

TITLE PAGE**STUDIES OF ADIPOSE TISSUE IN HUMANS WITH
SPECIAL REFERENCE TO INNERVATION BY THE
SYMPATHETIC NERVOUS SYSTEM**

A thesis submitted for the degree of Doctor of Philosophy
to the University of London

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Declaration and Statement of conjoint work

DECLARATION

I confirm that the work presented in the thesis is my own and that all references are cited accordingly. The thesis does contain work conducted jointly and a statement of the part played by myself and others is given below.

CONJOINT WORK

The work described in this thesis is my own except where otherwise described below or where so described in the text and formally referenced. This work was made possible as a result of a number of collaborations.

The studies performed in chapter III were performed by S.W. Coppack, M. Persson, S.M. Humphreys and J.M Miles in a collaboration between our unit at St Bartholomew's & The Royal London School of Medicine, London, U.K., the Endocrine Research Unit, Mayo Clinic, Rochester, MN and the Radcliffe Infirmary, Oxford University, Oxford, U.K. I analysed these data, tested the hypotheses and, with the assistance of my supervisor, SW Coppack wrote the report.

I recruited the volunteers, performed the cannulations, administered the isotopes, obtained and separated the blood samples for the studies presented in chapter IV in conjunction with J.N Patel and the assays were performed in the laboratory of Prof G Eisenhofer at the NIH. I performed the data analysis and wrote the report in conjunction with my supervisor, S.W Coppack.

The data presented in chapters V and VI were obtained as a collaboration between our unit at St Bartholomew's & The Royal London School of Medicine, London, U.K and those of Prof K Frayn, F. Karpe and B.A. Fielding at the Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital, Oxford OX3 7LJ and Prof I.A. Macdonald at the Centre for Integrated Systems Biology and Medicine, Institute of Clinical Research and School of Biomedical Sciences, University of Nottingham Medical School, Nottingham, U.K. The studies were shared between the London and Oxford units and the results pooled. Authorship of the data presented in chapter V was prospectively allocated to the Oxford unit and that of the data presented in chapter VI to ours. I performed pilot studies which established the experimental protocol for both chapters and was involved in the collaboration and study design from its inception. I performed the studies undertaken at St Bartholomew's and the Royal London School of Medicine. I proposed and designed the use of mock datasets to act as controls, proposed the use of a euglycaemic-hyperinsulinaemic clamp protocol to obviate the effect of spontaneous insulin pulses and used the pulse detection algorithm 'Cluster 7' to analyse the real and mock datasets. The analysis and writing of the data in chapter VI was mine in conjunction with my project supervisor, SW Coppack. The assays were performed by J Hinson and K Noonan at Queen Mary, University of London and Vera Ilic, Sandy Humphreys, Louise Dennis, Sarir Sarmad, and Sally Cordon assisted with the studies and performed sample analysis in Oxford.

The work reported in chapter VII was a collaboration between our unit and that of Prof John Priestley and colleagues (notably Sharon Averill) at the Neuroscience

Institute, Queen Mary, University of London. I am grateful for their help in learning and developing the techniques described to examine adipose tissue using immuno-histochemical techniques and confocal microscopy. I developed the ideas and hypotheses, performed the tissue preparation and histochemical analysis, obtained and interpreted the images and wrote the report in conjunction with my project supervisor, S.W Coppack.

I gratefully acknowledge the contribution of all these individuals and laboratories both to the data herein presented and also to my own training and scientific development.

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ABSTRACT

This thesis reports the effects *in vivo* of the sympathetic nervous system (SNS) in human subcutaneous abdominal white adipose tissue (WAT) and other tissues involved in energy storage and utilisation.

Cannulation of superficial veins draining skin, abdominal subcutaneous WAT and deep forearm muscle combined with isotope turnover methodology and tissue blood flow estimation was used to investigate the behaviour of these tissues under varying experimental conditions.

Glucose infusion study:

This examined differential substrate uptake and utilisation in the three tissues. WAT was responsible for only a small amount of glucose disposal and deep forearm muscle took up but did not release NEFA. Skin was a net exporter of lactate. Results confirm the relative purity of the venous effluent from these tissues.

Sympathetic Nervous System study:

This examined whole body, WAT and forearm muscle SNS activity in lean and obese individuals under fasting and postprandial conditions. Whereas whole body SNS activity was increased in the obese, regional heterogeneity of SNS activity was evidenced by reduced SNS activity in WAT. Adipose tissue blood flow was significantly reduced in the obese. This may underlie abnormal lipolysis and/or blood flow regulation in obesity.

Pulsatility Studies:

These studies examined whether lipolysis and leptin production in human WAT is uniform or pulsatile. Novel control datasets were used to test the robustness of a widely used pulse detection algorithm. Whereas NEFA release appeared truly pulsatile, apparent leptin ‘pulses’ occurred with similar frequency in the control datasets and appear likely simply to reflect variability.

Anatomical studies:

Confocal immuno-fluorescence microscopy was used to demonstrate innervation of WAT in man for the first time. Such innervation appears confined to the microvasculature and suggests that the defective SNS activity within WAT is likely to affect adipose tissue biology primarily through defective regulation of adipose tissue blood flow.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AT	Adipose tissue
ATBF	Adipose tissue blood flow
ATPF	Adipose tissue plasma flow
AUC	Area under the curve
BAT	Brown adipose tissue
BMI	Body mass index (weight/height ²)
Cal	Calorie
cAMP	Cyclic Adenosine monophosphate
CGRP	Calcitonin gene related peptide
Ci	Curie
COMT	Catechol-O-methyltransferase
CsI	Caesium iodide
CV	Coefficient of variation
DEXA	Dual energy X-ray absorptiometry
DNA	De-oxy ribonucleic acid
DOPA	Dihydroxyphenylalanine
dpm	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid

E.M	Electron microscope (microscopy)
ELISA	Enzyme linked immunosorbent assay
FBF	Forearm blood flow
HARS	HIV-associated adipose redistribution syndrome
³ H-NE	Tritiated Norepinephrine
HSL	Hormone sensitive lipase
LBM	Lean body mass
LH	Luteinising hormone
LPA	Lysophosphatidic acid
LPL	Lipoprotein lipase
MAO	Monoamine oxidase
MCT	Monocarboxylate transporter
mRNA	Messenger ribonucleic acid
MSNA	Muscle sympathetic nerve activity
MW	Molecular weight
NE	Norepinephrine
NEFA	Non esterified fatty acid(s)
NO	Nitric oxide
NPY	Neuropeptide Y
PGP	P Glycoprotein
PRV	Pseudo-rabies virus
QC	Quality control
QMW	Queen Mary and Westfield College, University of London
R _a	Appearance rate

Rs	Spearman's rank correlation coefficient
rt-PCR	Reverse transcriptase polymerase chain reaction
SA	Specific activity (of a radionucleide)
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SEM	Standard error of the mean
SNS	Sympathetic Nervous System
TAG	Tri-acyl glycerol
TBNE	Total body norepinephrine spillover
TH	Tyrosine hydroxylase
TNF- α	Tumour necrosis factor- α
UCP	Uncoupling Protein
VEGF	Vascular endothelial growth factor
w/v	Weight/volume
WAT	White Adipose tissue
WBFM	Whole body fat mass
WO	Washout
^{133}Xe	Xenon 133

CHAPTER I

Introduction and Literature Review

INTRODUCTION

The prevalence of obesity and of its complications is rising at an alarming rate not only in this country (where over one in five males are now obese according to the National Audit Office) but also in the USA and worldwide (Kopelman, 2000).

The importance of Sympathetic Nervous System (SNS) innervation of white adipose tissue (WAT) in myriad physiological and pathological processes is increasingly gaining recognition. Not only does the SNS have the potential to influence overall body mass through effects on energy expenditure but it may also directly or indirectly influence abnormalities of lipolysis, glucose homeostasis and blood pressure control which together form the cornerstones of the metabolic syndrome. It may therefore contribute not only to the development of obesity but also to its complications (Astrup & Macdonald 1998, Macdonald, 1995, Snitker *et al*, 2000, Webber *et al*, 1994).

Abnormalities of fat distribution as well as of total fat mass are now recognised to be important in obesity, particularly in relation to the development of visceral adiposity and consequent risk of insulin resistance. The observed variability in regional fat distribution between the sexes and between different ethnic groups convincingly demonstrates the regional and functional heterogeneity of adipose tissue, independent of its total mass, and the potential importance of understanding how this diverse tissue is regulated on a regional as well as whole body level (Arner, 1999).

The current state of knowledge relating to the contribution of the sympathetic nervous system (SNS) to the regulation of WAT physiology will be reviewed, touching upon some of the problems implicit in attempting to unravel its role. It will emerge that the presence of species differences makes assessment of SNS activity in humans particularly desirable. Unfortunately, however, obtaining good

in vivo human data has in the past been beset with important methodological limitations and conflicting conclusions have resulted (Grassi & Esler 1999, Young & Macdonald 1992, Snitker *et al.*, 2000).

The purpose underlying the studies presented in this thesis has been to develop, and apply robust anatomical and physiological techniques for use in humans to help unravel the role of the sympathetic nervous system in regulating this often neglected but metabolically pivotal tissue.

LITERATURE REVIEW

The role of the SNS in the regulation of adipose tissue physiology.

The review presented in this section will consider the evidence that the SNS is in a position to have an important influence on the regulation of body weight both directly through its innervation and regulation of WAT (and its microvascular environment) and also indirectly through its effects on other endocrine organs and metabolically active tissues such as skeletal muscle, the pancreas and the gut.

Discussion of its effects on lipolysis, thermogenesis, recruitment and differentiation of pre-adipocytes as well as consideration of the origins and destination of afferent and efferent signals to and from WAT will be presented leading to an evaluation of the evidence that SNS innervation of this tissue has relevance to the understanding and treatment of human obesity.

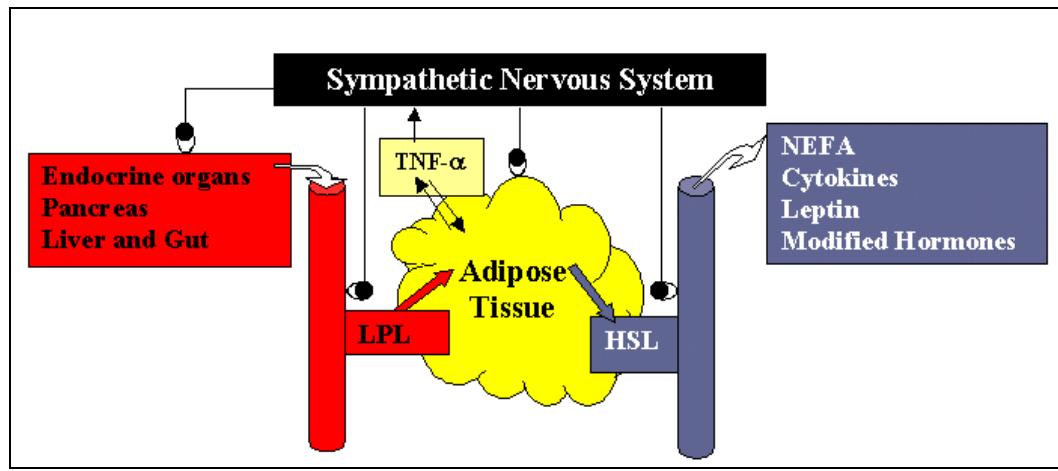
It will emerge from this discussion that: -

1. The SNS is potentially in a position to be able to regulate lipolysis, thermogenesis, adipose tissue endocrine function and pre-adipocyte recruitment and differentiation.
2. The role of the SNS in human obesity remains unclear.
3. Techniques for unravelling this complex system in humans are evolving but remain subject to important limitations.
4. Heterogeneity between different species and tissues and between the SNS innervation and catecholamine sensitivity of different adipose tissue depots may explain some of the divergence in the current literature relating to the role of the SNS in human obesity.

The SNS and regulation of body weight

The SNS is in a position both anatomically and physiologically to regulate virtually all aspects of nutrient partitioning, substrate disposal and, therefore, body weight homeostasis (Figure 1.1).

Fig. 1.1: The regulation of the adipocyte and its endocrine, nutrient and vascular microenvironment by the SNS.



Legend to Figure 1.1

Nutrients and hormones regulating WAT activity include catecholamines, insulin, glucose, triglycerides, sex steroids and gut-peptides. The SNS is potentially able to influence adipocytes not only by direct innervation but also by its modulation of signals from the pancreas, gut, liver and other endocrine organs as well as by regulation of its vascular perfusion.

In addition to this, the SNS may exert a further level of control by its effects on local vascular tone and permeability which may influence both the concentration and rate of presentation of such substrates (Samra *et al*, 1996). Not surprisingly, such levels of complexity have proved exceedingly difficult to unravel, particularly in humans, and a rather confusing literature has resulted (Snitker *et al*, 2000, Young *et al*, 1992).

Despite the complexities referred to above, there is now a substantial body of evidence that changes in SNS activity underpin many forms of obesity in animals (Bray 1989, Bray 1990, Bray 1991, Bray & York 1998, Bray 2000) and, furthermore, evidence of significant abnormalities in the local activity of the SNS in obesity that have long been described in animals are now finding correlates in human obesity. These include effects on lipolysis (Astrup & Macdonald 1998), thermogenesis (Schwartz *et al*, 1987) and futile cycling (Mercer & Williamson 1988), transcriptional regulation of uncoupling proteins (UCPs) (Berraondo *et al*, 2000), metabolic rate (Saad *et al*, 1991) and the novel effect on the ‘re-awakening’ of human brown AT (Weyer *et al*, 1999) as well as possible effects on appetite (Leibowitz *et al*, 1984) and spontaneous physical activity (Christin *et al*, 1993).

Receptor Physiology and Regulation of Lipolysis

An important feature of the SNS innervation of WAT is the fact that it involves 5 distinct adrenoceptor subtypes which are differentially recruited by epinephrine, norepinephrine (NE) and their synthetic agonists and antagonists (Lafontan *et al*, 1997). These receptors often display opposing effects on adipocytes in response to ligand binding and their relative cell membrane densities not only show marked regional heterogeneity (Arner 1999) but are also subject to rapid changes in response to different metabolic and endocrine/paracrine influences. Thus, insulin (Engfeldt *et al*, 1988), leptin (Breslow *et al*, 1997) and tumour necrosis factor- α

(TNF- α) (Berkowitz *et al*, 1998) can modulate the expression of adrenergic receptors in WAT. Catecholamine-induced lipolysis in human visceral WAT is increased in obesity due to increased function of β_3 -adrenoceptors, decreased function of α_2 -adrenoceptors and increased ability of cyclic AMP to stimulate lipolysis (Lafontan & Berlan 1980, Lafontan *et al*, 1997). When associated with reduced lipolysis in other depots, this leads to a relative redistribution of lipolysis to visceral depots and consequent exposure of the liver, via the portal vein, to increased concentrations of compounds known to induce a state of insulin resistance. Studies in rodent models have been able to demonstrate marked increases in insulin sensitivity following surgical removal of visceral WAT (Barzilai *et al*, 1999). Studies by Richelsen and colleagues (Richelsen *et al*, 1991) have demonstrated that the differential (up to five fold) responsiveness of visceral and subcutaneous adipose tissue to catecholamine induced lipolysis could be blocked in the peripheral fat by yohimbine, an α_2 -antagonist, implying that there is high activity of inhibitory α_2 -adrenoceptors in the subcutaneous depot.

In addition to the release of catecholamines, most sympathetic nerve terminals also co-secrete Neuropeptide Y (NPY) (Lundberg *et al*, 1985) which appears to have complex effects on SNS signalling. Whereas there is evidence for augmentation of catecholamine effects at the target receptor, it seems that its release may also act pre-synaptically to reduce the amount of NE released per action potential. It has been suggested that this might serve as a mechanism to conserve NE at times of maximal stimulation of the SNS (Potter 1988). To what extent this mechanism operates in human white adipose tissue (WAT) has not been determined although it may be of some potential interest given both the central role of NPY in the hypothalamic regulation of appetite and also that most attempts to assess the activity of the SNS in man use measurements of NE spillover into the circulation, the validity of which could be affected if this putative system is indeed operational in man. The other implication of the findings discussed above is that the concentration of catecholamines achieved within an adipose tissue depot could, through differential sensitivity of the 5

adrenoceptor sub-types for differing catecholamine concentrations, determine whether the net effect is one of inhibition or stimulation of lipolysis.

The SNS Regulation of Thermogenesis

The differential regulation of adrenoceptor-dependent effects both within adipocytes and also in other tissues is strikingly demonstrated in the induction of uncoupling proteins (UCPs). These appear to be involved in the dissipation of proton gradients and thus the ‘wasting’ of surplus energy in the form of heat (some of which ‘obligatory’ and some ‘adaptive’ or ‘facultative’), a concept with potential implications in the regulation of body weight (Ricquier 2006).

Facultative thermogenesis is mediated by at least two separate components, diet-induced thermogenesis and cold-induced thermogenesis. Both of these components appear to depend at least in part on the activity of the SNS in modulating UCP expression in a tissue-specific manner. Thus, the administration of a β_3 -agonist to KK-Ay mice for 21 days resulted in large (14-, 6- and 16-fold) increases in UCP 1, 2 and 3 respectively in the brown adipose tissue of these animals but there was no detectable change at all in WAT UCP 2 mRNA levels. Furthermore, UCP 3 mRNA levels in skeletal muscle and heart were reduced to approximately 10% of their baseline values (Yoshitomi *et al*, 1998). Similar increases in UCP 1 and 2 have been found in rats after cold exposure but they were reduced by prior denervation suggesting direct SNS involvement in this adaptive response although UCP3 appeared not to be under direct sympathetic control in this particular model (Denjean *et al*, 1999). There is also a rodent model with reduced levels of BAT which develops significant obesity (Lowell 1993) presumably as a result of reduced UCP1 mediated thermogenesis.

Thermogenesis in man is likely to be relatively more dependent on skeletal muscle as demonstrated by the fact that infusion of epinephrine into forearm tissues causes an approximately 90% increase in O₂ consumption which, if

representative of skeletal muscle throughout the body, extrapolates to approximately 40% of the total thermic response to the dose of epinephrine they administered (Simonsen *et al*, 1992). However, evidence is emerging to suggest that functional brown adipose tissue (BAT) exists in adult humans and is also inducible by the activation of the SNS. Patients with phaeochromocytomas for example develop adipose tissue with the morphological characteristics of BAT (Lean *et al*, 1986, Ricquier *et al*, 1982). Pharmacological studies of β_3 -adrenoceptor agonists such as CL 316,243 have resulted in recruitment of such cells in adult primates, a phenomenon referred to as the ‘re-awakening’ of dormant BAT (Weyer *et al*, 1999). Humans given synthetic β_3 -agonists showed several potentially important beneficial effects in terms of lipolysis, insulin sensitivity and fat oxidation without any untoward effects on the cardiovascular system, making such agents and the mechanisms upon which they act promising targets for further research and development (Weyer *et al*, 1999).

The clinical importance of energy dissipation in the form of heat is apparent in the observation that food restriction sufficient to maintain a 10% reduction in body weight is associated with a highly significant reduction in energy expenditure (Leibel *et al*, 1995) measured as resting metabolic rate. Acute starvation may reduce this by as much as 40% (Saris 1995). Whilst this homeostatic mechanism may usefully defend body weight in times of famine, it is highly counterproductive during attempts to lose weight by dietary modification and is probably at least partly responsible for the dismal failure of this strategy in clinical practice.

Thus, whilst uncertainty exists as to the actual role played by both obligatory and facultative thermogenesis in the regulation of body weight in humans, its further understanding and potential pharmacological manipulation may be a matter of some importance.

SNS Regulation of Adipose Tissue Microcirculation

Activation of the SNS may be able to influence lipolysis not only directly via β -adrenoceptor mediated amplification of the cAMP second messenger system but also indirectly via an effect on capillary permeability and blood flow regulation (Samra *et al*, 1996). An increase in capillary permeability and blood flow will tend to remove lipolytic end products, bound to plasma proteins, from the immediate vicinity of the adipocyte thus reducing any inhibition of lipolysis caused by local increases in NEFA concentrations (Burns *et al*, 1978). There are important although not fully characterised changes in AT blood flow following meal ingestion which differ between lean and obese humans. Whether these are predominantly related to differences in tissue insulin or SNS sensitivity is not currently clear (Summers *et al*, 1996). The SNS may play a longer term regulatory role in vascular tissue via the potent angiogenic stimulating factor, vascular endothelial growth factor (VEGF) which has been shown to be directly regulated by cold stress (which increases SNS activity) in rat BAT and also by β -adrenoceptor agonists and NE (Asano *et al*, 1997). Other aspects of the complex issue of AT microcirculation have been reviewed elsewhere (Crandall *et al*, 1997) in detail and are discussed further in relation to the studies presented in Chapter VII.

Trophic effects of the SNS

It is becoming increasingly acknowledged that the SNS appears to have trophic, anti-apoptotic and possibly differentiating effects on pre-adipocytes. This is implied by reports of reduced BAT apoptosis in the presence of cold stress or directly applied NE (Briscini *et al*, 1998) and doubling of fat cell number in intact rodent fat pads following sympathetic denervation (Youngstrom & Bartness, 1998). More recent work has identified a possible mechanism for these observations (Valet *et al*, 1998, Pages *et al*, 2000) by demonstrating that the

release of lysophosphatidic acid (LPA), an adipogenic factor in WAT, appears to be subject to control by α_2 -adrenoceptor activation. The role of adipocyte recruitment in the regulation of the overall fat cell mass in humans and some of the mechanisms underlying this has also been reviewed recently by O'Rahilly (Prins & O'Rahilly 1997). Further discussion of the role of the SNS in fat cell recruitment and differentiation is presented in chapter VII.

Anatomy

The precise ultra-structural anatomy of the innervation of WAT by the SNS is far from being fully characterised (Bartness & Bamshad 1998, Bartness & Song 2007) and is the subject of the studies reported in chapter VII. The only semi-quantitative estimate available from animal studies of the number of adipocytes that are actually innervated by SNS terminals reported a figure of 3% despite rich innervation of surrounding vascular tissue (Slavin & Ballard 1978). Such a low innervation density appears intriguing in the light of preliminary data suggesting that peripheral, but not visceral WAT, is rather unresponsive, at least in terms of lipolysis, to the effects of physiological levels of circulating catecholamines (Richelsen *et al*, 1991). Thus it may be speculated either that current methods lack the power to detect all of the synaptic interactions that exist (for example, *en passant* contacts lacking classical pre- and post-synaptic morphology) or that the innervated cells represent a pool of the most metabolically active adipocytes or that the SNS regulates these adipocytes largely via control of their perfusing vasculature and local tissue microenvironment.

The SNS reaches AT from diverse brain regions via ganglia in the spinal cord. Studies using the retrograde trans-synaptic pseudo-rabies virus (PRV) tracer method in rodent and Siberian hamster WAT (Bartness & Ballard 1998) reveal projections from the spinal cord, brainstem (nucleus of the solitary tract, C1 and A5 regions, and the rostroventro-lateral, rostroventro-medial and caudal raphe nuclei), forebrain hypothalamic nuclei (arcuate nucleus, dorsal and lateral

hypothalamic areas, zona incerta and paraventricular, suprachiasmatic and dorsomedial nuclei) and forebrain non-hypothalamic nuclei (zona incerta, medial amygdala, medial preoptic area, septum and bed nucleus of the stria terminalis). A particularly salient insight into the direct relationship between WAT and the SNS *in vivo* has been obtained recently by the work of Niijima (Niijima 1999) who dissected out and cut the afferent sympathetic nerve to white and brown AT depots in the rat. Electrical recordings of neuronal activity were made from the cut ends of these nerve trunks both before and after the local injection of leptin directly into the AT depot which they innervated. Recordings were also taken from the autonomic supplies to liver, pancreas and adrenal medulla. The results demonstrated that the effect of the leptin on AT was to increase the afferent SNS activity to that depot and also to the pancreas, liver and adrenal glands whilst at the same time reducing the parasympathetic tone to these organs. These findings would appear to demonstrate a direct link between local AT leptin receptors and the autonomic nervous system innervation of these tissues as part of a reflex arc.

Human Studies-Limitations and Results

It emerges from the above that the SNS is ideally placed in many ways to be an important regulator of body weight although at present the literature relating to its actual function in man, particularly in obesity, is sometimes conflicting (Grassi & Esler 1999, Young & Macdonald 1992, Snitker *et al*, 2000). No studies to date have actually demonstrated the presence of SNS axons in human white adipose tissue. The conflicting reports have undoubtedly arisen in part due to the difficulty that exists in assessing the local as opposed to whole body activity of the SNS and the assumptions that such methods must necessarily make.

Historically, the main methods employed to this end in humans (Grassi & Esler 1999) have been measurement of whole body indices of SNS activity such as plasma or urine NE concentrations. Such methods are constrained by the assumption that NE release into the circulation and then into the urine bears a linear relationship to SNS activity and, furthermore, are able to take account of

neither the regional heterogeneity of SNS activity nor of the fact that there may exist post-receptor modifications of adrenoceptor-mediated signals. A refinement of this method has been to combine superficial abdominal vein catheterisation with Esler's radio-labelled tracer methods (Esler *et al*, 1984) to study specific subcutaneous AT depots in man (Frayn *et al*, 1989). This too remains constrained by the assumption of a linear relationship between SNS activity and the spillover of NE into the circulation, albeit local circulation, but represents probably the best single currently available method. Yet even with this refinement, spillover methods remain unable to address the fundamental problem that it may not be possible to define the influence of the SNS on AT purely in terms of the amount of NE released into the circulation. It may be, for example, that changes in the innervation density, number of adrenoceptors, response to neurotransmitter binding, the relative contribution of often opposing effects of α - and β -subtypes, not to mention changes in NE reuptake and metabolism and even blood flow modulation (Rongen *et al*, 2000) may all cause dissociation of the measured NE concentrations from the actual effects exerted upon AT.

Another method which attempts to measure local SNS activity directly is muscle sympathetic nerve activity (MSNA) which records activity in SNS axons within the peroneal nerve. Whether MSNA activity in the peroneal nerve truly reflects SNS regulation of adipose tissue is debated. Thus for many reasons, human studies are subject to a number of limitations and must be interpreted with some degree of caution.

Notwithstanding the limitations discussed above, there is now a growing body of evidence to suggest that local abnormalities of the SNS may have relevance to obesity in humans. In order to circumvent the problem of deciding whether detected differences in SNS activity are cause or consequence of obesity, Pima Indians have been subjected to intense study as their very high propensity to develop obesity provides fertile ground for prospective study. The alternative method is to study people in the post-obese state although not only is this

notoriously difficult given the low success of weight reduction in the majority of studies but also due to problems related to the known metabolic perturbations associated with relative under-feeding and weight loss, including, for example, the previously described changes in metabolic rate associated with this (Leibel *et al*, 1995).

In a prospective study of male Pima Indians, lower levels of 24h urinary NE and epinephrine excretion were highly correlated with increased subsequent weight gain (Tataranni *et al*, 1997) and further studies by the same group have also demonstrated both lower β -adrenoceptor sensitivity to the administration of isoprenaline in Pima Indians relative to Caucasian controls (Tataranni *et al*, 1998) and also lower levels of SNS activity, estimated by muscle sympathetic nerve activity (MSNA) (Tataranni *et al*, 1999).

Genetic disturbances in the SNS have also been identified in this population which has a high rate of mis-sense mutations of the β_3 -adrenoceptor gene. Those with the mutation are prone to the development of obesity, type 2 diabetes, and diastolic hypertension (Silver *et al*, 1997). Studies of this mutation in other populations has yielded variable and sometimes contradictory results with no effects on obesity or insulin sensitivity in a German population (Buettnner *et al*, 1998), effects on obesity only in Chinese women with type 2 diabetes (Xiang *et al*, 1998) and over-representation amongst older Australian obese women but not men (Kurabayashi *et al*, 1996). One study though has shown that the mutation is a predictor of the failure to lose weight in obese Caucasian women with type 2 diabetes (Sakane *et al*, 1997).

Studies in age and fat-free body mass matched lean and obese Caucasian women using tracer balance methodology applied to subcutaneous AT have been able to demonstrate reduced levels of local AT, but not systemic, NE spillover into the circulation in the obese subjects (Coppock *et al*, 1998) and there is some recent evidence to suggest that the lipolytic response to infused catecholamines may also

be blunted in obese humans both under basal conditions (Horowitz *et al*, 2000) and after exercise (Borsheim *et al*, 2000). Studies in formerly obese humans again have yielded sometimes divergent results although Astrup has produced convincing evidence of a defect in the SNS in a study of obese patients before and after weight loss where the thermic response to glucose and the arterial NE concentrations were significantly reduced (by a factor of 4) in the obese relative to lean controls and remained abnormal (by a factor of 2) after a mean 30 Kg weight reduction (Astrup *et al*, 1990).

It is clear from the review above that under-activity of the sympathetic nervous system (SNS) in AT has the potential to cause obesity through several mechanisms including reductions in lipolysis, thermogenesis and also possibly appetite modulation (Astrup *et al*, 1998). It has been proposed that most animal forms of obesity, including even that of the ob/ob mouse, are characterised by SNS under-activity (Bray & York 1998, Bray 1991, Bray 1990, Breslow *et al*, 1997). To what extent this applies to humans has been hotly debated with some investigators citing raised plasma catecholamine levels and obesity-associated hypertension as evidence that obesity is, in fact, a state of increased rather than decreased SNS activity (Rumantir *et al*, 1999, Grassi 1999).

The motivation for the studies presented in this Thesis was to study aspects of the metabolism of a human WAT depot compared to other tissues (Chapter III), its SNS outflow compared to lean controls under fasting and post-prandial conditions (Chapter IV), its co-ordination in terms of lipolysis (Chapter V) and leptin production (Chapter VI) and its anatomical pattern of SNS innervation (Chapter VII) in order to address some of these questions. We anticipated that increased understanding of depot and tissue-specific heterogeneity of function would help to reconcile some of the divergent conclusions reached in the previous literature.

