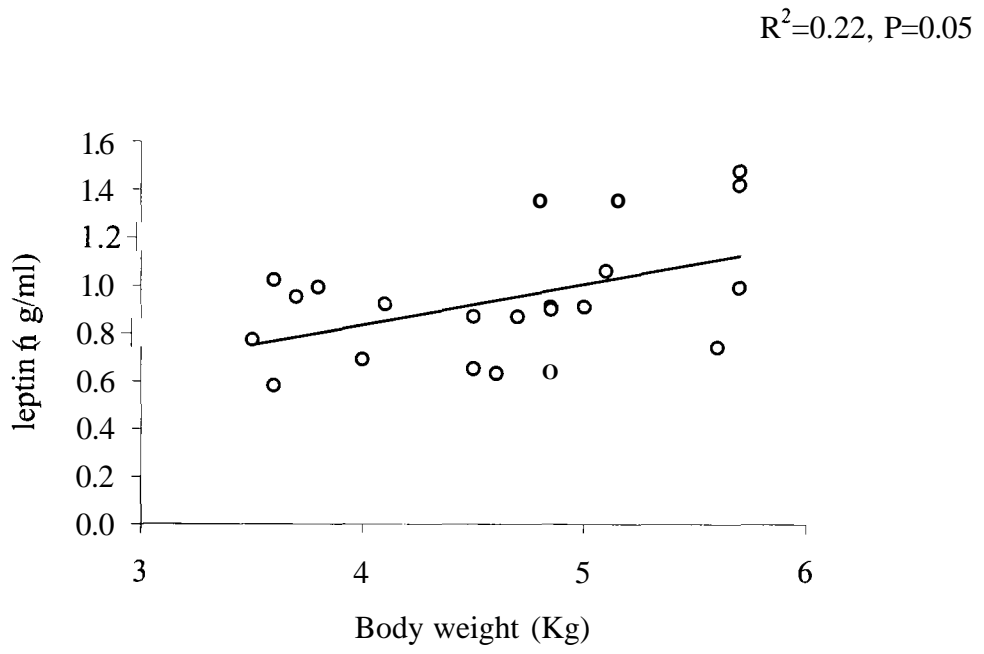


Figure 4.5

Relationship between plasma and fat per kg body weight

(vaginally delivered, n=22)

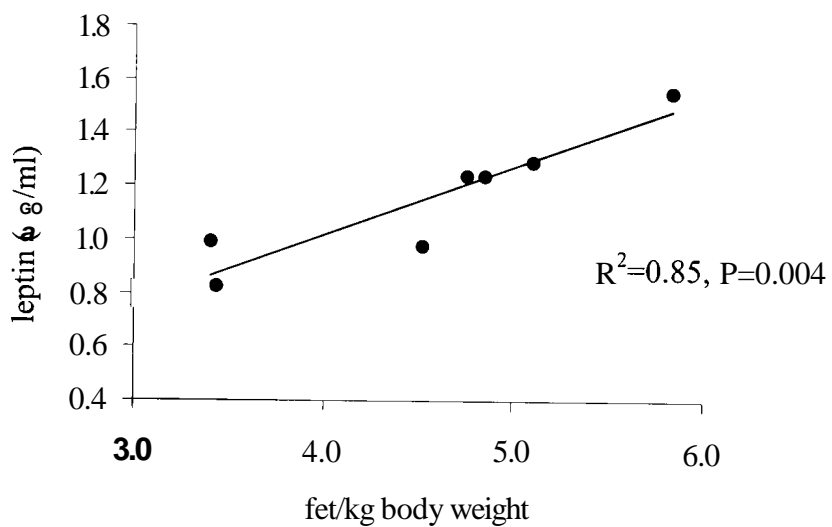


Figure 4.6

Relationship between plasma and body weight

(caesarean section delivered, male, n=7)

4.4 Discussion

4.4.1 Effect of Delivery on Plasma Leptin

Cord plasma leptin concentrations in the sheep were found to be significantly lower than values published for human placental studies. This is not surprising as I have shown ovine neonatal plasma leptin concentrations to be considerably less than those of human neonates (1-3 days of postnatal age) (Mostyn *et al.* 2001b). The birth of lambs in this particular study can be likened to an elective caesarean section delivery near term in humans, as labour was not initiated prior to caesarean section delivery.

Caesarean section delivery caused a delay in the "normal" decline in plasma leptin observed in the vaginally delivered animals resulting in significantly higher neonatal plasma leptin concentrations. This could be due to a number of factors that are known to alter in caesarean section delivery:

- sympathetic nervous activity
- thyroid hormones
- adipose tissue deposition and thermoregulation
- Cortisol

One of the major physiological differences in lambs born by caesarean section compared to vaginally delivered lambs, is the reduction in physical stress at birth associated with a decrease in the normal surge of catecholamines in the neonate (Falconer & Lake 1982; Faxelius *et al.* 1983; Hagnevik *et al.* 1984; Irestedt *et al.*

1982; Lagercrantz & Bistoletti 1973). This is due to a number of reasons including, passage through the birth canal compresses the head - this kind of mechanical trauma is known to stimulate catecholamine release. Brief reversible hypoxia during uterine contractions may also increase catecholamine production. Therefore, lack of labour is thought to cause the reduced neonatal plasma catecholamine concentrations observed after elective caesarean section delivery. Leptin has been shown to be negatively regulated by the sympathetic nervous system (Li *et al.* 1997; Mostyn *et al.* 1998; Trayhum *et al.* 1998), with less sympathetic activity, the caesarean section delivered animals would experience less inhibition of leptin secretion, thus higher plasma values.

Plasma T3 concentrations are low during most of gestation in the sheep but increase rapidly with the onset of parturition (Eraser & Liggins 1988; Wu 1990). This increase has been shown to occur in parallel with the increase in Cortisol observed at this time (Eraser & Liggins 1988; Eraser & Liggins 1989). Caesarean section delivered lambs experience a reduced increase in plasma Cortisol and T3 (Bird 1996). Thyroid hormones have previously been shown to regulate leptin mRNA expression although the nature of this regulation is complex (Flier *et al.* 2000). Hypothyroid rats have been shown to have higher plasma leptin concentrations and adipose tissue that releases more leptin than their euthyroid controls (Fain & Bahouth 1998). However, *in vitro* studies have shown that under conditions that mimic the fed state (i.e. high glucose, glucocorticoids and insulin) thyroid hormones will stimulate leptin expression. In contrast, conditions which mimic the fasted state (i.e. glucocorticoid alone or in the presence of a P3-

adrenoceptor agonist) T3 enhances the loss of leptin mRNA (Fain & Bahouth 1998). The conditions that match the situation of the vaginally delivered newborn lamb are those of the fasted state. Considering the two findings together, hypothetically, T3 should have an inhibitory effect on leptin. Vaginally delivered lambs and human infants have significantly higher plasma T3 concentrations than those delivered by caesarean section (Bird 1996; Clarke *et al.* 1997c) therefore this is a possible mechanism of suppression of leptin expression and an explanation for the higher plasma leptin in caesarean delivered lambs.

Route of delivery of the newborn is an important influence on ability to thermoregulate after birth. Increased T3 and SNS activity promote non-shivering thermogenesis in BAT of the newborn lamb, these factors are substantially reduced in caesarean section delivery (Bird 1996; Clarke *et al.* 1997c; Hagnevik *et al.* 1984), thus vaginally delivered lambs are better able to maintain body temperature after birth (Clarke *et al.* 1997c). Non-shivering thermoregulation in BAT is associated with an increase in lipolysis, thus a reduction in adipose tissue mass and an increase in circulating free fatty acids (Clarke *et al.* 1997c). The vaginally delivered lambs had significantly less adipose tissue per kg body weight than their caesarean section delivered counterparts, this is most likely due to increased BAT thermogenesis. Although there was no correlation between plasma leptin and BAT nor fat/kg body weight in the vaginally delivered group, previous studies have shown neonatal leptin to be positively influenced by body fat. It is possible that the reduced BAT abundance of vaginally delivered lambs had a negative regulatory effect on leptin secretion, resulting in the caesarean

section delivered lambs, who had significantly more adipose tissue, having higher plasma leptin immediately after birth. Although measurement of fat mass was taken at the end of the study when plasma leptin concentrations were similar between groups, it is possible that fat mass was lower in the vaginally delivered lambs from the first blood sample. It is possible that lambs delivered by caesarean section also have a suppressed metabolic rate compared to vaginally delivered lambs (Clarke *et al.* 1997c), this could impede the clearance of leptin from the kidney (Zeng *et al.* 1997), resulting in higher leptin concentrations in the plasma of caesarean section delivered animals.

Plasma glucocorticoid levels are thought to regulate circulating leptin concentrations in the adult (De Vos *et al.* 1995) although there is no evidence for this in the newborn. Plasma Cortisol levels peak at the time of birth and are significantly higher in cord plasma of vaginally, compared to caesarean section delivered neonates (Bird 1996). This is reversed postnatally with lambs delivered by caesarean section exhibiting higher plasma Cortisol concentrations (Clarke *et al.* 1997c). Cortisol therefore may be another factor involved in the maintenance of higher postnatal leptin in the caesarean section delivered lambs at one hour of postnatal age.

4.5 Conclusion

Plasma leptin concentrations decrease after birth and are positively correlated with adipose tissue depots and/or body weight dependent on route of delivery. The extent to which fetal leptin may regulate fetal growth remains to be established.

CHAPTER 5

THE EFFECT OF CORTISOL ON THERMOGENIC CAPACITY OF BAT IN THE LATE GESTATION FETAL LAMB

5.1 Introduction

Fetal plasma Cortisol levels, along with a number of hormones such as T3 and SNS activity, peak around the time of birth (Lagercrantz & Bistoletti 1973; Polk 1995; Slebodzinski *et al.* 1981). This is coincident with peak activity and expression of UCPI (Casteilla *et al.* 1989; Clarke *et al.* 1997a) which is critical for the initiation of non-shivering thermogenesis. Manipulation of thyroid hormones in the newborn lamb has been shown to improve thermoregulation of lambs following caesarean section delivery (Bird *et al.* 1998; Heasman *et al.* 2000). Administration of noradrenaline to lambs at delivery has also been found to diminish the drop in body temperature normally observed after birth through increased abundance of UCPI (Symonds *et al.* 2000a).

Route of delivery is a critical factor in determining postnatal thermogenesis.

Lambs that are delivered by caesarean section are not subjected to the stress of normal vaginal delivery and subsequently exhibit:

- lower colonic temperature
- reduced thermogenic activity of BAT
- reduced plasma Cortisol and T3 concentrations (Clarke *et al.* 1997c)

Dexamethasone treatment of pregnant ewes has been shown to improve the adaptation after birth following premature delivery of lambs by caesarean section (Clarke *et al.* 1998). The thermoregulation of these premature lambs was very similar to unfreated lambs delivered 1-2 days before term. Cortisol appears to be acting to mature the thermoregulatory capacity of the fetuses in this study. It is

not known whether manipulating the Cortisol status of the late gestation fetus would have a stimulatory influence on BAT development and activity.

The voltage dependent anion channel (VDAC) is a channel forming protein found in the outer mitochondrial membrane. VDAC has a major role in the delivery of ADP, ATP and other metabolic substrates into the mitochondria (Chapters 6 and 7). VDAC is present in fetal BAT and increases in abundance to peak at one day of postnatal age when UCPI is also highly abundant (Chapter 7). It is not known if Cortisol infusion would influence VDAC abundance in mitochondria.

5.1.1 Aim and Hypothesis

The aim of this study was to determine whether Cortisol, along with intact adrenal glands, influences UCPI abundance, expression and activity and leptin mRNA expression in perirenal BAT from sheep fetuses during late gestation. It was hypothesised that impaired Cortisol secretion would reduce the thermogenic capacity of fetal lambs. Conversely, increased plasma Cortisol concentrations were hypothesised to increase the thermogenic capacity of BAT of fetal lambs.

5.2 Methods

5.2.1 Experimental Design

All animal work was carried out at the University of Cambridge by Dr Abigail Fowden, full experimental procedures can be found in Li *et al* 1998 (Li *et al.* 1998) with the exception that BAT was sampled at after administration of a lethal dose of anaesthetic (sodium pentobarbitone, 200mg/kg intravenously). Briefly, Welsh Mountain ewes carrying twin fetuses of known gestational age were entered into the study and the fetuses were either treated with Cortisol or saline, or underwent adrenalectomy during late gestation. Figure 5.1 outlines the experimental procedure in a flow chart.

5.2.2 Laboratory Procedures

Mitochondria were prepared from PAT as described in chapters 2.3.1 and 2.3.6 in order to assess VDAC and UCPI abundance with western blotting. Total RNA was also prepared from BAT as described in Chapter 2.3.6 for Northern Blotting (Chapter 2.3.7) and RT-PCR (Chapter 2.3.8). Plasma Cortisol and thyroid hormone concentrations were analysed by Dr Abigail Fowden at the University of Cambridge (Fowden & Silver 1995). Hormone data presented in this thesis have been published previously by Dr Fowden and are reproduced here as necessary to allow full interpretation of my original data. Only the hormone data for animals included in thermogenic analyses are included.

5.2.3 Statistical Analysis

Statistical analysis with respect to significant differences ($p < 0.05$) between values obtained from the different treatment groups was carried out using Mann-Whitney U tests.

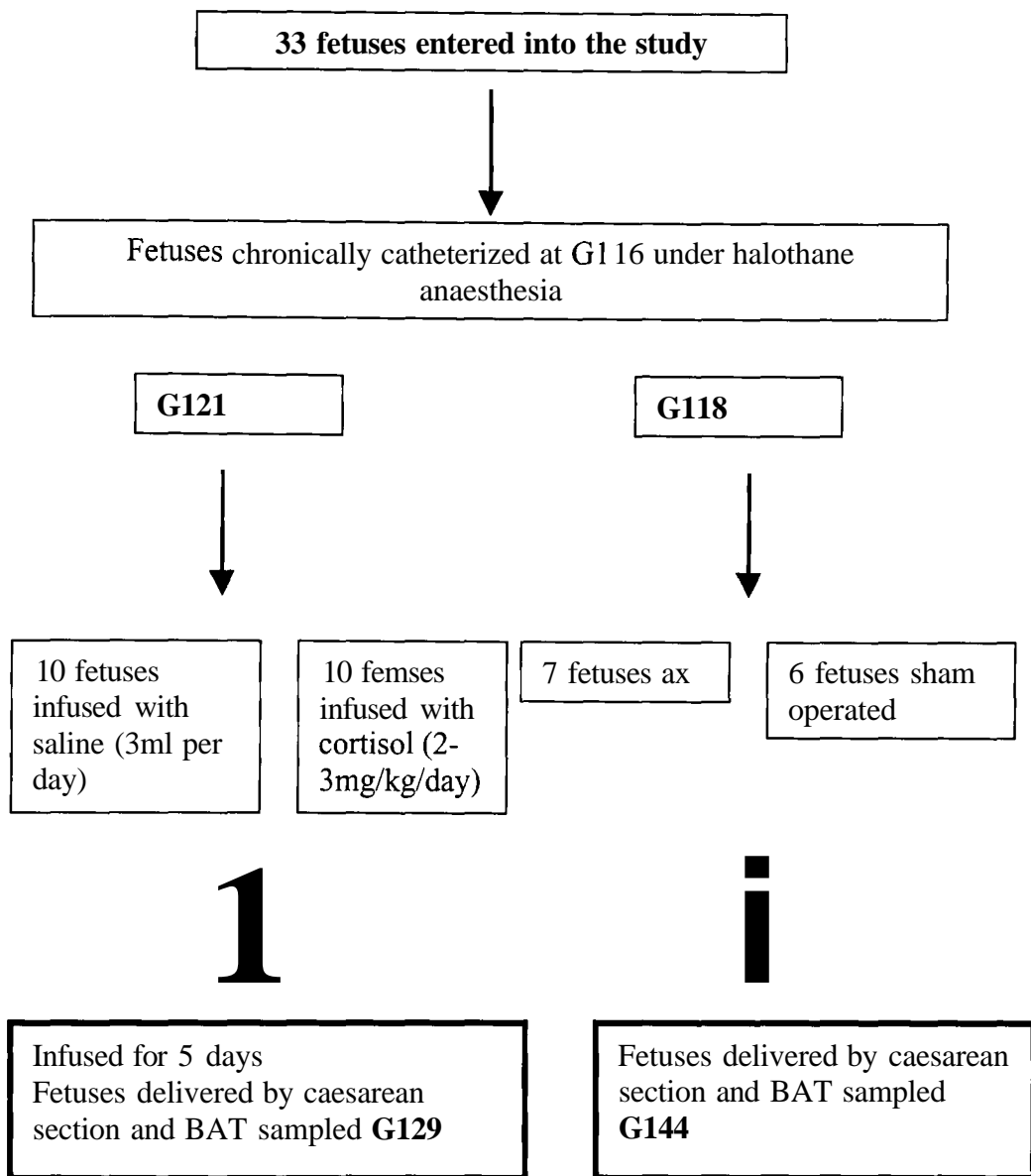


Figure 5.1

Flow chart of experimental procedures.

ax, adrenalectomised.

G121, 121 days gestation; G1 18, 118 days gestation etc.

5.3 Results

5.3.1 Plasma Hormones

Plasma Cortisol concentrations were found to be significantly higher in the Cortisol infused fetuses compared to those infused with saline ($P < 0.001$) (Figure 5.2). Adrenalectomised fetuses had significantly lower plasma Cortisol concentrations than fetuses that underwent sham operations ($P = 0.001$) (Figure 5.2). The plasma Cortisol levels in the 2 "control" groups were within the normal range for the fetal age and exhibited the expected increase with increasing gestation ($P < 0.001$) (Figure 5.2).

Triiodothyronine (T3) plasma concentrations were significantly higher in Cortisol treated fetuses compared to those treated with saline ($P = 0.004$) and in the sham operated compared to adrenalectomised fetuses ($P = 0.03$) (Figure 5.3) There was also a significant gestational increase in plasma T3 ($P = 0.04$). Plasma thyroxine (T4) concentrations were similar between groups (Figure 5.4).

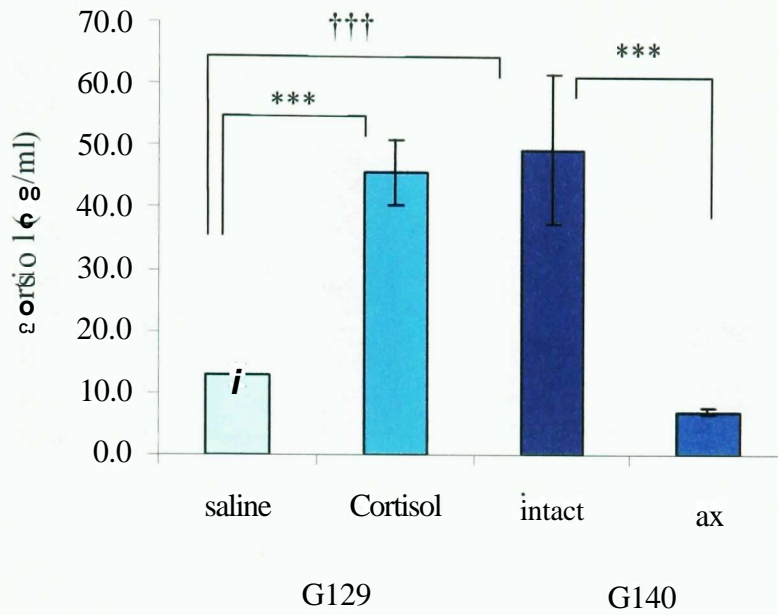


Figure 5.2

Effect of Cortisol status on plasma Cortisol concentrations in the late gestation fetus

Values are means \pm SEM (saline n=10, Cortisol n=10, intact n=6, ax n=7).

*** indicates $P < 0.001$ for treatment effect

††† indicates $P < 0.001$ for age effect

G129, 129 days gestation; G140, 140 days gestation.

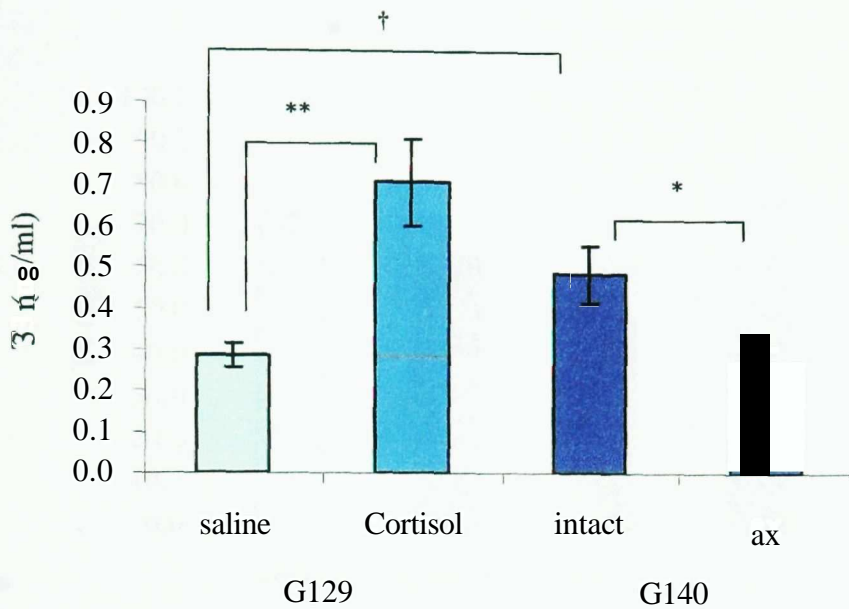


Figure 5.3

Effect of Cortisol status on plasma T3 concentrations in the late gestation fetus

Values are means \pm SEM (saline n=10, Cortisol n=10, intact n=5, ax n=7).

* indicates $P < 0.05$ for treatment effect

** indicates $P < 0.01$ for treatment effect

t indicates $P < 0.05$ for age effect

G129, 129 days gestation; G140, 140 days gestation

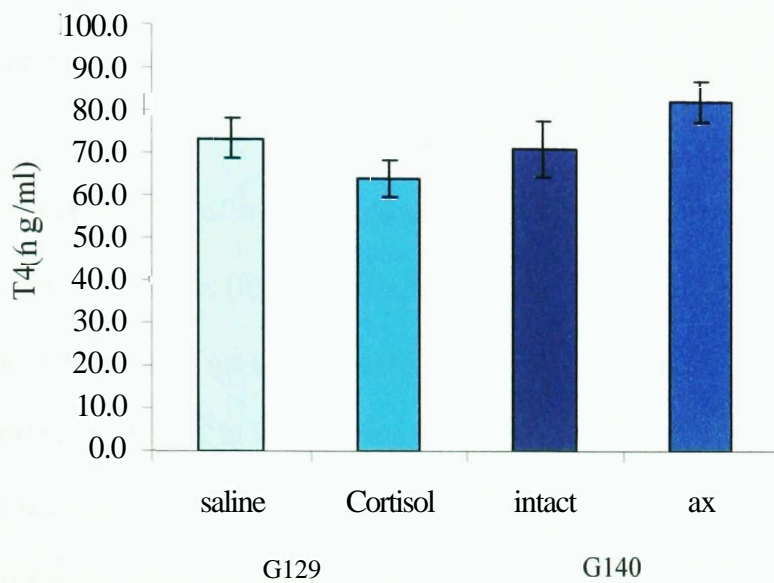


Figure 5.4

Effect of Cortisol status on plasma T4 concentrations in the late gestation fetus

Values are means \pm SEM (saline n=10, Cortisol n=10, intact n=5, ax n=7).

G129, 129 days gestation; G140, 140 days gestation

5.3.2 Lamb Body Weight and BAT Analysis

Lamb weights were assessed at the time of delivery, none of the treatments affected body weight (Figure 5.5) or crown mmp length (Figure 5.5). There was a normal increase in body weight and crown mmp length with gestational age (Figure 5.5).

The potential activity of BAT, as assessed by GDP binding was found to be similar between groups (Figure 5.6) although there was a trend for higher GDP binding in the Cortisol group compared to saline treated fetuses. UCPI abundance was found to be higher in lambs treated with Cortisol, although this was not statistically significant. UCPI protein was significantly increased in intact fetuses compared to the adrenalectomised group ($P=0.009$) (Figure 5.7). UCPI mRNA expression was assessed using Northern blotting, the results are shown in Figure 5.8. UCPI mRNA is expressed as a ratio of 18S, then as a percentage of a reference sample present on all gels, this is to remove inter-gel variation. There were no significant differences between the treatment groups although there was a trend towards higher UCPI mRNA in the intact fetuses compared to those that underwent adrenalectomy.

Voltage dependent anion channel (VDAC) abundance was measured in BAT using Western blotting. There were no significant differences between the treatment groups (Figure 5.9).

Due to the small numbers involved in the study, it was not possible to investigate correlations within individual groups for all thermogenic measurements. When

animals from all groups were considered together, Cortisol was found to be positively related to UCP1 protein abundance ($R^2=0.41$) (Figure 5.10), but not to UCP1 mRNA expression in BAT. Plasma Cortisol concentration was also found to be positively related to plasma T3 ($R^2=0.59$) (Figure 5.11). T3 was positively related to UCP1 protein abundance ($R^2=0.31$) (Figure 5.13). GDP binding was found to be positively related to UCP1 protein abundance ($R^2=0.59$) (Figure 5.12). There was only a very weak positive relationship between UCP1 protein abundance and UCP1 mRNA expression ($R^2=0.29$). Table 5.1 summarises all correlations between thermogenic and hormonal parameters.

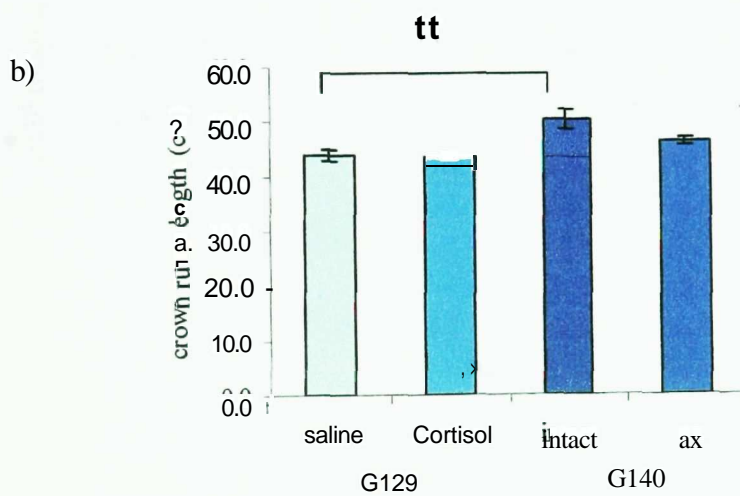
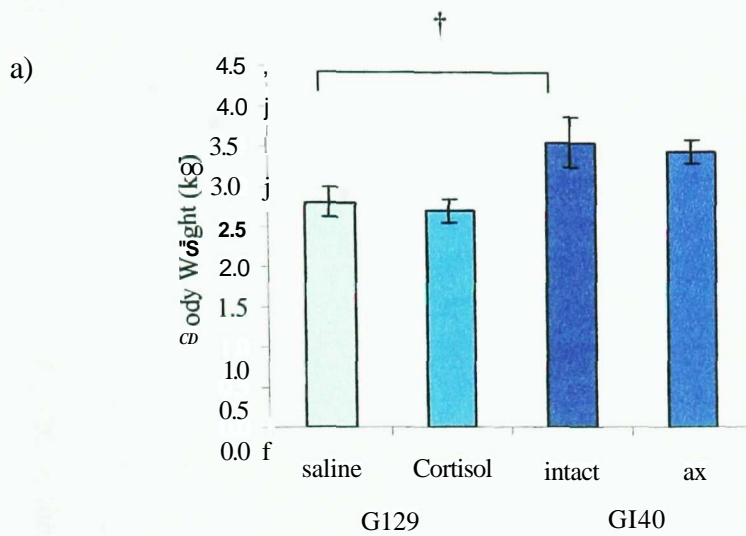


Figure 5.5

a) Effect of Cortisol status on body weight

b) Effect of Cortisol status on crown rump length

Values are means \pm SEM.

t indicates $P < 0.05$ for age effect

tt indicates $P < 0.01$ for age effect

G129, 129 days gestation; G140, 140 days gestation

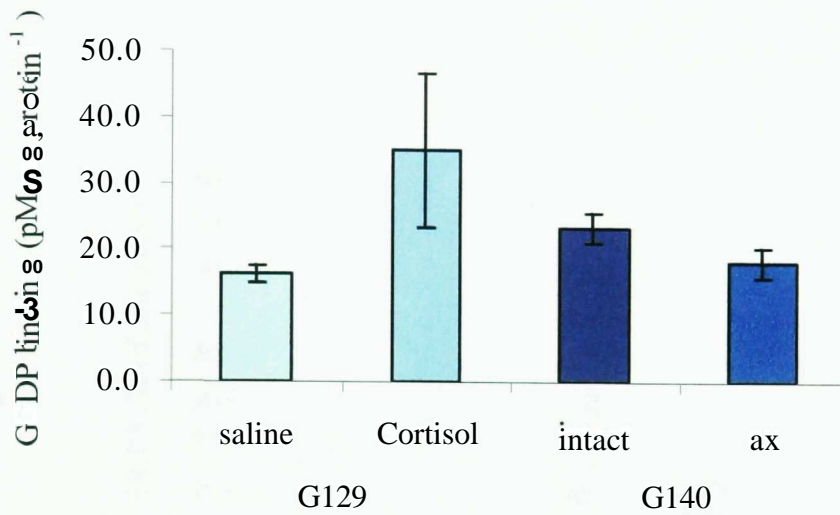


Figure 5.6

Effect of Cortisol status on GDP binding in BAT from fetal lambs

Values are means \pm SEM.

(saline, n = 5; Cortisol, n = 4; intact, n=5; adrenalectomised (ax). n=5)

G129, 129 days gestation; G140, 140 days gestation

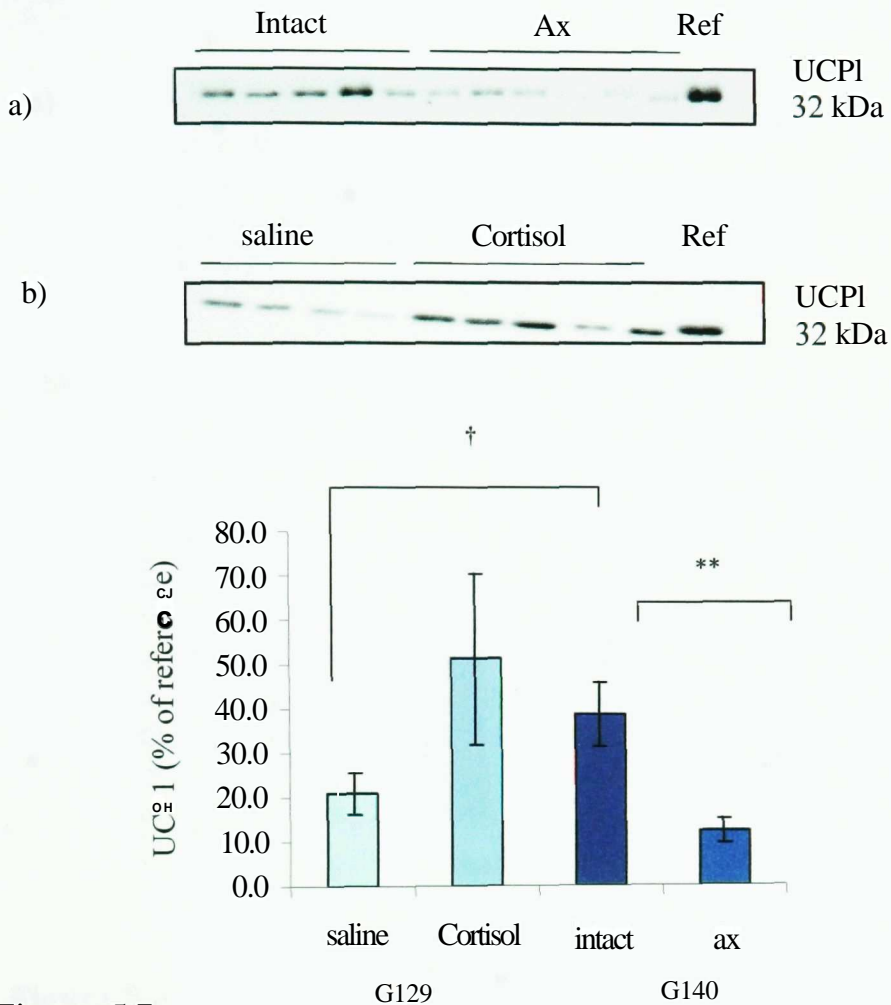


Figure 5.7

a) Representative image of a Western blot for UCP1, 10 µg MP each lane.

b) Effect of Cortisol administration on UCP1 protein abundance

Values are means ± SEM.