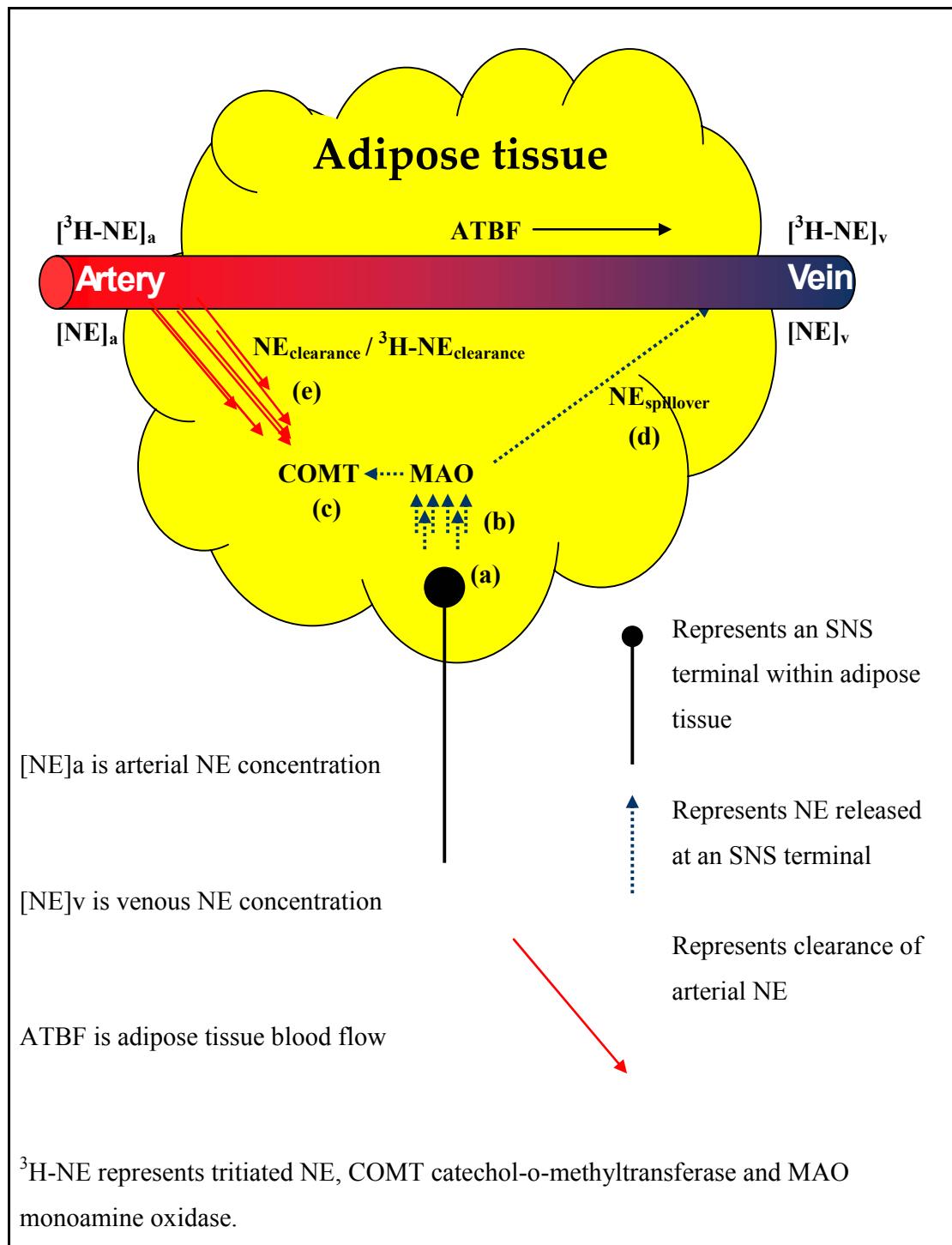
CHAPTER II

CORE EXPERIMENTAL METHODS

NOREPINEPHRINE SPILLOVER METHODOLOGY

We applied Esler's tracer balance approach which is based on the concept that the rate of entry of the SNS neurotransmitter, norepinephrine (NE), into venous blood (norepinephrine spillover, $NE_{\text{spillover}}$) is an index of local sympatho-neuronal activity over a wide range of firing rates (Grassi & Esler 1999). Figure 2.1 illustrates in diagrammatic form the key physiological principles of method used to assess SNS nerve activity within adipose tissue. The cannulations used, isotopes, experimental measurements, assumptions and calculations are described in the following sections.

Figure 2.1: Illustration of the source and fate of NE within adipose tissue



Legend to Figure 2. 1

Schematic illustration of the principals used to measure NE spillover. In essence, NE is released as a neurotransmitter at SNS terminals (a) but is also present in circulating blood. Following release, most is taken up in the terminal by monoamine oxidase (MAO, uptake 1) (b) or in the tissue by catechol-o-methyl transferase (COMT, uptake 2) (c). Some (perhaps 15%) will ‘spill-over’ ($NE_{\text{spillover}}$) into the vasculature having evaded these uptake mechanisms (d). $NE_{\text{spillover}}$ is a measure of NE added to the vasculature as a result of release from SNS terminals.

NE_{flux} is the net tissue uptake or release of NE. It is calculated as the product of the veno-arterial difference in NE ($[NE]_v - [NE]_a$) and adipose tissue plasma flow (ATPF).

However, NE transit is bi-directional and a quantitatively large proportion of the NE present in the arterial inflow leaves the vasculature and is cleared in the tissue ($NE_{\text{clearance}}$) (e). $NE_{\text{clearance}}$ is quantitatively larger than spillover, typically in the order of 50% depending on flow rates and other circumstances.

$NE_{\text{spillover}}$ is therefore the sum of NE_{flux} and $NE_{\text{clearance}}$ and is expressed in pmol.100g adipose tissue⁻¹.min⁻¹

$NE_{\text{clearance}}$ is calculated as the product of the fractional extraction of tritiated NE (³H-NE), arterial NE concentration $[NE]_a$ and ATPF under steady state infusion conditions. This relies on the assumption that infused ³H-NE is not released by SNS axons within the time course of the experiment and that it behaves in all other ways as unlabelled NE. Please see text for details of calculations of these quantities and for details of the assumptions made in using this methodology.

Cannulations

Following consent, screening and baseline anthropomorphic measurements, subjects were taken to a clinical area in which the temperature was maintained between approximately 18 and 22⁰C and cannulae were inserted into a superficial abdominal vein for WAT venous effluent sampling, radial artery (following an Allen test to confirm the presence of a viable ulnar arterial supply to the hand), and deep forearm vein (retrograde cannulation of the median cubital vein at the elbow). Additional cannulae were sited in order to maintain isotope infusions.

The abdominal vein cannulations were performed by means of the Seldinger technique whereby a paediatric 22/F gauge 20cm long cannula was passed over a guide wire inserted into a visible superficial abdominal vein and advanced so that its tip was positioned just above the confluence of the external epigastric vein and the sapheno-femoral junction in the groin as judged by surface anatomy. It has been previously shown that the venous blood from this site contains a mixture of effluent from superficial abdominal WAT and a lesser contribution from abdominal skin and does not contain any significant contribution from muscle as there are no known branches that perforate the barrier of the external oblique aponeurosis (Frayn *et al*, 1989). This cannulation was carried out under strict aseptic technique (using chlorhexidine surgical skin prep) and the initial puncture site (always at least 10cm from the actual sampling site due to the length of the catheter) was infiltrated with 1% lignocaine as local anaesthesia. In cases where veins were difficult to cannulate, a cold light source (Brian Reece Scientific Ltd, Berks, UK) and/or heating device (a small electric blanket placed on the abdomen) were used to aid visualisation/induce local vasodilatation but again, the illuminated or slightly warmed areas were anatomically distinct from the actual sampling site. Radial artery cannulation was performed in a standard manner

with a 20 gauge cannula using a minimal amount of 1% lignocaine local anaesthetic and an aseptic technique.

Forearm vein cannulation was designed so that the venous blood would be as far as possible obtained from forearm muscular elements rather than skin or hand circulation. Cannulation was therefore performed retrogradely using an 18 gauge cannula into a deep branch of the median cubital vein using the methods of Coles *et al* (Coles *et al*, 1958). All samples were taken with the hand circulation having been excluded for at least 2 minutes by the inflation of a blood pressure cuff to supra-systolic pressures at the wrist. The placement of the line was checked by blood gas analysis and accepted if the saturation of blood withdrawn was less than 60% (Møller *et al*, 1989). This is because blood draining the hand or skin is known typically to have saturations of greater than 70% and often as much as 80 or 90% due to higher flow rates, lower metabolic activity in the resting state and the presence of multiple functional or anatomical arterio-venous fistulae. However, despite the precautions described for both the forearm and abdominal vein cannulations, a degree of contamination of these effluents by blood draining skin and other tissues is impossible to exclude altogether.

Flow calculations

Forearm blood flow was determined by venous plethysmography (Hokanson Instruments, Bellevue, WA) (Greenfield *et al*, 1963). A mercury strain gauge was placed around the relaxed and supported forearm with hand circulation excluded for at least 2 minutes by a cuff inflated to supra-systolic pressures at the wrist. A further pressure cuff instantaneously inflated to a predetermined level intermediate between arterial and venous pressure (in practice, set at 40mmHg) was placed on the upper arm above the elbow. The change in electrical resistance in the mercury strain gauge was recorded for 3 cardiac cycles following the upper cuff inflation and the peak slope (in %/s) of the resulting trace recorded using Chart for Windows software (AD instruments, Hastings, UK). This figure

represents the volume expansion of the forearm per unit time at the level of the strain gauge when arterial inflow but not venous outflow is permitted relative to the resting volume of that part of the forearm. As one ml of blood and one ml of forearm muscle by definition occupy the same volume, the percentage volume increase due to arterial blood influx may be referred to as mls blood per 100mls of forearm tissue. As forearm tissue comprises mostly muscle with a fixed contribution from bone which will not expand, this figure is considered as blood flow per 100g forearm muscle. Some inaccuracy in these assumptions is inevitable given differences in forearm tissue composition between muscular and less muscular individuals and between those with relatively greater amounts of subcutaneous forearm adipose tissue.

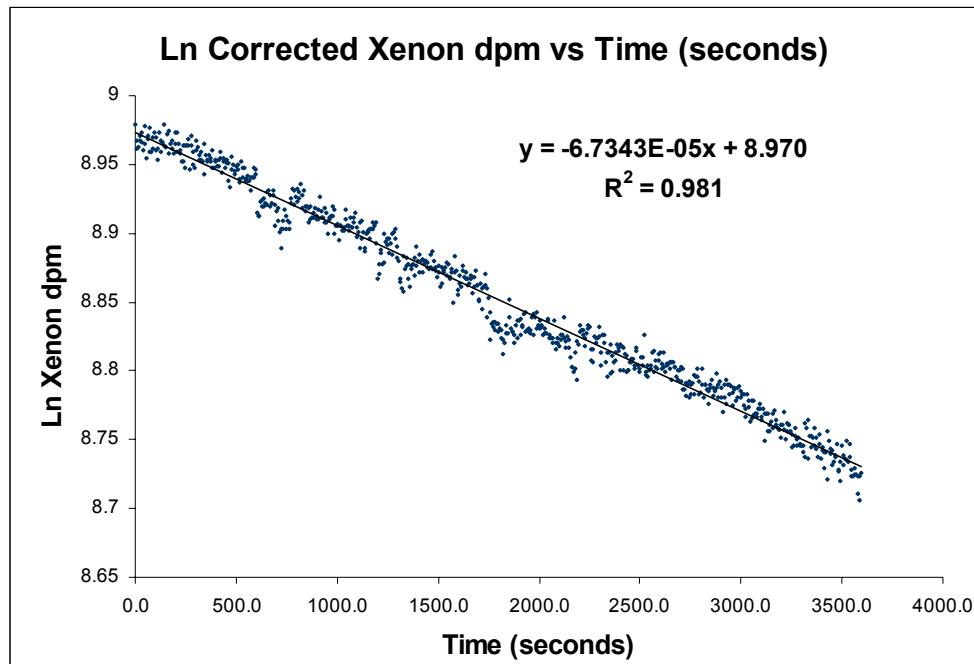
Adipose tissue blood flow was quantified using the ^{133}Xe dispersion method (Larsen *et al*, 1966, Coppack *et al*, 1990, Quaade *et al*, 1967, Nielsen 1972, Prinz *et al*, 1986). Approximately $150\mu\text{Ci}$ of ^{133}Xe (Mallinckrodt Medical, Le Petten, Holland) dissolved in a volume of approximately 0.05 to 0.15 mls saline depending on the specific activity of the isotope was injected using a 26 gauge needle into superficial abdominal AT approximately 3 cm lateral to the umbilicus using a minimally traumatic technique and no local anaesthetic. The injecting needle was carefully withdrawn over at least 45 seconds to avoid leakage along the needle track and the ^{133}Xe depot allowed to equilibrate with AT for at least an hour before measurements were taken. This is to allow the complete dissolution of the ^{133}Xe into AT according to its partition coefficient, λ (Jansson & Lonroth 1995). It has been shown that the gradient of the mono-exponential washout curve detected by a caesium iodide detector (Oakfield Instruments, Eynsham, UK) reflects the blood supply to that area of AT and the results have been validated in animals with micro-spheres and in humans with microdialysis and colour doppler techniques (Nielsen 1972). Prior to calculating the mono-exponential washout curve, the raw counts were corrected against a standard curve of the form

$$y = x / \{1 - (n/x)\}$$

supplied by the manufacturer where y denotes corrected counts, x raw counts and n the maximum raw count detection rate when a highly active source is placed close to the detector (determined experimentally as 40,000). This correction is necessary due to the fact that the number of counts that the Cs iodide cell can detect is finite and the error increased in importance as the maximum level of detection is reached.

The dose of ^{133}Xe administered was chosen as a compromise between this and the introduction of errors from random count variation and background radiation if too small a count rate were attempted. Values of approximately 5000-10,000 counts/s were typically obtained. A typical curve with the natural log of the corrected count rate plotted against time is presented in figure 2.2 below.

Figure 2.2: Log_e corrected ^{133}Xe count vs. time in a representative subject over 1 hour.



Legend to Figure 2.2

Shows natural logarithm (Ln) decay in dpm over time in a representative subject plotted in ‘Microsoft Excel’ to obtain the gradient of the mono-exponential decay curve. For calculations and assumptions used, please refer to text.

It may be shown that the rate constant k of the mono-exponential decay curve of the form $y=a+be^{-kt}$ may be calculated according to the formula

$$k = (\ln y_2 - \ln y_1) / t_2 - t_1$$

where y_1 and y_2 were the counting rates at times t_2 and t_1 min respectively. In practice, k is taken as the gradient of the line of best fit of a plot of $\ln {}^{133}\text{Xe}$ (dpm) vs. time as shown above. This experimentally derived rate constant may then be used to calculate the adipose tissue blood flow (ATBF, mls. 100g^{-1} adipose tissue. min^{-1}) using the formula

$$\text{ATBF} = -100k\lambda$$

where λ is the AT-to-blood partition coefficient for ${}^{133}\text{Xe}$. The value for λ was taken to be 10 ml/g as this has been shown to represent a close approximation in both lean and obese subjects (Jansson & Lonroth 1995).

Subcutaneous adipose tissue plasma flow (ATPF) was calculated as

$$\text{ATPF} = \text{ATBF} \cdot x \text{ (1-hematocrit).}$$

Although well validated, this technique is subject to a number of possible sources of error which are not possible to quantify exactly. These include the fact that some of the measured blood flow will be partitioned to skin, the counting geometry of the Xenon depot may vary over time or between individuals, the count rate is determined over a 30 minute period and the actual blood flow may not be entirely static over this time, tissue distortion by the presence of the volume of saline and/or damage caused by the injection may all influence local blood flow and diffusion of the Xenon may be influenced by unknown local anatomical differences such as the lobular structure of the AT and its microcirculation and interstitial fluid content.

Isotopes

Once the cannulae were in place, the ^{133}Xe was injected as described above and a constant rate infusion of ^3H -Norepinephrine (^3H -NE) was started, the subject being allowed to rest during the equilibration of these isotopes. The tritiated norepinephrine was necessary for the reasons discussed in the legend to figure 2.1. SNS activity was to be quantitated by measurement of a-v differences in NE concentrations multiplied by the appropriate tissue blood flow. This is possible because NE is released from SNS terminals and undergoes one of a number of fates (Esler *et al*, 1990). Much of it is taken back up into the nerve terminal that released it by a process (uptake 1) which results in its metabolism by the intraneuronal enzyme, monoamine oxidase (MAO). NE that escapes this reuptake mechanism is subject to degradation elsewhere in the tissue (uptake 2) by non-neuronal catechol-o-methyl transferase (COMT). However, a small proportion (estimated at around 15%) escapes both of these processes and diffuses into the perfusing blood allowing the measurement of a-v differences. It has been shown that the proportion of NE that ‘spills over’ (norepinephrine spillover) into blood is directly proportional to that released at SNS terminals over a wide range of SNS activity (Esler *et al*, 1990) thus permitting semi-quantitative assessment of SNS activity locally within a tissue by measurement of the NE flux (product of a-v difference and flow) from that tissue. However, this calculation alone is not sufficient as in a single passage through the human forearm, for example, a variable amount (up to 50%) of circulating NE is removed. Thus NE is both taken up from and simultaneously released into blood in passage through tissues and this will render assessment of NE production on the basis of net flux alone inadequate. However, the proportion of tissue uptake of NE may be calculated from the assessment of the simultaneous uptake of radio-labelled infused ^3H -NE. This is then converted into tissue clearance of NE by taking the product of perfusing arterial concentration and fractional uptake of NE. The net flux may then be converted into a true NE spillover measurement according to the formula

$$\text{NE}_{\text{spillover}} = \text{Net NE}_{\text{flux}} + \text{Tissue NE}_{\text{clearance}}$$

If $[\text{NE}]_v$ and $[\text{NE}]_a$ represent venous and arterial NE concentrations respectively and ATPF is adipose tissue plasma flow,

$$\text{Net NE}_{\text{flux}} = ([\text{NE}]_v - [\text{NE}]_a) \times \text{ATPF}$$

and

$$\text{Tissue NE}_{\text{clearance}} = \text{NE}_{\text{fractional extraction}} \times [\text{NE}]_a \times \text{ATPF}$$

$\text{NE}_{\text{fractional extraction}}$ ($f_{\text{ex}}\text{NE}$) is calculated with the use of the isotope enrichment and represents the fractional amount of isotope activity removed from the arterial plasma in a passage through the tissue. Thus, where SA_a and SA_v are NE specific activities in arterial and venous samples, respectively,

$$f_{\text{ex}}\text{NE} = \frac{([\text{NE}]_a \times \text{SA}_a) - ([\text{NE}]_v \times \text{SA}_v)}{[\text{NE}]_a \times \text{SA}_a}$$

Adipose tissue NE spillover rate, AT $[\text{NE}]_{\text{spillover}}$, was therefore calculated as

$$[\text{NE}]_{\text{spillover}} = \left\{ ([\text{NE}]_v - [\text{NE}]_a) + ([\text{NE}]_a \times \frac{([\text{NE}]_a \cdot \text{SA}_a) - ([\text{NE}]_v \cdot \text{SA}_v)}{[\text{NE}]_a \cdot \text{SA}_a}) \right\} \times \text{ATPF}$$

Some of the assumptions inherent in these calculations include the fact that radial artery [NE] concentration may not be exactly equal to that in the arteries actually perfusing abdominal AT and the possibility that isotope effects may exist (i.e. that the fact that the labelled NE is physically different to the ‘native’ molecule may affect its tissue metabolism). It must further assume that labelled NE is not taken up, transported and released by SNS axons. However, in addition to permitting

estimates of tissue clearance, this technique does permit assessment of contributions to NE metabolism by uptakes 1 and 2 if labelled moieties of metabolites are measured in subsequent assays as the intermediate and end products of these pathways are quite distinct (Esler *et al*, 1990).

The isotope used was levo-[ring-2,5,6-³H] (New England Nuclear, Boston, MA) provided in acetic acid/ethanol (9:1) with specific activity range 40-80 Ci/mmol, purity >97%, optical purity>99%, MW 169.2. This was diluted into aliquots with ascorbic acid preservative under aseptic conditions and the vials defrosted immediately prior to infusion. Whole body kinetics for NE are calculated according to the formula

$$\text{Whole body rate of appearance} = \frac{[{}^3\text{H}]\text{-NE isotope infusion rate (dpm/min)}}{\text{specific activity (dpm/nmol)}}$$

Rongen (Rongen *et al*, 2000) has observed that spillover assumptions appear to hold under steady conditions but may be affected by changes in blood flow in non-steady state conditions. He proposed a mathematical correction of spillover, namely appearance rate (AR), which appears robust to this where

$$\text{AR} = \frac{\text{Spillover}}{(1 - f_{ex}\text{NE})}$$

and calculations made using this correction are presented where appropriate alongside those using the assumptions of Esler (Grassi & Esler 1999).

Sampling and assays

Once all the isotope infusions have been allowed to reach steady state, samples were drawn simultaneously from all three sampling sites and were taken directly into cooled lithium-heparin tubes (Monovet, Sarstedt, Germany). They were stored on ice until centrifugation for 15 minutes at 3000 rpm whereupon the plasma was separated and aliquoted within 30 minutes of being drawn and plasma stored at -70°C until assay. All assays were performed in a single batch in the lab of Prof G Eisenhofer (NIH, Bethesda, Washington, USA) and appropriate internal controls and standards used. Plasma [NE] concentration was determined by a single isotope derivative radio-enzymatic method and plasma [^{3}H]-NE specific activity was determined after organic extraction (Shah *et al*, 1985, Eisenhofer *et al*, 1986). Samples of the infusates were taken in order to measure specific activities and the rates of isotope infusion noted in order to permit calculation of whole body kinetics.

METHODS FOR OTHER STUDIES

Cannulations were used in the other studies and the techniques used were similar to those outlined above. A euglycaemic hyperinsulinaemic clamp was employed to obviate spontaneous insulin pulses in the studies described in chapters V and VI.

Insulin Clamp Methodology

A hyperinsulinaemic-euglycemic clamp was established by infusing insulin, using a primed-constant infusion protocol (DeFronzo *et al*, 1979) with the final infusion rate $35 \text{ mU. m}^{-2}. \text{min}^{-1}$.

Histological Techniques

These are integral to the relevant chapter and are therefore described fully in Chapter VII.

CHAPTER III

GLUCOSE AND KETONE BODY METABOLISM IN HUMAN DEEP AND SUPERFICIAL FOREARM AND SUBCUTANEOUS ABDOMINAL TISSUE

INTRODUCTION

Peripheral (non-splanchnic) tissues are important sites of uptake of oxidative fuels such as glucose and ketone bodies. Skeletal muscle, brain and myocardium are regarded as the major sites of oxygen uptake (Ferrannini & DeFronzo 1997) but even in healthy lean subjects (e.g. ~70 kg man) the skin (~6 kg) and adipose tissue (~12 kg) are large organs which could contribute significantly to glucose and ketone body uptake, as well as lactate production (Ferrannini & Groop 1989, Buchalter *et al*, 1989). There are relatively few data *in vivo* about the uptake of oxidative fuels by skin and adipose tissue.

The Fick principle, or arterio-venous difference method, has been extensively used to study metabolism *in vivo* in man (Zierler 1961). However, arterio-venous differences studies require identification of suitable vascular beds for use as models of the tissue of interest. Both the deep forearm and the leg have been used to study skeletal muscle metabolism (Andres *et al*, 1956, Coles *et al*, 1958, Mottram & Butterfield 1961, Wahren 1966, Kaijser & Rössner 1975, Rössner *et al*, 1976, Jackson *et al*, 1986, Møller *et al*, 1989). For the study of subcutaneous adipose tissue, Zierler's group (Baltzan *et al*, 1961) proposed that the superficial forearm tissue was a suitable bed, and this model was used extensively (Andres *et al*, 1956, Wahren 1966, Kaijser & Rössner 1975, Rössner *et al*, 1976, Jackson *et al*, 1986, Jackson *et al*, 1987). Frayn suggested that a subcutaneous abdominal vein yields samples more closely reflecting adipose tissue metabolism (Frayn *et al*, 1989).

In many resting tissues glucose is utilised as a substrate for anaerobic glycolysis, lactate being the end-product released by the tissue (Buchalter *et al*, 1989, Andres *et al*, 1956, Kreisberg 1972). Both skin and adipose tissue appear to use anaerobic glycolysis more than resting muscle. Lactate production by skin has been shown by studies *in vitro* (Hsia 1971, Johnson & Fusaro 1972). Similarly, *in vitro* (Crandall *et al*, 1983, Newby *et al*, 1990) and microdialysis studies (Jansson

et al, 1990, Hagström *et al*, 1990, Simonsen *et al*, 1994) show lactate production by adipose tissue. Arterio-venous difference studies both of the superficial forearm (Kaijser & Rössner 1975, Jackson *et al*, 1987) and of subcutaneous abdominal tissue (Simonsen *et al*, 1994, Coppack *et al*, 1990a, Coppack *et al*, 1990b) show lactate production. However the quantitative importance of these peripheral tissues for whole body lactate production remains uncertain (Kerckhoffs *et al*, 1998, Coppack *et al*, 1996).

Although the superficial forearm has little subcutaneous adipose tissue and relatively thick skin compared to the superficial abdominal site, previous authors have considered the oxidative fuel uptake of adipose tissue and skin to be generally similar (Jackson *et al*, 1987, Johnson & Furaso 1972). The aim of our study was to examine glucose and ketone body metabolism in deep forearm and superficial tissues and in particular to compare the characteristics of the superficial forearm tissues and subcutaneous abdominal tissues as described by Zierler (Baltzan *et al*, 1961) and Frayn (Frayn *et al*, 1989), respectively. We therefore undertook a study combining arterio-venous difference techniques across superficial forearm tissue, subcutaneous abdominal tissue and deep forearm tissue (Andres *et al*, 1956, Coles *et al*, 1958, Mottram & Butterfield 1961, Wahren 1966, Kaijser & Rössner 1975, Jackson *et al*, 1986) in normal healthy subjects. Measurements were made in the post-absorptive state and during hyper-insulinaemia induced by glucose infusion. We wished to test the hypothesis that these two superficial tissue beds would have similar patterns of metabolism.

METHODS

Subjects

Approval for the study was given by the Local Research Ethics Committee. Thirteen healthy male volunteers gave written, informed consent. All volunteers were non-smokers and taking no medications. Subject data is shown in Table 3.1. All subjects were well nourished, weight stable and had normal biochemical screen, haematology screen and urinalysis.

Table 3.1: Characteristics of volunteer subjects

Characteristic	Median (inter-quartile range)
Age (years)	26 (23, 29)
Weight (kg)	73.0 (70.0, 82.6)
Body mass index (kg.m^{-2})	23.6 (22.8, 24.8)
Percentage body fat	17.4 (15.8, 17.8)
Total body muscle mass (kg)	31.5 (29.7, 32.5)
Total body skin mass (kg)	5.84 (5.59, 6.61)
Total body fat mass (kg)	13.0 (10.5, 14.7)

Legend to Table 3.1:

Baseline characteristics of the study subjects. Please see Methods for details of deriving body composition data.

Protocol

The subjects came to the Clinical Research Centre at 1700 h and on arrival ate a standard meal providing 20 kcal•kg lean body mass (LBM)⁻¹ with 50% of calories as carbohydrate, 30% as fat, and 20% as protein. Thereafter the subjects ate nothing and consumed only clear (decaffeinated and calorie-free) fluids. Body composition was determined by dual energy X-ray absorptiometry (Lunar Instruments, Madison, WI). We chose to study healthy, non-obese, young males since it was expected that they would have a relatively consistent thickness of subcutaneous tissue deep to the skin of the anterior forearm. This was the case, as skin fold thickness in this region was 2-4 mm in all subjects.

Subjects awakened at 0700 h. Blood sampling cannulae were inserted (using local anaesthesia) into a radial artery, a subcutaneous abdominal vein (Frayn *et al*, 1989), a superficial forearm vein (Wahren 1966, Rössner *et al*, 1976, Jackson *et al*, 1986, Baltzan *et al*, 1961, Jackson *et al*, 1987) and a deep forearm vein (Andres *et al*, 1956, Coles *et al*, 1958, Mottram & Butterfield 1961, Wahren 1966, Kaijser & Rössner 1975, Rössner *et al*, 1976, Jackson *et al*, 1986). The subcutaneous abdominal vein was sampled from a cannula whose tip was just inferior to the inguinal ligament (by surface anatomy) (Frayn *et al*, 1989, Coppock *et al*, 1989). The forearm sampling cannulae were contralateral to the arterial cannulae. The median antebrachial vein or another tributary of the basilic vein (Gelfand & Barrett 1987) was cannulated to obtain superficial drainage from the forearm, the cannula tip being 4-12 cm inferior to the medial epicondyle (Andres *et al*, 1956, Wahren 1966, Kaijser & Rössner 1975, Baltzan *et al*, 1961, Heaf *et al*, 1977). In 2 subjects superficial veins did not permit drawing of adequate samples and the superficial veins of these subjects were excluded from further consideration. Deep forearm (antecubital, usually the median cubital) veins were cannulated retrogradely (Andres *et al*, 1956, Coles *et al*, 1958, Mottram & Butterfield 1961, Wahren 1966, Kaijser & Rössner 1975, Rössner *et al*, 1976,

Jackson *et al*, 1986, Møller *et al*, 1989). In one subject, no anatomically suitable deep antecubital vein could be cannulated. Samples from forearm veins were always taken after exclusion of hand circulation for 2 minutes by applying a sphygmomanometer cuff inflated to 60-100 mmHg above arterial pressure.

Oxygen saturation was measured in each vein. If initial deep forearm venous O₂ was above 60 % (Møller *et al*, 1989) the cannula was moved in an attempt to achieve acceptable values. If subsequent deep venous O₂ saturations were not below 60 % then these samples were not considered further as recommended by Møller (Møller *et al*, 1989). The samples from an additional superficial vein were discarded because oxygen O₂ saturation was not consistently >70%.

An infusion cannula was inserted in a forearm vein, ipsilateral to the cannulated artery. All cannulae were kept clear with constant infusions of sterile 150 mM NaCl at 30 mL.h⁻¹.

After inserting the cannulae (at -90 min), 50 µCi ¹³³Xe in sterile saline was injected into the subcutaneous abdominal adipose tissue in the drainage of the abdominal vein being sampled to estimate adipose tissue blood flow as described in chapter 2 ‘Methods’ (Coppock *et al*, 1990, Quaade *et al*, 1967, Nielsen 1972, Prinz *et al*, 1986).

Blood samples were drawn from all sampling cannulae at -30, -20, -10 and -1 min. At time ‘0’ an infusion of sterile 50% glucose was infused at 8 mg.min⁻¹.kg body weight⁻¹. In some subjects, this infusion rate was associated with discomfort near the infusion site, and the rate was reduced when necessary. Infusion rates actually achieved were between 6-8 mg.min⁻¹.kg body weight⁻¹ for all subjects.

At 60 minutes a sample was taken from the arterial cannula. All cannulae were sampled at 90, 100, 110 and 120 min.

Adipose tissue blood flow was estimated between -30 and -1 min as well as

between 90 and 120 min. This was done by measuring the washout of $^{133}\text{Xenon}$ from the tissue depot, assuming a tissue partition co-efficient of 0.82 ml/g (Coppock *et al*, 1999, Nielsen 1972).

Forearm blood flow was measured using venous occlusion strain-gauge plethysmography (Hokansen, Bellview, WA) (Coppock *et al*, 1999, Whitney 1953, Cooper *et al*, 1955). Blood flow was apportioned to superficial and deep forearm tissues according to the data of Cooper who examined the effect of ‘switching’ off superficial flow with iontophoresed vasoconstrictor (Cooper *et al*, 1955).

Sample handling and analyses

Blood samples were collected in heparinized blood-gas syringes and kept on ice. Oxygen content was subsequently measured on a co-oximeter (Instrumentation Laboratories 282, Lexington KY). Blood pH was determined on a blood gas analyzer (Instrumentation Laboratories 1302). An aliquot was taken into 1% perchloric acid for analysis of ketone bodies and pyruvate.

Other blood samples were taken and immediately transferred to pre-cooled tubes containing EDTA and kept on ice until centrifuged at 4°C, which was done within 30 minutes of the sample being drawn. Plasma was then stored at -70°C.

Glucose, lactate, pyruvate, β -hydroxybutyrate and acetoacetate were measured by enzyme-linked fluorimetric analyses (Frayn *et al*, 1990).

Plasma insulin concentration was determined by radioimmunoassay (Coppock *et al*, 1999) and haematocrit by centrifugation. Assay results are reported in the fluid assayed e.g. plasma for NEFA, whole blood for oxygen. Conversions between plasma and whole blood are used for whole-body estimates, and other derived values (e.g. percentage of oxygen consumption accounted for by fuels).

Calculations and Statistics

Net arterio-venous difference was calculated by subtraction of the venous concentration from the arterial concentration. Thus a negative arterio-venous difference indicates net release, and a positive arterio-venous difference indicates net uptake. Net fluxes were calculated by multiplication of the arterio-venous difference by the tissue whole blood or plasma flow, depending upon which was appropriate.

We estimated, by extrapolation, the lactate production that could be accounted for by the whole-body muscle mass (WBMM), whole-body skin (WBS) and whole-body fat mass (WBFM) in these subjects. To do this we measured the WBFM by DEXA and took the WBMM to be 50% fat free mass (Elia *et al*, 1988). We assumed that the whole of the WBMM behaved the same as the deep forearm tissue studied here and that the whole of the WBFM behaved the same as the subcutaneous abdominal tissue studied here. These assumptions are considered in the Discussion. The density of muscle was taken as 1.10 g.ml^{-1} (Clasey *et al*, 1999).

Because we could not be sure that the observed values were normally distributed, results are given as medians (interquartile range). Comparisons between groups were done using Mann-Witney and Wilcoxon's tests for unpaired and paired data respectively. A probability value of $p<0.05$ was taken to be statistically significant.