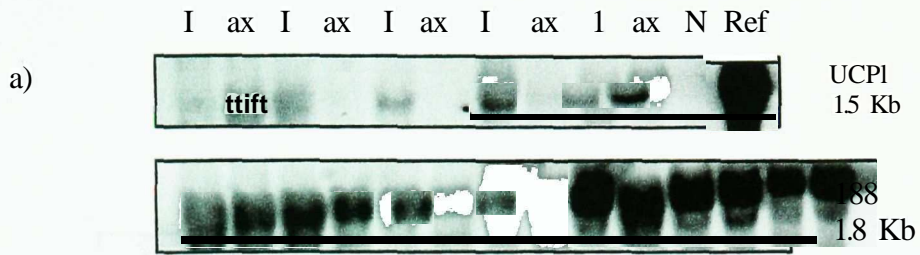
(saline, n = 4; Cortisol n = 4; intact, n=5; adrenalectomised (ax), n=6)

MP, mitochondrial protein; Ref reference sample (4 hour old lamb PAT mitochondria); ax, adrenalectomised.

** indicates $P < 0.01$ for treatment effect

† indicates $P = 0.063$ for age effect

G129, 129 days gestation; G140, 140 days gestation



b)

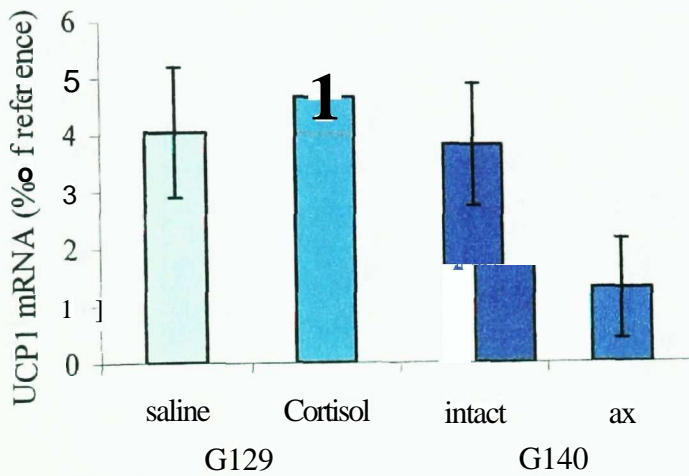


Figure 5.8

a) Representative image of a Northern blot for UCP1 and 18S, 10 µg RNA each lane.

b) Effect of Cortisol administration on UCP1 expression.

Values are means ± SEM.

(saline, n = 5; Cortisol, n = 5; intact, n=5; adrenalectomised, n=5)

Ref, reference sample (4 hour old lamb PAT mRNA); N, negative control (liver mRNA); I, intact; ax, adrenalectomised.

G129, 129 days gestation; G140, 140 days gestation

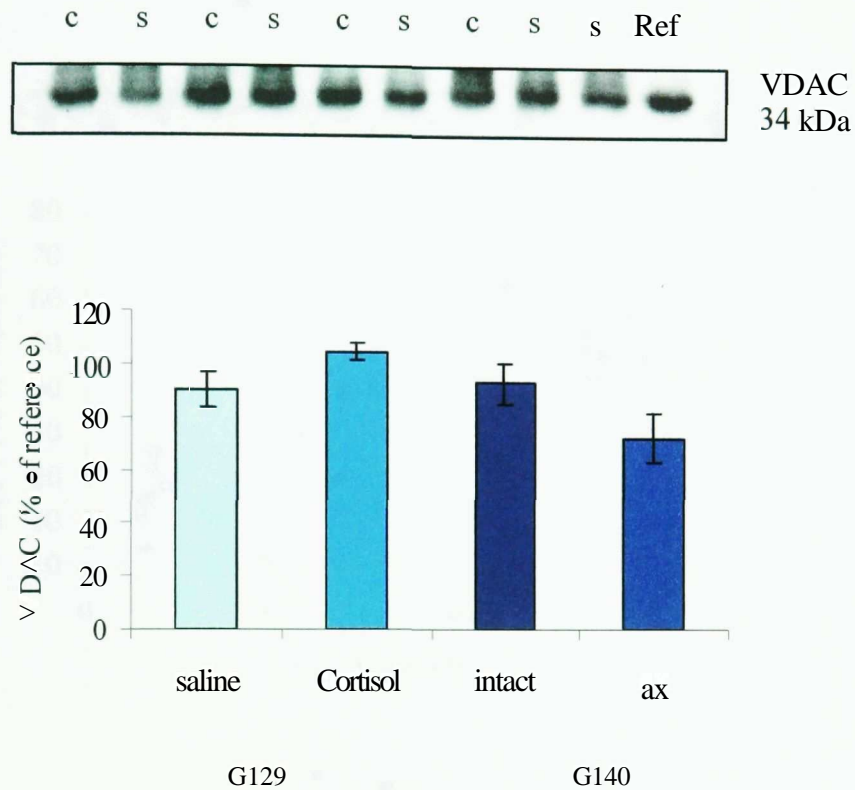


Figure 5.9

a) Representative image of a Western blot for VDAC, 10 µg MP each lane.

b) Effect of Cortisol administration on VDAC protein abundance

Values are means \pm SEM.

(saline, n = 5; Cortisol, n = 4; intact, n=5; adrenalectomised (ax), n=5)

MP, mitochondrial protein; Ref reference sample (7 day old lamb muscle mitochondria); ax, adrenalectomised.

G129, 129 days gestation; G140, 140 days gestation

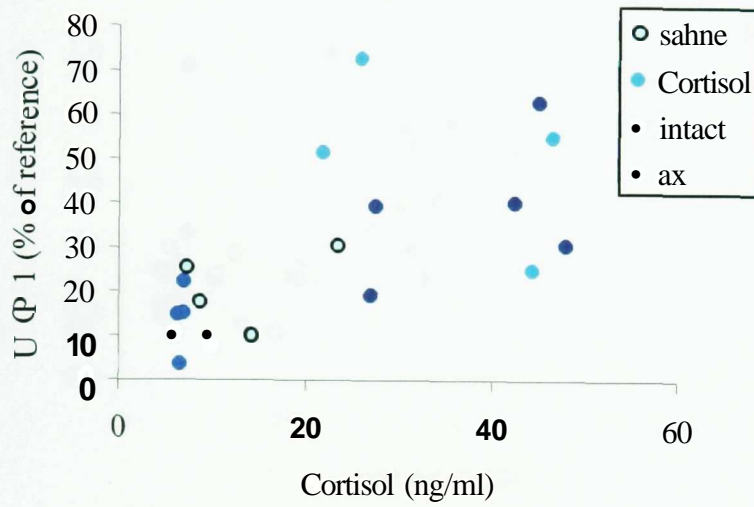


Figure 5.10

Relationship between Cortisol and UCPI protein abundance in fetal lambs.

(saline, n=4; Cortisol n=4; intact, n=5; ax, n=6)

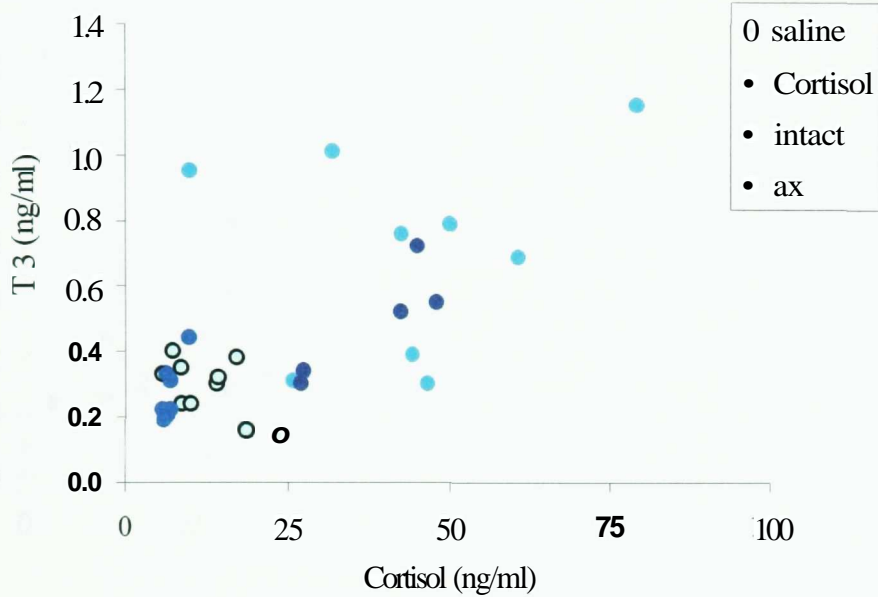


Figure 5.11

Relationship between Cortisol and T3 in fetal lambs.

(saline, n=10; Cortisol n=9; intact, n=5; ax, n=7)

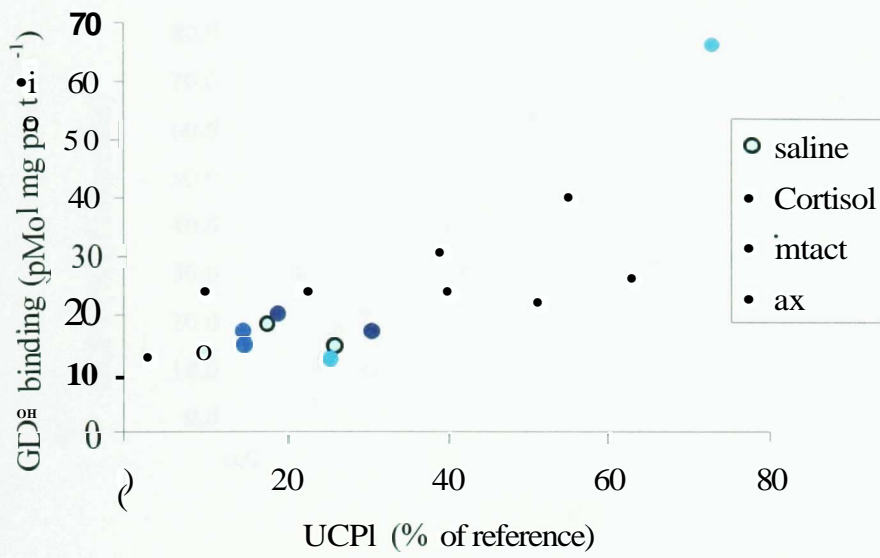


Figure 5.12

Relationship between GDP binding and UCPI in fetal lambs.

(saline, n=3; Cortisol n=3; intact. n=5; ax, n=5)

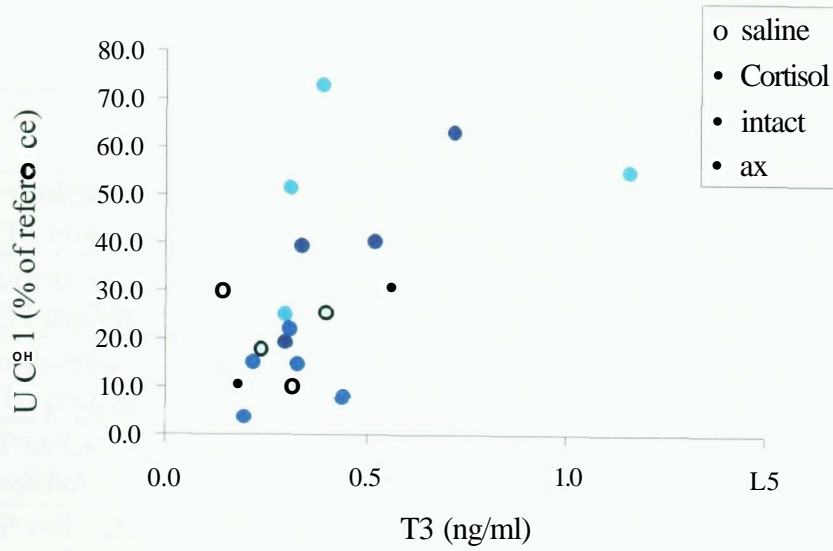


Figure 5.13

Relationship between plasma T3 concentrations and UCP1 in fetal lambs.

(saline, n=4; Cortisol n=4; intact. n=5; ax, n=6)

Table 5.1

Associations between measured thermogenic and hormonal parameters in the four treatment groups.

vs, versus; NS, non significant result; NA, result not available.

	saline	Cortisol	intact	ax	all groups
UCPI mRNA vs UCPI protein	R ² = NA NS	R ² = 0.65 NS	R ² = 0.31 NS	R ² = 0.10 NS	R ² = 0.29 P = 0.043 *
Cortisol vs UCPI mRNA	R ² = 0.0013 NS	R ² = 0.08 NS	R ² = 0.25 NS	R ² = 0.023 NS	R ² = 0.05 NS
Cortisol vs UCPI protein	R ² = 0.14 NS	R ² = 0.28 NS	R ² = 0.25 NS	R ² = 0.045 NS	R ² = 0.40 P = 0.001***
GDP vs UCPI mRNA	R ² = 0.11 NS	R ² = 0.95 NS	R ² = 0.36 NS	R ² = 0.020 NS	R ² = 0.04 NS
GDP vs UCPI protein	R ² = 0.03 NS	R ² = 0.84 P = 0.01 **	R ² = 0.30 NS	R ² = 0.39 NS	R ² = 0.58 P = 0.001***
T3 vs UCPI mRNA	R ² = 0.12 NS	R ² = 0.31 NS	R ² = 0.03 NS	R ² = 0.08 NS	R ² = 0.018 NS
T3 vs UCPI protein	R ² = 0.14 NS	R ² = 0.05 NS	R ² = 0.64 NS	R ² = 0.05 NS	R ² = 0.31 P = 0.019*
T3 vs GDP	R ² = 0.74 NS	R ² = 0.05 NS	R ² = 0.003 NS	R ² = 0.005 NS	R ² = 0.14 P = NS
Cortisol vs GDP	R ² = 0.08 NS	R ² = 0.09 NS	R ² = 0.15 NS	R ² = 0 NS	R ² = 0.07 P = NS

5.4 Discussion

Cortisol is known to have a role in the maturation of many tissues and organ systems, however, this is the first time a direct role in fetal adipose tissue development and function has been reported.

5.4.1 Effect of Cortisol Status on Plasma Hormones

Plasma concentrations of Cortisol in the saline and intact groups were within the normal range for the particular gestational age and exhibited the expected increase towards parturition. Cortisol is known to "auto-amplify" production of glucocorticoids by causing maturation of the adrenal gland (Naaman Reperant & Durand 1997). This may explain the rapid increase in fetal Cortisol levels prior to parturition. Both Cortisol and saline infused groups were studied at a gestational stage prior to the normal "surge" in plasma Cortisol levels. The increase in plasma Cortisol in the infused group mimicked the normal increase observed up-to term, producing Cortisol concentrations similar to those in the intact group who were approximately 11 days older, and experiencing the late gestational rise in Cortisol. Conversely, plasma Cortisol concentrations in the adrenalectomised group were markedly reduced.

As expected, plasma T3 concentrations were influenced by the Cortisol status of the fetus which has been well documented previously (Stein 1994; Thomas *et al.* 1978). During late fetal life, Cortisol induces a switch in thyroid hormone metabolism that prepares the fetus for extrauterine life. Fetal plasma Cortisol and T3 concentrations increase concurrently as Cortisol stimulates outer ring

deiodination of T4 (Wu *et al.* 1978). This explains the divergent T3 levels in the different groups of fetuses. Thyroxine levels were not significantly altered by Cortisol treatment, although values were slightly higher in the fetuses that underwent adrenalectomy and lower in the fetuses that received Cortisol. This can also be explained by the positive influence of Cortisol on T4 deiodinase. Thyroxine concentrations will therefore decline as T4 is transformed into T3 by the loss of an outer ring iodine, a reaction which is up-regulated by Cortisol (Wu 1990).

When the relationship between T3 and Cortisol is investigated, a positive correlation would be expected, however, if the two "control" groups are examined individually, a negative relationship between Cortisol and T3 is seen in the saline treated fetuses. It is possible that there is an age, or Cortisol concentration, "cut-off point above which Cortisol regulates T3 concentrations. It is therefore possible that above a certain plasma threshold, Cortisol along with other as yet unknown factors regulate T3 levels.

5.4.2 Effect of Cortisol Status on Thermoregulation

Although there were no statistically significant differences in GDP binding and UCP1 abundance in the Cortisol treated fetuses, there was a strong trend towards higher potential activity of BAT and more UCP1 protein. The adrenalectomised group however, exhibited a significantly lower UCP1 abundance than intact controls. Despite the change in protein abundance, there was only a trend towards less UCP1 mRNA in the adrenalectomised group. The mechanism of up-regulation by Cortisol is unknown, but possible explanations are:

- increased β_3 - adrenoceptor abundance and activity on BAT
- increased T3 concentrations up-regulating UCP1 expression
- induction of enzymes necessary for thermogenesis.