RESULTS

Blood oxygen content

In all 13 subjects, the oxygen saturations always ranked arterial (highest), abdominal vein, superficial forearm vein, deep vein (lowest) at all time points (Table 3.2). However, since O₂ saturations were not consistently below 60% in the deep vein samples of 3 subjects, and not consistently above 70% in the superficial forearm vein samples of 1 subject, these venous samples were not analysed further and are excluded from further presentation. Thus data presented relate to n=13 for arteries and abdominal veins, n= 9 for deep forearm veins and n=10 for superficial forearm veins.

The oxygen contents of the arterial blood and the arterio-venous differences across deep forearm, superficial forearm and subcutaneous abdominal tissue are shown in Table 3.2.

Post-absorptive conditions

Arterial concentrations were steady during the post-absorptive measurement period (time -30 to -1 in Figure 3.1)

Net arterio-venous differences in the post-absorptive state are shown in Table 3.2 and the fluxes (i.e. arterio-venous difference multiplied by the blood/plasma flow) in Figures 3.2 and 3.3. Subcutaneous abdominal tissue tended to take up less glucose than deep forearm ($p =0.07$), but there were no significant differences in the post-absorptive glucose uptake between tissues. Lactate output was seen consistently in all subjects for the two superficial tissue beds in the post-absorptive state, being significantly greater from superficial forearm than from either of the other two depots ($p<0.05$). Deep forearm lactate metabolism showed

net release in some subjects and net uptake in others. Pyruvate flux was significantly different ($p<0.05$) between deep forearm tissue (net uptake in most subjects) and the two superficial tissues (which released pyruvate in all subjects). Pyruvate release was not significantly different between the superficial forearm and subcutaneous abdominal tissue beds.

From the results shown in Table 3.3, it can be calculated that the net proportion of glucose uptake that was released as lactate (i.e. anaerobic glycolysis) was 95.2 (50.9,137) % for superficial forearm tissue, a significantly higher fraction ($p<0.05$) than those for deep forearm (24.6 (3.7, 52.4) %) or subcutaneous abdominal tissue (20.9 (10.0, 40.0)%). Net glucose uptake (allowing for lactate and pyruvate balance) could account for only 5.3 (-6.2, 36.0) % of superficial forearm oxygen uptake. The equivalent value for deep forearm tissue and subcutaneous abdominal tissue tended to be greater at 27.8 (16.5, 49.1)% and 66.3 (17.3, 110)%, respectively (not significant).

Post-absorptive NEFA efflux was significantly different between all three tissues (i.e. deep forearm less than both other tissue beds ($p<0.02$), superficial forearm release was less than subcutaneous abdominal tissue ($p<0.01$)). Ketone body uptake was greater in both forearm tissues than in subcutaneous abdominal tissue ($p<0.05$).

Table 3.2: Arterio-venous differences during the Post-absorptive state

		Net a-v differences		
	Arterial concentration	Forearm muscle	Forearm skin	Adipose tissue
Glucose	4338 (4025, 4408)	195 (163, 351)	151 (57, 263)	121 (23, 368)
Lactate	869 (705, 1050)	-152 (-240,-14)	-214 (-426,-167)*	-118 (-194,-65)
Pyruvate	30 (27, 50)	2 (-5, 19)	-8 (-14,11)	-4 (14,-2)*
NEFA	521 (381, 651)	24 (7, 62)	-209 (-69, -326)*	-665 (-379, -870)*†
β-Hydroxy-butyrate	153 (61, 249)	44 (21, 80)	40 (12, 51)	7 (2, 10)*†
Aceto-acetate	81 (48, 103)	29 (-7, 58)	19 (-2, 35)	10 (3, 20)
Oxygen	8092 (7387, 8674)	3949 (3213, 4532)	1437 (1098, 1747)*	575 (488, 653)*†

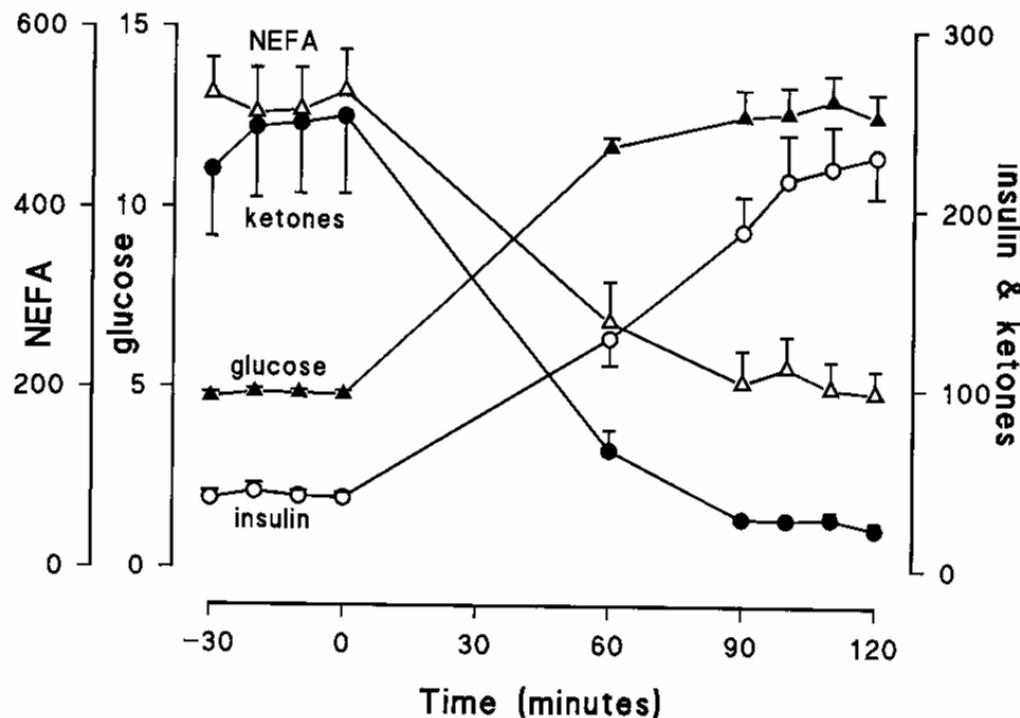
Legend to Table 3.2:

A positive value indicates uptake. A negative uptake value indicates release. All values are medians (interquartile range) and in $\mu\text{mol.l}^{-1}$. NEFA are plasma concentrations, all other values are whole blood concentrations.

* = significantly different from forearm muscle, $p<0.05$

† = significantly different from forearm skin, $p<0.05$

Figure 3.1: Changes in NEFA, Ketones, Glucose and Insulin in response to Glucose infusion



Legend to Figure 3.1:

Changes with time in concentrations of plasma non-esterified fatty acids (Δ), whole blood ketone bodies (●), whole blood glucose (\blacktriangle) and plasma insulin (○). Glucose infusion ($6\text{-}8 \text{ mg.kg body weight}^{-1}.\text{min}^{-1}$) commenced at time zero. Values are medians (\pm interquartile range).

For glucose, values are in mmol.l^{-1} . For NEFA and ketone bodies, values are in $\mu\text{mol.l}^{-1}$. For insulin, values are in pmol.l^{-1} .

Change during glucose infusion

As expected, arterial glucose, lactate, pyruvate and insulin concentrations rose in all subjects whereas NEFA and ketone body concentrations declined ($p<0.02$ for all). Figure 3.1 shows the changes of arterial glucose, total ketone bodies and insulin with time during glucose infusion. For ketone bodies, the values were near to the limits of assay detection, so caution is necessary for interpretation of these results.

Adipose tissue blood flow declined significantly ($p<0.05$) from 4.67 (3.79, 6.03) to 3.33 (2.53, 5.35) ml.min $^{-1}$.100g tissue during the glucose infusion. Estimated muscle blood flow and skin blood flow changed little from the post-absorptive value of 3.27 (3.04, 3.44) to 3.64 (2.99, 3.94) ml.min $^{-1}$.100 ml tissue and from 7.09 (3.77, 8.20) to 8.20 (4.40, 11.04) ml•min $^{-1}$ •100 ml tissue $^{-1}$, respectively, during the glucose infusion.

Table 3.3: Arterio-venous concentration differences during glucose infusion

		Net a-v differences		
	Arterial concentration	Forearm muscle	Forearm skin	Adipose tissue
Glucose	11523 (11227, 12796)	1174 (782, 2120)	697 (216, 1231)	447 (167, 1450)*
Lactate	1105 (935, 1311)	-46 (-6, -86)	-204 (-135, -263)*	-104 (-58, -229)*
Pyruvate	78 (57, 105)	15 (-3, 30)	-1 (-14, 9)	-3 (0, 13)*
NEFA	182 (104, 275)	32 (-27, 98)	-63 (-1, -75)	-12 (9, -43) †
β-hydroxy butyrate	7 (6, 13)	-1 (-2, 2)	-3 (-4, -2)	-1 (-3, 1)
Aceto-acetate	15 (11, 22)	2 (-4, 11)	-2 (-7, 9)*	-3 (-8, 3)

Legend to Table 3.3:

A positive value indicates uptake. A negative value indicates release. All values are medians (interquartile range) and in $\mu\text{mol.l}^{-1}$. NEFA are plasma concentrations, all other values are whole blood concentrations.

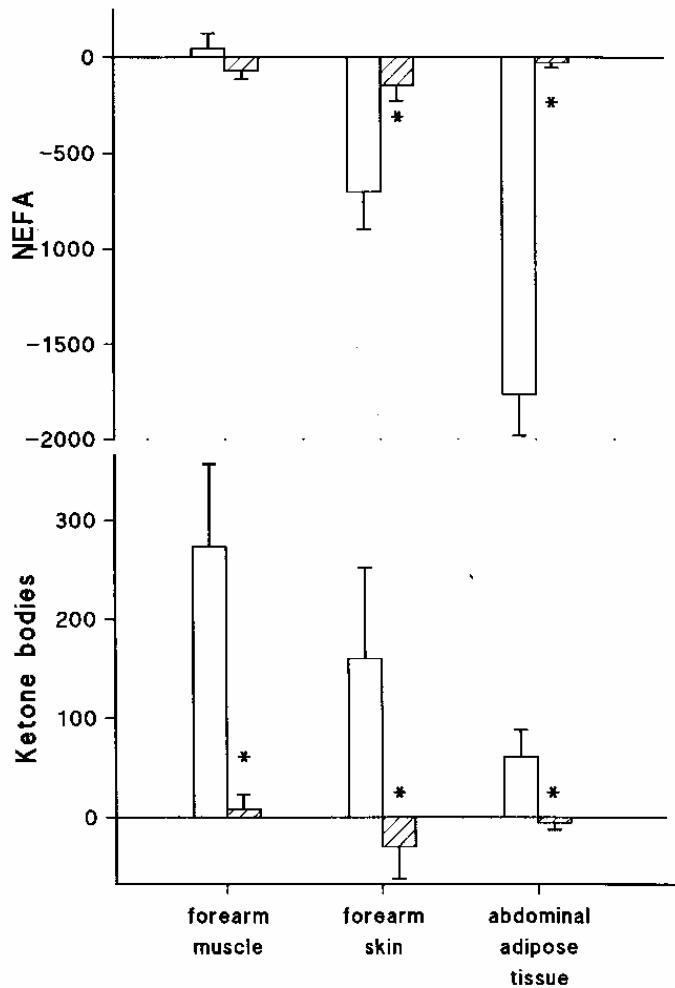
* = significantly different from forearm muscle, $p<0.05$

† = significantly different from forearm skin, $p<0.05$

The arterio-venous differences during glucose infusion are shown in Table 3.3 and the fluxes during glucose infusion in Figures 3.2 and 3.3. As indicated, the glucose flux for deep forearm and superficial forearm tissue increased significantly, whilst the fluxes for fatty acids and ketone bodies declined across all tissues. Notably, in subcutaneous abdominal tissue, the glucose flux across subcutaneous abdominal tissue tended to increase, but the change did not reach statistical significance. During the glucose infusion, deep and superficial forearm tissues both took up significantly more glucose than did subcutaneous abdominal tissue ($p<0.05$).

The suppression of fatty acid release was greater ($p<0.02$) in subcutaneous abdominal tissue than in the superficial forearm tissue both in absolute and percentage terms (Figure 3.2).

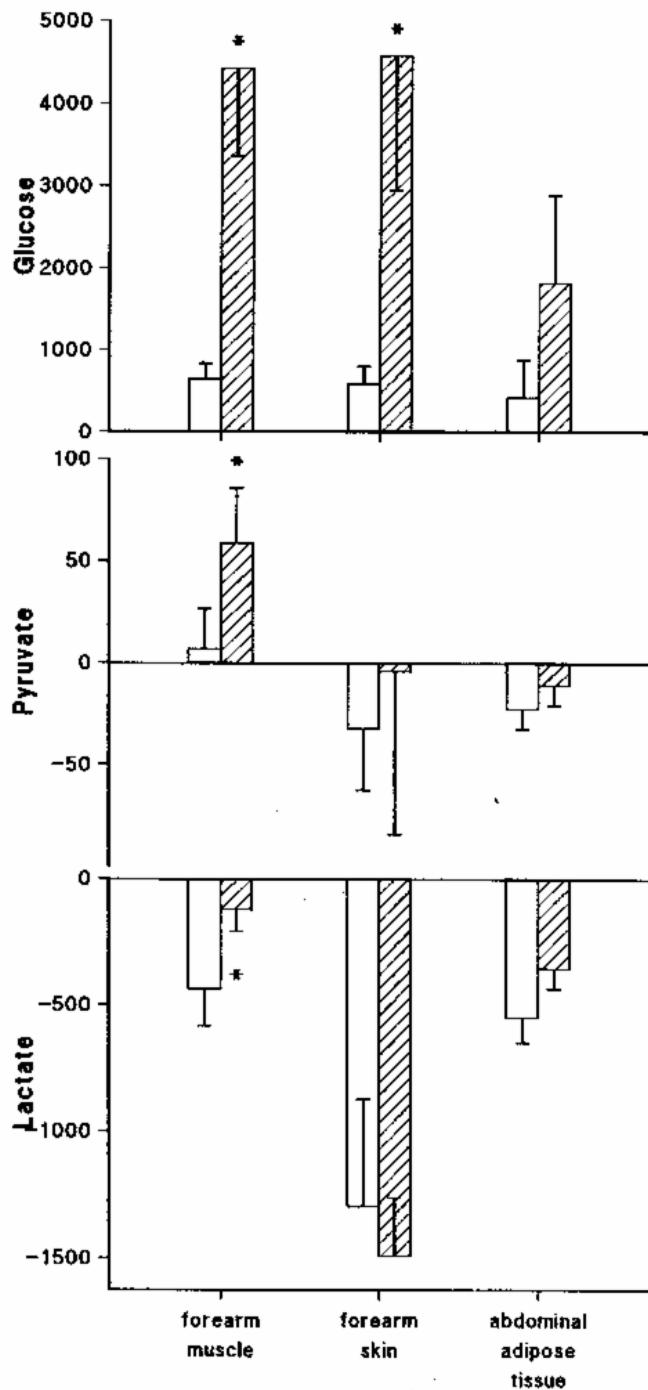
Figure 3.2: NEFA and Ketone fluxes in the 3 tissues before and during glucose infusion



Legend to Figure 3.2:

Median (\pm interquartile range) values of fluxes (in $\text{nmol.}100 \text{ g}^{-1}.\text{min}^{-1}$) for non-esterified fatty acids (upper panel) and ketone bodies (lower panel). The open bars indicate results during the post-absorptive state, the hatched bars indicate results during glucose infusion. Positive flux values indicate uptake, negative values indicate release. Values during glucose infusion that are significantly different ($p < 0.05$) from post-absorptive are indicated (*).

Figure 3.3: Glucose, Pyruvate and Lactate Fluxes in the 3 tissues before and during Glucose infusion



Legend to Figure 3.3:

Median (\pm interquartile range) values of fluxes (in $\text{nmol.}100 \text{ g}^{-1}.\text{min}^{-1}$) for glucose (upper panel), pyruvate (middle panel) and lactate (lower panel). The open bars indicate results during the post-absorptive state, the hatched bars indicate results during glucose infusion. Positive flux values indicate uptake, negative values indicate release. Values during glucose infusion that are significantly different ($p<0.05$) from post-absorptive are indicated (*).

Whole body extrapolations

Assuming the whole body muscle mass behaved like the deep forearm tissue in these subjects, the whole body muscle mass would consume 186 (154, 272) $\mu\text{mol}.\text{min}^{-1}$ of glucose and release 127 (14, 252) $\mu\text{mol}.\text{min}^{-1}$ of lactate.

Extrapolation of the current superficial forearm tissue results to the whole body skin mass would suggest that skin consumes less glucose (33 (25, 96) $\mu\text{mol}.\text{min}^{-1}$) than whole body muscle ($p<0.05$), but produces a comparable amount of lactate (84 (44, 198) $\mu\text{mol}.\text{min}^{-1}$). Likewise, whole body estimates of glucose consumption by adipose tissue 53 (8, 220) $\mu\text{mol}.\text{min}^{-1}$ were less ($p<0.05$) than those of whole body muscle mass. The estimated whole body fat mass lactate production rate was 70 (32, 120) $\mu\text{mol}.\text{min}^{-1}$.

DISCUSSION

As expected, the deep forearm tissue, which has been extensively studied as a model for muscle metabolism (Andres *et al*, 1956, Coles *et al*, 1958, Mottram & Butterfield 1961, Wahren 1966, Kaijser & Rössner 1975, Jackson *et al*, 1986), behaves differently from the two superficial tissue beds. The novelty of the current study is in the differences seen between these superficial tissues, notably (a) in the post-absorptive state the superficial forearm tissue tended to take up more glucose than the subcutaneous abdominal tissue; (b) superficial forearm glucose uptake was mostly converted to lactate; (c) superficial forearm glucose uptake increased more during glucose infusion and became significantly greater than that in the abdominal tissue; (d) conversely, the superficial forearm tissue released less fatty acids post-absorptively; but (e) this release was less completely suppressed during glucose infusion.

Results in this study are consistent with the interpretation that forearm superficial tissue in lean men drains primarily skin with little contribution from subcutaneous fat, whilst the subcutaneous abdominal tissue is predominantly adipose tissue with a proportionately smaller amount of skin. This supports previous suggestions (Kaijser & Rössner 1975, Simonsen *et al*, 1994), although a direct comparison has not been reported.

As mentioned above, in the post-absorptive state, the glucose arterio-venous differences and fluxes were not significantly different between the three sites but the superficial abdominal tissue tended to take up least glucose (Table 3.2 & Figure 3.3). Previous work has consistently reported a lower uptake (usually significantly lower) of glucose by abdominal subcutaneous tissue compared to deep forearm tissue (Coppock *et al*, 1990, Coppock *et al*, 1996, Horowitz *et al*, 2001). Previous studies showed lactate production by superficial forearm tissue (Kaijser & Rössner 1975, Jackson *et al*, 1987). The current study showed this

lactate production by superficial forearm tissue was higher than that of the other two tissues (Table 3.2, Figure 3.3). This superficial forearm lactate production could account for most of that tissue's glucose uptake which would be compatible with the high conversion rates of glucose to lactate seen in studies *in vitro* of skin (Newby *et al*, 1990, Newby *et al*, 1988, Mårin *et al*, 1987). As expected from previous work (Baltzan *et al*, 1961, Coppack *et al*, 1999), both superficial tissues released significant amounts of fatty acids (NEFA), whilst the deep forearm took up NEFA. The NEFA arterio-venous difference and NEFA flux were both significantly greater ($p<0.02$) in abdominal subcutaneous than forearm superficial tissue (Table 3.2, Figure 3.2), reinforcing the idea that this depot contains more adipose tissue than skin.

During glucose infusion, the glucose uptake of both the forearm tissues increased significantly ($p<0.05$), whilst that of the subcutaneous abdominal tissue did not. This led to the glucose arterio-venous difference in deep forearm and the glucose fluxes for both deep and superficial forearm tissue being greater ($p<0.05$) than that of the abdominal tissue during glucose infusion (Table 3.3, Figure 3.3). It is well-known that hyperglycaemia and hyperinsulinaemia increases deep forearm glucose uptake. The lack of such an increase in the abdominal tissue that we believe to represent mostly adipose tissue is surprising in relation to some previous *in vitro* work (Green & Newsholme 1979, Zeuzem & Taylor 1985). However, this observation is in line with previous arterio-venous difference findings (Coppack *et al*, 1996, Coppack *et al*, 1989) and other studies suggesting that insulin produces only a modest increase in glucose uptake by adipose tissue (Mårin *et al*, 1987, Hjöllund *et al*, 1985).

Also as expected (Kerckhoffs *et al*, 1998, Coppack *et al*, 1999, Coppack *et al*, 1989, Frayn *et al*, 1994), the insulinaemic response to glucose infusion reduced lipolysis in subcutaneous abdominal tissue. During the same period the release of NEFA from superficial forearm tissue also declined significantly ($p<0.05$). However the inhibition of NEFA release in both absolute and percentage terms

was less in forearm than abdominal tissue. This observation perhaps parallels the observation that lipolysis in the upper-body is more resistant to insulin's anti-lipolytic effect than is lipolysis in the lower-body (Jensen 1991, Martin & Jensen 1991, Roust & Jensen 1993).

The importance of these tissues to whole body glucose metabolism can be estimated by extrapolation. Our data suggest that postabsorptive skin and adipose tissue are only minor consumers of glucose ($\approx 4\%$ and $\approx 7\%$ of whole body, respectively). Such assumptions should be interpreted conservatively because of heterogeneity between adipose tissue depots (Jensen 1991, Jansson *et al*, 1992). Estimates of adipose tissue glucose uptake based upon uptake of ^{14}C -labelled glucose into a single adipose depot are low (Mårin *et al*, 1987, Mårin *et al*, 1992). However, that method may underestimate glucose uptake because it only measures ^{14}C -glucose remaining in the tissue at the time of biopsy and takes no account of isotope taken up and then re-released as lactate, glycerol or CO_2 . Previous arterio-venous estimates of post-absorptive whole body adipose tissue glucose uptake have been $11 \pm 2\%$ for lean female subjects (Horowitz *et al*, 2001) and $\sim 5\%$ in lean subjects of mixed sex (Coppock *et al*, 1996). In obese sex-matched groups, the equivalent estimates were $21 \pm 4\%$ and $\sim 7\%$ (Coppock *et al*, 1996, Horowitz *et al*, 2001), but those studies share the same limitation as the current one in terms of extrapolation from a single depot to the whole body.

Transport of monocarboxylates such as lactate and pyruvate across the plasma membrane has a role in the metabolism and pH regulation of most cells (Manoharan *et al*, 2006). Monocarboxylate transport is mediated by a family of monocarboxylate transporters (MCTs) of which there are now known to be 14 members, although only for members 1–4 has lactate and pyruvate transport been demonstrated directly (Halestrap & Meredith 2004). To be translocated correctly to the plasma membrane, MCTs 1–4 require the ancillary protein basigin (also known as CD147) or its homologue, embigin (also known as gp70) (Fanelli *et al*, 2003). Adipose tissue and skin have been proposed (Jansson *et al*, 1990,

Hagström *et al*, 1990, Kerckhoffs *et al*, 1998, Mårin *et al*, 1987) as important sources of lactate production. Our data confirm that these two tissues do produce lactate. However, whole-body extrapolation suggests that net adipose tissue production is a small proportion (~ 8%) of whole body lactate production (as reviewed by Buchalter (Buchalter *et al*, 1989)). The equivalent contribution of skin would be only slightly greater (~ 9%). The hyperlactatemia seen after glucose infusion is accompanied by no significant change in the lactate release by either superficial tissue. The lactate output by deep forearm tissue was reduced during glucose infusion. This was also seen following an oral glucose load in Jackson's study (Jackson *et al*, 1987) but not in other studies (Coppock *et al*, 1990, Kerckhoffs *et al*, 1998).

The subcutaneous abdominal tissue had the largest arterio-venous difference for NEFA and appeared the better tissue bed for study of a relatively pure adipose tissue depot. Superficial forearm tissue contains some lipolytic elements (presumably adipose) but showed the greatest arterio-venous difference for lactate and pyruvate production, suggesting skin elements are predominant in the arterio-venous differences seen.

In summary, these findings suggest that superficial forearm veins drain a predominantly cutaneous (rather than adipose) bed with modest NEFA release and high conversion of glucose to lactate (~90%). During glucose infusion, skin glucose uptake increases significantly and NEFA release is reduced (by ~70%). In contrast the superficial abdominal vein drains a predominantly adipose tissue bed with greater lipolysis but less conversion of glucose to lactate. During glucose infusion adipose glucose uptake changes little whereas NEFA release is markedly (>90%) inhibited. Whole body extrapolations suggest that both skin and adipose tissue are each minor (<10%) contributors to whole body glucose uptake and lactate production.

These studies confirmed the relative purity of venous drainage from 3

metabolically distinct body tissues. We then sought to examine the role of the SNS as a putative mechanism underlying the different metabolic characteristics of two of these tissues, adipose tissue and deep forearm skeletal muscle under post absorptive and postprandial conditions in individuals of differing body composition. The Sympathetic Nervous System (SNS) is thought to regulate lipolysis (and thereby, ketogenesis (Bahnsen *et al*, 1984)), adipose tissue blood flow (Ardilouze *et al*, 2004) and secretion of cytokines from adipose tissue (Haynes 2000). Dysfunction of both skeletal muscle and adipose tissue metabolism is described in insulin resistant states and obesity. Catecholamine infusions elevate blood glucose levels, suppress plasma insulin and differentially affect glucose metabolism to lactate or pyruvate (Pernet *et al*, 1984). We therefore hypothesised that abnormalities of the SNS innervation of these tissues may be found in human obesity and have the potential either to cause or maintain the obese state or to underlie some of its metabolic consequences.

CHAPTER IV

DYSFUNCTION OF SYSTEMIC AND REGIONAL SYMPATHETIC NERVOUS SYSTEM OUTFLOW IN HUMAN OBESITY

INTRODUCTION

The sympathetic nervous system contributes to body weight regulation via influences on appetite (Bray 2000, Tsujii & Bray 1998), resting energy expenditure (Webber & Macdonald 2000) and thermogenesis (Bachman *et al*, 2002, Yoshitomi *et al*, 1998). Sympathetic nerves innervating adipose tissue also regulate lipolysis (Collins & Surwit 2001, Youngstrom & Bartness 1998, Christin *et al*, 1989, Carey 1998) and other endocrine/paracrine functions, such as secretion of leptin and other cytokines (Trayhurn *et al*, 1996, Rayner 2001, Trayhurn *et al*, 1998, Lu *et al*, 1998, Orban *et al*, 1999), as well as adipocyte differentiation (Loncar 1991) and apoptosis (Valet *et al*, 1998). Since the above actions influence overall energy balance and adipose tissue behaviour, it seems plausible that dysfunction of the sympathetic nervous system (SNS) might result in a tendency for weight gain. Indeed, as pointed out by Bray (Bray 1991), this view is supported by many animal models of obesity.

Conversely, a number of clinical studies have shown that the SNS is systemically activated in obesity and it is widely thought that sympathetic activation may be a major factor in obesity-related hypertension (Daly & Landsberg 1991, Landsberg 2001, Carlson *et al*, 2000, Corry & Tuck 1999, Esler 2000, Kunz *et al*, 2000, Masuo *et al*, 2000, Vaz *et al*, 1997). This view is corroborated by the observation that SNS traffic is reduced after weight loss and this is accompanied by a reduction in blood pressure (Grassi *et al*, 1998).

Despite this, many of the changes in the behaviour of adipose tissue seen in obesity would be better explained in terms of reduced rather than increased sympathetic drive to this tissue. For example, increased leptin secretion, increased fat depot size and decreased lipolysis per unit fat mass (Klein *et al*, 1988) are all features of obesity yet each of these changes can also be caused by

measures that reduce sympathetic activity such as surgical sympathectomy (Youngstrom & Bartness 1998) or adrenergic receptor blockade (Rayner 2001).

Despite the advent of a new generation of sympathomimetic anti-obesity agents with specificity for the β_3 adrenoceptor (Weyer *et al*, 1999), little is known about the function of sympathetic nerves innervating white adipose tissue, the principal location of this receptor in man. The need for specific information on the innervation of adipose tissue is further underlined by the fact that many studies (Vaz *et al*, 1997, Esler *et al*, 1984, Cox *et al*, 1995, Muntzel *et al*, 1994) have demonstrated that the SNS shows considerable regional and tissue specific heterogeneity.

We therefore hypothesized that sympathetic outflow to adipose tissue would be reduced in obesity independent of the systemically increased SNS outflow previously reported in this condition.

We examined differences in sympathetic outflow after an overnight fast and in response to feeding in 11 obese subjects ($BMI>30\text{ Kg.m}^{-2}$) and 11 lean controls ($BMI<25\text{ Kg.m}^{-2}$). Sympathetic outflow was assessed systemically and regionally using radiotracer-dilution methodology, involving intravenous infusions of ^3H -labelled norepinephrine and measurements of the rate of entry of endogenous norepinephrine into the general circulation and into blood draining subcutaneous abdominal adipose tissue and forearm skeletal muscle.

METHODS

A detailed account of the methods, assumptions and calculations is available in Chapter II ‘Core Experimental Methods’.

Subjects

Eleven obese ($\text{BMI} > 30 \text{ kg.m}^{-2}$) and 11 lean ($\text{BMI} < 25 \text{ kg.m}^{-2}$) age and sex-matched controls (Table 4.1) were recruited for the study. Full, informed and written consent was obtained from each volunteer prior to participation. Medical history and physical examination (including an Allen test- digital occlusion of the radial artery to ensure adequate ulnar arterial blood supply to the hand before radial artery cannulation) were performed and routine laboratory blood tests sent for analysis (including full blood count, haematocrit, urea and electrolytes, liver function tests, fasting glucose, fasting lipids and thyroid function tests). Urine was tested for protein and blood and a urine pregnancy test performed before isotope administration in all women of childbearing potential. Subjects were weight stable for at least 3 months prior to participation in the study.

Anthropomorphic tests were performed on each subject including resting metabolic rate (Deltatrac), height, weight, waist-hip ratio, skin fold thickness, blood pressure and pulse rate. Subjects attended the clinical research centre at approximately 0800 hrs after consuming a standardised meal and snack the previous evening and fasting overnight from midnight. The meal contained 15 kcal/Kg lean body mass (LBM) calculated from the formula

$$\text{LBM} = (0.3281 \times \text{weight/Kg}) + ((0.33929 \times \text{height/cm}) - 29.5336)$$

and delivered energy from carbohydrate, fat and protein in the proportions 50%, 30% and 20% respectively, figures approximating to a healthy, eu-caloric diet (although perhaps somewhat different to that actually consumed by an average

Western population). The snack was of identical macronutrient composition but comprised 5kcal/Kg calculated lean body mass.

All subjects refrained from vigorous physical activity or alcohol for at least 36 hours and from caffeine or smoking (2 smokers in each group) for 12 hours prior to the study. Subjects taking drugs known to affect the SNS such as antidepressants, diuretics, α - or β -blockers, false transmitters, cocaine, cannabis, sibutramine or amphetamines were not recruited nor were those with a history of significant ($> 5\%$) weight loss in the 3 months prior to the experiments. The presence, proven or suspected, of pathological states such as obstructive sleep apnoea which are known to be capable of affecting SNS activity were carefully noted but as degrees of sleep disturbance are so prevalent amongst the morbidly obese and would tend to lead to increased rather than decreased SNS activity, subjects with suggestive features were not excluded on the basis of this alone. Known history of phaeochromocytoma, other SNS disease such as the Steele-Richardson syndrome or reflex sympathetic dystrophy and history of chemical or surgical sympathectomy were excluded from taking part in these experiments.

Table 4.1: Baseline characteristics of study subjects

TABLE 4.1. BASELINE CHARACTERISTICS *		
CHARACTERISTIC	LEAN	OBESE
	(N=11)	(N=11)
Age (years)	33.3 ± 2.86	38.4 ± 2.50
Sex – no. (%)		
Male	6 (54%)	5 (45%)
Female	5 (45%)	6 (54%)
Body Mass Index (Kg.m ⁻²)	23.1 ± 0.24	44.2 ± 0.84
Blood Pressure‡		
Systolic - mmHg	120.3 ± 2.89	135.3 ± 3.68
Diastolic - mmHg	75.5 ± 3.17	89.6 ± 3.35
Weight – Kg	67.9 ± 2.38	135.7 ± 9.23
Height – cm	171.2 ± 2.89	171.7 ± 2.02

Legend to Table 1

* Values are mean ± SEM.