Evidence for an increased number β_3 -adrenoceptors comes from publications indicating that Cortisol causes maturation and development of these receptors *in utero* (Fowden *et al.* 1988). However, *in vitro* studies of mouse 3T3-F442A cells (a mouse cell line with the phenotype of WAT) have shown glucocorticoids to suppress expression of β_3 adrenoceptors. This evidence however, is not from a whole animal study, nor from a BAT cell line and definitely not a fetal model. Interestingly, combined Cortisol and thyrotrophin releasing hormone (TRH) infusion of fetal lambs has been shown to have a stimulatory effect on lung β adrenoceptor binding capacity, despite a lack of effect of Cortisol alone (Fowden *et al.* 1988). This might explain the results from the *in vitro* study mentioned above, the Cortisol infusion of adipocytes in culture occurred under non-physiological circumstances, as Cortisol would normally increase circulating T3 concentrations.

Cortisol is also known to contribute to increased catecholamine concentrations during labour, delivery and cutting of the umbilical cord. Increased plasma catecholamine concentrations *in utero* could up-regulate UCP1 through increased activity of β_3 -adrenoceptor activity i.e. adenylyl cyclase, this could upregulate UCP1 activity without an increase of UCP1 mRNA. This would occur via up-regulation of free fatty acid liberation from triglycerides regulated by increased

cAMP and hormone sensitive lipase in BAT, thus, "unmasking" UCP1. Although no statistically significant changes in UCP1 activity were observed, a strong trend for higher GDP binding was observed in the Cortisol treated group.

Catecholamines have also been shown to directly increase UCP1 protein (Mory *et al.* 1984). In the rat, catecholamines, thyroid hormones and other factors have been shown to regulate UCP1 expression via a 211-bp enhancer upstream of the UCP1 gene, although it is not known if such an enhancer is present on the ovine UCP1 gene (Cassard-Doulcier *et al.* 1998).

Enhanced T3 concentrations in the Cortisol treated and intact group may also have influenced thermoregulation. As outlined in Chapter 5.4.1, Cortisol up-regulates plasma T3 concentrations via increased activity of T4 deiodinase. This occurs normally during late gestation, causing a peak of plasma T3 around one day of age. Thyroid hormones have been shown previously to be important in the initiation of thermoregulation in newborn lambs (Bird *et al.* 1998; Heasman *et al.* 2000; Schermer *et al.* 1996), with higher concentrations being associated with enhanced thermoregulation and thyroidectomy associated with significantly lower thermogenic activity (Schermer *et al.* 1996). Triiodothyronine is thought to upregulate and stabilise UCP1 expression (Guerra *et al.* 1996; Stein 1994) and abundance (Heasman *et al.* 2000) in BAT. Although no statistically significant changes were observed in UCP1, there was a trend towards higher UCP1 expression in the Cortisol infused and intact groups and T3 was found to have a positive correlation with UCP1 protein. Triiodothyronine has been documented to up-regulate activity of mitochondrial carriers, proton leak and electron transport chain components (Goglia *et al.* 1999) via responsive sequences on the

UCP1 gene (Rabelo *et al.* 1995) (Cassard-Doulcier *et al.* 1998), all of which would influence thermoregulation positively. It is possible that Cortisol is acting in a "permissive" way, not directly increasing UCP1 abundance and expression, but acting via increased plasma T3 concentrations.

Cortisol is known to modulate the maturation of various enzymes during fetal development, including those for gluconeogenesis in the liver. It is not known if Cortisol acts on lipolytic enzymes in adipose tissue, although TRH administration to newborn caesarean section delivered lambs has been shown to increase plasma NEFA concentrations (Bird *et al.* 1998). If Cortisol did up-regulate triglyceride oxidative enzymes in BAT, this would further potentiate thermogenesis by providing extra free fatty acids to "unmask" the GDP binding sites of UCP1.

5.5 Conclusion

Enhanced plasma Cortisol concentrations in the late gestation sheep fetus have been shown to increase UCP1 protein abundance and UCP1 expression in association with increased plasma T3. In contrast, impaired Cortisol secretion resulted in significantly reduced UCP1 protein abundance, UCP1 expression in association with increased plasma T3.

In conclusion the increase in UCP1 mRNA expression and protein abundance observed in ovine perirenal adipose tissue during late gestation is mediated, in part, by the prepartum rise in plasma Cortisol.

CHAPTER 6

PRODUCTION OF

POLYCLONAL ANTIBODIES

AGAINST A SPECIFIC

MITOCHONDRIAL PROTEIN

6.1 Introduction

The discovery of the UCP1 homologues, UCP2 and 3 (Fleury *et al.* 1997; Riquier 1997; Vidal-Puig 1997) has provided another line of research into thermoregulation and energy regulation. However, there are no commercially available antibodies for ovine UCP2 or 3 and serious doubts have been raised against the sensitivity of those available for rodents (Jezek *et al.* 1999). Knowledge of the presence and relative abundance of the UCP homologues in neonatal tissues would provide us with more information on their role in the production of heat at birth and possibly explain further the results of Chapter 3.

Purification of UCP1 relies on detergent solubilisation from mitochondria followed by purification by hydroxyapatite column chromatography as described by Lin and Klingenberg (Klingenberg & Lin 1986; Lin & Klingenberg 1980; Lin & Klingenberg 1982) and Tiselius as described by Bemardi (Bemardi 1973). The detergents used for extraction of UCP1 have been selected and optimised according to the following parameters:

1. solubilisation
2. retention of nucleotide binding
3. stability of binding and homodispersity (Klingenberg & Lin 1986)

An important step in the purification of UCP1 is separation from ADP/ATP carrier, which has certain similar properties and is of a similar molecular weight (30 Kda) (Lin & Klingenberg 1982). The purification method developed by Lin and Klingenberg (Lin & Klingenberg 1980; Lin & Klingenberg 1982) utilised the observation that UCP1 was more easily detached from the mitochondrial

membrane than the ADP/ATP carrier, therefore less detergent was needed and very little salt. Triton-x-100 has been shown to be the most suitable detergent for purification of UCP1 as it solubilises the protein effectively, while retaining binding capacity (Lin & Klingenberg 1982). Before extraction with triton-x-100, soluble proteins and those peripheral to the membranes can be removed by pre-treatment with a non-solubilising detergent such as Polyoxyethylene ω -1 (PEW-1), however, the sedimented membranes should be washed free of PEW-1 by resuspension in a sucrose buffer in order to use minimum amounts of triton-x-100 for the subsequent solubilisation. This is due to PEW-1 sequestering triton-x-100 by forming mixed micelles (Lin & Klingenberg 1982).

Hydroxyapatite column chromatography has been shown to be the most effective method of purification of UCP1 (Klingenberg & Lin 1986). When the triton-x-100 extract of mitochondria is applied to a hydroxyapatite column, a rapid and high degree of purification is obtained, UCP1 is confined to the breakthrough whereas most other solubilised proteins are adsorbed. If the hydroxyapatite column is run at room temperature, the ADP/ATP carrier is denatured and adsorbed (Lin & Klingenberg 1982).

Antiserum against ovine UCP1 has been successfully produced in our laboratory for use in western blotting and immunohistochemistry (Schermer *et al.* 1996) using the above purification technique. The predicted amino acid sequence of human and rodent UCP2 is 59% homologous to the sequence of human and rodent UCP1 (Fleury *et al.* 1997) and the predicted sequence of human UCP2 shows a triplicated structure common to UCP1 and other mitochondrial carriers

(Vidal-Puig 1997). It was therefore proposed that a method known to isolate UCP1 and the ADP/ATP carrier would be suitable for the purification of UCP2.

6.1.1 Aim

The aim of this study was to produce specific antibodies against UCP2 that could be used in immunohistochemistry and western blotting in order to assess the postnatal ontogeny and hormonal regulation of UCP2 in the sheep.

6.2 Methods

6.2.1 UCP2 Peptide Purification

Mitochondrial Preparation

Mitochondrial fractions were prepared as previously described (Symonds *et al.* 1992) from 50g of one - month old lamb kidney. Kidney was chosen as at the time, it has been shown to express only the UCP2 homologue (Fleury *et al.* 1997; Fleury & Sanchis 1999) although this has recently been questioned (Pecqueur *et al.* 2001). The final mitochondrial pellets (see Chapter 2.3.1a) were resuspended in approximately 1 ml of MOPS buffer (20 mM MOPS, 20 mM sodium sulphate anhydrous, 1 mM EDTA (pH 6.7)) and pooled to give a total volume of ~20 ml. A 1 ml portion of this mitochondrial fraction was saved at -20 °C for total protein assessment, the remaining mitochondrial suspension was mixed with an equal volume of 5 % (w/v) Polyoxyethylene -w-1 (PEW-1) and incubated at 0 °C for 30 minutes prior to centrifugation at 28,000 rpm for 30 minutes at 4 °C. The resulting mitochondrial pellet was resuspended in approximately 20 ml of tris-sucrose buffer (0.3 M sucrose, 10 mM Tris-HCl, 2

mM EDTA (pH 6.7)) and further centrifuged at 28,000 rpm for 30 minutes at 4 °C. The pellet was this time resuspended in 20 ml 5 % (w/v) triton-x-100 and incubated at 0 °C for 30 minutes followed by centrifugation at 28,000 rpm for 30 minutes at 4 °C. The resulting supernatant was stored for hydroxyapatite chromatography. Figure 6.1 demonstrates this method in flow chart format.

Hydroxyapatite Column Chromatography

Hydroxyapatite (HA) (Bio - gel Hydroxyapatite gel, Biorad Laboratories) was equilibrated with the MOPS buffer (100 ml HA added to 600 ml MOPS buffer) with gentle mixing for 10 minutes then allowed to settle. The supernatant containing "fines" was removed and the HA equilibrated with a further 600 ml MOPS buffer, allowed to settle and the supernatant removed. This procedure was repeated once more and the resulting sediment was resuspended in 100 ml MOPS buffer and poured into a 50 cm³ burette. Once the column was completely settled the void and breakthrough volume were estimated using 0.05 % (w/v) methyl orange dye. Twenty ml of dye was loaded onto the column and the volume at which dye was first eluted, and when dye was no longer eluted were measured. The void volume represents the volume of buffer eluted prior to peptides and the break-through volume is an estimate of the volume to be collected. These values are approximate as they represent the movement through the column of a dye and do not account for the slower movement of protein sample.

The triton-x-100 supernatant was applied to the column at room temperature and allowed to move through under normal air pressure, MOPS buffer was added to the top of the column at the same rate of elution. The break-through fraction was collected on ice and stored at - 20 °C until further analysis.

Ultrafiltration

The protein samples obtained were further purified and concentrated using spin ultrafiltration. Centriprep 10 (Amicon, Millipore Corporation, UK) spin concentrators were initially used to remove excess buffer from the peptide fractions. These have a specific molecular weight cut off point of 10 Kda and any peptides smaller than this are removed from the solution through the pressure of centrifugation and the remaining peptides become increasingly concentrated as the operation continues. The resulting solution was then applied to a 100 KDa spin concentrator (Centrex UF-2 Centrifugal Filter; Schleicher and Schnell Uk Ltd) to remove any large molecular weight particles.

Preparation of mitochondria (Chapter 2.3.1.a).

i

Pre-treatment with a non-solubilising, nonionic detergent (PEW-1). This opens membranes and removes soluble and loosely bound proteins, thus reducing the requirement for solubilising detergent.

1

Solubilisation with a nonionic, solubilising detergent (triton-x-100). This recovers membrane components of the mitochondria.

i

Purification on HA column. UCP1 has been shown not (in contrast to other extracted proteins) to be adsorbed into the HA.

1

Concentration using ultrafiltration. Specific molecular weight cut-off spin concentrators remove excess buffer and proteins with an undesired molecular weight.

1

Gel electrophoresis to determine molecular weight and purity. SDS-PAGE is used to visualise the purified peptide.

Figure 6.1

Flow diagram of UCP2 purification method

6.2.2 Protein Concentration Determination

Initially, protein concentrations were determined using the Lowry Assay (Chapter 2.3.2), however, this provided unsatisfactory results. It has been shown that the Lowry assay is sensitive to detergents (Yeang *et al.* 1998), therefore an alternative method of protein concentration, the bicinchoninic acid assay (BCA assay), was utilised. The BCA assay was developed by Smith *et al.* 1985 and modified by Wiechelman *et al.* 1988 (Smith *et al.* 1985; Wiechelman *et al.* 1988) and is used to determine total protein concentrations of mitochondria prepared using detergents.

6.2.2.a Principle of the Assay

The BCA assay measures the formation of Cu^{+1} from Cu^{+2} by the Biuret complex in alkaline solutions of protein. It was originally thought that the mechanism of the assay was the same as in the Lowry assay, but it has since been determined that there are two distinct reactions that take place with copper ions unique to the BCA assay. The first reaction occurs at lower temperatures and is the result of the interaction of copper and BCA with the following residues: cysteine, cystine, tryptophan and tyrosine. At elevated temperatures it has been shown that the peptide bond itself is responsible for colour development. This is why performing the assay at 37 °C increases the sensitivity and reduces the variation in the response of the assay to protein composition. The BCA reagent replaces the Folin-Ciocalteu reagent used in the Lowry assay with bicinchoninic acid. The BCA reagent forms a complex with Cu^{+1} , which has a strong absorbance at 562 nm. BCA is advantageous in that it does not interact with as many substances as the Folin-Ciocalteu reagent, especially detergents and buffers.

6.2.2.b Assay Procedure

Protein samples were diluted between 1 in 5 and 50 in 0.9 % saline to ensure concentrations fell within the linear range. A standard curve (0 - 1 mg/ml) was prepared using bovine serum albumin (BSA) in 0.9 % saline. Solutions A (1 % BCA, 2 % sodium carbonate, 0.16% sodium tartrate and 0.4 % NaOH, pH 11.25 with 10% sodium bicarbonate) and B (4 % copper sulphate) were prepared and a ratio of 100A:2B was mixed on the day of assay. Two ml of A:B mix was added to 100 μ l sample/standards in duplicate and were incubated at 60 °C 30 minutes following which absorbance at 570 nm was measured. Protein concentrations were determined following linear regression analysis of the standard curve and corrected for the initial dilution. The intra- and inter-assay coefficients of variation were 4.9 % (n=3) and 2.0 % (n=3) respectively.

6.3.2 Protein Visualisation

In order to visualise the proteins isolated from hydroxyapatite column chromatography, and determine their molecular weight, SDS-PAGE was carried out and the resulting gels stained using either Coomassie Brilliant Blue or silver nitrate.

6.3.2.a Coomassie Blue Staining

SDS-PAGE was carried on purified proteins as described in Chapter 2.3.4. In this case, proteins were not blotted onto a nitrocellulose membrane, but were irreversibly stained with Coomassie Brilliant blue (CBB) dye. CBB is a commonly used staining procedure for the detection of proteins. It is the method

of choice if SDS is used in the electrophoresis of proteins, and is sensitive for a range of 0.5 to 20 pg of protein.

CBB Staining Procedure

After SDS-PAGE the gel was fixed in fixing solution (40 % (v/v) methanol and 7 % (v/v) acetic acid) for 1 hour, this immobilises the proteins. Proteins are then stained with CBB (0.025 % CBB, 40 % methanol, 7 % acetic acid made up to volume with dH₂O) for up to 2 hours with mixing. Excess CBB was removed by incubating with destain solution 1 (25 % (v/v) methanol, 10 % (v/v) acetic acid made up to volume with dH₂O) for approximately 20 minutes followed by up to 24 hours in destain solution 2 (25 % (v/v) methanol made up to volume with dH₂O) until the desired resolution is obtained. The gel was washed several times in dH₂O prior to drying. Gels were placed upside - down onto a sheet of saran wrap™ then inverted onto two pieces of blotting paper, the gel "sandwich" was then dried for 3 hours using a (Scie-Plas Gel Drier (GD4534); Heto Laboratory Equipment). Figure 6.2 shows the 34 KDa peptide band obtained from CBB staining.

6.2.3.b Silver Staining

Silver staining was also used to visualise proteins after SDS-PAGE. Silver staining is a more complex protocol but gives much greater sensitivity. Silver staining uses colloidal silver to stain proteins. The silver ions (Ag⁺) bind to protein bands and are deposited as metallic silver (Ag⁰). The silver stains the bands black/brown. Silver stains can detect protein bands containing levels as low as 1 ng and thus are more sensitive than CBB stains. This procedure was

carried out according to the method of Hariow and Lane (Hariow & Lane 1988) and Sambrook *et al* (Sambrook *et al.* 1989).