‡ Both systolic and diastolic blood pressures were significantly higher in the obese group ($p<0.008$ and $p<0.01$ respectively). Otherwise, there were no significant differences between the groups except in weight/BMI.

Protocol

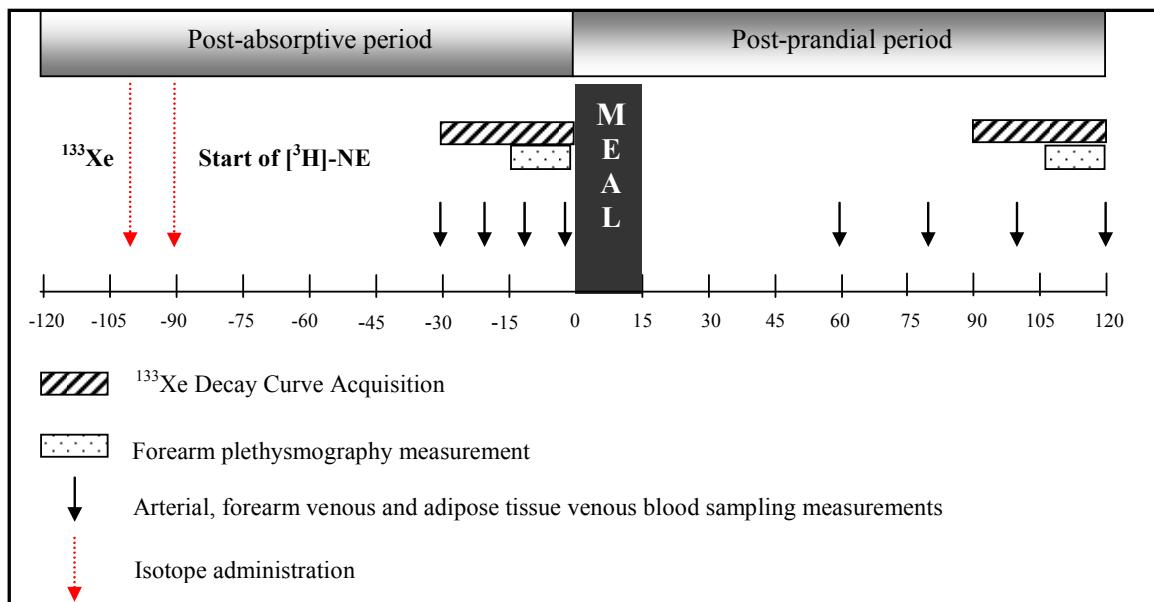
Subjects attended the Clinical Research Centre at 0800hrs after the overnight fast and standard meal and snack the previous evening discussed above.

Arterial blood was obtained from a radial artery catheter inserted under aseptic conditions using local anaesthesia (lidocaine, 1% w/v). Venous blood from subcutaneous abdominal adipose tissue was obtained from an indwelling catheter in a branch of the superficial epigastric vein (Frayn *et al*, 1993) and that draining deep forearm tissue (principally muscle) was drawn from a retrogradely placed forearm venous cannula as previously described (Coppock *et al*, 1996). All lines were maintained with a constant rate saline infusion of 30mls/h and left for at least 45 mins prior to sampling.

In order to determine tissue norepinephrine uptake, a constant infusion of tracer amounts ($10 \text{ nCi} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) of levo-[ring-2,5,6- ^3H]-norepinephrine (40–80 Ci/mmol; New England Nuclear, Boston, MA) was used. This infusion was continued for at least 60 min to ensure steady state plasma concentration before blood sampling commenced.

Abdominal subcutaneous adipose tissue blood flow (ATBF) was measured by the ^{133}Xe dispersion technique (Larsen *et al*, 1966, Jansson & Lonroth 1995) and forearm blood flow was determined by mercury strain-gauge plethysmography (Hokanson, Bellevue, WA) (Greenfield *et al*, 1963).

After 4 pairs of arterial and venous blood samples from each site in the fasting state had been obtained, the subject then ingested a meal containing 15kcal/Kg lean body mass with the same macronutrient profile as the previous evening. Post-prandial measurements were taken at 60, 80, 100 and 120 minutes after the start of meal ingestion as shown in Figure 4.1 below.

Figure 4.1: Experimental Design**Legend to Figure 4.1:**

Time course of isotope administration and equilibration, post-absorptive measurements (blood sampling, forearm plethysmography and adipose tissue blood flow (ATBF) estimation), meal ingestion, and finally post-prandial measurements (blood sampling, forearm plethysmography and ATBF estimation) are shown. Keys are presented in the Figure and the protocol is described further in the text.

Analyses

Blood was collected directly into cooled, heparinised containers and were centrifuged within a few minutes before being frozen at -70°C until analysis. Plasma norepinephrine concentration, ³H-norepinephrine specific activity and epinephrine concentrations were determined as described previously (Eisenhofer *et al*, 1986).

Calculations

We applied Esler's (Esler *et al*, 1984) tracer balance approach which is based on the concept that the rate of entry of the SNS neurotransmitter, norepinephrine (NE), into venous blood (norepinephrine spillover, NE_{spillover}) is an index of local sympathoneuronal activity over a wide range of firing rates (Esler *et al*, 1990).

Thus, at steady state isotope concentrations, systemic (whole body) norepinephrine spillover (NE_{Systemic spillover}) was calculated as

$$\text{NE}_{\text{Systemic spillover}} = \frac{[{}^3\text{H}]\text{-NE isotope infusion rate (dpm/min)}}{[{}^3\text{H}]\text{-NE specific activity (dpm/nmol)}}$$

and local NE spillover as $\text{NE}_{\text{spillover}} = \text{ATPF} \times ([\text{NE}]_v - [\text{NE}]_a) + \text{Tissue NE}_{\text{clearance}}$

$$\text{where Tissue NE clearance} = \frac{({}^3\text{H}[\text{NE}]_a - {}^3\text{H}[\text{NE}]_v) \times [\text{NE}]_a}{{}^3\text{H}[\text{NE}]_a}$$

and ${}^3\text{H}[\text{NE}]_a$ and ${}^3\text{H}[\text{NE}]_v$ are the tritiated norepinephrine counts in arterial and venous samples respectively, $[\text{NE}]_v$ and $[\text{NE}]_a$ represent venous and arterial norepinephrine concentrations respectively and ATPF is adipose tissue plasma flow.

Local spillovers were also determined using Chang's (Chang *et al*, 1991) modification of these calculations which has been advocated (Rongen *et al*, 2000) as being less vulnerable to changes in tissue perfusion. Further details of the methods, calculations and assumptions used in this study are presented in Chapter II.

Terminology

Direct and absolute quantification of SNS activity *in vivo* would require measurement of many factors including the temporal pattern and frequency of action potentials together with the quantity of neurotransmitter release. It is not possible to measure all these factors together *in vivo* in man. We have measured global (systemic) and regional 'norepinephrine spillover', the best available index of SNS activity but have used the term 'sympathetic outflow' rather than 'sympathetic nervous system activity' throughout the text in acknowledgement of these issues.

Statistical Analysis

Values are presented as means \pm SEM. Differences between the 2 groups were assessed by ANOVA (SPSS ver. 10, SPSS inc Chicago, Ill.). Data were logarithmically transformed where necessary to normalise the distribution. Correlations were assessed using Spearman's Rank Correlation Coefficient. Results were considered significant at $p < 0.05$.

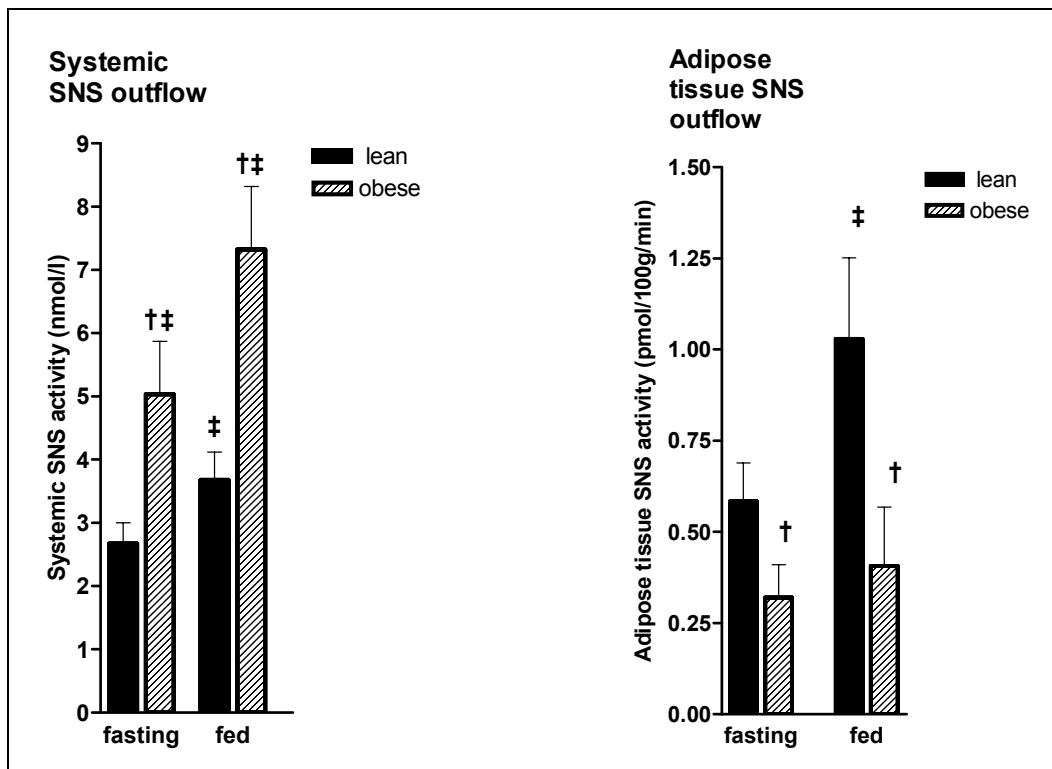
RESULTS

Adipose tissue norepinephrine spillover

Local norepinephrine release from sympathetic nerves in adipose tissue was significantly lower in the obese subjects than in lean controls in both fasting (0.33 ± 0.09 vs. 0.58 ± 0.11 pmol. $100\text{g}^{-1}.\text{min}^{-1}$) and post-prandial (0.38 ± 0.15 vs. 1.08 ± 0.22 pmol. $100\text{g}^{-1}.\text{min}^{-1}$) states ($p=0.041$ and $p=0.008$ respectively by ANOVA after logarithmic transformation). This is shown in Figure 4.2.

The transition to the post-prandial state was not associated with any significant increase in sympathetic outflow to adipose tissue in the obese group ($p=0.657$ by ANOVA). However, in marked contrast to this, post-prandial adipose tissue norepinephrine spillover increased in the lean subjects by over 80% ($p=0.013$ by ANOVA). These data are also shown in Table 4.2 and Figures 4.2 and 4.4.

These differences were little affected by recalculation using Chang's index (Ra_{Chang}) (Chang *et al*, 1991, Rongen *et al*, 2000), and remained statistically significant suggesting that the observed changes were not attributable to changes in blood flow (Table 4.2).

Figure 4.2: Systemic and Adipose tissue SNS outflow

Legend to Figure 4.2

† represents a significant difference ($p<0.05$) between lean and obese groups.

‡ represents a significant difference ($p<0.05$) between fasting and fed states within either lean or obese groups.

Bars represent systemic (left) and adipose tissue (right) SNS outflow in the lean (filled black) and obese (hatched) subjects. For each individual, 4 fasting and 4 post-prandial values are averaged. Values shown are means \pm SEM.

‘Systemic SNS outflow’ is the rate of secretion of norepinephrine into the circulation, measured in nmol per minute. ‘Adipose tissue sympathetic nerve outflow’ is the release rate of norepinephrine into venous blood draining 100g adipose tissue per minute.

Systemic SNS outflow was higher in the obese in both fasting and fed states whereas adipose tissue SNS outflow was significantly lower in the obese and failed to respond to feeding.

Table 4.2: Other results

TABLE 4.2. OTHER RESULTS			
	Arterial NE concentration (pmol.l⁻¹)		
	Fasting	Fed	
Lean	1,249.6 ± 107 [†]	1,653.7 ± 123 ^{†,‡}	
	1,996.5 ± 226	2,371.6 ± 138 [‡]	
	Arterial Epinephrine concentration (pmol.l⁻¹)		
	Fasting	Fed	
Lean	259.3 ± 22 [†]	218.3 ± 62	
	149.6 ± 26	169.8 ± 37	
	Adipose tissue NE spillover (pmol.100g⁻¹.min⁻¹)		
	Fasting	Fed	
Lean	0.58 ± 0.11 [†]	1.08 ± 0.22 ^{†,‡}	
	0.33 ± 0.09	0.38 ± 0.15	
	Adipose tissue NE appearance rate § (pmol.100g⁻¹.min⁻¹)		
	Fasting	Fed	
Lean	1.38 ± 0.26 [†]	1.87 ± 0.331 ^{†,‡}	
	0.80 ± 0.22	1.09 ± 0.40	
	Forearm muscle NE spillover (pmol.100g⁻¹.min⁻¹)		
	Fasting	Fed	
Lean	2.04 ± 0.26	4.01 ± 0.42 [‡]	
	2.80 ± 0.42	4.55 ± 1.16 [‡]	
	Forearm muscle NE appearance rate § (pmol.100g⁻¹.min⁻¹)		
	Fasting	Fed	
Lean	6.66 ± 0.59 [†]	11.38 ± 0.90 [‡]	
	9.85 ± 1.00	10.00 ± 1.30	

Legend to Table 4.2

Values are means of 4 fasting ($t-30, -20, -10, 0$) or fed ($t+60, +80, +100, +120$) sampling points \pm SEM. Statistical significance is tested by repeated measures ANOVA.

† represents a significant difference ($p<0.05$) between lean and obese groups.

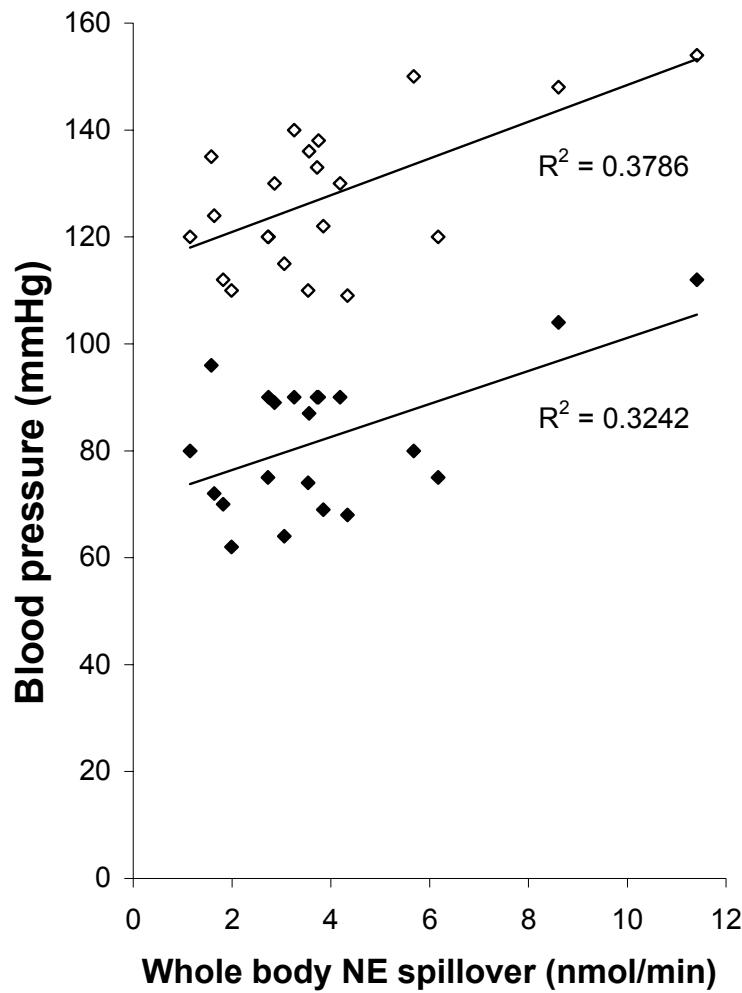
‡ represents a significant difference ($p<0.05$) between fasting and fed states within either lean or obese groups.

§ Appearance rate (Chang Index) is spillover with a correction for changes in the fractional extraction of NE as a result of changes in blood flow (see methods for details).

Systemic spillover and its relationship to Blood Pressure

In marked contrast to the lower sympathetic outflow to adipose tissue, systemic SNS outflow in the fasting state (Figure 1) was significantly greater ($p<0.02$, by ANOVA) in the obese ($5.03\pm 0.84 \text{ nmol}\cdot\text{min}^{-1}$) than in the lean ($2.67\pm 0.33 \text{ nmol}\cdot\text{min}^{-1}$) subjects. This difference was accentuated by meal ingestion when SNS outflow increased in both groups ($p<0.05$ by ANOVA) to $7.32\pm 1.0 \text{ nmol}\cdot\text{min}^{-1}$ and $3.68\pm 0.44 \text{ nmol}\cdot\text{min}^{-1}$ respectively, and remained significantly higher in the obese ($p=0.007$ by ANOVA).

Significant correlations existed between fasting total body norepinephrine spillovers and both systolic and diastolic blood pressures ($R_s=0.57$, $p<0.006$ and $R_s=0.676$, $p<0.001$ respectively). This relationship is shown in Figure 4.3.

Figure 4.3: Relationship between Blood Pressure and Whole Body NE Spillover**Legend to Figure 4.3**

Correlation between whole body NE spillover (nmol/l) and systolic (\diamond) and diastolic (\blacktriangle) blood pressure. Spearman rank correlation coefficients were highly significant for both relationships ($p<0.006$ and $p<0.001$ respectively). R squared values for the relationships are presented adjacent to the regression lines.

Arterial plasma catecholamine concentrations

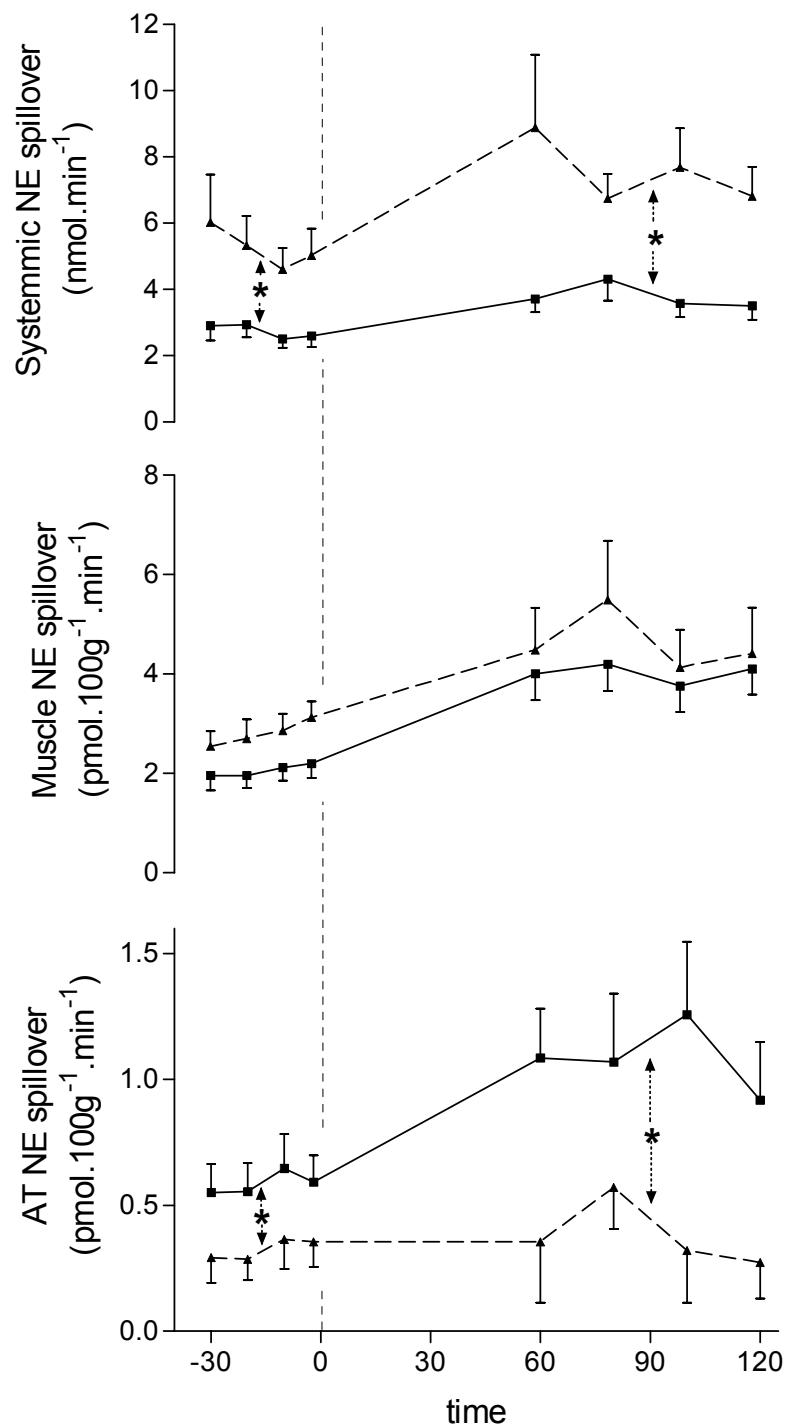
Although concentrations of epinephrine, secreted by the adrenal medulla, were significantly greater in the lean subjects in the fasting state ($p<0.02$, Table 4.2), there was no significant difference in the post-prandial state ($p=0.77$) and no significant response to the meal in either group.

In contrast, arterial concentrations of norepinephrine (Table 4.2) reflected systemic release and thus were significantly higher in the obese in both fasting ($p=0.02$ by ANOVA) and fed ($p<0.02$ by ANOVA) states, increased in both groups in response to meal ingestion ($p<0.05$ by ANOVA) and, like systemic SNS outflow, showed positive correlations with both systolic and diastolic blood pressures in both fasting and fed states ($p<0.05$ for both).

Forearm muscle norepinephrine spillover

Although the release of norepinephrine from forearm muscle exceeded that of adipose tissue in both groups ($p<0.0001$ by ANOVA) and increased in both groups in response to meal ingestion ($p<0.05$ by ANOVA), there were no significant differences between the lean and obese groups in either the fed or fasting state (see Figure 4.4 for comparison of systemic, muscle and forearm muscle NE spillover at each time point before and after eating).

Figure 4.4: Systemic, Muscle and Adipose Tissue NE Spillover at each time point.



Legend to figure 4.4:

Graphs of adipose tissue (bottom panel), muscle (middle panel) and systemic (upper panel) NE spillover at each experimental time point (error bars represent SEM). Hatched lines joining triangular markers (\blacktriangle) represent obese subjects and continuous lines joining square markers (\blacksquare) are lean controls. The vertical hatched line represents the time of meal ingestion ($t=0$). Significant differences between lean and obese groups are denoted by asterisk (*) and vertical arrows (\Downarrow).

Whereas systemic NE outflow was greater in the obese subjects, local SNS outflow to adipose tissue was reduced in the obese group. These differences in adipose tissue and systemic spillovers between the two groups were significant both before and after the meal ($p<0.05$ by ANOVA).

Adipose tissue NE spillover increased in the lean ($p=0.013$) but not the obese groups ($p=0.657$) in response to meal ingestion.

Systemic and muscle NE spillovers increased in both groups in response to eating ($p<0.05$ by ANOVA) but systemic NE spillover remained significantly higher in the obese ($p=0.007$ by ANOVA).

Muscle norepinephrine spillover did not differ between groups either before or after meal ingestion.

DISCUSSION

The principal findings of the current study are that there is a local defect in sympathetic outflow to adipose tissue in obesity both in fasting and the fed states. This local adipose tissue defect is in contrast to the increased systemic sympathetic outflow in obesity and the lack of any significant difference in skeletal muscle. Whereas systemic sympathetic responses to feeding are intact in obese subjects, those regulating adipose tissue are impaired or absent.

These divergent abnormalities of sympathetic outflow, further evidenced by the dissociation between epinephrine derived from the adrenal medulla and systemic sympathetic outflow, illustrate the principle of heterogeneity in SNS outflow to different tissues and organs (Vaz *et al*, 1997, Esler *et al*, 1984, Cox *et al*, 1995).

Consistent with Landsberg's hypothesis (Reaven *et al*, 1996) relating obesity hypertension to high SNS outflow driven by hyperinsulinaemia, and with other studies of systemic SNS outflow in obesity (Vaz *et al*, 1997), there were significant correlations in our study between total body norepinephrine spillover and both systolic and diastolic blood pressures suggesting that obesity associated hypertension might be mediated via adrenergic mechanisms in addition to the known effects of insulin resistance and/or hyperinsulinaemia *per se* (Johnston *et al*, 1992).

Catecholamines not only regulate lipolysis within adipose tissue but also influence many other adipose tissue functions including leptin (Trayhurn *et al*, 1996, Lu *et al*, 1998) and adipocytokine (Orban *et al*, 1999) production, cell differentiation and apoptosis (Valet *et al*, 1998), and uncoupling protein (UCP) expression (Yoshitomi *et al*, 1998). The importance of this SNS dependent regulatory system was elegantly demonstrated in a study (Youngstrom & Bartness

1998) in which experimental sectioning of sympathetic nerves to hamster white adipose tissue resulted in a 200% increase in the number of adipocytes.

Until now, it has been thought that resistance of adipose tissue to the actions of catecholamines (Reynisdottir *et al*, 1994, Schiffelers *et al*, 2001, Arner 1999, Hoffstedt *et al*, 1997, Dodt *et al*, 2000) may be responsible for the observed pathophysiology of adipose tissue in obesity such as the increased cell number, reduced lipolysis per unit mass of adipose tissue (Klein *et al*, 1988), and increased leptin production (Maffei *et al*, 1995). However, we now demonstrate that any resistance to catecholamines is further compounded by reduced sympathetic outflow, at least to the adipose depot we examined, in obesity. Further studies will be required to establish whether these apparent abnormalities in innervation may explain how abnormal glucose and NEFA metabolism may be encountered in individuals at risk of diabetes despite them having preserved responses *in vivo* to infused catecholamines, matched weight and similar insulin sensitivity and insulin levels to control subjects (Frobes *et al*, 2006). Other studies by the same group (Kousta *et al*, 2002) have demonstrated delayed or impaired metabolic and thermogenic responses to mixed meal ingestion in subjects with previous gestational diabetes which are potentially consistant with our finding of blunted meal stimulated sympathetic responses in adipose tissue in obese subjects who also are at risk from, but do not currently have, diabetes.

Several (Cox *et al*, 1995, Patel *et al*, 1999, Fagius & Berne 1994), but not all (Matsumoto *et al*, 2001), groups have demonstrated increased systemic SNS outflow in response to meal ingestion in lean humans and a reduction of SNS outflow in response to underfeeding in lean subjects has also been described (Gohler *et al*, 2000, Young & Landsberg 1997). The current study, whilst confirming these findings, reveals for the first time a complete absence of this increase in post-meal SNS outflow to adipose tissue in obese subjects.

Certain limitations are inherent in the methods used in this study. Norepinephrine rate of entry into blood is an indirect measurement of the rate of release of neurotransmitter at the synaptic cleft and other factors such as firing frequency, receptor disposition and post-receptor signal transduction will undoubtedly have a bearing on the effect of the SNS upon adipose tissue. Nevertheless, the spillover methodology that we have used has been shown to be correlated to local electrical activity in animal preparations (Esler *et al*, 1990) and is widely considered to be the best available method for studying local SNS outflow *in vivo* (Grassi & Esler 1999). In addition, the present study has only investigated subcutaneous abdominal adipose tissue and, given the regional heterogeneities of both the SNS and of adipose tissue, it would be important for future studies to examine other adipose tissue depots. It is not possible on the basis of our results to determine for certain whether the observed defects in SNS outflow are primary or secondary to obesity. However, it has previously been demonstrated that low 24h norepinephrine excretion presaged future weight gain (Tataranni *et al*, 1997) and that subcutaneous abdominal adipocyte resistance to catecholamines was at least partially reversed by weight loss (Reynisdottir *et al*, 1994). Whichever the case, reduced sympathetic outflow to adipose tissue would certainly appear to be a maladaptive response to obesity whether or not it contributes to its causation, as it would tend to perpetuate rather than counteract the condition (Macdonald 1995). Whether or not a reduction in sympathetically mediated lipolysis may constitute a defence against the development of diabetes by reducing 'lipotoxicity' (Unger 2002) is perhaps teleologically plausible but this remains an issue which is subject to considerable debate and one which can not be resolved on the basis of the current data.

Selective β_3 -adrenoceptor agonists have shown initial promise as a novel class of anti-obesity agent (Weyer 1999) despite the fact that no clinical studies have investigated the function of sympathetic nerves within adipose tissue, the principal location of this receptor subtype. The defects we report in adipose tissue SNS outflow suggest that pharmacological correction may provide a rationale for

restoring adipocyte function and ultimately reducing adipocyte mass. On the other hand, our demonstration of raised systemic SNS outflow and its positive correlation with blood pressure in obesity emphasizes the need for such agents to act specifically to avoid unwanted cardiovascular sympathomimetic effects.

We conclude that in human obesity, regional sympathetic outflow to adipose tissue is reduced whereas sympathetic outflow is increased. Although sympathetic neural responses to feeding are intact overall, those specifically regulating adipose tissue are impaired. The increases in sympathetic outflow may contribute to hypertension, whereas hypofunction of sympathetic nerves innervating adipose tissue may contribute to body fat accumulation in obesity via a variety of specific local mechanisms including abnormal lipolysis and disordered adipocytokine secretion.

We therefore proceeded to investigate the possible role of SNS innervation of adipose tissue in co-ordinating NEFA and adipokine secretion with studies assessing the pulsatile release of these substances from within the same subcutaneous abdominal adipose tissue depot. We aimed to test the hypothesis that SNS innervation of this tissue is involved in qualitative as well as quantitative control of lipolysis and adipokine secretion. Specifically, we hypothesised that release of free fatty acids and cytokines would be pulsatile. If this were shown to be the case, we reasoned that pulsatile release would be entrained to either SNS or insulin pulses. We therefore studied the effects of euglycaemic hyperinsulinaemia NEFA and leptin concentrations reasoning that, under hyperinsulinaemic clamp conditions, any effects due to spontaneous endogenous insulin pulses would be attenuated.

CHAPTER V

OSCILLATIONS OF FATTY ACID AND GLYCEROL RELEASE FROM HUMAN SUBCUTANEOUS ADIPOSE TISSUE IN VIVO

INTRODUCTION

It is increasingly recognized that metabolic processes do not operate at steady rates. Oscillations in glycolytic flux, synchronized across cells, have long been recognized (Dano *et al*, 1999). Pulsatile secretion of hormones is also well established, and for insulin, for example, there is a clear oscillatory pattern with a characteristic period of 12–13 min observable in systemic plasma (Lang *et al*, 1979, Hansen *et al*, 1982). Among the metabolic processes regulated by insulin is adipose tissue lipolysis. Recently, rapid oscillations in lipolysis have been observed in omental adipose tissue in dogs (Getty *et al*, 2000). These oscillations appeared to be independent of insulin, however, and were partially blocked by propranolol. More recent work in dogs has shown that oscillations of lipolysis, detected in systemic plasma non-esterified fatty acid (NEFA) concentrations, are superimposed on a steady background level of NEFA, and only the oscillatory component is blocked by specific blockade of the β_3 -adrenoceptor, which is responsible for lipolysis in dogs (Hücking *et al*, 2003). In humans, adipose tissue lipolysis, as measured by microdialysis of interstitial glycerol, is not affected by propranolol after an overnight fast (Arner *et al*, 1990). This seems surprising but might be understandable if β -adrenergic activation were responsible for only a small component of lipolysis; rapid oscillations might be lost with the limited time resolution of microdialysis. If pulsatile lipolysis were detectable in humans, it might considerably alter our view of NEFA release and its interaction with insulin action and glucose metabolism. Therefore, we have sought evidence for pulsatility of lipolysis in human adipose tissue.