Silver Staining Procedure

As with CBB staining, the gel is removed from the electrophoretic equipment and not blotted. The gel was washed for 30 minutes in prefixing solution 1 (50 % (v/v) methanol, 10 % (v/v) acetic acid made up to volume with dH₂O) followed by a 30 minute wash in prefixing solution 2 (5 % (v/v) methanol, 7 % (v/v) acetic acid made up to volume with dH₂O). The gel was then fixed for 30 minutes in 10 % (v/v) gluteraldehyde followed by over night rinsing in a large volume of dH₂O. The following day the gel was soaked in reducing solution (5 µl/ml dithiothreitol (DTT) made up to volume with dH₂O) for 30 minutes followed by 30 minutes treatment with 0.1 % (w/v) silver nitrate solution. The gel was then rinsed once in dH₂O then twice rapidly with developing solution (0.0185 % (v/v) formaldehyde, 3 % (w/v) sodium carbonate made up to volume with dH₂O) followed by soaking in developing solution until a desired stain is achieved. Staining was then stopped by the addition of 5 ml 2.3 M citric acid. The gel was washed several times in dH₂O prior to drying (described in Chapter 6.2.3.a). Figure 6.3 shows the single 34 KDa peptide band obtained with silver staining.

6.2.4 Immunisation of Rabbits

Three female New Zealand White rabbits were immunised with the putative UCP2 protein. An initial 100 pg immunisation was given in non-ulcerative Emends Adjuvent (Guildhay Ltd, UK) at 2 sites either side of the spine, 4 cm away from the spine between the shoulders and the hips. Rabbits were given 3 booster immunisations of 75 pg UCP2 peptide in the same way. All blood

samples were taken from the marginal ear vein. Nine months after the initial immunisation, the rabbit with the most promising antisemm was humanely euthanased with an overdose of sodium pentobarbitone (Euthatal) given via the marginal ear vein prior to exsanguination.

6.2.5 Antibody Screening

The specificity and quality of the antisera obtained was assessed using western blotting (see Chapter 2 for details). This method was chosen, as it is the assay for which the antibodies are required. Unfortunately, none of the UCP2 antibodies or positive control peptides that are available commercially are ovine specific and there is concern about the cross reactivity of the available UCP2 antibodies with other members of the uncoupling protein family (Jezek *et al.* 1999; Pecqueur *et al.* 2001). For this reason I was unable to obtain adequate positive and negative controls at the time of antisera screening. To assess non-specific antibody binding a pre-immunisation bleed was taken from all rabbits. This plasma was used along side post-immunisation plasma to determine specific antibody binding. The purified protein and the kidney mitochondrial fraction from which it was purified were used as positive controls on all gels. Western blotting was carried out following each antisemm sampling to determine which titre was most immunoreactive.

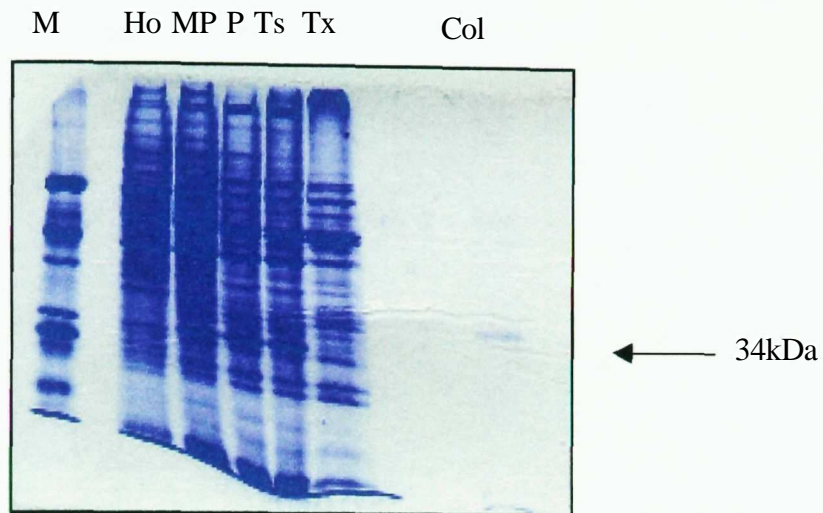


Figure 6.2

Protein Gel stained with CBB dye

M, molecular weight markers; Ho, kidney mitochondrial homogenate; MP, kidney mitochondrial protein; P, PEW-1 extract; Ts, tris sucrose buffer extract; Tx, triton-x-100 extract; Col, column breakthrough fraction.

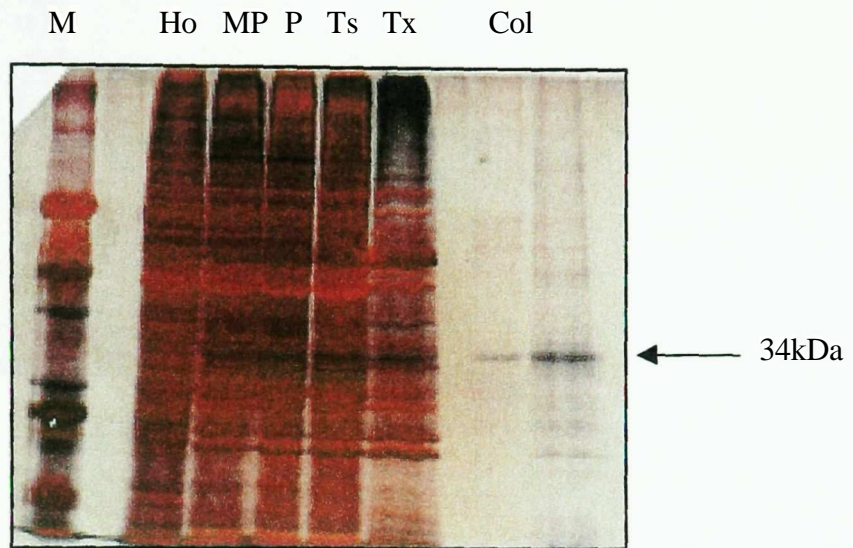


Figure 6.3

Protein Gel stained with silver

M, molecular weight markers; Ho, kidney mitochondrial homogenate; MP, kidney mitochondrial protein; P, PEW-1 extract; Ts, tris sucrose buffer extract; Tx, triton-x-100 extract; Col, column breakthrough fractions.

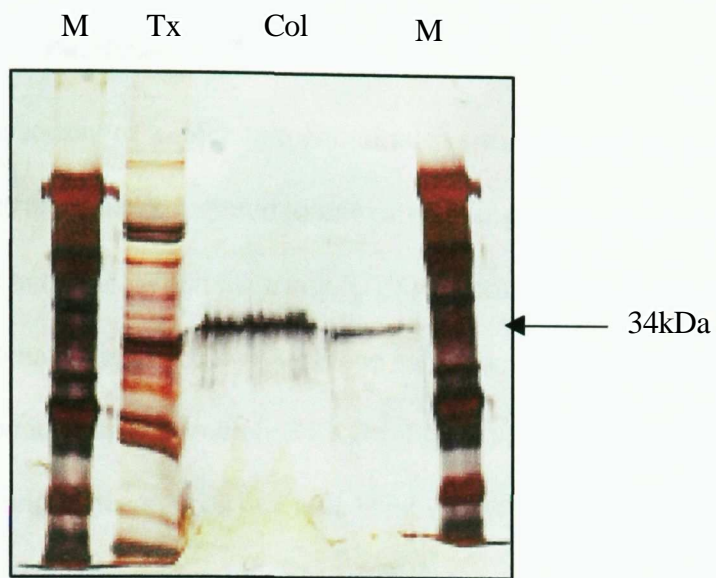


Figure 6.4

Protein Gel stained with silver

M, molecular weight markers; Tx, triton-x-100 extract; Col, column breakthrough fractions.

6.3 Results

6.3.1 Purification of UCP2

Detergent treatment of kidney mitochondrial suspensions produced a different protein spectrum when compared to untreated suspensions on SDS-PAGE (Figures 6.2 and 6.3). When the triton-x-100 supernatant was subjected to HA column chromatography, SDS-PAGE and subsequent staining revealed a significant band at approximately 34 KDa (Figures 6.2 and 6.3). With further purification and concentration, a single band was visible at this molecular weight (Figure 6.4). The purity of this band was thought to be suitable for antibody production.

6.3.2 Antibody Selection

Serum from only one of the immunised rabbits showed immunoreactivity against the peptide and kidney mitochondrial suspension. Figure 6.5 shows the results from western blots with serum from the fourth booster injection sample from rabbit 498. It was decided that this serum would be specific enough for western blotting and the rabbit was humanely euthanased so that as much serum as possible could be obtained. Western blots with serum taken prior to immunisation did not detect any bands at the desired molecular weight (Figure 6.6). When further western blots were carried out it was clear that the sera detected a band at 34 KDa in not only kidney, but also BAT and muscle. This was encouraging, as these tissues had been shown to express UCP2 at a high level in previous papers (Fleury *et al.* 1997; Fleury & Sanchez 1999).

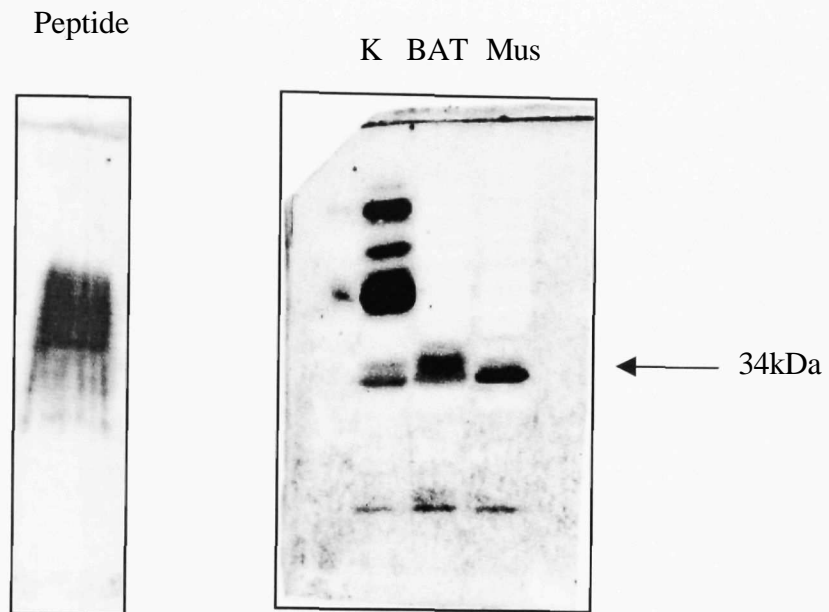


Figure 6.5

Western blots detected with semm from rabbit 498 (1 in 1000 dilution) after fourth booster immunisation, 8 months after initial immunisation. (peptide and samples were not run on the same gel)

K, kidney MP; BAT, 7 day old lamb BAT MP; Mus, 7 day old lamb skeletal muscle MP

M P K Mus Liv M

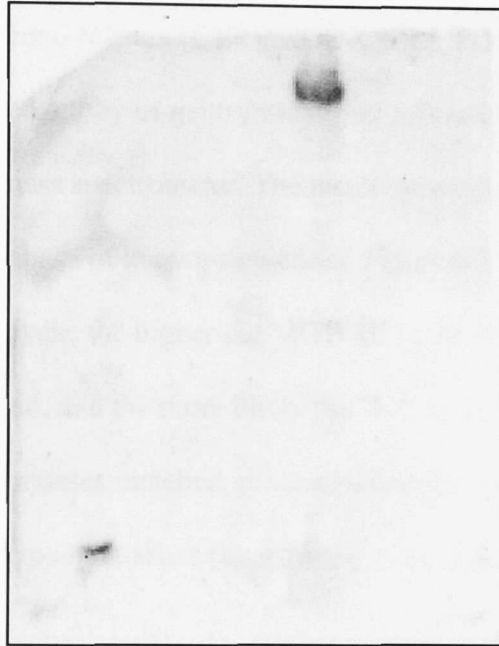


Figure 6.6

Western blots detected with semm from rabbit 498 (1 in 800 dilution) prior to immunisation

M, molecular weight marker; P, UCP2 peptide; K, kidney MP; Mus, 1 day old lamb skeletal muscle MP; Liv, liver MP.

6.3.3 Sequencing and Confirmation

Subsequently, I had the opportunity to have the purified protein sequenced and have a western blot containing the purified protein detected with a specific UCP2 antibody. These analyses were carried out by Martin Brand (MRC Dunn Human Nutrition Unit) and Bmno Miroux (CEREMOD-CNRS, France) respectively. Sequencing was carried out by in-gel tryptic digest followed by extraction and run on a MalDI TOE mass spectrometer. The resulting sequence was then matched against a database of known sequences. Figure 6.7 shows the results from the database analysis, the higher the MOWSE score, the higher number of peptide masses matched, and the more likely the "hit" is. In Figure 6.7, the percentage of peptide masses matched, protein molecular weight, accession number from NCBI protein database (<http://www.ncbi.nlm.nih.gov/entrez/>), species and protein name are listed. Obviously, this sort of confirmation is optimally carried out prior to antibody production, but was not available at this stage. However, I thought it would provide definitive confirmation of the peptide sequence. The results of the sequencing were clear that the peptide was not UCP2, but the voltage dependent anion channel (VDAC) isoform 1 (Figure 6.7). Similar results were obtained from Bmno Miroux who confirmed that his sensitive UCP2 antibody did not detect my purified peptide and suggested that it was VDAC.

Entry	MOWSE score	Match	% cover	Mass	Description
POR1_RABIT	1.02E+08	14	51.35	30509	(Q9TT15) VOLTAGE-DEPENDENT ANION-SELECTIVE CHANNEL PROTEIN
POR1_BOVIN	5.84E+07	13	60.99	30591	(P45979) VOLTAGE-DEPENDENT ANION-SELECTIVE CHANNEL PROTEIN
BAB13473	3.12E+07	13	56.85	30755	(BAB13473) Mitochondrial T0tally dependent anion c
AAF00115	3.12E+07	13	56.89	30755	(AAF00115) Voltage-dependent anion channel 1.
POR1_MOUSE	2.97E+07	13	54.59	32352	(Q69902) VOLTAGE-DEPENDENT ANION-SELECTIVE CHANNEL PROTEIN
POR1_HUMAN	1.60E+07	13	54.61	30541	(P21796) VOLTAGE-DEPENDENT ANION-SELECTIVE CHANNEL PROTEIN
AAF78903	1.60E+07	13	54.42	30727	(AAF78903) Voltage-dependent anion channel 1.
AAO54939	1.60E+07	13	54.42	30773	(AAO54939) Voltage-dependent anion channel.
POR1_RAT	6.04E+05	11	41	32445	(Q9Z2L0) VOLTAGE-DEPENDENT ANION-SELECTIVE CHANNEL PROTEIN

Figure 6.7

Results from Mass Spectrometry and database analysis of putative UCP2 protein.

mavpptyadl gksardvftk gygfglikld lktksengle ftssgsante ttkvtgslet
kyrwteyglf ftekwndnt lgteitvedq larglkltd sspntgkk nakiktgykr
ehinlgcdvd fdiagpsirg alvlyegwl agyqmnfeta ksrvtqsna vgyktdefql
htnvdgtf ggsiyqkvnk kletavnlaw tagsntrfg iaakyqidpd acfsakvnns
sliglytqt lkpgiklts alldgknvna gghklgle fqa

Figure 6.8

Peptide sequence for POR1Rabbit, the most closely matched protein to the putative UCP2 peptide. Taken from <http://www.ncbi.nlm.nih.gov/> accession number GI: 10720225 (Q9TT15).

6.4 Discussion

The reason for the unsuccessful production of UCP2 is most likely that it is not present in the kidney. Some of the factors that may have been important are:

- The tissue used for protein purification
- The method of protein purification

Kidney was chosen for UCP2 protein purification as published data demonstrated that it was a tissue with a high expression of UCP2 (Fleury *et al.* 1997; Gong 1997) and that it contained no other UCP homologue. However, as discussed in Chapters 1 and 3, mRNA expression has been shown not to be conclusive evidence that the uncoupling protein in question is translated and incorporated into mitochondrial membranes. More recent data suggests that UCP2 mRNA levels do not correlate with the variation of the protein and that UCP2 protein is expressed at very low levels *in vivo* (Pecqueur *et al.* 2001). Quantitative analysis of mouse tissues by the same group has shown that UCP2 protein is four and ten times less abundant in lung and stomach, respectively, than spleen mitochondria and that UCP2 is approximately 160-fold less abundant in spleen mitochondria than UCP1 is in BAT mitochondria (Pecqueur *et al.* 2001). This equates to approximately 313 ng UCP2/mg of mitochondria in spleen and 78 ng/mg in lung (Stuart *et al.* 2001b). The peptide purified in this study had an abundance of approximately 0.11 mg/mg of mitochondria, this equates to 11 % of mitochondrial protein.