The subcutaneous upper-body adipose tissue makes the largest contribution to the systemic plasma NEFA pool (Guo *et al*, 1999), so we measured arterio-venous differences for the products of lipolysis, NEFA and glycerol, across the subcutaneous abdominal fat depot every 2 min at steady state. Adrenergic stimulation of lipolysis in humans is brought about mainly by $\beta 1$ -and $\beta 2$ -adreceptors (Barbe *et al*, 1996), so it is not possible to block it systemically

without causing cardiovascular side effects. We therefore measured plasma norepinephrine concentrations in arterial and adipose tissue venous plasma as an index of systemic and local sympathetic activity and sought relationships between these measurements and lipolysis. Because it seems intrinsically likely that oscillatory insulin concentrations will affect lipolysis, we also used a constant insulin infusion with the euglycaemic hyperinsulinaemic clamp technique to remove this possible stimulus.

Rapid oscillations of insulin secretion are seen in obese as well as normal-weight subjects (Hansen *et al*, 1982) but are attenuated in relatives of patients with type 2 diabetes (O'Rahilly *et al*, 1988) and absent in type 2 diabetes (Matthews 1996). They are somewhat restored with weight loss (Matthews 1996). To obtain some preliminary information on the effects of obesity on any lipolytic pulsatility observed, we included subjects with a wide range of adiposity.

METHODS

Nine healthy subjects (five men), covering a range of adiposity levels (BMI 19.7–50.8, median 23.8 kg/m²), were recruited. Their ages ranged from 21 to 61 years (median 43). The subjects were healthy and receiving no medication. The protocol was approved by the Oxfordshire Clinical Research Ethics Committee and the East London and City Ethics Committee, and all subjects gave written informed consent.

Subjects were studied after an overnight fast. A 10-cm, 22-gauge Hydrocath catheter (Becton Dickinson, Oxford, U.K.) was introduced over a guidewire into a superficial vein on the anterior abdominal wall and threaded toward the groin so that its tip lay just superior to the inguinal ligament. This provided access to the venous drainage from the subcutaneous abdominal adipose tissue, which was uncontaminated by muscle drainage, with a relatively minor contribution from skin (Frayn & Coppack 2001). A retrograde cannula was placed in a vein draining the hand, which was warmed in a hot-air box maintained at 60°C to obtain arterialised blood (n=4) or into the radial artery (n=5). The term ‘arterialised’ is used to mean blood drawn by either method in this study. The cannulae were kept patent by a slow infusion of 0.9% (wt/vol) saline. After a 30-min resting period, blood was taken with careful timing in order to ensure simultaneous sampling from the arterialised and adipose tissue venous lines and was repeated every 2 min for 60 min. After this 60-min sampling period, a hyperinsulinaemic-euglycemic clamp was established by infusing insulin, using a primed-constant infusion protocol (DeFronzo *et al*, 1979), with the final infusion rate 35 mU. m⁻². min⁻¹. The arterialised blood glucose concentration was maintained at 5 mmol/l by measurement of glucose using a reflectometer (Hemocue, Sheffield, U.K.) at 5-min intervals. Once the blood glucose concentration was stable, blood sampling was again started simultaneously from the arterialised and adipose tissue venous lines, and this was repeated every 2 min for 60 min. Clamps were not performed in three subjects.

Adipose tissue blood flow (ATBF) was measured during the entire experiment by registering the washout of ^{133}Xe injected into the subcutaneous abdominal adipose tissue as previously described (Samra *et al*, 1995). Blood samples were taken into heparinised syringes. A portion of each blood sample was rapidly deproteinized with 7% (wt/vol) perchloric acid for glycerol determination. Samples were kept on ice, and plasma was separated rapidly by centrifugation at 4°C. One aliquot was stored with preservative at -70°C for subsequent norepinephrine analysis. Plasma glucose was measured within 24 h on fresh plasma to avoid the variable loss of glucose on freezing (Clark *et al*, 1990). Plasma NEFA and blood glycerol concentrations were measured on samples stored at -20°C. Plasma NEFA, glucose concentrations, and blood glycerol concentrations were measured using enzymatic methods on an IL Monarch centrifugal analyzer (Instrumentation Laboratory, Warrington, U.K.). Plasma insulin was measured using a double-antibody radioimmunoassay (Pharmacia and Upjohn, Amersham, U.K.). Plasma norepinephrine was measured by high-performance liquid chromatography with electrochemical detection (Forster & Macdonald 1999) on samples stored at -70°C.

Data analysis.

Glycerol and glucose were not measured in some samples for practical reasons, so for these measurements, $n = 6$. Given that some subjects did not undergo the clamp and some adipose venous samples were not taken, a total of 2,190 potential data points were available for NEFA, glycerol, and norepinephrine. In fact 2,119 measurements were available (97% of potential total). Sixteen individual missing values were filled by interpolation for analysis of pulsatility. We sought evidence for pulsatility in arterialised and adipose tissue venous concentrations, as well as in the veno-arterialised difference for plasma NEFA and blood glycerol concentrations, which we took as a simple measure of the release from adipose tissue. We did not attempt to calculate absolute flux rates (veno-arterialised

difference x blood flow) because, as described in ‘Results’, measurements of ATBF over 2-min intervals turned out to be unreliable. For norepinephrine, there was generally net uptake across adipose tissue, and we calculated arterio-venous differences. For ATBF, Mediscint software (Oakfield Instruments, Eynsham, U.K.) was used to record counts per second (cps) over 20s intervals. We took these data into Microsoft Excel and calculated averages over 2-min periods to correspond with blood samples and over 10-min periods to calculate mean ATBF values. ATBF is proportional to the (negative) slope of the log(cps) versus time plot.

Evidence for pulsatility was sought as follows. The program ‘Cluster 7’ (http://mljohnson.pharm.virginia.edu/pulse_xp/downloads.php) (Veldhuis & Johnson 1986) was used following the guidelines supplied with the program. This program has been used previously for similar studies (Hücking *et al*, 2003) and validated for this purpose against an alternative program, ULTRA. The program searches for peaks and nadirs defined by changes above and below mean baseline that are outside of the limits expected from analytical variation. Analytical variation was measured as described below. The default settings of the program were not altered, except that the minimum number of points needed to define peaks and nadirs (default setting 2) was changed as follows. For most measurements, we set 2 for the number for a peak and 1 for a nadir. This was done because our samples were at 2-min intervals, and we discovered that we were looking at some events with a period ~6–7 min, so often only 1 point defined a nadir (or peak). In addition, the half-life of plasma NEFA in humans, for instance, is ~3min (Bonadonna *et al*, 1990, Meyer *et al*, 1997), so a peak might convert to a nadir within a few minutes. We tested the effect of choosing 2/2 points (peak/nadir) against 2/1 with several sets of data. The latter made the program more sensitive at finding some peaks but did not consistently alter the characteristics of the peaks found (width, interval, etc.).

The half-life of plasma norepinephrine in normotensive subjects is ~2 min (Silverberg *et al*, 1978, Young *et al*, 1980), so for norepinephrine concentrations, we set the number of points at 1/1. Then, for each subject, ‘Cluster 7’ calculated the number of significant peaks discovered and their characteristics. This was done separately for the fasting and clamp periods (i.e., 30 points each).

We tested the reliability of this approach in a number of ways. First, we applied ‘Cluster 7’ to 30 repeated analyses of plasma NEFA from one fasting sample, and similarly to 30 repeated analyses of blood glycerol. In the latter case, 30 separate aliquots of one blood sample were extracted with perchloric acid (as described earlier) for analysis.

We also created “mock” veno-arterialised differences for NEFA by subtracting one subject’s arterialised concentrations from the next subject’s venous NEFA concentrations and tested these for pulsatility. We created two mock datasets (subject A venous minus subject B arterialised, subject A venous minus subject C arterialised) for each real dataset (subject A venous minus subject A arterialised). This was done to reduce the impact of apparent pulses seen when pairing data from mismatched subjects (e.g., lean man with obese woman, etc.). Second, we attempted to validate the observed pulsatility using the technique of cross-correlation analysis. Cross-correlation was also used to test for significant temporal relationships between variables. In cross-correlation, two datasets are lined up and correlated to each other. One dataset is then “moved” one time point relative to the other (2 min with our data), and the correlation is repeated; this is repeated several times. If there are significant temporal relationships between the datasets, significant correlations will be observed with a “lag” (number of points moved) corresponding to the time delay between maximal coincidence. If there is a similar baseline trend in both variables (e.g., downward drift with time), then spurious significant cross-correlations will be generated. To avoid this, we transformed the data for cross-correlations by “differencing” (replacing each point with the difference between it and the previous point). We argued that if we

applied cross-correlation analysis to independently measured variables, it would test whether the variations we observed were simply random or were indeed related to some underlying process. Cross-correlations were performed using SPSS for Windows (release 11.5.0; SPSS, Chicago, IL).

The within-batch analytical variation of the methods was assessed for ‘Cluster 7’. For plasma NEFA, the coefficient of variation (CV) was 1.4% based on the 30 replicate measurements (each in duplicate) of a plasma sample described above. We used a conservative value of 3% for ‘Cluster 7’. For blood glycerol, the CV was 7.2%, again based on the 30 replicate measurements (each in duplicate) described above. We used a value of 10% for ‘Cluster 7’. For plasma insulin and norepinephrine, we used a value of 5%, based on experience of variation between duplicates.

RESULTS

Plasma and blood concentrations.

Plasma and blood concentrations, averaged over the 60-min fasting and clamp periods in each individual are shown in Table 5.1. As expected, there was marked output of both NEFA and glycerol from subcutaneous adipose tissue in the fasting state, and this was suppressed to a large degree during the clamp. Arterialised plasma norepinephrine concentrations were not altered by the clamp.

ATBF.

Detailed analysis of ATBF showed that estimates over 2-min periods have a CV ~50%. It is not, therefore, possible to generate reliable results on ATBF using ‘Cluster 7’. Thus, we did not further analyze ATBF for evidence of pulsatility. Mean ATBF was not altered by the clamp (Table 5.1).

Table 5.1: Mean concentrations in blood and plasma

	Arterial NEFA ($\mu\text{mol/l}$)	Venous NEFA ($\mu\text{mol/l}$)	Arterial Glycerol ($\mu\text{mol/l}$)	Venous Glycerol ($\mu\text{mol/l}$)
Fasting	521 ± 88 (9)	1,198 ± 161 (8)	62.9 ± 13.8 (6)	213.1 ± 27.4 (5)
Clamp	103 ± 59 (7)	211 ± 166 (6)	28.9 ± 8.0 (5)	59.6 ± 17.8 (5)
P*	0.002	0.004	0.054	0.003
	Arterial NE (nmol/l)	Arterial Insulin (pmol/l)	Arterial Glucose (mmol/l)	ATBF ($\text{ml} \cdot \text{min}^{-1} 100 \text{ g}^{-1}$)
Fasting	0.65 ± 0.16 (5)	46 ± 12 (9)	5.15 ± 0.12 (6)	3.6 ± 0.6 (9)
Clamp	0.62 ± 0.22 (4)	294 ± 17 (6)	4.75 ± 0.12 (6)	4.0 ± 0.9 (7)
P*	NS	0.000	NS	NS

Legend to Table 5.1:

Data are means ± SE (n). NEFA, norepinephrine (NE), glucose, and insulin were measured in plasma, and glycerol was measured in whole blood.

*P value for fasting vs. clamp (paired t test). Arterial, arterialised; Venous, adipose tissue venous.

Evidence for pulsatility

Analysis with ‘Cluster 7’, as described in ‘Methods’, showed inconsistent evidence for pulsatile behaviour of arterialised NEFA and glycerol concentrations. Not all subjects showed significant pulses, and the estimated period was extremely variable from person to person. However, there was much more consistent evidence for pulsatile release of NEFA and glycerol from the subcutaneous abdominal adipose depot, as measured by the veno-arterialised difference: seven of nine and five of six subjects showed more than one peak for NEFA and glycerol release, respectively (Table 5.2). The period was ~12–14 min.

Norepinephrine showed clear evidence of repeated peaks in every subject for arterialised, adipose venous, and the arterio-venous difference, with a period~6–8 min (Table 5.2). Arterialised insulin concentrations showed clear pulsatility in all subjects, as expected, with a typical period of 12 min (Table 5.2).

Table 5.2: Analysis of pulsatility using ‘Cluster 7’ during the fasting period

Measurement	Subjects available (n)	>1 peak found (n)	Median no. of peaks	Median inter-peak interval (Range)/min
NEFA (arterialised)	9	5	2	15.4 (8–30)
NEFA (adipose venous)	9	6	3	11.5 (8–24)
NEFA (V-A difference)	9	7	3	12.0 (10–17)
Mock NEFA (V-A difference)	18	10	2*	14 (9–28)†
Glycerol (arterialised)	6	3	2.5	12.7 (12–22)
Glycerol (adipose venous)	6	0		
Glycerol (V-A difference)	6	5	2	14 (6–30)
Norepinephrine (arterialised)	5	5	6	7.1 (6.3–11)
Norepinephrine (adipose venous)	4	4	6	8.0 (6.3–13)
Norepinephrine (V-A difference)	4	4	6.5	6.0 (5.6–8.0)
Glucose (arterialised)	6	0		
Insulin (arterialised)	9	9	4	12 (8–14)

Legend to Table 5.2

V-A difference, veno-arterialised difference (adipose tissue venous minus arterialised concentration). Mock NEFA (V-A difference) created by subtracting one subject's arterialised concentration from another subject's venous (see Methods). Median interpeak interval and range (minutes) presented for datasets in which > 1 peak was found.

* Number of peaks detected less for “mock” than for real data ($P < 0.05$)

† significantly greater variation in peak interval compared with true NEFA (V-A difference) ($P < 0.01$).

To test whether the apparent pulsatility was indeed real for NEFA and glycerol, we examined cross-correlations between independent measurements, arguing that random fluctuations in the data would not result in significant cross-correlations. For this, we used ‘differenced’ data to eliminate baseline trends as described in ‘Methods’. We examined the cross-correlation of arterialised with adipose venous NEFA concentrations in nine people. Five showed significant positive cross-correlations with no lag ($P < 0.05$ in two subjects, $P < 0.01$ in one subject, and $P < 0.001$ in two subjects).

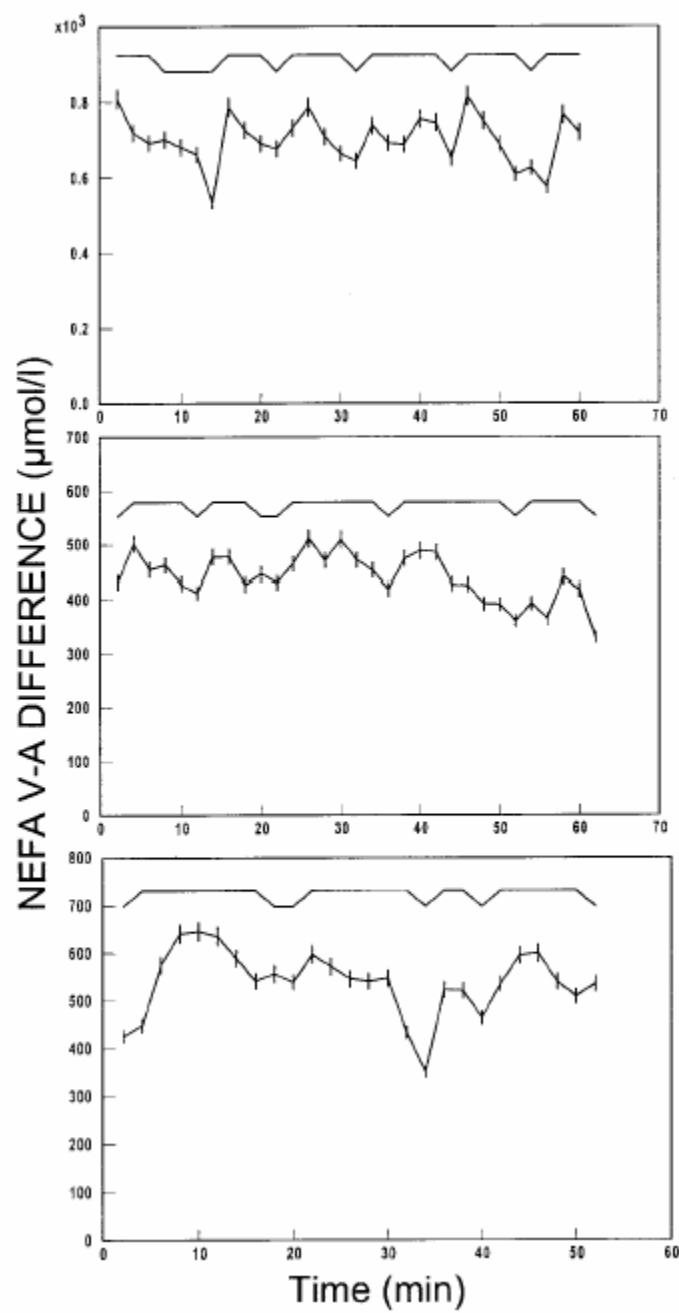
For arterialised and adipose venous norepinephrine (four subjects available), three subjects showed significant cross-correlation with no lag ($P < 0.05$ in two subjects and $P < 0.01$ in one subject).

Finally, to examine completely independent metabolic variables, we looked at the veno-arterialised differences for NEFA and glycerol. Data were available in six subjects. In three subjects, there were significant positive cross-correlations with

no lag ($P < 0.05$ in one subject and $P = 0.001$ in two subjects), and in two others, there were significant positive cross-correlations with lag ~ 10 min ($P < 0.05$).

We attempted to use cross-correlation analysis to show the physiological origin of the pulsatility observed. We cross-correlated veno-arterialised differences for NEFA and glycerol with arterialised norepinephrine and insulin concentrations. No consistent patterns were observed for norepinephrine. There was a tendency to a positive cross-correlation between veno-arterialised NEFA and arterialised insulin concentrations. Of seven people analysed, six showed positive cross-correlations (of whom four were at $P = 0.05$), but one showed a significant negative cross correlation ($P < 0.02$).

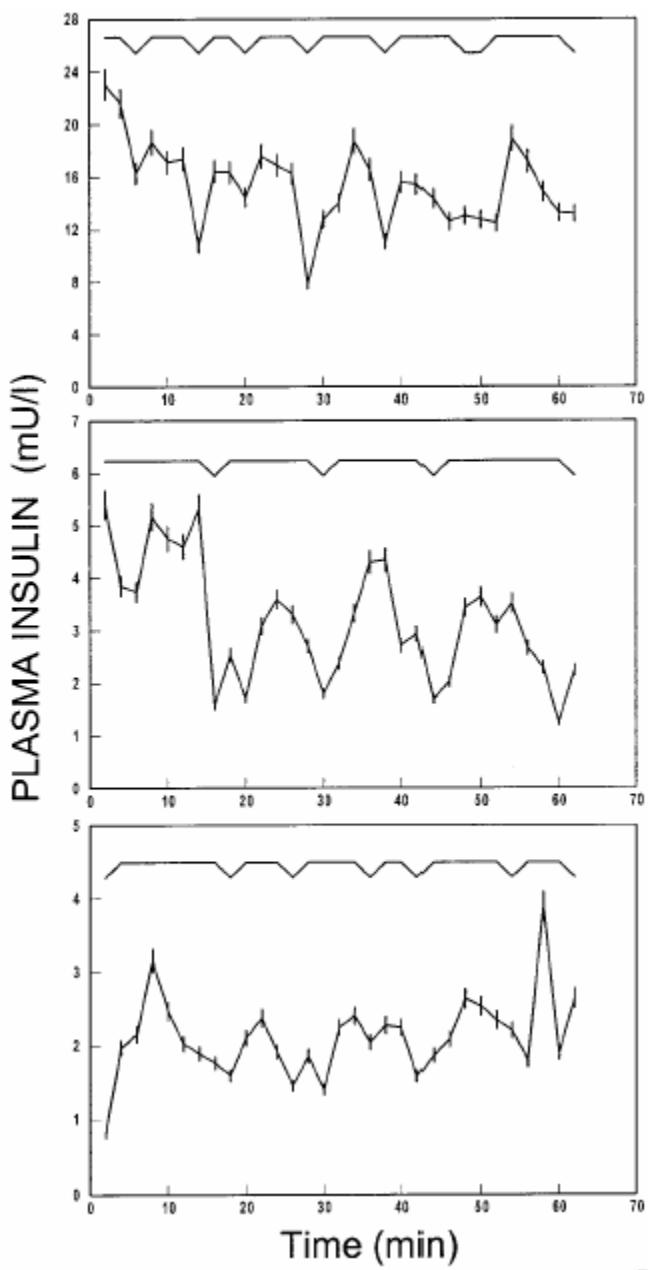
There was no obvious relationship between pulsatility and BMI. Pulses were observed in both lean and obese subjects (Figs. 5.1 and 5.2). If the subjects were divided into tertiles of BMI, the median number of pulses for NEFA veno-arterialised difference was the same (three) in the lowest tertile (median BMI 21.1 kg/m²) and the upper tertile (median BMI 37.3 kg/m²).

Figure 5.1: V-A differences for NEFA in the fasting state

Legend to Figure 5.1:

Representative traces for NEFA release from subcutaneous adipose tissue expressed as veno-arterialised (V-A) difference. The data are veno-arterialised differences for NEFA in the fasting state from three subjects, with BMI (from top to bottom) 50.8, 23.8, and 21.1 kg/m². The pictures are the graphical output from ‘Cluster 7’. The error bars represent analytical variation (a conservative estimate, see Methods). The top line in each panel shows the interpretation of peaks and troughs by ‘Cluster 7’.

Figure 5.2: Arterialised plasma Insulin concentrations in the Fasting State



Legend to Figure 5.2:

Representative traces for arterialised plasma insulin concentrations.

The data are from the same three subjects as the data in Figure 5.1. For explanation, see Figure 5.1.

Checks for reliability.

We applied ‘Cluster 7’ to 30 repeated measurements of plasma NEFA and blood glycerol from a single blood sample, as described in Methods. The program did not detect any pulses, and there was no cross-correlation detected between NEFA and glycerol measurements. ‘Cluster 7’ was also applied to the “mock” NEFA veno-arterialised differences created by subtracting one subject’s arterialised concentrations from the next subject’s venous concentrations (Table 5.2).

‘Cluster 7’ detected fewer pulses in the “mock” NEFA veno-arterialised differences than in the true data ($P < 0.05$, Mann Whitney test), and the characteristics of the pulses found were far less consistent (Table 5.2), with significantly greater variation in the reported peak width ($P = 0.006$) and peak interval ($P = 0.004$) (F tests from SPSS).

Hyperinsulinaemic clamp.

During insulin infusion, there was surprisingly more consistent evidence for pulsatile behaviour of arterialised NEFA and glycerol concentrations, with all subjects showing more than one peak during the period of observation and with reasonably consistent periods of ~12–14 min (Table 5.3). There was again consistent evidence for pulsatile release from the subcutaneous abdominal adipose depot, as measured by the veno-arterialised difference (six of seven and six of six subjects showing more than one peak for NEFA and glycerol, respectively). The

period was again ~10–15 min. As in the baseline period, norepinephrine showed clear evidence of repeated peaks in every subject for arterialised, adipose venous, and the arterialised-venous difference, with a period~7–10 min.

Table 5.3: Analysis of pulsatility using ‘Cluster 7’ during the hyperinsulinemic-euglycemic clamp

Measurement	Subjects available (n)	>1 peak found (n)	Median no. of peaks	Median inter-peak interval (Range/min)
NEFA (arterialised)	7	7	4	12 (8–14)
NEFA (adipose venous)	7	7	3	13 (10–36)
NEFA (V-A difference)	7	6	4	15 (10–20)
Glycerol (arterialised)	6	6	2.5	13 (10–32)
Glycerol (adipose venous)	6	1	1	18 —
Glycerol (V-A difference)	6	6	3	11.5 (9–17)
Norepinephrine (arterialised)	4	4	6.5	7.10 (6.9–22)
Norepinephrine (adipose venous)	4	4	6	9.5 (7–11.5)
Norepinephrine (V-A difference)	4	4	7	7.03 (6–8)

Legend to Table 5.3:

V-A difference, veno-arterialised difference (adipose tissue venous minus arterialised concentration). See also legend to Table 5.2.

Once again, we applied cross-correlation using ‘differenced’ data during the clamp period to assess the reliability of the observed pulsatility. Cross-correlations were generally not so consistent as in the fasting state, but that was to be expected given the smaller concentrations measured. For the cross-correlation of arterialised with adipose venous NEFA concentrations, there were data from seven people, three of whom showed significant positive cross-correlations with no lag or a lag of 2 min ($P = 0.05$ in one subject, $P < 0.01$ in one subject, and $P < 0.001$ in one subject). For the veno-arterialised differences for NEFA and glycerol, data were available in six subjects. In two, there were significant cross-correlations with no lag or 2-min lag ($P = 0.05$ in one and $P < 0.001$ in the other).

DISCUSSION

Our main finding was that there were multiple pieces of evidence for pulsatile release of NEFA and glycerol from human subcutaneous adipose tissue *in vivo*. In contrast, we found inconsistent evidence for pulsatility of systemic NEFA and glycerol concentrations, even though this has been found for NEFA in dogs (Hücking *et al*, 2003). This may imply that in humans, the different adipose depots that contribute to the systemic plasma NEFA pool are not co-ordinated in their pulsatility. We must also consider, however, that an even more precise NEFA assay, perhaps applied over a longer period, might have revealed pulsatility of systemic concentrations. In terms of NEFA and glycerol release, we also have to remember that the transit time for blood through the tissue bed is finite and that simultaneous blood sampling (arterialised and adipose venous) might not be absolutely appropriate. However, we believe the transit time is probably short in relation to the time it takes to draw a sample: blood flow is high in relation to tissue fluid content.

We sought evidence for the main regulator of lipolytic pulsatility, but our results do not give firm conclusions in this respect. While the period of pulsatility of NEFA release was strikingly similar to that of insulin concentrations, pulsatility of NEFA release was maintained even at the suppressed levels seen during the hyperinsulinaemic clamp, when insulin concentrations were steady. This seems to rule out insulin as the main stimulus. It would have been interesting to clamp insulin at fasting concentrations using somatostatin, but at this stage of investigation, we did not want to alter the secretion of other peptide hormones potentially involved. Although there would have been some residual endogenous (and potentially pulsatile) insulin secretion during the clamp, this would have been minor compared with the exogenous insulin.

Taking plasma norepinephrine concentrations as an index of sympathetic nervous system activity, there was clear and consistent evidence for pulsatility, and it would seem likely in principle that this could be a major driver of lipolytic pulsatility, as in dogs (Getty *et al*, 2000, Hücking *et al*, 2003). However, the period of norepinephrine pulsatility was consistently shorter than that for lipolysis, and no strong cross-correlations were observed between the two variables. At the whole-body level, there is a correlation between plasma norepinephrine concentration and sympathetic nerve activity recorded in the peroneal nerve in the resting state (Wallin *et al*, 1981). However, subsequent studies have shown a regional inhomogeneity of sympathetic nervous system responses to interventions such as feeding, where increased norepinephrine spillover is observed in some tissues or organs but not others (Cox *et al*, 1995). The lack of a relationship between oscillations in plasma norepinephrine and in veno-arterialised differences for glycerol or NEFA indicates that any pulsatility in sympathetic nervous system outflow to abdominal subcutaneous adipose tissue is not in synchrony with the oscillations seen in arterial norepinephrine. This is not too surprising, since the major source of arterial norepinephrine is likely to be spillover from sympathetic nerves innervating the vasculature, and one would expect any physiological variation in norepinephrine release to be related to variations in blood pressure rather than in metabolic variables such as adipose tissue lipolysis.

The alternative explanation is that there is some communication between adipocytes that leads to co-ordinated lipolysis. This type of communication is well established in other cell types. Yeast cells in suspensions show co-ordinated oscillations in glycolytic flux (Dano *et al*, 1999). Pancreatic islets in perfusion systems or in a perfused pancreas will show co-ordinated oscillations in glycolysis, intracellular Ca^{2+} concentrations, and insulin secretion (Cunningham *et al*, 1996, Kennedy *et al*, 2002, Gilon *et al*, 2002). However, it is difficult to imagine how signals could diffuse between adipocytes that may be several centimetres apart sufficiently rapidly to produce the periodicity observed here.

One concern in interpreting such data is the detection of spurious pulsatility. Our checks with repeated measurements of one sample gave us confidence that ‘Cluster 7’ will not always detect pulses, as did the lack of pulsatility found in plasma glucose concentration. It was also clear in the fasting state that arterialised NEFA and glycerol concentrations gave less consistent pulsatility than did the corresponding veno-arterialised differences.

We also tried to test validity of the reported pulses by various types of randomisation. For instance, we took each subject’s data (e.g., for arterialised NEFA concentrations) and randomised the time points (data not shown). We also created mock veno-arterialised differences by subtracting one subject’s arterialised concentrations from another subject’s venous concentration. However, we found that such randomisation usually led to ‘Cluster 7’ detecting some pulses, albeit significantly less consistent in character than with the original data. The reason for this must be that a truly pulsatile dataset contains values that are significantly above or below mean values (i.e., by more than analytical variation), and even after randomisation, these values will sometimes by chance associate to give peaks or nadirs.

We consider the data and statistical tests suggest real pulses were present, however, because of the strong independent evidence from cross-correlation analysis. The significant cross-correlations observed between veno-arterialised differences for NEFA and glycerol, for instance, can only easily be explained by true pulsatile lipolysis in the subcutaneous abdominal adipose depot.

We had hoped to test the pulsatility of ATBF, but when the data were examined in detail, it became clear that estimates of ATBF over 2-min intervals from ^{133}Xe -washoutcurves are highly variable and not satisfactory for pulsatility analysis. It remains a possibility that the pulsatility of NEFA and glycerol release that we observed in fact represents pulsatility of ATBF superimposed on a constant rate of

lipolysis. It will be difficult to test this with existing methodologies as the time resolution is inadequate. We believe it is unlikely to be the explanation because it would leave open the question of what drives pulsatility of ATBF. Insulin itself does not regulate ATBF (Karpe *et al*, 2002). In the fasting state, local β -adrenergic blockade does not alter ATBF and the major regulator appears to be the nitric oxide pathway (Ardilouze *et al*, 2004). Adrenergic stimuli only become the predominant regulator of ATBF in response to carbohydrate ingestion (Karpe *et al*, 2002, Ardilouze *et al*, 2004). Of course, even if our observations were a reflection of pulsatility of ATBF, the observation of pulsatile release of NEFA and glycerol still stands, although the interpretation as a reflection of pulsatile lipolysis would need re-evaluation.

The nature of the analysis we have performed does not permit us to conclude whether there is regular oscillation or irregular pulsatility, but to distinguish these two would require longer periods of observation during which it would be extremely difficult to maintain a metabolic steady state.

We did not observe any differences in pulsatility of lipolysis between lean and obese subjects. As outlined above, insulin pulsatility is retained in obesity but lost in type 2 diabetes. It would be interesting to extend these studies to diabetic patients and also to patients with disorders of lipid metabolism in future work.

We conclude that there is pulsatile release of NEFA and glycerol from human subcutaneous adipose tissue *in vivo*. We are unable to pinpoint the stimulus for this pulsatility, although insulin seems to be excluded because of the retention of pulsatility during a hyperinsulinaemic clamp. Pulsatility of NEFA release is maintained in obese subjects, although we have not yet examined patients with type 2 diabetes. Our results have implications for understanding the regulation of lipolysis in humans and raise questions as to the nature of pulsatile release of other adipose tissue products such as leptin. Leptin is known to have a diurnal rhythm entrained by meal ingestion. Defects in leptin production or action are

rare but well recognised causes of obesity in animals and humans. We therefore sought direct evidence of pulsatile leptin production in humans.