If the above information regarding UCP2 tissue abundance had been available at the time of the study it would have been clear that the purified peptide was a mitochondrial protein other than UCP2. VDAC is known to make up around 20 % of mitochondrial protein (Gottlieb 2000), which correlates well with the

abundance I observed. There may also be species differences, UCP2 mRNA appears to be highly expressed in the kidney of human and rodents, however, UCP2 may be expressed to a lesser extent in ovine sample.

The kidney is a highly metabolic tissue, involved in many physiological processes, receiving approximately 25 % of total cardiac output (Ganong 1995) this suggests that kidney mitochondria are highly active, containing many proteins involved in substrate metabolism. One of the main functions of the kidney is the formation and excretion of urine; this involves the reabsorption and secretion of solutes across the tubules. The role of VDAC is highly compatible with this function so it is not surprising that VDAC was found to be highly abundant in this organ, perhaps at such a high abundance, that any UCP2 was "quenched" by VDAC. If this attempted isolation of UCP2 were to be repeated, the lung currently appears to be a better candidate tissue for UCP2 production (see Figure 7.6).

The purification technique employed was chosen for a number of reasons; it was cheap, quick and produced a protein from an ovine tissue rather than a recombinant peptide sequence as the ovine UCP2 sequence was not available at that time. The method was well documented and verified for the purification of UCP1 in ovine adipose tissue (Schermer *et al* 1996). The sequence homology between UCP1 and UCP2 is 59 % (Fleury *et al.* 1997) and both share the triplicate structure of 100 amino acids found for all mitochondrial carriers of the inner mitochondrial membrane (Fleury & Sanchis 1999). It was hoped that such similarities would allow UCP2 to be extracted in the same way as UCP1. UCP2

may not be adsorbed onto the HA column in the same way as UCPI, but as the exact method of protein adsorption and interaction with HA is unknown (Bernardi 1973), it may not be possible to investigate this further.

6.5 Conclusion

Despite the unsuccessful production of UCP2 antibodies, serendipity prevailed and a specific antibody was raised against the equally interesting protein, VDAC. There appears to be a lack of knowledge regarding VDAC in the neonatal and fetal period, thus much more work can be carried out to investigate its role in the transition from fetal to neonatal life and its endocrine and nutritional regulation. Further work may be carried out in order to produce a specific ovine UCP2 antibody as there is still some concern regarding the specificity of the mouse UCP2 antibody we are currently utilising.

CHAPTER 7

ONTOGENY OF OVINE

BROWN AND WHITE ADIPOSE

TISSUE MITOCHONDRIAL

PROTEINS AND TISSUE

DISTRIBUTION OF VDAC

7.1 Introduction

In light of the results of Chapter 6 I carried out an extensive literature review on VDAC and its role in fetal and perinatal development. The overwhelming outcome was that very little was known about the role of VDAC, a mitochondrial channel protein, at these critical times when the need for thermogenesis is greatest.

VDAC is a 283 amino acid protein that forms an anion selective channel, generally considered to be the main pathway for metabolite and ion diffusion across the outer mitochondrial membrane due to its propensity to form pores (Colombini 1979). Mitochondrial respiration is influenced by changes in the permeability of the outer membrane regulated by VDAC (McEnery *et al.* 1993). VDAC has been shown to have a number of additional functions and is also thought to be present in other cellular membranes (Bathori *et al.* 2000; Reymann *et al.* 1998; Shinohara *et al.* 2000; Thinnes & Reymann 1997). Hexokinase and other mitochondrial enzymes bind to VDAC to obtain selective access to mitochondrially generated ATP, this may control delivery of glucose-6-phosphate into the glycolytic pathway (McEnery *et al.* 1993). Cytochrome *c* also binds to VDAC, but the physiological significance of this is unclear. It has been suggested, however, that VDAC serves as part of the undefined pathways for release of cytochrome *c* from the inter-membrane space, a process that has been implicated in the chain of events involved in apoptosis (Cai *et al.* 1998; Crompton 1999; Crompton *et al.* 1998; Desagher & Martinou 2000; Vander

Heiden *et al.* 2001).

7.1.1 Aim

The aim of this study was to utilise the specific ovine VDAC antibody to investigate the postnatal ontogeny of VDAC and to compare it with other important mitochondrial proteins namely, cytochrome *c* and UCP1 and to investigate the tissue distribution of UCP2 and VDAC.

7.2 Methods

7.2.1 Experimental Design

BAT was sampled from the perirenal region of fetal lambs at 145 days gestation (n=4) and postnatal lambs at 4 hours (n=5), 1 (n=5), 7 (n=5) and 30 days (n=5) of life after a lethal dose of anaesthetic (200 mg kg⁻¹ pentobarbital sodium; Euthatal: RMB Animal Health, UK). Tissue samples were also taken from the following regions for tissue distribution studies; hind limb skeletal muscle, heart muscle (left ventricle), brain (cortex), liver, spleen, pancreas (all from 7 day old lamb), late gestation whole placentome, mammary gland (late gestation ewe) and white adipose tissue (30 day old lamb).

7.2.2 Laboratory Procedures

Mitochondria were prepared from PAT as described in chapters 2.3.1 and 2.3.6 in order to assess VDAC, UCP1 and cytochrome *c* abundance with western

blotting. Mitochondria were also prepared from a number of different tissue types (see Figures for details) in order to assess the distribution of VDAC and UCP2. UCP2 was detected using a specific mouse UCP2 antibody that was provided by Dr Bmno Miroux.

7.2.3 Statistical Analysis

Statistical analysis with respect to significant differences ($p < 0.05$) between values obtained from the different ages of lambs was carried out using Mann-Whitney U test.

7.3 Results

7.3.1 VDAC Ontogeny

VDAC protein abundance peaked at day 1 of postnatal age in BAT as shown in Figure 7.1. VDAC was present in fetal brown adipose tissue at an equivalent abundance to that of 4 hour - old lambs. Between 4 hours of age and 1 day, there was a significant increase in VDAC ($P=0.009$). Following the peak abundance at 1 day of age there was a significant reduction in VDAC up to 7 days ($P=0.008$) and a further reduction up to 30 days of postnatal age ($P=0.009$). Between 1 and 30 days of postnatal age there was a 90 % reduction in VDAC abundance.

7.3.2 UCPI Ontogeny

UCP1 abundance increased significantly in BAT between late fetal life and 4 hours of day of age in the lamb ($P=0.008$) (Figure 7.2). Like VDAC, UCP1 also peaked at day 1 ($P=0.003$), and declined up to day 7 of postnatal age ($P=0.001$). However, by 30 days of age there was no detectable UCP1 protein in PAT ($P=0.006$ compared with day 7) although UCP2 was still present (Figure 7.5).

7.3.3 Cytochrome *c* Ontogeny

The cytochrome *c* ontogeny was similar to that of VDAC and UCP1. There was no difference in cytochrome *c* between late fetal and early neonatal values (Figure 7.3). However, abundance of cytochrome *c* significantly increased to peak at day 1 of postnatal age ($P=0.021$). There was then a reduction in cytochrome *c* up to 7 ($P=0.021$) and 30 days of age ($P=0.02$) although

cytochrome *c* was still detectable.

7.3.4 UCP2 Tissue Distribution

UCP2 was found to be highly expressed in lung, spleen and white adipose tissue (Figure 7.6). It was not possible to analyse UCP2 abundance in BAT, heart and skeletal muscle, as the antibody is known to cross-react with UCP1 and UCP3.

Unlike UCP1, UCP2 was still highly abundant in 30-day-old lamb adipose tissue (Figure 7.5).

7.3.5 VDAC Tissue Distribution

VDAC was found in a wide variety of fetal, neonatal and also adult tissues (Figure 7.7). The highest abundance of VDAC appeared to be in heart, brain, skeletal muscle (although not shown) and kidney from 7 day old lambs. VDAC was found to be present in the placenta, lung, spleen, liver and pancreas, but at a reduced abundance. No VDAC was detectable in non-lactating mammary gland from a 140-day gestation ewe. Immunohistochemistry was carried out by Victoria Wilson (Experimental Officer, Child Health), to further investigate the location of VDAC in certain tissues. Figure 7.8 shows the location of VDAC in a kidney section from an adult ewe, the VDAC is clearly present in the tubules of the kidney. Figure 7.9 confirms the presence of VDAC in human placenta and Figures 7.10, 7.11 and 7.12 show VDAC in adult sheep PAT, skeletal muscle and placenta respectively. Human placental sections were included in the analysis in order to establish if the antibody would cross react with human VDAC so that it could be utilised in further studies. Immunohistochemistry was carried out on 6

pm sections tissues with Hams's Haematoxylin as a counter stain using the Dako Envision system (Dako, UK).

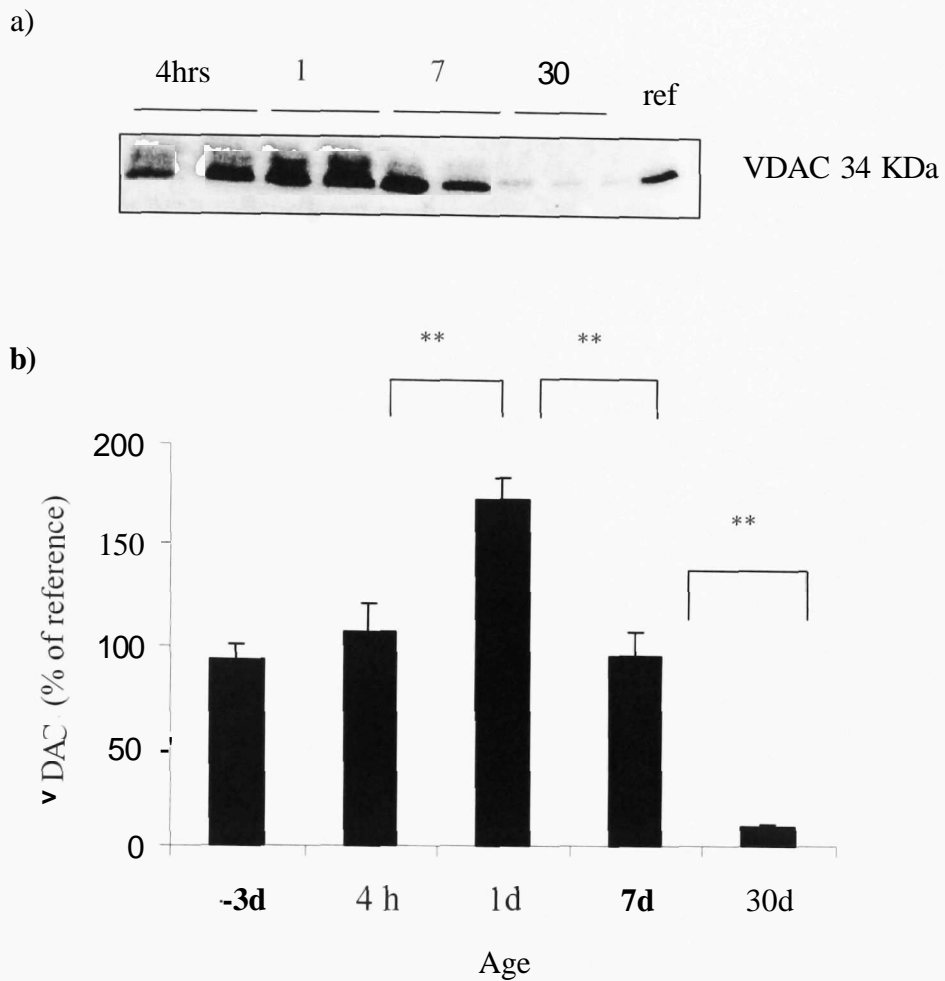


Figure 7.1

Abundance of VDAC in BAT from late gestation, to one month of age in the lamb.

a) Representative image of a Western blot for VDAC, 10 μ g MP each lane.

b) Effect of postnatal age on VDAC protein abundance

Values are means \pm SEM (n=5 each age).

MP, mitochondrial protein; Ref reference sample (7 day old lamb skeletal muscle mitochondria)

** indicates $P < 0.01$ for age effect

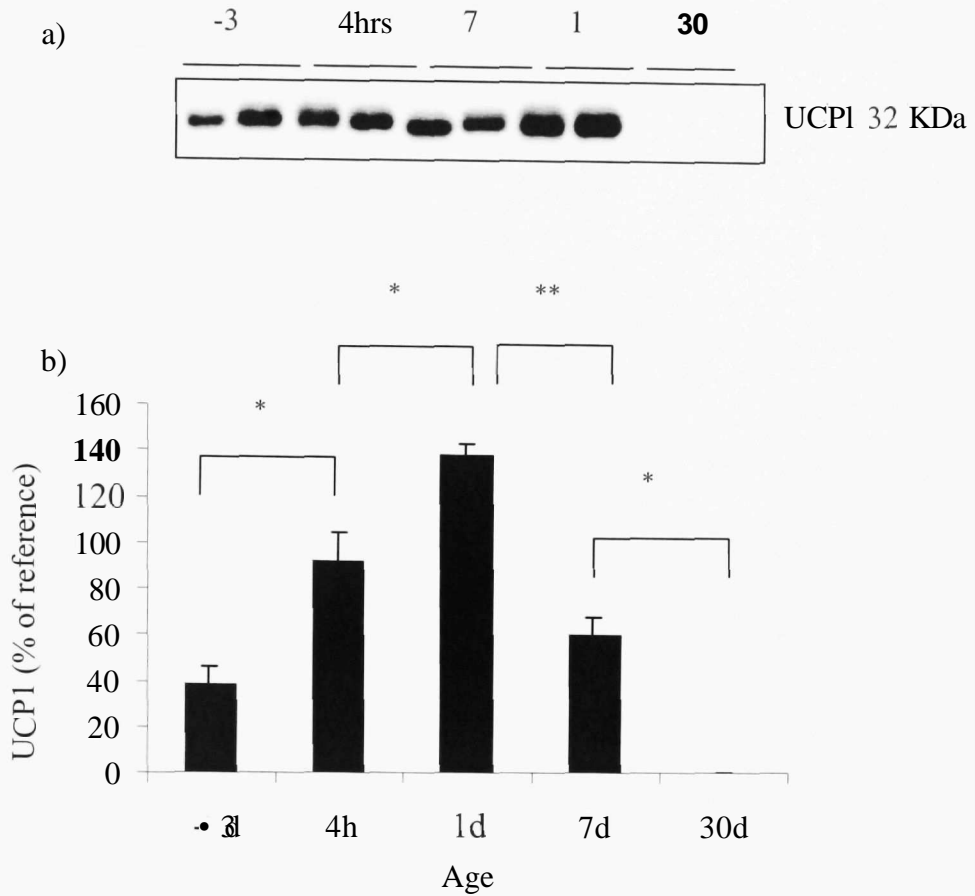


Figure 7.2

Abundance of UCPI in BAT from late gestation, to one month of age in the lamb.

a) Representative image of a Western blot for UCPI, 10 µg MP each lane.

b) Effect of postnatal age on UCPI protein abundance

Values are means ± SEM (n=5 each age).