CHAPTER VI

OSCILLATIONS IN LEPTIN RELEASE FROM ADIPOSE TISSUE IN THE FASTING STATE AND IN RESPONSE TO EUGLYCAEMIC- HYPERINSULINAEMIA

INTRODUCTION

Adipose tissue was once thought to be a metabolically inert fat repository. However, in recent years it has become clear that it subserves myriad functions including but not limited to fertility, hormonal modulation, immunity, systemic inflammation, thermogenesis and insulin resistance. It has even been ventured that it functions as an endocrine ‘organ’ with all the co-ordination and regulation of structure and function that this term implies in other endocrine organs (Mohamed-Ali *et al*, 1998, Lawrence & Coppack 2000). This concept arose in part following the positional cloning of leptin by Friedman’s group in 1994 (Zhang *et al*, 1994), a landmark in the understanding of adipose tissue biology.

Leptin is a peptide produced in human white adipose tissue which has important effects in the maintenance of body weight through NPY and non-NPY mediated effects on appetite and also through its effects on energy expenditure (Coll *et al*, 2007, Ahima *et al*, 2006, Stephens *et al*, 1995, Erickson *et al*, 1996). Although the absence of leptin is known to be associated with extreme and reversible obesity (Farooqi *et al*, 1999), most obese subjects are not deficient in leptin but instead have elevated circulating leptin concentrations (Farooqi & O’Rahilly 2006). However, in the obese, elevated leptin concentrations do not have the anorexogenic or metabolic effects that might be expected. The concept of obesity being a leptin resistant state has therefore been advanced (Eikelis *et al*, 2007, Scarpase & Zhang 2009).

Leptin resistance

Experimental data in animals suggests that this resistance to leptin action may be induced by 16 days of high (45%) fat feeding (Trayhurn *et al*, 1995). These data suggest that leptin resistance may not be an obligatory feature of obesity *per se*

but may be induced at some point before or during the development of the obese state. Treatment of obesity with leptin has not, with the exception of patients with documented leptin gene mutations, been considered worthwhile due to this resistance.

Many hormones (e.g. LH, insulin) have pulsatile patterns of release and defects in their co-ordinated pulsatile secretion are found in hormone resistant states, an example being the loss of insulin pulses during the progression of insulin resistance to type 2 diabetes (Porksen *et al*, 2002). Such defects have even been detected in non-diabetic relatives of patients with type 2 diabetes suggesting that abnormalities of pulsatility may be encountered early in the pathogenesis of syndromes of hormone resistance (O'Rahilly *et al*, 1988).

It has been suggested (Saad *et al*, 1998) that 'obesity is associated not only with higher leptin levels but also with blunted diurnal excursions and dampened pulsatility.... this abnormal rhythmicity may contribute to leptin resistance in obesity'.

Leptin pulsatility and the SNS

Serum leptin levels in humans and other animals undergo circadian variability with levels highest at night and after meal ingestion and lowest in the morning and when fasting (Sinha *et al*, 1996, Schoeller *et al*, 1997). Superimposed upon this circadian pattern, distinct pulses have been detected although the frequencies reported vary widely between 3.25 (Sinha *et al*, 1996), 3.6 (Saad *et al*, 1998), 7 (Koutkia *et al*, 2003), 10 (Ahmad *et al*, 2001), 30 (Licinio *et al*, 1998) and 32 (Licinio *et al*, 1997) pulses per 24 hours. By reanalysing their data originally sampled at 7 minute intervals at simulated 14, 21, 28, 35, 42, 49 and 56 minute intervals, Licinio (Licinio *et al*, 1998) demonstrated elegantly how much the sampling frequency used will affect the number of pulses detected. Using these derived datasets, the observed 24 hour pulse frequency declined from 30 with the

raw data to 11.5, 8, 6, 3, 4, 1.5 and 0 respectively. Much of the variance evident in the reports cited above may therefore be explicable in terms of divergent methodologies and sampling protocols. Pulsatile concentrations are most evident at meal times and during sleep (Mingrone *et al*, 2005) but the potential extrinsic oscillator has not been ascertained. Using cross-correlation techniques, some have pointed to a relationship between insulin concentration (Saad *et al*, 1998), possibly with a time lag of as long as 275 minutes (Koutkia *et al*, 2003). Others (Wellhoener *et al*, 2000) have suggested that insulin action in terms of insulin-stimulated glucose disposal is responsible on the basis of hypoglycaemic-clamp techniques. Others consider that neither insulin, corticosteroid action (Himms-Hagen 1999), nor an intact pituitary gland (Kousta *et al*, 1998), appears responsible. One report has concluded that leptin pulses may be entrained to SNS variability (Himms-Hagen 1999).

In support of the concept of SNS regulation, connections to candidate brain regions have been suggested by retrograde neuronal tracer studies which have shown that pseudorabies virus injected into WAT is transported to the intermediolateral cell group and thence on to the nucleus of the solitary tract (which is known to be involved in voluntary food intake) and thence to the paraventricular nucleus which stimulates lipolysis. In addition, the suprachiasmatic nucleus (SCN), a hypothalamic region involved in circadian rhythmicity also appears to innervate white adipose tissue suggesting a possible mechanism whereby circadian signals could be transmitted to adipose tissue via its SNS innervation (Bartness & Song 2007). These observations concord well with evidence for leptin excursions being primarily linked to food intake and time of day (Sinha *et al*, 1996, Schoeller *et al*, 1997). Further data supporting a role for the SNS in mediating leptin pulsatility has come from experimental axonal interruption studies that have attenuated leptin pulses by disconnection of the afferent SNS innervation of adipose tissue (Nijjima 1999) and from studies that show that beta agonist therapy may affect leptin production (Ricci *et al*, 2005).

Pulsatility as a marker of adipose tissue co-ordination as an ‘organ’

Variability in the plasma concentration of a hormone or cytokine may be regulated by an external oscillator although the possibility that circadian oscillations may be generated by genetic mechanisms intrinsic to adipose tissue with or without external modification (Zvonic *et al*, 2006) has recently been advanced. There is little agreement as discussed above as to where the external oscillator, if any, lies although either insulin, the SNS or both have some evidence in support of them in this role.

Whatever its origin, variability in plasma concentrations does not necessarily imply co-ordinated secretory pulses. Changing binding characteristics (e.g. to leptin soluble receptors or specific or non-specific (e.g. albumen) binding proteins in plasma), changes in the rate of clearance, hydrolysis or receptor mediated uptake as well as changes in production (Gavrilova *et al*, 2003) may lead to variability in arterialised or venous plasma concentrations over time. Conversely, for similar reasons, the absence of co-ordinated pulsations in venous or arterialised plasma does not *ipso facto* rule out the presence of co-ordinated output from adipose tissue. Evidence for co-ordinated pulsation would support the view of adipose tissue as an ‘organ’ rather than simply a collection of similar cells. Although mathematical modelling techniques such as deconvolution analysis may be used in an attempt to estimate production from plasma concentrations and decay and distribution characteristics, they are inherently less satisfactory than direct measurement of output where this is possible.

In summary, although most studies report diurnal and prandial variability of leptin concentrations, the presence, periodicity and control of leptin release directly from adipose tissue has not been characterised. Nor has the question of whether leptin resistance may be related to defective pulsatility been fully resolved.

We therefore sought evidence for the pulsatile production of leptin in individuals of differing body weight using adipose tissue venous cannulation and veno-arterial difference methodology. We sought to determine whether leptin release or concentration is in fact pulsatile and, if so, whether the patterns of release differed between lean and obese individuals suggesting that this might explain observed differences in leptin action. Finally, we sought evidence that the release of leptin from adipose tissue might be entrained by insulin pulses and thus would be attenuated under hyperinsulinaemic-euglycaemic clamp conditions as a result of blunting of insulin pulses.

Our hypothesis was that leptin production demonstrates co-ordinated pulsatility and that leptin pulses would continue despite attenuation of spontaneous insulin pulsatility. We reasoned that, if proven, this would lead to proposal of a further hypothesis that disorders of leptin pulsatility might affect tissue responses and therefore contribute to the leptin resistance seen in obesity.

METHODS

Eight healthy subjects (five men), covering a range of adiposity levels (BMI 20.5–50.8, median 23.8 kg/m²), were recruited. Their ages ranged from 21 to 61 years (median 43). The subjects were healthy and receiving no medication. The protocol was approved by the Oxfordshire Clinical Research Ethics Committee and the East London and City Ethics Committee, and all subjects gave written informed consent. Subjects were studied after an overnight fast.

The methods used were identical to those in Chapter V above and will not therefore be reiterated in detail. Subjects were cannulated using the techniques described in chapter II ‘Core Methods’.

Sampling proceeded at 2 minute intervals for 60 minutes from abdominal subcutaneous and arterial (n=5) or arterialised (n=3) blood. A hyperinsulinaemic-euglycemic clamp was established by infusing insulin, using a primed-constant infusion protocol (DeFronzo *et al*, 1979), with the final infusion rate 35 mU. m⁻². min⁻¹ as before. Once the blood glucose concentration was stable, blood sampling was again started simultaneously from the arterialised and adipose tissue venous lines, and this was repeated every 2 min for 60 min. Clamps were not performed in five subjects for technical reasons. Samples were kept on ice, and plasma was separated rapidly by centrifugation at 4°C. Plasma leptin analysis was performed using a commercially available enzyme linked immunosorbent assay (ELISA) method with a sensitivity of 2 ng/ml. Standard curves were constructed and 2 replicates were used for each measurement. Intra-assay coefficient of variation was reported as 6.3% and the mean difference between replicates was less than 2%. Adipose tissue blood flow (ATBF) was measured as described in Chapter II ‘Core Experimental Methods’. Of all potential leptin samples, 97% were available for analysis (those missing were due to technical difficulties e.g. blocked cannula). Missing values were filled by interpolation of the mean value

of adjacent results before data analysis. There were no instances of consecutively missing data points. Data were not available for some subjects during the hyperinsulinaemic-euglycaemic clamp and veno-arterial differences were not obtained from some subjects due to technical problems with cannulation.

We sought evidence for pulsatility as described above in Chapter V. We used similar tests of reliability to examine whether the pulse detection programme ‘Cluster 7’ (Veldhuis & Johnson 1986) was detecting real pulses or simply random variability. These tests of robustness included creation of “mock” veno-arterialised differences for leptin by subtracting one subject’s arterialised concentrations from the next subject’s venous leptin concentrations which were then tested for pulsatility.

In addition, we created further ‘control’ data by randomisation of the time order of both venous concentration and veno-arterialised differences in an attempt to determine whether ‘pulses’ detected in ‘Cluster 7’ represented truly co-ordinated pulses or whether variability for any one of a number of reasons was more likely. For adipose tissue venous leptin concentrations, the random number generator in ‘Microsoft Excel’ was used to create a random number between 0 and 1 and these numbers were put alongside the true time-ordered results. Columns were then sorted according to increasing randomly generated number so that the time order of the true results became randomised. Data were then re-analysed using ‘Cluster 7’ to attempt to detect pulses. For data presented in this chapter, the default settings of 2 points each to define a peak or a nadir were used within ‘Cluster 7’ due to the longer half life of leptin (24.9 ± 4.4 minutes) compared to NE or NEFA in plasma (Klein *et al*, 1996).

Statistical methods and assumptions are as for Chapter V above and data are mean \pm SD except where otherwise stated.

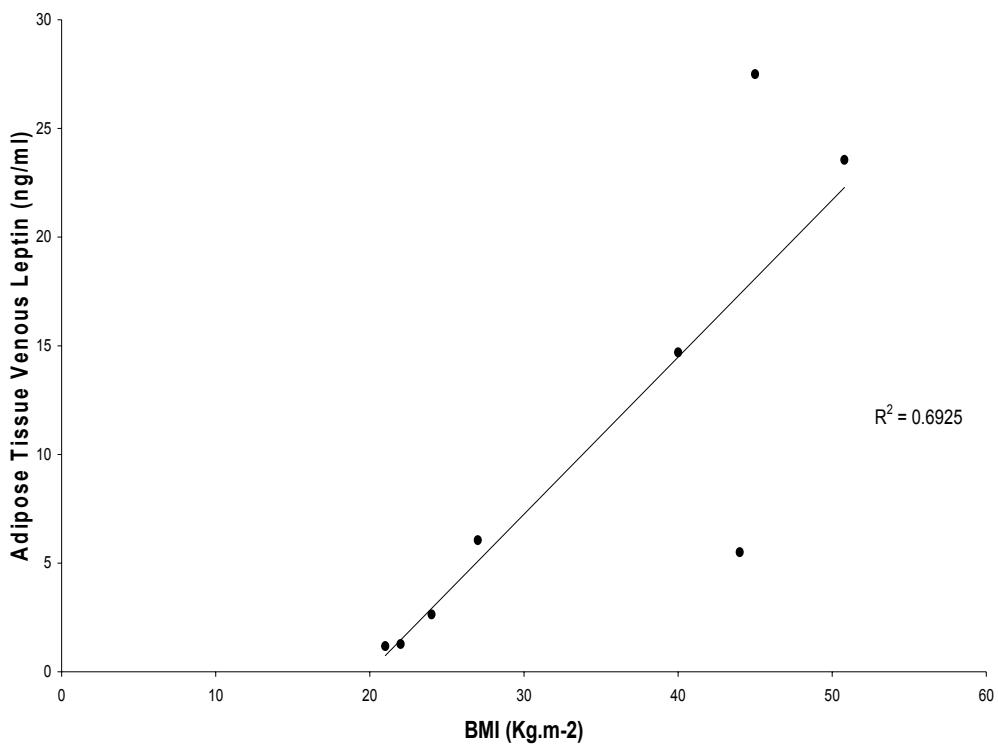
RESULTS

POST-ABSORPTIVE STATE

Venous Concentrations

Fasting leptin levels were higher in the more obese subjects as expected ranging from mean values of 1.18 to 27.5 ng/ml as BMI increased from 20.5 to 50.8 Kg.m^{-2} ($p<0.005$). This relationship is demonstrated in Figure 6.1.

Figure 6.1: Relationship between adipose tissue venous leptin concentration (ng/ml) and Body Mass Index (BMI, Kg.m^{-2})

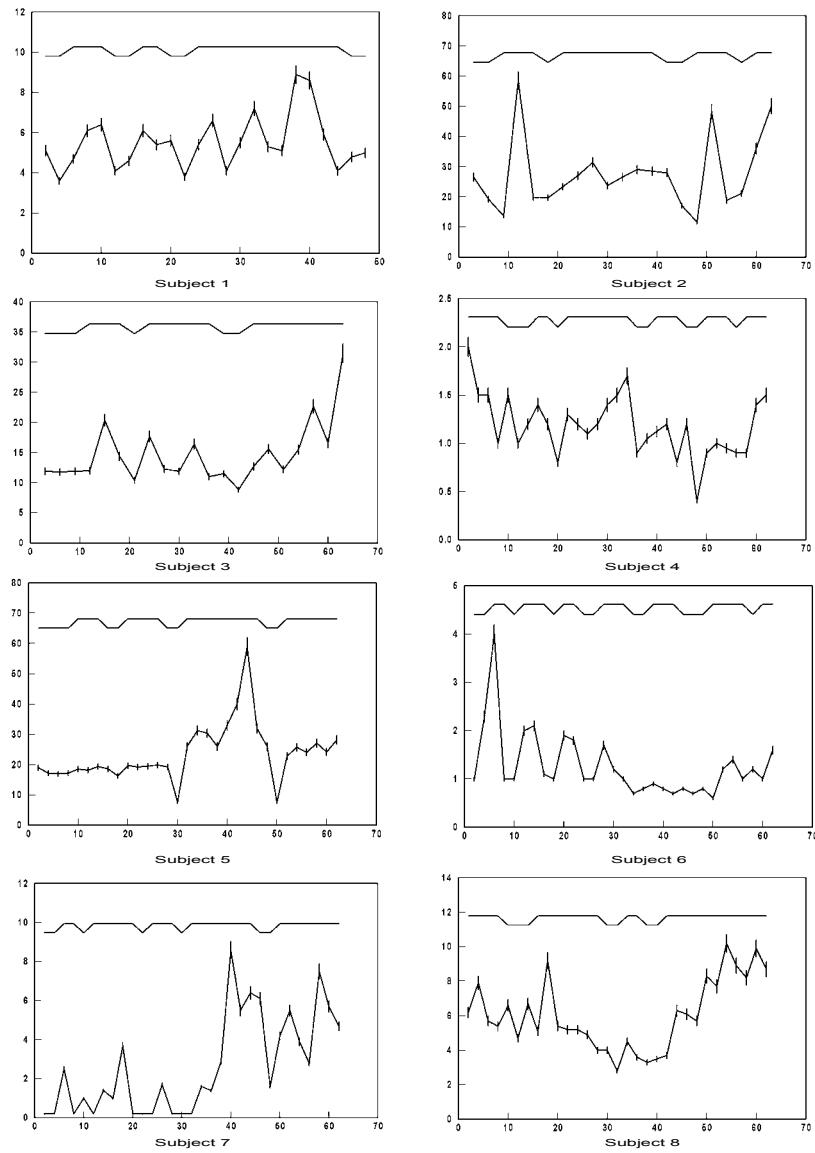


Legend to Figure 6.1

Shows the significant increase in mean plasma leptin (ng/ml) for subjects of increasing BMI (Kg.m^{-2}). The correlation co-efficient (non-parametric Spearman's test as samples from a non-normal distribution) was $p<0.005$.

Variability in adipose tissue venous leptin concentrations was apparent in all 8 subjects and 2-6 (3.25 ± 1.39) peaks and 2-6 (3.50 ± 1.41) troughs were detected by the 'Cluster 7' programme as pulses (Table 6.1). There was no relationship between the number of peaks and troughs and body mass index. Figure 6.2 below shows the graphs generated using this programme with the settings described in Methods.

Figure 6.2: Post-absorptive Venous leptin concentration (ng/ml) vs. time



Legend to Figure 6.2

Shows raw outputs of venous leptin concentrations (ng/ml) vs. time (min) from 8 subjects as detected using ‘Cluster 7’. The horizontal line above each leptin-time plot represents peaks or troughs identified by ‘Cluster 7’.

Table 6.1: Adipose tissue venous leptin in the post-absorptive state

Subject	1	2	3	4	5	6	7	8
Mean leptin	5.50	27.50	14.70	1.18	23.55	1.27	2.64	6.05
AUC	253.9	1621.5	862.8	69.9	1413.3	76.0	158.5	360.3
Peaks (n)	2	3	2	4	3	6	4	2
Troughs (n)	2	3	2	5	3	6	4	3

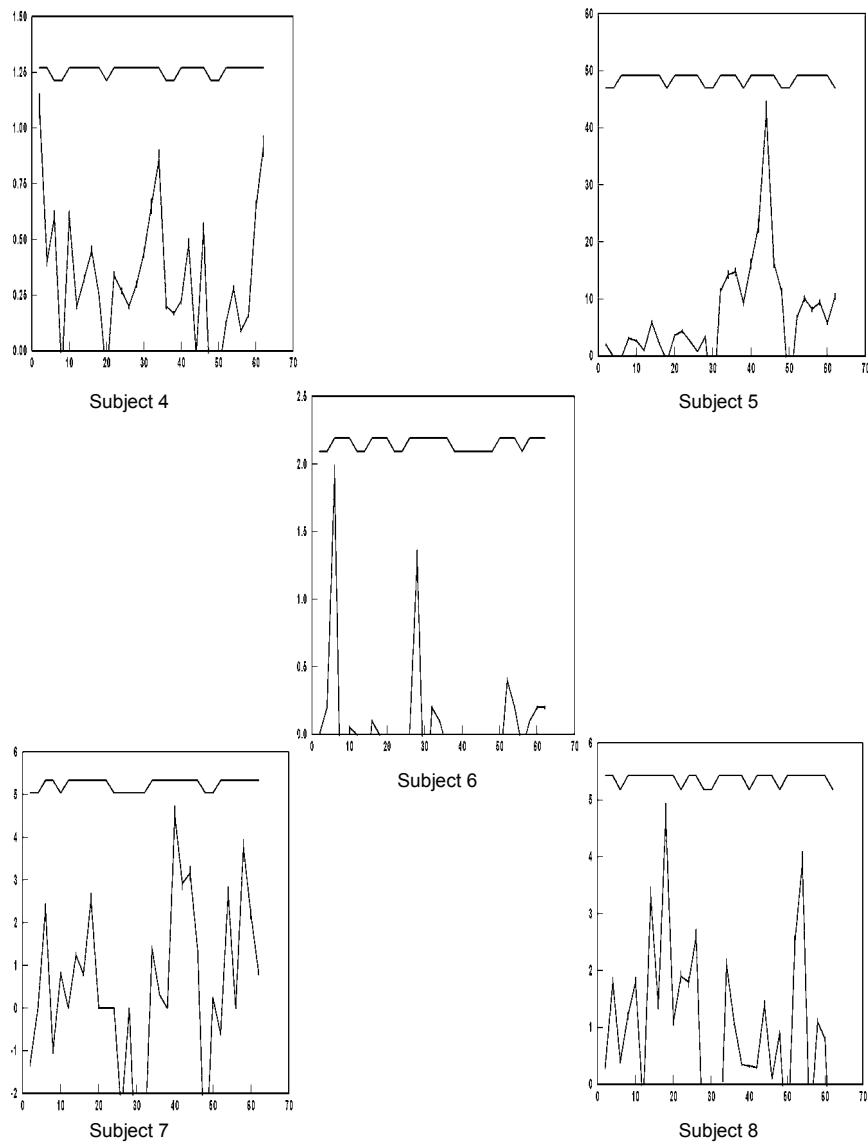
Legend to Table 6.1

Individual adipose tissue venous leptin values (ng/ml) for 8 subjects (numbered 1,2...8) showing mean adipose vein concentration, area under the leptin-time curve (AUC) and number (n) of peaks and troughs determined by ‘Cluster 7’ over 60 minutes prior to insulin clamp.

V-A Differences

Similarly, ‘Cluster 7’ detected between 3 and 5 pulses in v-a differences in the post absorptive state in all subjects (Table 6.2). Again, the number or peaks (3.6 ± 0.54) and troughs (4.0 ± 0.7) detected by ‘Cluster 7’ did not differ between lean and obese subjects. Figure 6.3 shows the graphs generated using the pulse detection programme with the settings described in ‘Methods’.

Figure 6.3: Leptin v-a differences (ng/ml) in the post-absorptive state



Legend to Figure 6.3

Shows the raw 'Cluster 7' outputs with pulse detection (horizontal line) of leptin v-a difference (ng/ml) vs. time (minutes) from 5 subjects in the post-absorptive state using the settings described in 'Methods'.

Table 6.2: V-a differences (ng/ml) for 5 subjects' 'true' leptin datasets

Subject	4	5	6	7	8
Mean v-a difference	0.328	7.174	0.00163	0.457	0.876
AUC of v-a difference	18.32	432.3	0.3	28.84	56.8
Peaks	3	4	4	3	4
Troughs	4	4	4	3	5

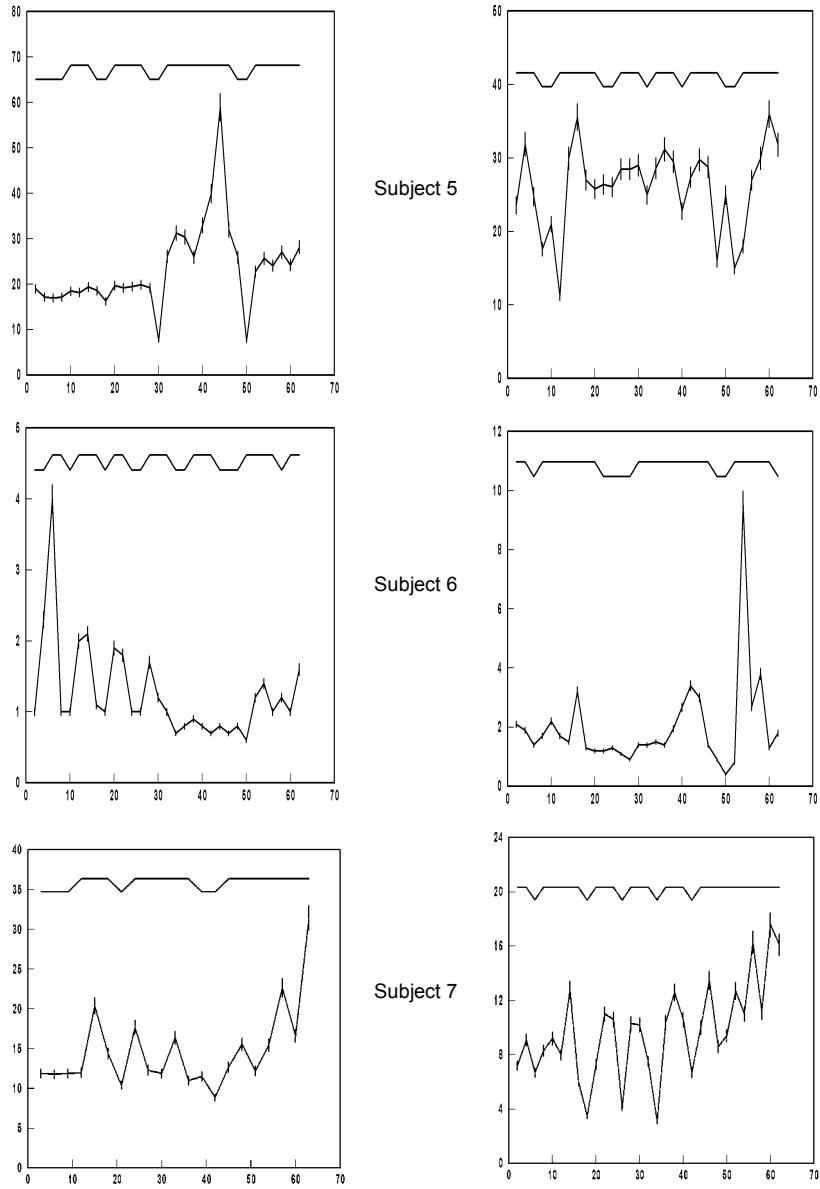
Legend to Table 6.2

Shows mean veno-arterial difference (ng/ml) and area under the curve or veno-arterial difference in leptin (ng/ml) for 5 subjects in the post absorptive state.

CLAMP STUDIES

Venous leptin concentrations did not increase significantly before vs. during euglycaemic hyperinsulinaemia (9.15 ± 12.5 ng/ml vs. 12.48 ± 12.29 ng/ml, $p=0.21$) but there was a non-significant trend towards increased v-a differences (2.54 ± 4.01 vs. 4.35 ± 4.89 ng/ml, $p=0.07$). The area under the leptin venous concentration curve (AUC) also showed a trend towards increase which just failed to reach statistical significance (153.8 ± 241.6 vs. 256 ± 285.6 , $p=0.05$). There was no significant difference between the number of venous leptin peaks before (4.33 ± 1.53) or during (3.33 ± 1.15) the euglycaemic hyperinsulinaemic clamp. Neither was there a difference in the number of venous leptin troughs before (4.3 ± 1.53) or during (4.3 ± 1.15) the insulin clamp ($p=1.0$).

Figure 6.4: Adipose tissue venous leptin concentrations (ng/ml) in 3 subjects during euglycaemic-hyperinsulinaemic clamp



Legend to Figure 6.4:

Shows the raw 'Cluster 7' outputs of adipose tissue venous leptin concentrations (ng/ml) in 3 subjects during euglycaemic-hyperinsulinaemic clamp conditions using the settings described in 'Methods'.

Table 6.3: Adipose tissue venous leptin concentrations (ng/ml) during euglycaemic-hyperinsulinaemic clamp for 3 subjects

Subject	5	6	7
Mean leptin	26.09	2	9.73
Leptin AUC	1562.3	120.15	579
Peaks	4	2	4
Troughs	5	3	5

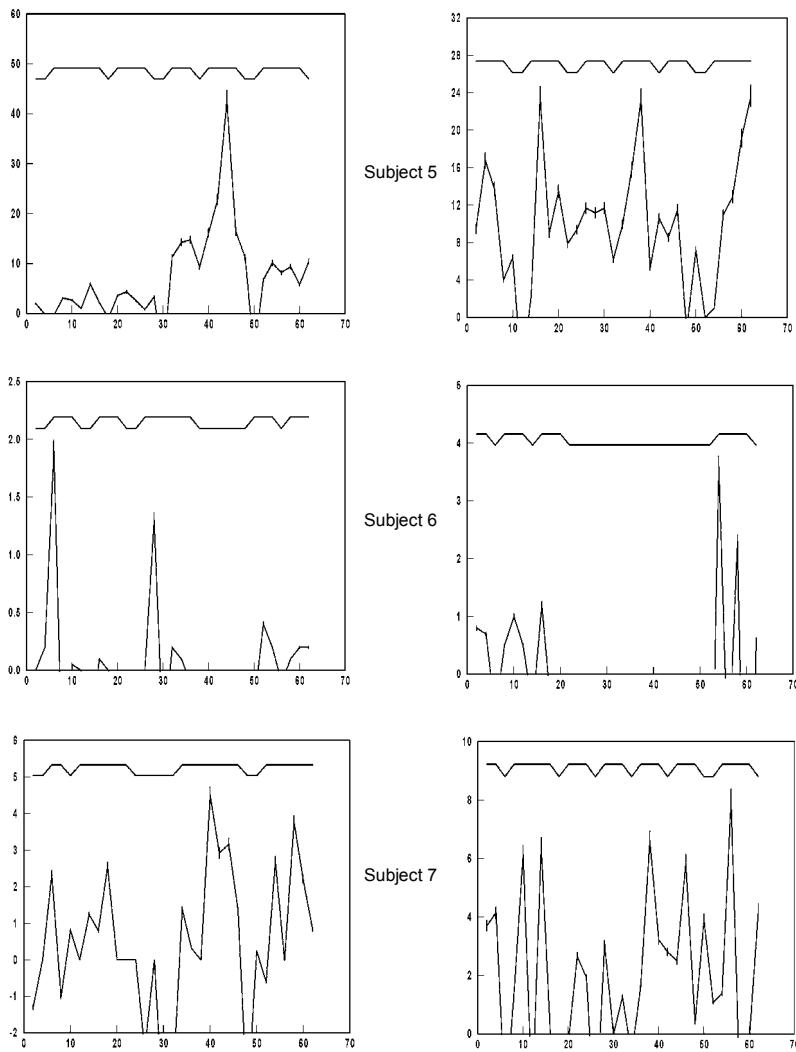
Legend to Table 6.3

Shows mean leptin concentrations (ng/ml) and area under the curve of leptin concentrations (Leptin AUC, ng/ml) for 3 subjects under conditions of euglycaemic hyperinsulinaemia.

V-a leptin differences (ng/ml) during euglycaemic-hyperinsulinaemic clamp

There was a trend towards an increase in leptin v-a differences in response to insulin (2.54 ± 4.01 before vs. 4.35 ± 4.89 ng/ml after insulin, $p=0.07$ (paired t test, 2 tailed) which just failed to reach statistical significance. This data is shown in Figure 6.5 and Table 6.4. The number of v-a difference peaks did not differ before (3.66 ± 0.33) or during (3.66 ± 2.33) the insulin clamp ($p=1.0$). Neither was there a difference in the number of troughs in v-a differences before (3.66 ± 0.58) and during (4.66 ± 1.53) the insulin clamps ($p=0.47$) (paired t test, equal variance not assumed).

Figure 6.5: Leptin v-a differences (ng/ml) before (left) and during (right) euglycaemic-hyperinsulinaemic clamp



Legend to Figure 6.5:

Graphical output from 'Cluster 7' for 3 subject's leptin v-a differences (ng/ml) before (left) and during (right) euglycaemic-hyperinsulinaemic clamp are shown over a 60 minute period after reaching steady state conditions and using the settings for 'Cluster 7' described in 'Methods'.

Table 6.4: Leptin v-a differences (ng/ml) during the insulin clamp for 3 subjects

Subject	5	6	7
Mean leptin v-a difference	9.98	1.17	1.9
Leptin v-a difference AUC	585.7	73.9	110.31
Peaks	4	2	5
Troughs	5	3	6

Legend to Table 6.4:

Mean leptin veno-arterialised differences for 3 subjects during steady state conditions of euglycaemic hyperinsulinaemia.