MP, mitochondrial protein

* indicates $P < 0.05$ for age effect

** indicates $P < 0.01$ for age effect

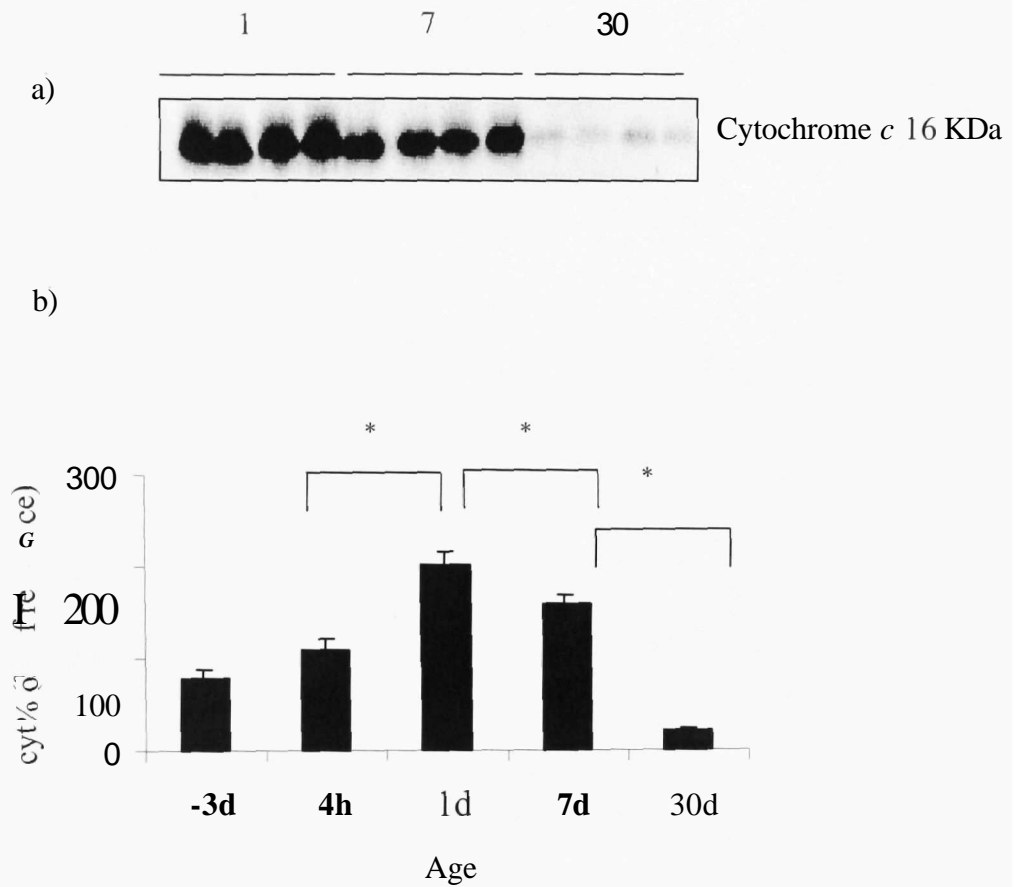


Figure 7.3

Abundance of cytochrome *c* in BAT from late gestation, to one month of age in the lamb.

a) Representative image of a Western blot for cytochrome *c*, 10 pg MP each lane.

b) Effect of postnatal age on cyt *c* protein abundance

Values are means \pm SEM (n=4 each age).

MP, mitochondrial protein

* indicates $P < 0.05$ for treatment effect

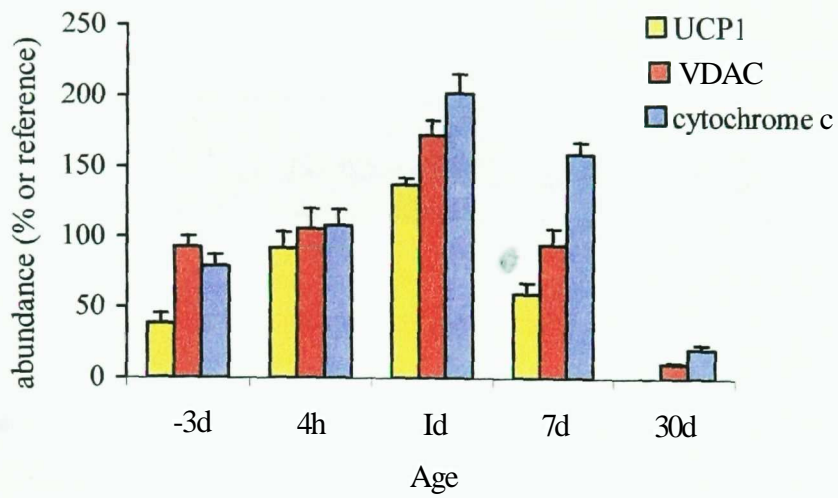


Figure 7.4

Effect of postnatal age on VDAC, UCP1 and cytochrome *c* protein abundance in BAT

Values are means \pm SEM.

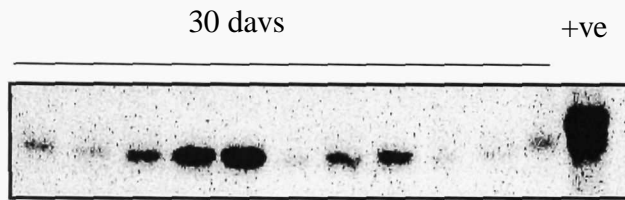


Figure 7.5

Representative image of a Western blot for UCP2, 10 pg MP each lane.

30 days (WAT tissue); +ve, UCP2 peptide (5ng).

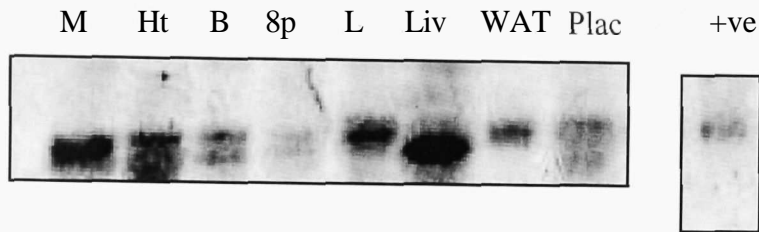


Figure 7.6

Representative image of a Western blot for UCP2, 10 pg MP each lane.

MP, mitochondrial protein; M, skeletal muscle; Ht, heart; B, brain; L, lung; Liv, liver; Plac, placenta (145 day gestation, "A" type whole placentome); WAT, white adipose tissue (30 day old lamb); Sp, spleen (7 day old lamb); +ve, UCP2 peptide (5ng).

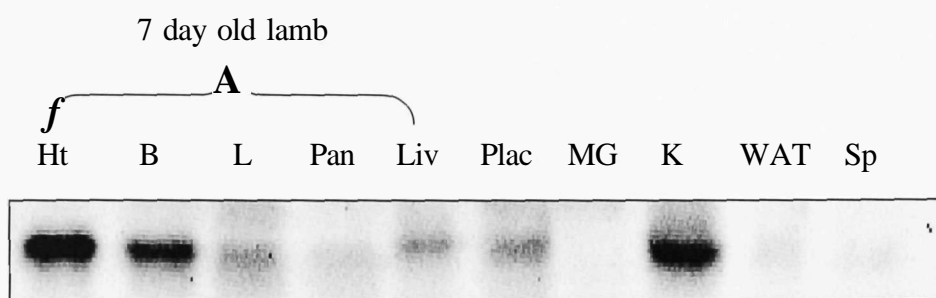
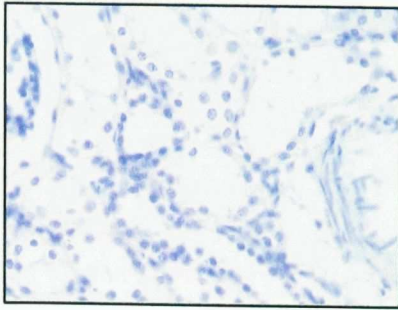


Figure 7.7

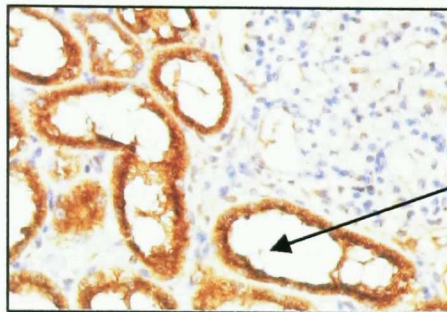
Representative image of a Western blot for VDAC, 10 µg MP each lane.

MP, mitochondrial protein; Ht, heart; B, brain; L, lung; Pan, pancreas; Liv, liver; Plac, placenta (145 day gestation, "A" type whole placentome); MG, mammary gland (late gestation ewe); K, kidney; WAT, white adipose tissue (30 day old lamb); Sp, spleen (7 day old lamb).

a)



b)



Kidney
tubule

Figure 7.8

Images of ovine kidney detected whh VDAC (1 in 400 dilution of antibody). All longitudinal sections.

a) Negative control section, not incubated whh antibody. Kidney from aduh ewe.

140 X magnification

b) Aduh ewe kidney section incubated whh VDAC (1 in 400) 140 X

magnification

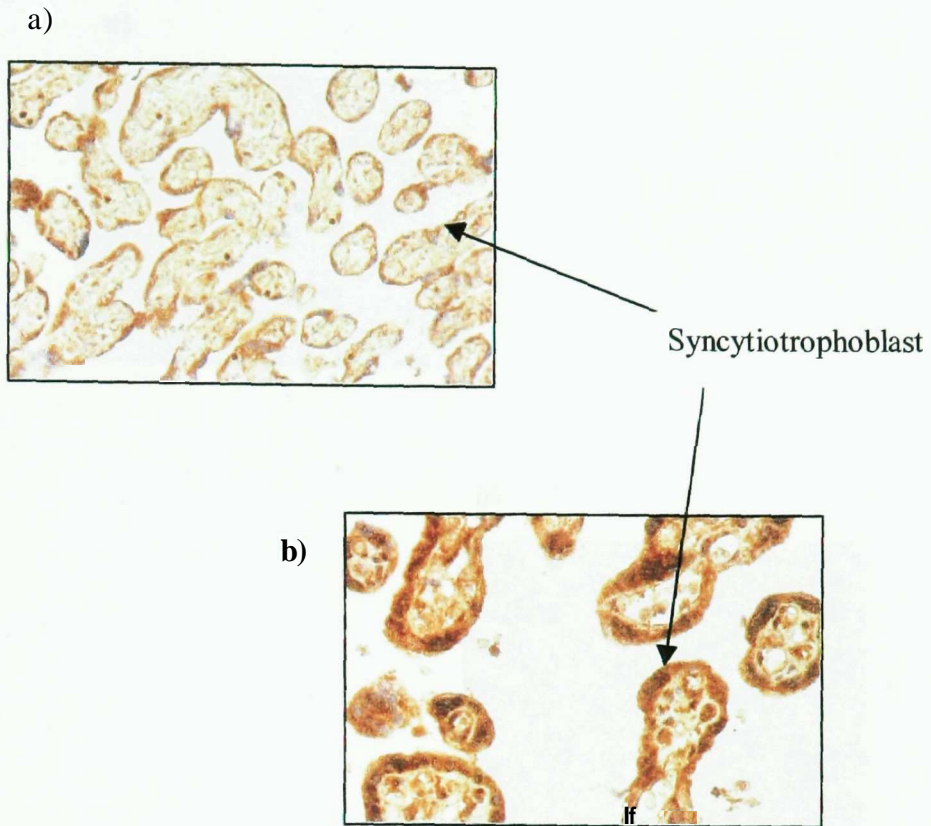


Figure 7.9

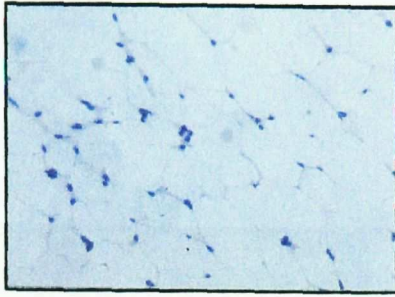
Images of human placenta detected with VDAC. All longitudinal sections.

a) Human placental section incubated with VDAC (1 in 100) 70 X magnification

b) Human placental section incubated with VDAC (1 in 100) 140 X

magnification

a)



b)

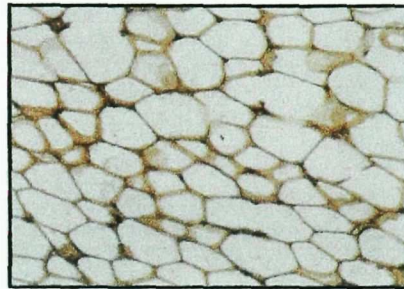


Figure 7.10

Images of ovine adult PAT detected with VDAC. All longitudinal sections.

a) Negative control section, not incubated with antibody. PAT from adult ewe.

70 X magnification

b) Ovine adult PAT section incubated with VDAC (1 in 100) 70 X magnification

a)



b)



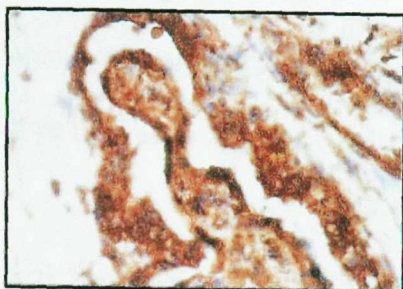
Figure 7.11

Images of ovine adult skeletal muscle detected with VDAC. All longitudinal sections.

a) Ovine skeletal muscle section incubated with VDAC (1 in 400) 17.5 X magnification

b) Negative control section, not incubated with antibody. Skeletal muscle from adult ewe 70X magnification

a)



b)

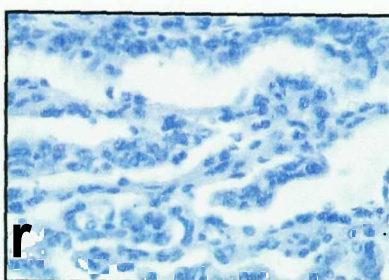


Figure 7.12

Images of ovine placentome detected with VDAC. All longitudinal sections.

a) Ovine placentome section incubated with VDAC (1 in 100) 140 X magnification

b) Negative control section, not incubated with antibody. Ovine placentome 140X magnification

7.4 Discussion

7.4.1 BAT Ontogeny of VDAC, Cytochrome *c* and UCPI

The ontogeny's of VDAC, cytochrome *c* and UCPI were found to be similar, all with a peak in abundance at day 1 of age.

The peak abundance of UCPI at day 1 of postnatal age is critical for the production of heat through non-shivering thermogenesis for the neonatal lamb who relies on BAT thermogenesis for heat production before the appearance of subcutaneous WAT and a thick fleece. UCPI mRNA and protein have previously been shown to peak at around the time of birth in the lamb (Clarke *et al.* 1997b, Finn, 1998 #105) and the current results correlate well with these published findings. UCPI is then down-regulated in the first month of age when the adipose tissue adopts the characteristics of WAT, that is: no UCPI, unilocular fat droplets with few cellular organelles (Casteilla 1989; Gemmel *et al.* 1972), however, UCP2 is still abundant in WAT at this time.

The postnatal ontogeny of VDAC was found to be similar to that of UCPI. VDAC had high antenatal abundance in ovine BAT, the function of VDAC during the fetal period is unknown, although it may be involved in the supply of ATP and ADP to the mitochondria of developing fetal tissues. The reduction in VDAC with increasing postnatal age was also similar to the ontogeny of UCPI. This may be related to the changing metabolic requirements of brown adipose

tissue. The metabolic requirements of BAT decline with increasing postnatal age as its role changes from that of an active heat producing tissue, to one with a major role in the storage of energy as fat. VDAC is known to control ATP flux across the outer mitochondrial membrane (Rostovtseva & Colombini 1996) and this role correlates well with the reduced abundance of VDAC as the energy requirements of BAT decline with increasing postnatal age.

VDAC is also a component of the mitochondrial permeability transition pore (MPTP) which is thought to be involved in apoptosis and also necrosis (Crompton 1999). The MPTP is formed from a complex of VDAC, the adenine nucleotide translocase and cyclophilin-D at contact sites between the outer and inner mitochondrial membrane (Crompton *et al.* 1998). There is evidence to suggest that the MPTP is utilised in some capacity during apoptosis, possibly in the release of cytochrome *c* from the mitochondria (Gottlieb 2000). The exact role of VDAC and the MPTP in apoptosis is not yet known. However, if an increased abundance of VDAC is associated with the initiation of apoptosis, one might speculate that on day one of postnatal age apoptosis is being initiated in BAT which would lead to the loss of adipocytes. This could be further investigated with specific assays of apoptosis.

The cytochrome *c* ontogeny was again similar to that of UCPI and VDAC, with a peak at one day of postnatal age. Cytochrome *c* was abundant antenatally, with levels similar to that of a four -hour old lamb. The function of cytochrome *c* in

fetal BAT is likely to be the same as that in the adult. Cytochrome *c* is involved in complex III and IV of the electron transport chain (Lehninger *et al.* 1993) where it acts as a mobile electron transporter. The reason for the significant reduction of cytochrome *c* with increasing postnatal age is likely to be the same as that for VDAC; as the adipose tissue adopts the characteristics of WAT there is less requirement for aerobic energy production through the electron transport chain.

7.7.3 Tissue Distribution of VDAC

VDAC was found to be highly abundant in skeletal muscle, kidney, brain, BAT and heart mitochondria from ovine samples. These tissues are all highly metabolically active; receiving 57.5 % of total cardiac output between them and 57.2 % of total oxygen consumption (Ganong 1995). This is in accordance with the role of VDAC as the major pathway for the transport of metabolites - especially adenine nucleotides (Kirk & Strange 1998). Metabolic enzymes such as hexokinase, glucokinase and creatine kinase may also bind to VDAC, this is thought to provide the enzymes with preferential access to mitochondrial substrates and ATP (Kirk & Sfrange 1998). VDAC has also been shown to exist in **extra-mitochondrial** localisations such as endosomes, sarcoplasmic reticulum, astrocytes and caveolae (Bathori *et al.* 2000; Dermietzel *et al.* 1994; Jakob *et al.* 1995).

It cannot be determined if VDAC in the tissue sections shown in Figures 7.14 and 7.15 are localised to mitochondria or cellular membrane, as the

magnification is not great enough to visualise mitochondria. However, the specific tissue locations give us a clue as to the position of the VDAC. In the kidney, VDAC is found in the walls of the tubules. Tubules function to absorb and secrete water and solutes in order to produce urine. Many of these transport mechanisms are ATP dependent, such as the potassium:sodium transporter, a high abundance of VDAC in the mitochondria of the tubular cells would provide ATP for such active transport. In the human placenta, VDAC is located in the syncytiotrophoblast. The syncytiotrophoblast constitutes the maternal fetal interface and has highly differentiated cytoplasm that is filled with vacuoles, multivesicular and dense bodies, lipid droplets, mitochondria and other organelles (Ross *et al.* 1995). One of the roles of the syncytiotrophoblast is steroidogenesis; cholesterol is mobilised into mitochondria and transformed into oestrogen and progesterone (Bonenfant *et al.* 2000; Ross *et al.* 1995). This type of activity would require energy; thus a high abundance of VDAC in these cell types is not unsurprising. It is also possible that VDAC is in an extra-mitochondrial location in these cells.

7.7.4 UCP2 Tissue Distribution

Initial work on UCP2 found that it had a wide tissue expression, but more recent work has identified a much lower tissue protein distribution, that is, the mRNA for UCP2 is present in many tissues, but the functional protein is only present in a fraction of them. This study found UCP2 to have highest abundance in lung and WAT mitochondria. UCP2 protein was found at a lower level in brain, spleen and placental mitochondria. This is slightly different to findings from

Pecqueur et al who found spleen mitochondria to have significantly more UCP2 than the lung (Pecqueur *et al.* 2001), although these experiments were carried out in mice. This could be a species variation or an age difference, the spleen mitochondria in my study were isolated from seven days old lambs, and the mice in the published study were seven to ten weeks old. The band obtained from liver mitochondria is of a slightly lower molecular weight, so it is thought not to be UCP2. With the limited evidence available regarding UCP2 protein, it seems unlikely that it has a role in thermogenesis, as physiological levels of the protein are unable to uncouple yeast mitochondria (Cadenas *et al.* 1999; Smart *et al.* 2001a; Stuart *et al.* 2001b). However, as discussed in Chapter 3, UCP1 knock-out mice survive the neonatal period without UCP1 to produce heat, no data has been published on UCP2 activity or tissue abundance at this critical time. It has been postulated that UCP2 has a role in reactive oxygen species (ROS) production (Arsenijevic *et al.* 2000) (Faggioni *et al.* 1998; Pecqueur *et al.* 2001; Stuart *et al.* 2001b). This correlates well with the high abundance of UCP2 observed in the spleen and lung as these tissues are actively involved in the immune response to infection.

7.8 Conclusion

The similar ontogeny's of UCP1, VDAC and cytochrome *c* suggests that these mitochondrial proteins may all be important in ensuring BAT maintains a maximal rate of thermogenesis at the critical time after birth. The parallel changes in mitochondrial proteins are coincident with the transition of BAT to WAT. The

fact that UCP2 is still abundant in WAT after 30 days of age suggests a different role than UCP1 as non-shivering thermogenesis is not relied upon at this stage in hfe.

CHAPTER 8

CONCLUSION

8.1 General Conclusions

The aim of this thesis was to examine the endocrine regulation of BAT in the fetal and neonatal lamb, in order to understand better the mechanisms involved in the transition from fetus to neonate. This was achieved by investigating the fetal or postnatal regulation of adipose tissue by Cortisol and leptin, respectively. Further insight into the metabolic regulation of adipose tissue was achieved by the development of specific ovine antibodies against VDAC. Below is a summary of my findings along with future perspectives.

8.1.1 Leptin and Neonatal Adipose Development

The main findings of Chapter three were the differential effects of leptin regulation of UCP1 of large mammals and rodents. Contrary to previous studies in rats and mice demonstrating that administration of leptin increases body temperature via UCP1 expression (Pelleymounter *et al.* 1995; Scarpace & Matheny 1998; Scarpace *et al.* 1997), the present studies found that administration of leptin to neonatal lambs caused a modest initial temperature maintenance, despite a reduction in the expression and abundance of UCP1. The effects on temperature occurred with no effect on lamb growth, body or BAT weight. The mechanism of leptin in this case, is as yet unknown as it did not act through UCP1. Possible mechanisms initiated by leptin to increase body temperature are outline in Figure 3.26, these include increased interleukin-1 (Luheshi *et al.* 1999) and upregulation of other uncoupling proteins (Cusin *et al.* 1998; Scarpace *et al.* 1998). The doses of leptin used in Chapters 3 produced plasma leptin levels above the normal physiological range, although they were considerable lower than concentrations observed in previous rodent studies. It is

possible that the doses of leptin used produced pharmacological effects, although all physiological, metabolic and hormonal values assessed were within the normal ranges. It was not possible to measure T3 or catecholamines in plasma in all studies in this thesis. T3 and catecholamines are important markers of thermogenesis and thus future work investigating the role of leptin in neonatal thermogenesis should include

It is likely that increased SNS activity had a major contribution in leptin maintenance of body temperature in the treated animals. Plasma NEFA concentrations were significantly higher in the treated animals at the time of maintained temperature, suggesting leptin was acting to increase sympathetic outflow to BAT thus increasing lipolysis. As described in Chapter 1.3.2 fatty acids can act to "unmask" UCPI, this increases the number of active sites available, increasing thermogenic activity with no increase in UCPI transcription. However, this does not explain the unusual finding of reduced UCPI expression and abundance. I suggest that leptin is acting as a signal to promote the neonatal transition from BAT to WAT in the sheep, with the high plasma levels observed at birth acting as a "switch", promoting the loss of UCPI mRNA and thus the transformation of BAT to WAT. There is evidence that leptin acts to modulate adipose tissue mass in the rodent by apoptosis (Qian *et al.* 1998), further investigations will have to be made to verify apoptosis in these ovine samples.

The impact of the route of delivery was investigated in Chapter 4; the major finding was that the neonatal decline in plasma leptin was attenuated in lambs

delivered by caesarean section. The factors responsible for higher plasma leptin after birth in caesarean section delivered lambs include the SNS and thyroid hormones, both of which are markedly reduced after caesarean section delivery in the sheep (Bird 1996; Clarke *et al* 1997c; Hagnevik *et al* 1984; Irestedt *et al* 1982). Reduced SNS activity and T3 contribute to an inferior ability to thermoregulate in caesarean section delivered lambs. When the results from Chapter three are considered, the slower decline in plasma leptin in these lambs may represent a compensatory response in order to restore body temperature.

8.1.2 Cortisol and Adipose Development

Chapter five investigated the role of Cortisol in the development and preparation of ovine fetal BAT for neonatal thermogenesis. Intact adrenals are required for the surge in Cortisol and the normal increase in UCPI in BAT leading up to birth. Cortisol treatment to mimic the normal late gestation increase was found to increase UCPI protein and GDP binding in BAT. Adrenalectomy of late gestation fetuses, in contrast, resulted in a reduction of UCPI and GDP binding. These changes were correlated with T3 concentrations suggesting that T3 and Cortisol are both required for the normal *in utero* development of BAT in sheep. This finding has important clinical implications, glucocorticoids are often used to treat pregnant women in premature labour to mimic the late gestational surge in Cortisol and aid development of the unborn child. This treatment has been carried out since the 1950's to promote surfactant production and lung development and improve overall outcome of the premature neonate. The results of this study suggest that Cortisol may also be improving neonatal thermogenic capabilities via increased development of BAT.

8.1.3 Postnatal Ontogeny of Mitochondrial Proteins

The production of UCP2 antibodies was unsuccessful, but a specific ovine VDAC antibody was produced for use in the investigation of BAT development and activity. VDAC was found to peak at day one of postnatal age, along with UCPI and cytochrome *c* in BAT. The functional significance of this high abundance of VDAC is unknown, although it is likely to be involved in the delivery of metabolic substrates to the mitochondria during the period of high metabolic requirement at birth. The reason for the peak abundance of cytochrome *c* at day one is likely to be similar to that of VDAC, that is, to ensure that the high demand for metabolic substrates is met. The peak activity of UCPI shortly after birth is well documented, and acts to maintain neonatal body temperature via NST. Mutations in the VDAC gene have been reported (Anflous *et al* 2001; Huizing *et al.* 1996) and result in altered sensitivity for ADP, it is not known if alterations in VDAC abundance would modify the thermogenic efficiency of the newborn given that the postnatal ontogeny is so similar.

Leptin has the potential to play a role in the transition from fetal to neonatal life, although its exact function remains unknown. Concentrations decline rapidly after birth as the infant moves from a state of passive nutrient uptake, to one of active enteral feeding, levels are known to decrease in response to starvation (Ahima 1996) and this may be one way of facilitating the infants' intake of milk. As the infant or lamb commences feeding and increases food intake, fat deposition will occur, as less energy is needed for maintaining body temperature. The neonate will therefore have less requirement for non-shivering thermogenesis in BAT as heat production from dietary induced and shivering thermogenesis in muscle plus increased insulation, act to maintain body temperature (Symonds *et al.* 1989a; Symonds *et al.* 1989b).

It is hypothesised that with increasing age plasma leptin increases in response to feeding and at the same time promotes loss of UCP1, as was observed in Chapter three. Concurrently, abundance of UCP2 and UCP3 may be increased, the activity of which may be stimulated during periods of nutritional stress (e.g. starvation (Ahima 1996)). This will enable the neonate to maintain a basal metabolic rate and prevent hypothermia despite the loss of UCP1.

The nutritional and endocrine regulation of VDAC remains to be established but may represent one of the factors involved in ensuring a smooth transition from fetus to neonate.

More studies are required to fully elucidate the role of leptin in the fetus and neonate. The emergence of sheep transgenic models (McCreath *et al.* 2000) in which under and over-expression of UCPs can be accomplished are likely to provide more information regarding the effect of leptin on thermogenesis at birth. Both acute and chronic leptin treatment reduce UCPI, whether a lack of leptin at birth will allow UCPI to function for longer than the usual first two weeks of life remains to be established. The potential therapeutic use of leptin to promote thermoregulation and postnatal adaptation remain an intriguing possibility.

Appendix I

List of suppliers

Aida Raytek Scientific Ltd, 26 Norton park View, Sheffield, S8 8GS, UK

Alpha Laboratories Ltd. 40 Parham Drive, Eastleigh, Hampshire, SO50 4NU,
UK

Ambion, Inc. AMS Biotechnology (UK Ltd), 185A & B, Milton Park, Abingdon,
Oxfordshire, OX14 4SR, UK

Amersham Pharmacia Biotech UK Ltd. Amersham Place, Little Chalfont,
Buckinghamshire, HP7 9NA, UK

Amicon, Millipore UK Ltd, The Boulevard, Blackmoor Lane, Watford, WD1
8YN, UK

Analytical Development Company. Pindar Road, Hoddeson, Hertfordshire,
EN11 0AQ, UK

Avery Berkel Ltd. Foundry Lane, Smethwick, West Midlands, B66 2LP, UK

Baxter Healthcare Ltd. Wallingford Road, Compton, Newbury, Berkshire,
RG20 7QW, UK

Bio-Rad Laboratories Ltd. Bio-Rad House, Maylands Avenue, Hemel
Hempstead, Hertfordshire, HP2 7TD, UK

BOC Edwards Ltd. Manor Royal, Crawley, West Sussex, RH10 2LW, UK

Cambridge Electrophoresis Ltd, 84 High Street, Cherry Hinton, Cambridge,
CB1 9HZ, UK

Dako Ltd. 16 Manor Courtyard, Hughenden Avenue, High Wycombe,
Buckinghamshire, HP13 5RE, UK

Diagnostic Systems Laboratories Ltd. Unit C121, 89 Bickerseth Road, Tooting,
London, SW17 9SH, UK

Fisher Scientific UK Ltd. Bishop Meadow Road, Loughborough, Leicestershire,
LE11 5RG, UK

Frank Wright Ltd. Blenheim House, Blenheim Road, Ashboume, Derbyshire,
DE6 1HA, UK

Fuji Photo Film (UK) Ltd Graphic Systems Imaging Centre, Unit 15, St
Martins Way, St Martins Business Centre, Bedford, MK42 OLE, UK

Guildhay Ltd, 6 Riverside Business Centre, Walnut Tree Close, Guilford, Surrey,
GU1 4UG, UK

Heto-Holten Ltd, PO Box 31, Camberiey, Surrey, GU15 1TN, UK

Hoefler Pharmacia Biotech Inc. 23 Grosvenor Road, St Albans, Hertfordshire,
AL1 3AW, UK

Hybaid Ltd, Action Court, Ashford Road, Middlesex, TW15 1XB, UK

Light Laboratories. 10 Princes Street, Brighton, BN2 1RD, UK

LKB Produkter AB. Box 305, S-161 26 Bromma, Sweden

Microsoft Corp. Microsoft Campus, Thames Valley Park, Reading, RG6 1WG,
UK

National Diagnostics. Unit 3, Chamberlain Road, Aylesbury, Buckinghamshire,
HP 19 3DY, UK

Packard Instruments. 14 Station Road, Pangbourne, berkshire, RG8 7DT, UK

Premier Beverages UK Ltd, Knighton, Adbaston, Stafford, ST20 0QJ, UK

Promega UK Ltd, Delta House, Chilworth Research Centre, Southampton, SO16
7NS, UK

Roche Diagnostic Products Ltd. PO Box 8, Welwyn Garden City, Hertfordshire,
AL7 3AY, UK

Salter Weigh-Tronix Ltd. George Street, West Bromwich, West Midlands, B70
6AD, UK

Sant Cruz. c/o Autogen BioclearUK Ltd, Holly Ditch Farm, Mile Elm, Calne,
Wiltshire, SN11 OPY, UK

Sarstedt Ltd. 68 Boston Road, Beaumont Leys, Leicester, LE4 1 AW, UK

Schleicher & Schuell GmbH, PO Box 4 D-37502, Dassel, Germany

Scientific Laboratory Supplies Ltd. Units 26-27, Wilford Industrial Estate,
Ruddington Lane, Wilford, Nottingham, NG11 7EP, UK

Sigma-Aldrich Company Ltd. Fancy Road, Poole, Dorset, BH12 4QH, UK

SIP Analytical Ltd. Goodwin Park, Sandwich Industrial Estate, Sandwich, Kent,
CT13 9LN, UK

SPSS UK Ltd. 1st Floor St. Andrew's House, West Street, Woking, Surrey,
GU21 1EB, UK

Techne (Cambridge) Ltd, Duxford, Cambridge, CB2 4PZ, UK

Vygon UK Ltd. Bridge Road, Cirencester, Gloucestershire, GL7 1PT, UK

Appendix II

Abbreviations

ADP	Adenine diphosphate
ATP	Adenine triphosphate
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CoA	Coenzyme A
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EtBr	Ethidium bromide
"G"	Gestational day
GC	Glucocorticoid
GDP	Guanosine diphosphate
HRP	Horseradish peroxidase
JAK	Janus kinase
MACP	Mitochondrial anion carrier protein
MOPS	3-(N-morpholino)propanesulfonic acid
NA	Noradrenaline
NaCl	Sodium chloride
NEFA	Non-esterified fatty acids
NST	Non-shivering thermogenesis

Ob-R	Leptin reading frame
OD	Optical Density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
RNA	Ribonucleic acid
SDS	Sodium dodecylsulphate
SNS	Sympathetic nervous system
SSC	Standard saline citrate
STAT	Signal transducer and activator of transcription
T3	Triiodothyronine
T4	Thyroxine
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TES	N-tris methyl-2-aminoethanesulphonic acid
TTBS	Tween-tris buffered saline
UCP	Uncoupling protein
UV	Ultra violet
WAT	White adipose tissue

Appendix III

Antibody Dilutions

Antibody	Supplier	Dilution
UCPI	in house	1/8000
UCP2	Bmno Miroux	1/10,000
VDAC	in house	1/1000
Cytochrome c	Santa Cmz (sc-7159)	1/1000
Swine anti rabbit IgG	Dako (PO 217)	1/1500

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