'CONTROL' DATASETS

In order to test whether or not these data were robust, we went on to create control datasets against which to test the true datasets so that we could be sure that what 'Cluster 7' was detecting was true pulsatility rather than random biological variability.

As described in 'Methods', mock datasets were created by either randomising the time order of plasma concentrations or v-a differences or by creating falsely paired v-a differences subtracting one subject's arterial concentration from the other subject's venous concentration.

Mock venous leptin datasets

Each of the control datasets for the 5 subjects studied demonstrated between 2 and 4 apparent peaks (3.2 ± 0.84) and between 3 and 4 apparent troughs (3.8 ± 0.45). The mean number of peaks ($p=0.46$) and troughs ($p=0.54$, t test, 2 sample, not assuming equal variance) did not differ significantly from the number using correctly time ordered datasets i.e. although 'pulses' were detected by 'Cluster 7' in all subjects' venous leptin concentrations and v-a differences, a similar number of pulses was also detected when the 'true' datasets were converted into control datasets both by false pairing and randomisation of their time order.

Mock v-a difference datasets

Again, mock or ‘control’ datasets were constructed as described in ‘Methods’.

1) False pairs

Variability was evident in the mock datasets and once again ‘Cluster 7’ recognised between 3-7 peaks (4.16 ± 1.47) and troughs (4.16 ± 1.60) in each subject. The number of peaks ($p=0.411$, t test assuming unequal variance) and troughs ($p=0.825$, t test assuming unequal variance) did not differ between the true and the mock v-a difference datasets.

Because this raised the possibility that ‘Cluster 7’ was detecting peaks that were due to the inherent variability of the data rather than due to the presence of true pulsatility, we went on to investigate this further with additional mock datasets. These were created by randomising the order of true v-a differences and then testing the resultant dataset with ‘Cluster 7’.

2) Random order v-a datasets

Randomised datasets were created in Microsoft Excel by using the random number generator to generate a random number between 0 and 1 in a column adjacent to the true time ordered values for leptin v-a differences. The values were then sorted according to increasing random number value to generate random time ordered leptin v-a differences. Again, 2-5 peaks (3.2 ± 1.3) and 2-6 troughs (3.8 ± 1.64) were found in each subject after randomisation of the time order of their v-a leptin differences. The number of peaks ($p=0.55$) and troughs ($p=0.81$, 2 tailed 2 sample t test) did not differ significantly between the true dataset and the randomised time order dataset in each subject.

3) Change in assay CV using cluster

As a result of discovering that cluster was detecting pulses in mock datasets, we investigated the possibility that apparent pulses were being detected because we underestimated the assay CV for leptin and we therefore changed the detection criteria of ‘Cluster 7’ to assume a more conservative assay CV of 10% which comfortably exceeded estimates of the assay CV of 6.3% which were based on repeated QC replicates as described in ‘Methods’.

This would allow for the fact that greater apparent CV would occur when an arterial value with its own CV were subtracted from a venous value with an inherent CV and also for variable CV at different. This had no significant effect on the pulse detection using cluster in the subjects studied.

DISCUSSION

We used direct adipose tissue venous sampling and a-v difference methodology seeking evidence of pulsatile leptin secretion. Using these methods, we studied release of leptin from subcutaneous abdominal adipose tissue in humans of differing body composition. We are not aware of any previous studies using this methodology to investigate secretion rather than venous concentration in man nor are we aware of the use of 2 minute sampling in order to be able to detect oscillations with a frequency that might be expected if pulsatility were, for example, entrained to insulin or SNS pulsations with inter-peak intervals in the order of 7-15 minutes (see Chapter V results). As expected, leptin levels and leptin production as assessed using veno-arterial differences was greater in the more obese subjects. In accordance with previous reports (Boden *et al*, 1997), the effect of euglycaemic insulin infusion was to increase the secretion of leptin although our results just failed to reach statistical significance for v-a difference ($p=0.07$) and area under the leptin curve ($p=0.05$). This may be explicable given previous reports suggesting a time lag between insulin and leptin excursions (Koutkia *et al*, 2003) or by the loss of power we experienced as a result of small numbers of completed clamp studies. The trend to increase we did see in v-a differences measured in adipose tissue venous effluent render any insulin mediated increase in leptin concentrations to be more likely to be due to changes in the rate of production, at least from the human subcutaneous adipose tissue depot than to a reduction in clearance. It has previously been shown that apparent insulin-mediated effects are blunted during hypo-glycaemic hyperinsulinaemia suggesting that glucose uptake per se rather than insulin binding may be the trigger for leptin release under these conditions (Wellhoener *et al*, 2000).

Although our data revealed variability in the secretion and v-a differences of leptin, our application of mock datasets to act as controls gave us reason to doubt

that this represented truly co-ordinated pulses. This is despite the fact that a commonly accepted programme for pulsatility detection suggested that the concentrations and production contained more variability than could be accounted for by assay variability alone. The strength of our method was that rapid (2 minute) sampling over 1 hour allowed us to look for rapid oscillations but also constrained us in that it only permitted us to make observations over this short time period. Oscillations with periods already reported in the literature variously between 3.25 (Sinha *et al*, 1996), 3.6 (Saad *et al*, 1998), 7 (Koutkia *et al*, 2003), 10 (Ahmad *et al*, 2001), 30 (Licinio *et al*, 1998) and 32 (Licinio *et al*, 1997) pulses per 24 hours are perfectly consistent with our negative findings in that no more than one oscillation with an inter-peak interval similar to those reported in these studies would occur in its entirety over our sampling period. Our methods were not designed to examine any form of diurnal variability, meal response or circadian rhythm: such influences on leptin production have been long established (Sinha *et al*, 1996) and repeatedly observed (Licinio *et al*, 1998, Migrone *et al*, 2005). We did not ‘smooth’ our data using techniques such as ‘differencing’ or passage thorough ‘filtering’ algorithms to remove unstable baseline characteristics or oscillations taking place over a longer period than we were measuring because the ‘Cluster 7’ programme methodology is thought to be inherently robust to such effects (see below).

‘Cluster 7’

As stated in its product literature (Veldhuis & Johnson 1986), ‘Cluster 7’ is less prone than previous methods to the effects of inconsistent pulse frequency estimates, fluctuating baseline hormone concentrations, variable pulse amplitudes, lack of consistent definition of what exactly constitutes a peak and a trough or lack of an explicit statistical basis for the analysis. Definition of a peak requires that a region of significant increase be associated with nadirs on both sides. A nadir is defined as a decrease followed by an increase, with all else representing a peak. In performing the analysis, the operator specifies individual test cluster

sizes for the nadir and peak (i.e., number of points to be used in testing nadirs against peaks), a minimum and maximum intra-series coefficient of variation, a t statistic to identify a significant increase, and a t statistic to define a significant decrease. The programme does assume that measurement error (essentially assay CV) is distributed in a Gaussian fashion although it is robust to skewed distribution. In determining the CV for the assay, the programme can either calculate it based on the variability of replicate assay results on the clusters of values used to define a particular peak or trough or can be set to use the measured CV of the assay as a whole based on the testing of replicated assays of a typically larger number of data points. On theoretical grounds, which they demonstrate mathematically, and with support from ‘stress testing’ the programme on data series of different hormones with known pulsatility, the authors conclude that a constant ‘external’ CV performs better and we therefore adopted this method analysing our results. The authors state that the program ‘should provide the following advantages in pulse analysis: 1) consistent pulse detection despite drifting base-line hormone concentrations; 2) lack of requirement for uniform pulse amplitudes within the series under analysis; 3) an ability to use multiple-point criteria to delineate significant nadirs or peaks; 4) a statistically explicit definition of significant increases and decreases in the data series and hence accurate demarcations of peak width; 5) sensitivity to assay precision within the actual experimental data series; and 6) substantial freedom from variable false-positive errors associated with a wide range of intra-assay coefficients of variation. Thus, we suggest that cluster analysis may be applicable to investigations of a wide variety of endocrine pulse signals.’

Variability vs. Pulsatility

Several potential mechanisms may account for variability without truly coordinated pulsatility. These include time dependent changes in soluble receptor mediated clearance, in renal leptin clearance (the predominant method (Cumin *et al*, 1996)) or in other factors such as adipose tissue blood flow. Adipose tissue

blood flow changes are known, for example, to affect patterns of NEFA export from adipose tissue by promoting re-esterification when the products of lipolysis are able to accumulate locally (Burns *et al*, 1978). For this reason, we had intended to measure leptin flux- that is the product of v-a difference and blood flow as this, using the Fick principle (Zierler 1961), would have given us true rates of production. As reported in Chapter V, we found our methods for ATBF quantification were not able to resolve changes any more rapidly than over approximately 20-30 minute periods of observation so this was not possible in practice. We might however suppose that if blood flow were truly pulsatile, the product of a variable and a pulsatile number (e.g. sinusoidal) series might be expected to produce a variable rather than truly pulsatile number, mathematically at least.

Detecting pulses in biological data is inherently problematic because of factors such as noise (which may or may not be Gaussian), unpredictable frequency, assay variability, biological variability (caused by haemolysis or degradation in the syringe and tubing or during storage), variability introduced by the very act of sampling (e.g. changes in blood flow due to the negative pressure applied to draw the sample), infrequent sampling (e.g. failing to find the apex of true peaks), statistical tests prone to what is in effect multiple hypothesis testing, moving baseline characteristics of the data, variable size of peaks, superimposition of slower (e.g. circadian) and faster (e.g. pulsatile) variability and the sometimes unknown plasma kinetics of the substance under study which may be multiple (e.g. receptor mediated and renal clearance) and concentration dependent.

In the case of adipose tissue, there are a number of adipose tissue depots (including the brain which may make a significant contribution to whole body leptin production (Eikelis *et al*, 2004) and random variations in the secretion from each could at times be in phase and summate and at others be out of phase with each other and not summate. The signal detected will depend upon many factors including the site and the frequency used. Insulin pulsatility serves as a good

illustration of these concepts. Insulin has an ultradian rhythm detectable with, for example, hourly blood sampling. It also has meal time excursions which may be similarly detectable. However, insulin also has a superimposed faster component of pulsatility with a period of approximately 5-15 minutes which is far better detected with sampling every 1-2 minutes directly from the portal vein than with less frequent sampling from peripheral veins. Each of these components is likely to have biological relevance and require different study designs to resolve. The diurnal variation may be related to diurnal changes in insulin sensitivity and the mealtime excursions are clearly related to carbohydrate disposal. The most rapid component is lost early in the pathogenesis of insulin resistance (O'Rahilly *et al*, 1988) and may thus be important in understanding this condition.

Conclusions

We conclude that although ‘Cluster 7’ and similar programs offer a number of distinct advantages in deciding whether variability within a time ordered dataset can be explained by assay variability alone, it does not confer the ability to disentangle co-ordinated pulsatility from other causes of variation such as biological variability or random noise. Thus, a cautious approach should be taken when interpreting claims to have detected pulsatility using methods such as ‘Cluster 7’. This is not to imply criticism of the use of the programme itself which is explicit and effective about what it does: it makes a probabilistic decision about whether a data point differs from those preceding and following it as a result of assay variability and defines an operator specified number of successive significant excursions in a positive or negative direction as a peak or a trough respectively. Using a series of control constructs derived from re-pairing or re-ordering the real data, we conclude that leptin concentrations show greater variability than can be accounted for by assay variability alone but that pulses detected by ‘Cluster 7’ are not more co-ordinated than random re-arrangements of the data. We did not find convincing evidence of differences (other than in amplitude and mean levels) between the characteristics of leptin production

assessed by v-a differences and adipose tissue venous concentrations between lean and obese subjects. We therefore reject our hypothesis that pulses of leptin production are subject to co-ordinated regulation and would be abnormal in obesity.

Our results are consistent with the concept that leptin acts as a medium to long term signal related to repletion of fat stores rather than as a minute to minute signal where pulsatility might be thought to be of greatest importance. For this reason, it is perhaps not altogether surprising that we failed to find convincing evidence of co-ordinated pulsatility. We advance our use of control datasets as a method by which truly co-ordinated hormone or cytokine pulsatility may be distinguished from any of the other reasons that variability may be encountered in time ordered sequences of biological datasets.

We sought next to examine the anatomy of the SNS within adipose tissue in order better to understand the consequences of the study in Chapter IV suggesting defective release of norepinephrine from SNS terminals within this tissue in obesity. We hoped that a greater understanding of the anatomy might give clues to the likely functional consequences of our findings. Specifically, we wished to confirm that SNS axons were present in human abdominal subcutaneous white adipose tissue and to determine whether they were predominantly peri-vascular in distribution or whether they made direct contact with adipocytes spatially distant from blood vessels.

CHAPTER VII

MORPHOLOGICAL STUDIES ON THE SNS INNERVATION OF ADIPOSE TISSUE IN MAN

INTRODUCTION

From function to structure

It has been known for over a century that the SNS is able to control the formation and dissipation of lipid stores through activation of its receptors within white adipose tissue (Dogiel 1898). Its ability to do this does not primarily depend upon changes in the concentration of circulating catecholamines as removal of their primary source, the adrenal medulla, does not attenuate the lipolytic response to experimentally induced hypoglycaemia (Nishizawa & Bray 1978, Spiegelman & Flier 2001). Local release of catecholamines within adipose tissue itself is further implied by studies comparing the effects of infusion of either glucose or the same amount of the glucose utilisation blocker, 2 deoxyglucose which produces a state of acute functional neuroglycopenia (Bartness & Bamshad 1998). Whereas infusion of glucose results in decreased firing rates of SNS axons innervating epididymal WAT, the same amount of intravenous 2 deoxyglucose results in increased firing rates in the same tissue bed. The mechanism by which catecholamines are delivered to the vicinity of adipocytes is significant because of the different concentrations, and as a consequence, the different receptor-mediated effects that may result. Whereas alpha 2 receptor activation (decreasing lipolysis) has been reported to predominate at low catecholamine concentrations, beta 3 activation and increased net lipolysis occurs at higher concentrations (Carmen & Victor 2006, Lafontan & Berlan 1995) If even the response to a metabolic emergency (hypoglycaemia) where circulating catecholamines are present in high concentration is mediated primarily by catecholamines released from SNS axons within adipose tissue, it must therefore follow that the routine autonomic control of lipolysis will also be dependent on local adipose tissue innervation rather than circulating catecholamines. Such an arrangement prevents the organism having to endure the effects of widespread catecholamine secretion

(the ‘fight or flight’ response) whenever lipolysis is required e.g. after overnight fasting. Whether high local catecholamine levels are achieved primarily by direct synaptic contact between SNS axons and adipocytes or alternatively via diffusion of Norepinephrine from the microvasculature is not currently clear (Wirsén 1964, Slavin & Ballard 1978, Rebuffe-Scrive 1991, Bartness & Bamshad 1998). Other functions of the SNS within adipose tissue including the possibility that it may also be able to influence lipolysis indirectly through regulation of adipose tissue blood flow are discussed below.

Catecholamines, ATBF and lipolysis

Direct stimulation of SNS axons in a dissected adipose tissue depot bathed in physiological medium causes lipolysis which is prevented by ganglionic blockade (hexamethonium) or SNS fibre destruction (6 hydroxydopamine) (Correll 1963). However, this does not necessarily reflect the situation in the intact animal where blood flow and capillary permeability may be simultaneously affected by the actions of the SNS. Both of these are important as they will influence local concentrations of released NEFA which are a potent end product inhibitor of lipolysis (Burns 1978). In conditions of increased lipolysis and increased blood flow, net export of NEFA will ensue. However, with increased lipolysis and reduced blood flow, there would be the potential for futile cycles of lipolysis and re-esterification using energy but not resulting in net release of NEFA into the circulation. Such futile cycling has the potential to be a significant determinant of the overall energy economy of the organism with obvious consequences for the understanding of some obese states (Hammond *et al*, 1987). Wirsén (Wirsén 1965), in an early series of studies in adipose tissue biology noted that norepinephrine infusion in general causes initial alpha-mediated constriction of vessels in addition to lipolysis with vasodilatation later ensuing. Infusion of catecholamines, alpha and beta adrenoceptor blockade and other manipulations of the SNS alter adipose tissue blood flow in animals and man (Rosell 1969, Ballard 1973, Ballard 1978, Dodt 1999). Under such influences, ATBF may vary by as

much as ten fold from 3-30 mls/100g/min adipose tissue depending on species, location of the depot and the prevailing metabolic circumstances (Ballard 1978). These and other data serve to illustrate the complexity of the regulation of adipose tissue by the SNS and suggest that blood vessels as well as, or even instead of, adipocytes are innervated by the SNS and that changes in flow and capillary permeability to albumen which permits removal of the products of lipolysis may be just as important as simple up-regulation of lipolysis *per se* (Rosell 1975).

SNS and fat cell differentiation

A number of animal studies suggest a role for the SNS innervation of adipose tissue in longer term energy homeostasis. There is evidence that the SNS may be involved in regulating differentiation and recruitment of pre-adipocytes (Giordano *et al*, 1998) and also in promoting trans-differentiation of adipocytes with predominantly white characteristics to those with the classical morphology and function of brown adipocytes (Himms-Hagen *et al*, 2000). This raises the intriguing possibility that the SNS may be closely involved in the longer term regulation of adipose tissue morphology and function as well as, or even instead of, in the minute-to-minute control of lipolysis. If this is indeed the case, understanding the nature of the SNS innervation of white adipose tissue may also underlie some of the observed inter-species and inter-depot differences in adipose tissue behaviour (Bartness & Song 2007).

Domestic fowl for example exhibit little if any catecholamine responsiveness (Carlson *et al*, 1964) whereas hibernating mammals such as the Siberian hamster demonstrate exquisite sensitivity to catecholaminergic stimulation, presumably related to the need to repeatedly and rapidly store and then mobilise adipose tissue during periods of fasting and re-feeding. Thus, denervation of a Siberian hamster fat pad whilst leaving the contralateral pad intact as an internal control leads to a doubling of the size of that fat pad. This appears to be due to an increase in the number of adipocytes rather than to an increase in any individual cell's lipid

storage (Youngstrom & Bartness 1998). Denervation additionally attenuates the lipolytic response to other stimuli such as fasting, oestrogens or changes in the light-dark cycle (Bartness & Song 2007). Conversely, the increased visceral adiposity of Cushing's syndrome has been suggested to derive from enhanced glucocorticoid-mediated catecholamine insensitivity (Rebuffe-Scrive 1991). Such observations, if interpreted to mean that one of the functions of the SNS is to act as a brake on adipose depot hyperplasia (Turtzo & Lane 2002), have obvious and far reaching potential consequences in our understanding of the development and maintenance of obesity.

Adipose tissue anatomy

Anatomical studies of adipose tissue have a long history although much detail remains unresolved (Bartness 2007, Bartness and Bamshad 1998). Animal studies have demonstrated what are thought to be SNS axons within adipose tissue, particularly in fasted animals (perhaps because the smaller adipocytes permit visualisation of axons otherwise concealed between cells) although the methodologies have only permitted tentative differentiation between vascular and direct innervation (Slavin & Ballard 1978) or have not been clear about innervation of WAT vs. innervation of regions with the characteristics of BAT within WAT. It has frequently been observed that the apparent innervation density appears highly variable even within the same adipose tissue depot (Bartness & Song 2007). Those studies that have reported direct contact either histochemically (Ballard 1974, Diculescu & Stoica 1970, Rebuffe-Scrive 1991, Slavin & Ballard 1978) or in electron microscopic (e.m.) studies (Slavin & Ballard 1978) generally report direct contact with only a minority of adipocytes. The only semi-quantitative estimate available from animal studies of the number of adipocytes that are actually innervated by SNS terminals reported a figure of 3% despite rich innervation of surrounding vascular tissue (Slavin & Ballard 1978). If such patchy and sparse direct innervation is truly the case, it would raise the intriguing possibility that either there is a metabolically active pool of

adipocytes with SNS connections (Ballard 1974) or alternatively that, through some kind of electrochemical coupling as observed in differentiating adipocytes *in vitro* (Yanagiya *et al*, 2007) these cells act as pacemakers for the entire adipose depot.

Descending innervation

The origin of descending SNS innervation might provide a clue to its function. If SNS fibres do more than simply regulate the provision of oxygenated blood to adipose tissue, its fibres might be expected to originate in areas of the brain involved in energy homeostasis. Anterograde and retrograde tracer studies reviewed recently (Bartness 2007) have demonstrated that the SNS afferents to adipose tissue do indeed arise from brain regions known to be involved in appetite and weight regulation and seasonal fat storage. This would support the hypothesis that their influence is not limited to the regulation of vascular tone *per se*. The high degree of co-localisation of SNS axons destined for adipose tissue and MC4 receptors has been proposed to explain how MC4 mutations change body weight more than pair feeding experiments would predict (Raposinho *et al*, 2003). This data suggests a role for the SNS in mediating the energy expenditure effects of MC4 mutations, the commonest monogenic form of obesity yet described (Farooqi *et al*, 2003).

Methodological considerations

Dogiel in 1898 was the first to report staining of nerves innervating WAT (Dogiel 1898). However, early studies using methylene blue lacked specificity for SNS fibres and conversely may have missed direct innervation of adipocytes in freely fed animals due to obscuration within a tightly packed adipose tissue matrix. It is difficult to be certain that the direct innervation reported in some studies (Ballard *et al*, 1974, Diculescu & Stoica 1970, Rebuffe-Scrive 1991, Slavin & Ballard 1978) has not been affected by the methodological difficulties inherent in

studying a tissue which is largely removed by standard histological techniques. A further potential difficulty in determining whether there is direct innervation of white adipose tissue lies in the ‘contamination’ of white adipose tissue with cells with a more ‘brown’ phenotype. Brown adipose tissue is densely innervated by the SNS and discrimination between white and brown adipose tissue is usually considered straightforward (see Colour Plate 7.1 for example). However, it is now clear that trans-differentiation between white and brown adipocytes does occur (as judged by UCP-1 staining), that the characteristics used in making these distinctions are not necessarily ‘all or nothing’ in nature and that brown and white adipose tissue are mixed in the same depots (Cinti 2009) (see also Colour plate 7.3). Even in adult humans thought to possess very little brown fat, patients with catecholamine-secreting tumours (phaeochromocytoma) have increased amounts of brown type fat in the perinephric adipose tissue depot (Lean *et al*, 1986). Similarly, infusion of catecholamines has been shown to increase the ‘brown’ characteristics of white adipose tissue in laboratory animals (Himms-Hagen *et al*, 2000). For these reasons, one of the main criteria used in distinguishing brown from white adipose tissue, innervation density, risks being a circular argument. Other discriminating features such as mitochondrial organisation have a degree of potential subjectivity. No previous study has used combined vascular and neuronal staining with three dimensional image reconstruction to examine in detail the precise anatomical relationship between neuronal and vascular elements in adipose tissue.

The role of SNS innervation of Adipose Tissue in Man

Studies of adipose tissue in animals may not be easily transferable to humans who have, at most, small amounts of brown adipose tissue and do not share with animals certain SNS dependent adipose tissue functions such as BAT thermogenesis and hibernation. The need for a human study is further underpinned by evidence suggesting that abnormal SNS activity within adipose tissue in man may be associated with clinical obesity. Our own studies outlined

in Chapter IV suggest that adipose tissue SNS activity is both reduced in human obesity in the post-absorptive state and also that it fails to respond normally to meal ingestion. Others have shown with microdialysis techniques that intraneuronal stimulation of WAT in obese women does not trigger lipolysis in subcutaneous WAT, whereas the same stimulation produces a profound increase in lipolysis in their lean counterparts (Dodd *et al*, 2000). Lipolysis in denervated limbs in patients with spinal cord injury is also impaired compared with contralateral unimpaired limbs (Karlsson *et al*, 1997). It is hard to understand the true significance of these observations without data confirming that SNS axons do indeed innervate adipose tissue in man.

In conclusion, it seems likely that the SNS is organised in such a way as to be able to deliver catecholamines at high concentration into the vicinity of adipose tissue adrenergic receptors. SNS effects, whether related to lipolysis or to pre-adipocyte differentiation and recruitment may potentially occur either through direct synaptic contact, through diffusion of catecholamines into adipose tissue from microvascular innervation or from changes in local NEFA concentration mediated by changes in blood flow or endothelial permeability to binding proteins such as albumen. Species differences exist in relation both to adipose tissue function (e.g. hibernation and thermogenesis) and innervation (e.g. lack of catecholamine induced lipolytic response in domestic fowl (Carlson *et al*, 1964)). No study has reported data on the existence or pattern of SNS innervation in white adipose tissue in man despite data suggesting abnormal SNS activity within this tissue in the obese state.

Our hypothesis therefore was:

- a) That techniques could be developed to visualise SNS axons within human adipose tissue for the first time
- b) That SNS axons would co-localise with blood vessels rather than exist independently

METHODS

Samples

Human adipose tissue samples were obtained from human visceral and subcutaneous adipose tissue during routine surgical procedures after approval from the East London Regional Ethical Committee [P01/039] having obtained fully informed consent from each subject. Animal material (rat and mouse) was used from tissue banks and from the discarded carcasses of animals kept and sacrificed by individuals with appropriate licenses during unrelated experiments thought unlikely to affect adipose tissue. None of the human subjects were known to have HIV, cancer or other conditions which would make serious abnormalities in adipose tissue likely.

Samples were kept in physiological saline and collected direct from the operating theatre or freshly killed animal before being placed in 4% paraformaldehyde for overnight fixation. Tissue was then frozen on dry ice (we found that liquid nitrogen tended to fracture the delicate tissue) and sectioned to between 10 and 100 μ m thickness at -10 to -50 deg C before preparation for immuno-fluorescence microscopy. Thin sections yielded the clearest staining but the blood vessels and tissue were prone to fracture and less three-dimensional information was available with confocal microscopy than with thicker sections of up to 100 μ m. Both human and animal adipose tissue was used to determine optimal staining techniques. Variations of tissue sectioning, antibody specificity, slide preparation, and imaging were used to determine the best methods of obtaining reliable and consistent staining with optimisation of signal to noise ratio. Permutations of the methods used to optimise visibility of SNS axons and endothelia included variations on the thickness of sections, time in paraformaldehyde, temperature of the cryostat and inclusion or omission of

antigen retrieval. In addition, incubation of sections with primary antibodies in sequence or together, use of primary antibodies raised in rabbit, sheep and goat, use of polyclonal rather than monoclonal primary antibodies, use of differing dilutions of the primary antibodies and variation of the length of primary antibody incubation were all used in reaching the optimised techniques described below.

Primary antibodies

The primary antibodies used were tyrosine hydroxylase (TH) at an optimal dilution of 1:10 and Q BEND-10 (CD 34) at optimal dilution of 1:50. Incubation times were varied from 30 minutes to 8 hours overnight. Longer times were in general found to yield more abundant SNS staining, although at the cost of increased non specific background immuno-fluorescence and were generally used as the non-specific background noise could be recognised as occurring on non-neuronal or vascular structures. We reasoned that it would be more important to avoid under-recognition of TH immuno-reactivity within the adipose elements than to avoid some element of unwanted noise that could later be compensated for in analysis. Rat aorta obtained from a tissue bank was used as a positive control for both primary antibodies containing both endothelial and SNS elements. Restriction of staining to tubular structures with the characteristics of vascular elements was used as an internal negative control for Q-BEND-10 and to beaded neuronal structures with the typical characteristics of SNS axons for anti-TH primary antibodies. This approach was confirmed by using PGP, a pan-neuronal marker in initial experiments to show that TH immuno-reactivity was limited to a subsection of all neuronal elements stained within the section.

TH

Mouse monoclonal IgG2a Kappa Anti-Human Tyrosine Hydroxylase (Novo Castra Laboratories, Newcastle upon Tyne, UK) was the primary antibody used to identify SNS axons within human adipose tissue. This antibody is raised against a

pro-karyotic recombinant protein corresponding to a portion of the carboxyl-terminal end of the tyrosine hydroxylase molecule. TH is the rate limiting enzyme in the biosynthesis of norepinephrine within SNS axons (catalyzing the conversion of the amino acid L-tyrosine to dihydroxy-phenylalanine (DOPA) which is a precursor for dopamine, itself a precursor of norepinephrine). TH is not known to be present in adipose tissue outside of catecholaminergic neuronal tissue.

CD 34

Mouse monoclonal IgG-1 anti-Human CD 34 (QBEND-10, Chemicon International Temecula, CA 92590) was used to stain vascular endothelia. The antigen is predominantly associated with the baso-luminal surfaces of endothelial membranes. QBEND-10 also immuno-precipitates a glycoprotein with a relative MW 110kD expressed on haematopoietic cells and on the established myeloid leukaemic cell line KG1A.

Slide preparation

Sections were picked up on coated slides and dewaxed in xylene (x2) and dehydrated in alcohol (x2). Endogenous peroxidase activity was removed using a solution containing 97 mls methanol and 3 mls H₂O₂ for 15 minutes. The slides were rinsed again in tap and then distilled water at room temperature before immersion in 20% acetic acid for 10 minutes.

Antigen exposure was enhanced by microwaving the slides on full power for 18 minutes in buffer solution (900 mls distilled water and 100 mls citrate buffer titrated to pH=6.0 with 0.1M NaOH) and then washed in Tris buffered saline. We found that, particularly when using thinner sections, omission of this step yielded better staining. Background (non-specific) immuno-fluorescence was blocked with normal horse serum (50µl of normal horse serum to 5 mls antibody diluent,

not removed from the slide). Sections were then washed in TBS and incubated with biotinylated secondary reagent from an Elite Kit. Avidin complex solution (fluoroscein avidin DCS 1:200 diluted in TBS and left to stand for 30 mins) was then added for 20 minutes, washed off and then DAB solution was applied for 5 minutes. This was followed by washing in running tap water for 5 minutes, brief air drying and mounting in Vectashield mounting medium. The slides appeared to be stable for several days if kept at +4 deg C especially if the cover slip was sealed with nail varnish to prevent desiccation.

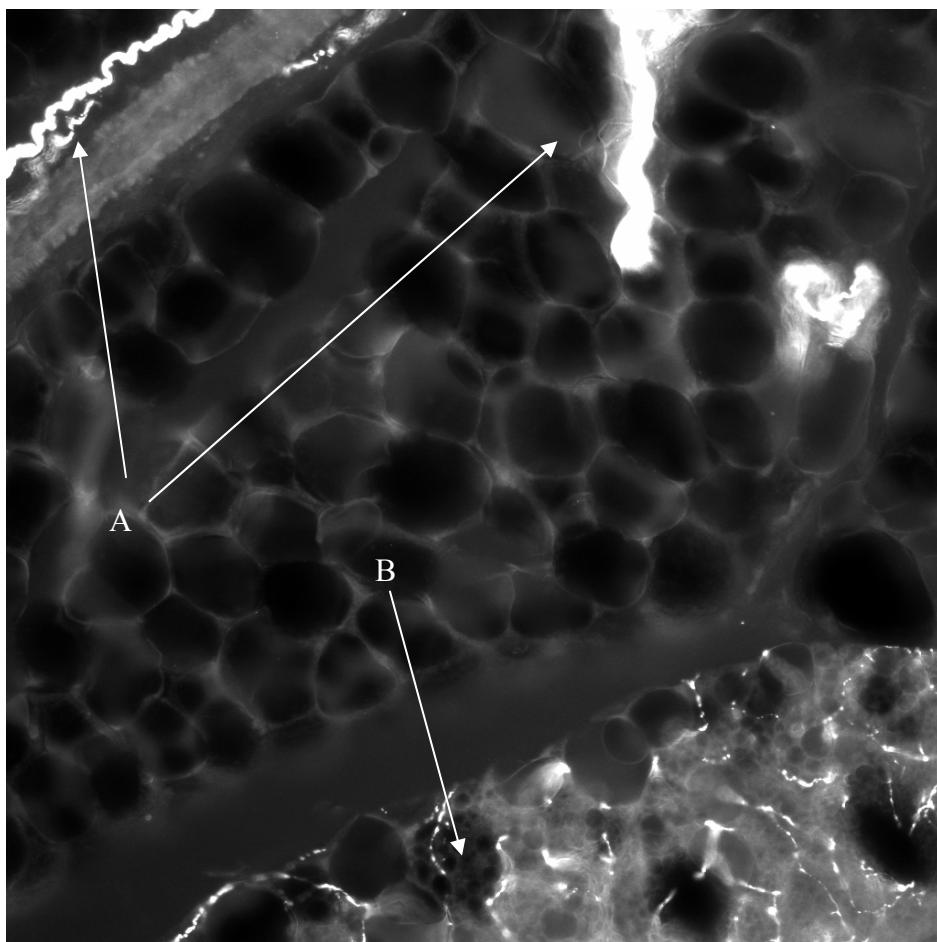
Observation was carried out using a fluorescent confocal laser scanning microscope (LSM510 Meta; Carl Zeiss) equipped with 10x dry, 20x dry, and 40x oil objectives. Stacks of images at regular intervals along the optical axis were collected by exciting the tissue using multiple colour laser lines and collecting emitted light through appropriate narrow band-pass filters. Each image was produced from an average of 8 frames after which the acquired images were processed to produce a surface-rendered 3-dimensional model using LSM Image Browser software (available from <http://www.zeiss.de/C12567BE0045ACF1/Contents-Frame/CAA2EF638EC5F0D3C1256ADF0050E2F1>).

RESULTS

The colour plates below are representative of the images obtained using the methods outlined above in both human and animal adipose tissue depots. The findings are not presented in a quantified manner for the reasons discussed in 'discussion' below but are based on observation of many sections.

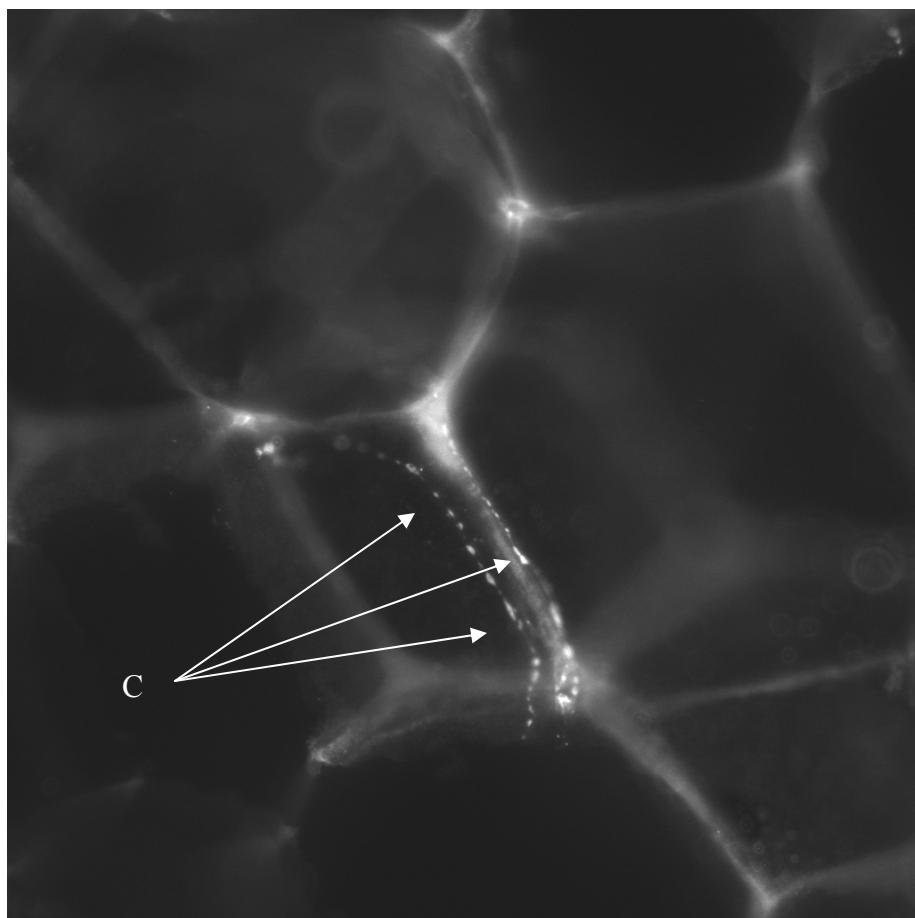
The principal findings were that there is evidence of SNS innervation of both visceral and subcutaneous adipose tissue in man although the axons appear to travel in association with blood vessels and none were encountered independently of endothelial staining. We propose that fracturing of blood vessels during the slide preparation and issues relating to plane of focus could potentially have been responsible for the apparent observation of independent SNS axons in older studies that did not use three-dimensional image reconstruction techniques or endothelial staining.

We did not find evidence to support the notion that adipocytes situated close to SNS axons would be smaller than other adipocytes due to the pro-lipolytic effects of the SNS.

Colour Plate 7.1: Rat inguinal adipose tissue x 10.**Legend to Colour Plate 7.1:**

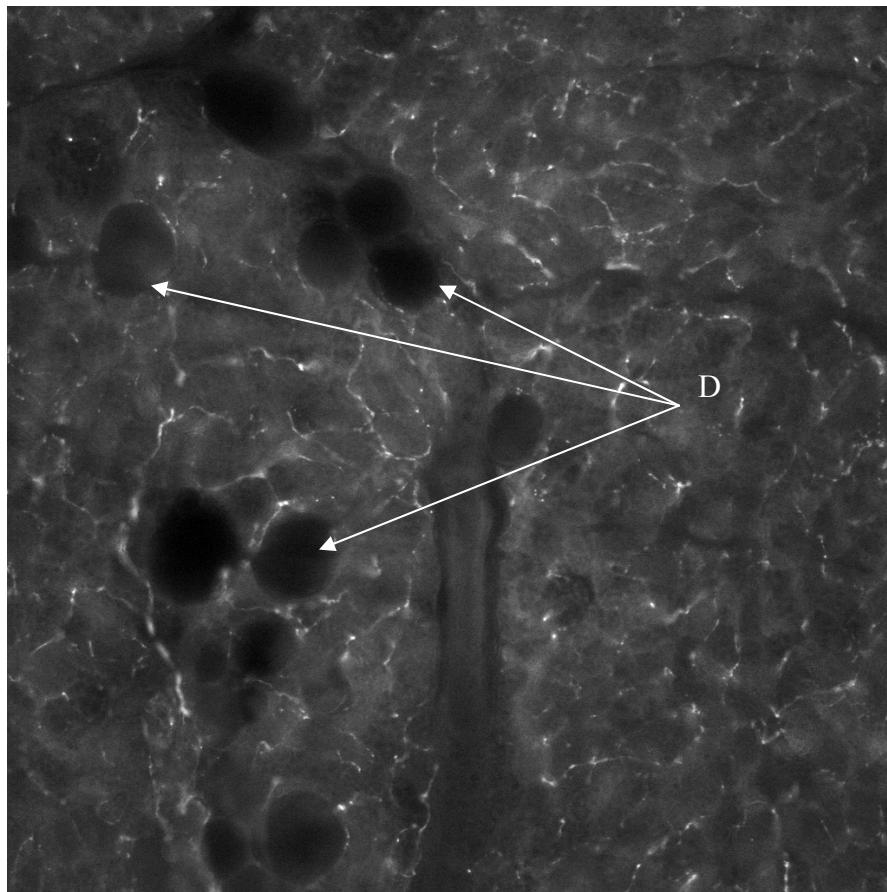
Interface between brown and white adipose tissue showing the different pattern of innervation, that in WAT being confined to vessels (relatively large vessels with dense innervation shown, labelled A). This demonstrates the classical distinction between multi-lobular, densely innervated brown adipocytes below (B) and larger, uni-lobular white adipocytes above.

Colour Plate 7.2: Human subcutaneous adipose tissue x 40.

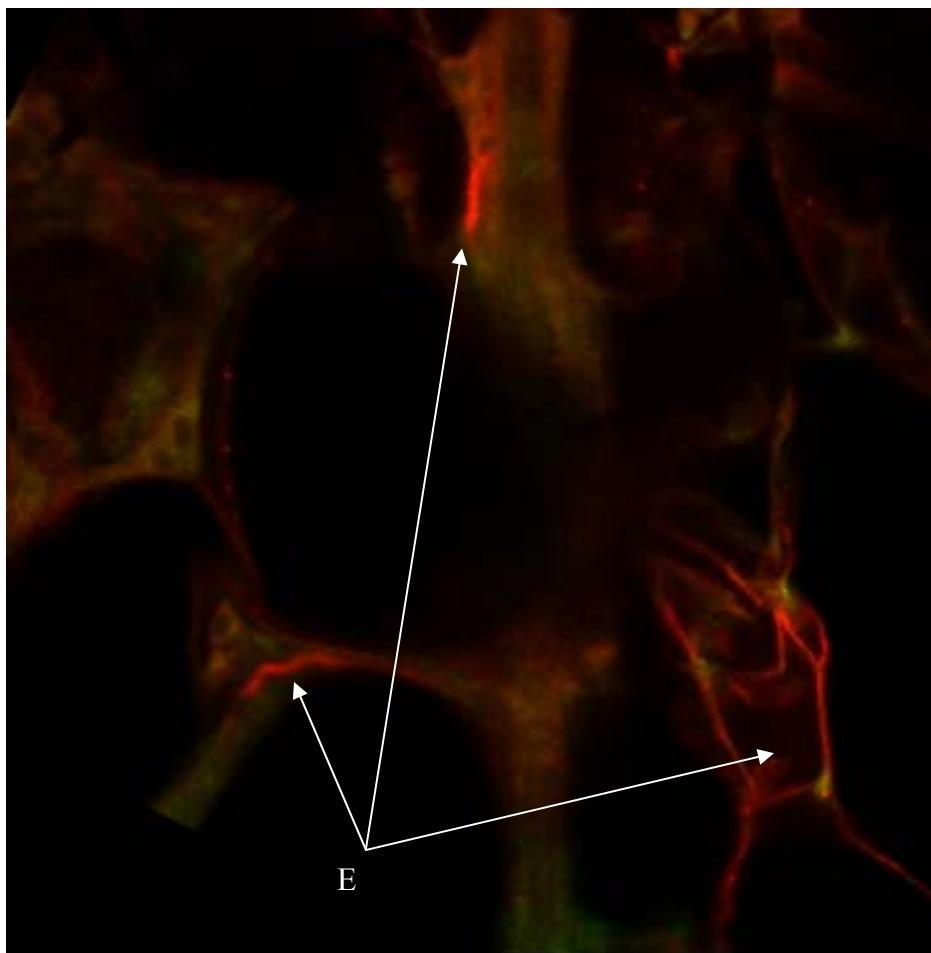


Legend to Colour Plate 7.2:

Without vascular staining, it is hard to determine whether or not these typical SNS axons (labelled C) are associated with a blood vessel. The use of endothelial counterstaining permitted us to conclude that all SNS axons we encountered were in close association with a blood vessel.

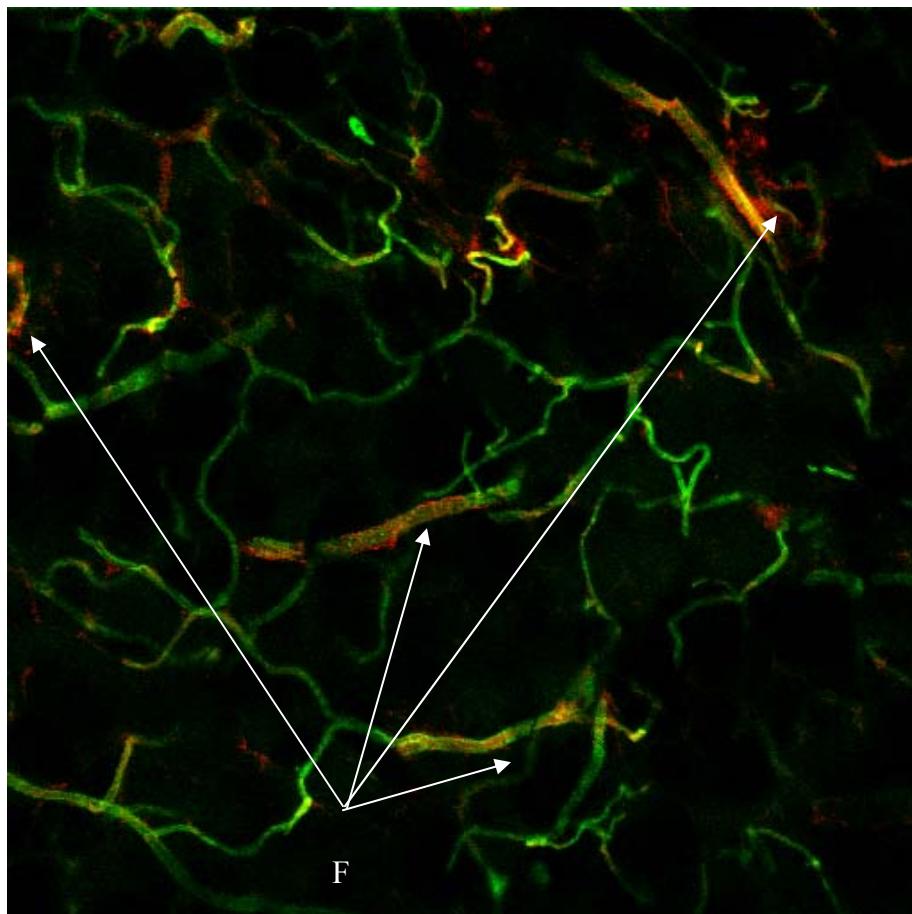
Colour Plate 7.3: Rat Perinephric Brown Adipose Tissue x 20**Legend to Colour Plate 7.3:**

This demonstrates an area of predominantly brown adipose tissue with dense innervation around virtually all of the small multi-lobular cells. However, it can be seen that there are ghosts of larger cells with a more typical white adipocyte morphology (labelled D). This demonstrates the complexity in labelling a region brown or white adipose tissue. The ability of individual adipocytes to have more or less brown adipose characteristics is discussed in the text.

Colour Plate 7.4: Human Subcutaneous White Adipose Tissue x 40**Legend to Colour Plate 7.4:**

Although not seen clearly in this 2 dimensional representation, the neural structures (labelled E, red) were seen clearly to be vascular with three dimensional analysis (TH red, QBEND-10 green).

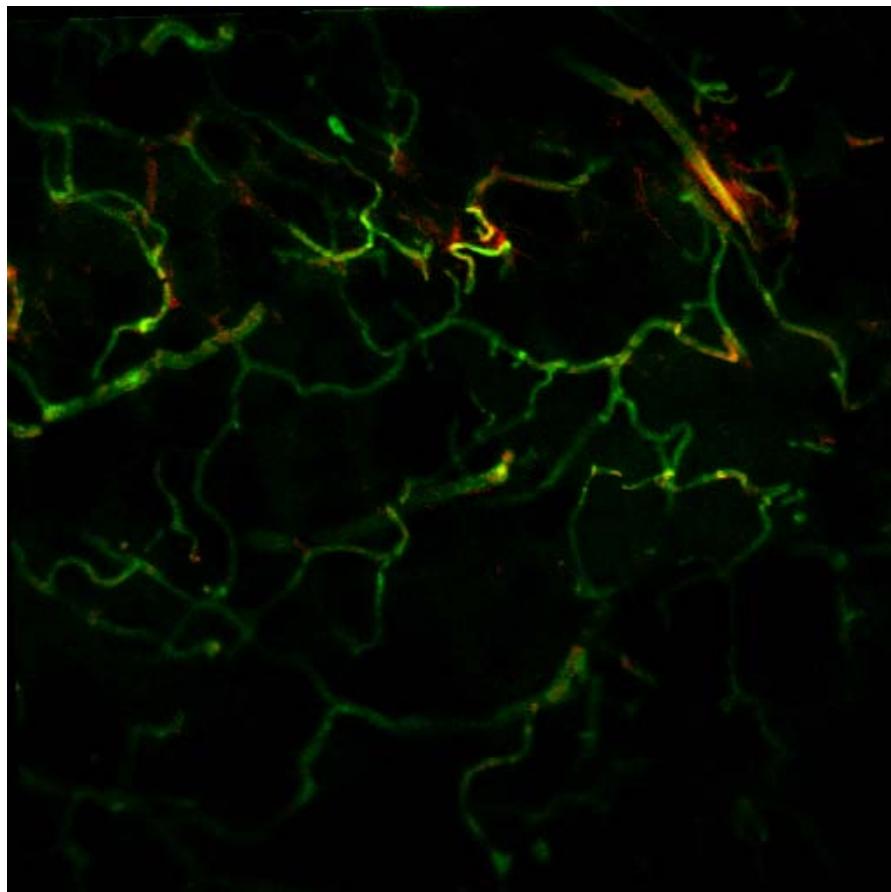
Colour Plate 7.5: Mouse inguinal adipose tissue (x 10).



Legend to Colour Plate 7.5:

This low power image demonstrates the rich latticework of blood vessels carrying SNS innervation (labelled F). Where nerves appear to be independent of vessels, further 3 dimensional imaging reveals a fractured vessel (e.g. top right of picture) or one out of the plane of focus (TH red, QBEND-10 green).

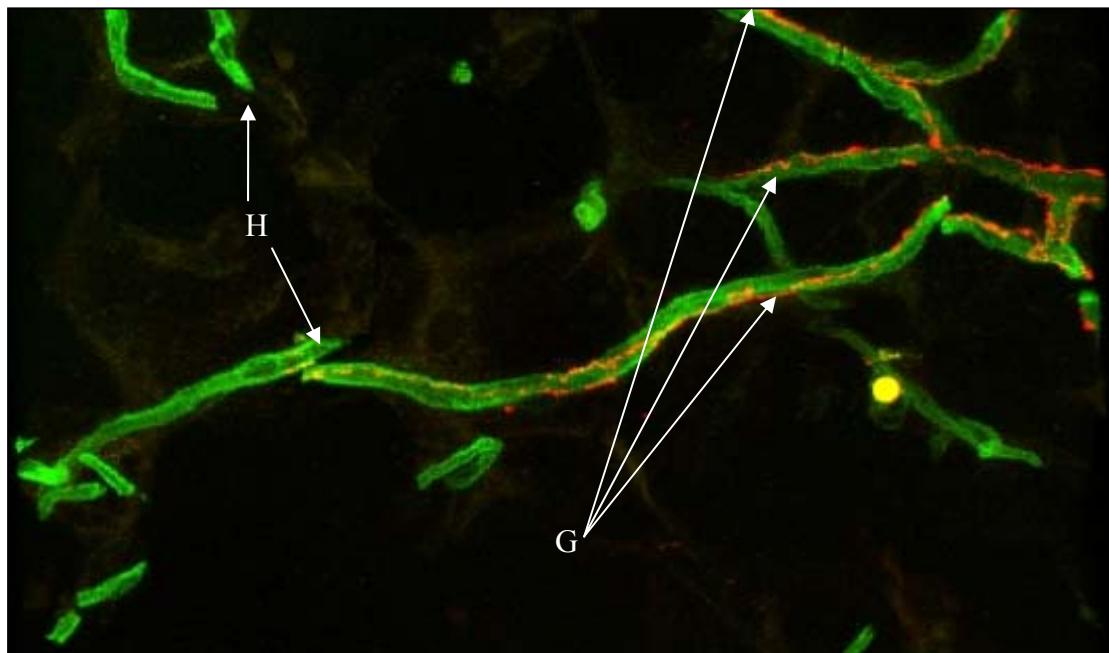
Colour Plate 7.6: Mouse inguinal adipose tissue (x 10).



Legend to Colour Plate 7.6:

Further view of the above at a different plane of section using the three dimensional image reconstruction software described in 'Methods'. It is apparent in this region that most, if not all, adipocytes are closely apposed to at least one SNS axon, especially when the images are viewed using three-dimensional techniques (TH red, QBEND-10 green).

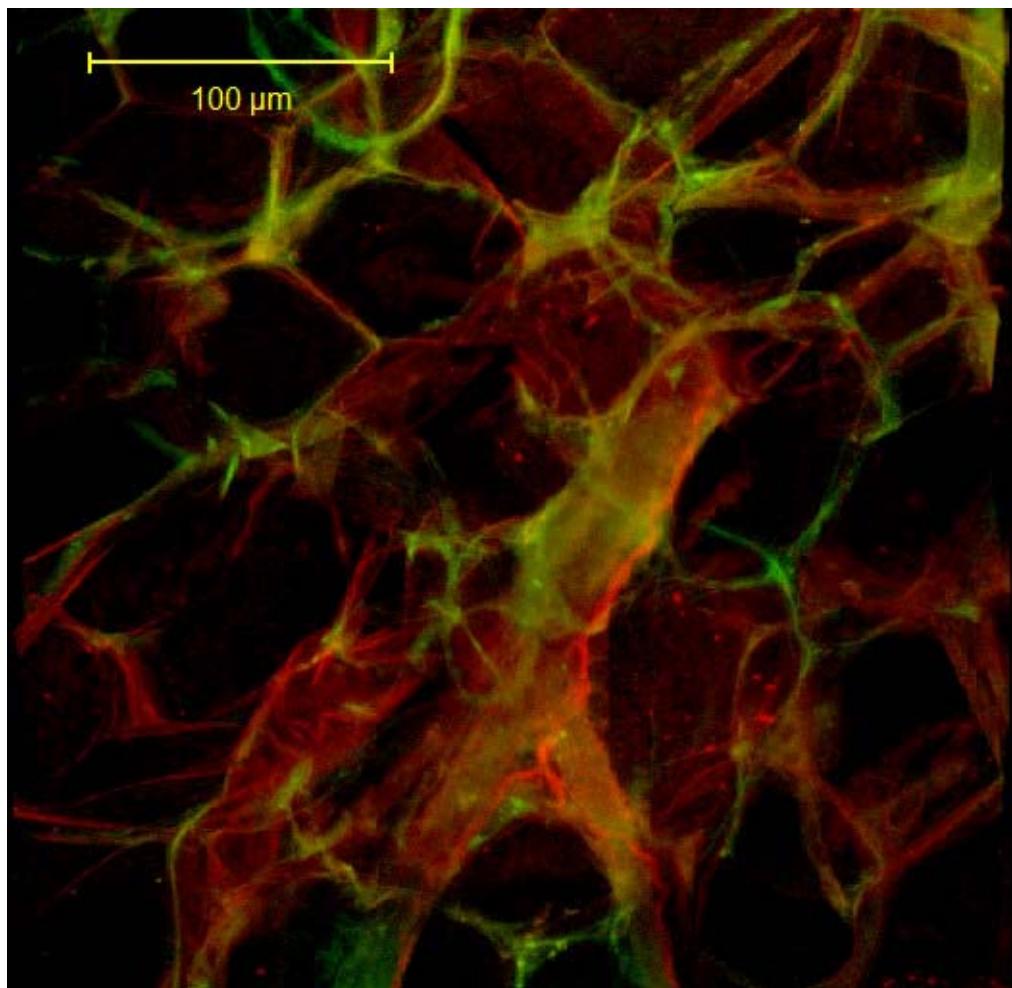
Colour Plate 7.7: Mouse inguinal adipose tissue (x 10).



Legend to Colour Plate 7.7:

Magnified view of part of the material shown in Colour Plate 7.6 demonstrating the close anatomical relationship between blood vessels and SNS axons (labelled G) and the widespread presence of fractured blood vessels (labelled H). As discussed in the text, much of the substance of adipose tissue may be lost in histochemical tissue preparation (TH red, QBEND-10 green).

Colour Plate 7.8: Human visceral white adipose tissue x 40.



Legend to Colour Plate 7.8:

Demonstrating vascular innervation within a rich latticework of vessels in human visceral (omental) adipose tissue (TH red, QBEND-10 green).

DISCUSSION

SNS axons exist within WAT

We used double immuno-fluorescence techniques and confocal microscopy to identify the anatomical relationship between endothelia and SNS axons in human white adipose tissue. We demonstrate directly for the first time in man that SNS fibres are present throughout both subcutaneous and visceral adipose tissue depots although nerve fibres were only encountered in relation to blood vessels.

Although perhaps expected, this conclusion was by no means inevitable given well established species differences in the innervation of white adipose tissue in animals. Many SNS dependent adipose tissue functions in animals are not shared in man such as hibernation and the use of brown adipose tissue for thermogenesis and therefore there is no *a priori* reason to have expected that sympathetic innervation would be similar.

Strengths and limitations of methods used

The main advantages of the techniques we used were related to the optimisation of double labelling immuno-fluorescence techniques, the use of three dimensional image reconstruction allowing us to observe the course of a single axon through the tissue for some distance and the use of QBEND-10 counterstaining to distinguish blood vessels from other cellular structures in the spaces between adipocytes. We were thus able to examine in three dimensions the anatomical relationships between vascular endothelia and SNS axons. Like all histological studies of adipose tissue, we were constrained by the fact that the lipid within adipocytes is removed by the staining process and by the fact that much of the structural integrity of the tissue may be lost as a result. We were not able using our endothelial stain to determine whether a particular blood vessel was an artery, arteriole, capillary, venule or vein or between arterioles situated at the opening of

arteriovenous anastomoses or capillary beds. Specific innervation of such structures might imply a predominant role in vascular innervation with effects on either lipolysis or fat cell recruitment and differentiation being secondary either to diffusion of catecholamines throughout adipose tissue or due to *en passant* synapses of axons primarily innervating blood vessels with adjacent adipocytes. It was, however, possible to distinguish capillaries from larger vascular structures on the basis of size either in relation to the scaling bar of the reconstruction or in relation to residual red blood cells in the lumen.

We consistently observed that not all vascular elements carried varicose TH immunoreactive neural structures. It may be that only larger vessels on the arterial side of the vascular tree carried SNS axons but our techniques did not permit us to examine this point specifically. Further studies with H&E counterstaining will be needed to determine whether these vessels were indeed arterioles and if so, whether all arterioles carry SNS axons or whether this function may have been limited to a defined subset. Adipocytes are large cells (sometimes larger than 100 μm in diameter) and thus it is difficult to section the tissue thickly enough to see whole cells whilst at the same time permitting sufficient antibody-antigen interaction and sharp focus with the confocal microscope. Tracking a single adipocyte with multiple sections was not feasible due to edge effects and the fact that a particular adipocyte will not tend to have any particular features that would help distinguish it from adjacent adipocytes. Thus our techniques did not enable us to quantify precisely the exact proportion of adipocytes that are immediately adjacent to an SNS axon although further refinement of the methods might well permit this in the future.

What can be inferred from this about adipose innervation?

In some species (e.g. ferret), gap junctions have been described which could potentially serve as a coupling mechanism so that the electrical stimulation of one adipocyte could be transmitted to its neighbours (akin to a ‘pacemaker’ cell). If

this were the case, it might be expected that groups of similarly sized adipocytes would be arranged in clusters. However, no such electrochemical coupling could be inferred from this observational study where it appeared that the size of any one cell could not be inferred from the size of its neighbours. Neither was it obviously the case that adipocytes adjacent to SNS fibres were smaller than others in their locality as might be expected if the function of the SNS innervation of adipose tissue were to bring about the direct control of lipolysis in a subpopulation of adipocytes. This anatomical observation accords well with evidence in Siberian hamsters suggesting that SNS denervation of a fat pad causes a change in the size of the fat pad via changes in fat cell number without influencing fat cell size (Youngstrom & Bartness 1998). Further studies will be needed to address the question of what determines adipocyte size and, for the moment at least, the answer to this question remains elusive.

SNS axons appear vascular

We did not find evidence to support the notion that there is both SNS innervation of blood vessels and also a distinct sub population of SNS axons that directly innervate white adipocytes. Our use of vascular counterstaining and three dimensional image reconstruction afforded the opportunity to track individual nerve fibres through the adipose tissue so that we could determine for some distance the origin and course of the nerve. We frequently observed situations where, for example, an SNS axon has become detached from a fractured blood vessel during tissue preparation and speculate that such fracturing might account for at least some of the previous reports of SNS axons visualised as being distinct from vascular innervation. The other possible explanation for previous reports that we periodically encountered was a situation where a blood vessel carrying an SNS axon coursed just below the plane of focus with the axon precisely in the plane of focus. Without the ability to visualise this in three dimensions, it could have appeared that the nerve was coursing through the tissue without an accompanying blood vessel.

Functionally, such a distinction may well be unnecessary. Changes in adipose tissue blood flow and/or vascular permeability have a profound influence on whether there is net export of the products of lipolysis (NEFA and glycerol) from adipose tissue to the general circulation or whether the accumulation of NEFA itself inhibits net lipolysis (Burns *et al*, 1978). In the latter circumstances, the potential for energy dissipation in futile cycles of lipolysis and re-esterification is realised. Such futile cycling has the potential both to generate heat and also to regulate the storage and release of lipid and thus body weight (Hammond *et al*, 1987).

It is possible therefore to reconcile SNS effects on lipolysis with essentially vascular innervation but other SNS functions such as trophic effects (Youngstrom & Bartness 1998) still need to be explained if this model is to be plausible. In fact, it has been established that differentiating pre-adipocytes are found in close proximity to vascular stem cell progenitors (Nishimura S *et al*, 2007) and this inter-relationship appears necessary for adipose tissue hyperplasia. Given this, one might even expect SNS innervation to be peri-vascular within adipose tissue rather than independent of it if this is an important role.

In conclusion, our finding of TH immunoreactivity limited to neural elements carried on vascular structures appears fully consistent with the known vascular, lipolytic and trophic roles of the SNS within adipose tissue.

Further studies

Our aim was to develop a technique adequate to study differences in innervation of white adipose tissue both within and between specific human adipose tissue depots under differing conditions. Much valuable insight has been gained by studies in the Siberian hamster, a species chosen for its ability to store and then mobilise large amounts of adipose tissue during hibernation, seemingly signalled

by changes in the light-dark cycle. These and other studies have told us a great deal about the response of adipose tissue depots to changing circumstances (cold, hunger, preparation for and emergence from hibernation etc) and clearly establish SNS innervation of white adipose tissue as a major mediator not only of lipolysis (both in its own right and as a facilitator of other signals such as oestrogen) but also of adipocyte recruitment, differentiation and trans-differentiation. Future use of our methods may enable studies to be carried out examining the effect on adipose tissue of, for example, agents which modulate SNS activity (beta stimulants and blockers), disorders of lipolysis such as HIV associated adipose redistribution syndrome (HARS) and the effects of changing body weight in different adipose tissue depots.

Issues of Quantification

Having established that SNS axons are indeed visible in human adipose tissue although apparently always in association with blood vessels, the next step will be to develop methods of quantification which will enable comparisons of different adipose tissue depots under differing experimental or environmental conditions. This does however present a considerable challenge. Whilst, for example, measuring the length of SNS fibres per high power field would be perfectly feasible, subjective bias could easily be introduced by choice of the plane of focus or whether or not to count an axon that is only faintly seen just below the plane of sharp focus. Then the question arises as to whether to count more than one axon on a blood vessel separately or not without knowing what the biological consequences of this are, if any, and how to allow for differing adipocyte size under varying experimental circumstances (e.g. should the denominator be per unit area or volume of a section or per adipocyte or per blood vessel). Alternative strategies might be to count the area of a slide staining positively for TH but this would then depend on the brightness and contrast settings used in image reconstitution and it is not at all clear what the biological relevance of surface area

of a neuron is. Then there are difficulties in quantification of neural structures without cell bodies in a tissue that is largely removed during the slide preparation.

For these and other reasons, no previous investigators have, to our knowledge, attempted histological quantification during immuno-fluorescence studies of adipose tissue SNS innervation. The only quantification we are aware of to date has been subjective grading on a scale of 1-5 for the apparent innervation density (Giordano A *et al*, 1996).

In summary, we demonstrate SNS innervation within white adipose tissue for the first time in man although in this study it appeared that SNS axons were invariably linked to blood vessels. Our methods should lend themselves to future use in examining human adipose tissue depots under different prevailing or experimental conditions.

We did not find evidence in support of a distinct subset of SNS axons which coursed through adipose tissue independent of blood vessels. We can not entirely discount the possibility that this was because of their loss during tissue preparation although the fact that the nerve integrity was sometimes preserved despite fracture of the blood vessel it was associated with would suggest that this is unlikely. We also can not completely discount the possibility that had our subjects been fasting for some time, depletion of adipocyte lipid droplets may have rendered such nerves visible, a phenomenon that has been suggested to occur in animal studies (Bartness & Bamshad 1998). However, in view of the fact that changes in vascular permeability and flow are capable of regulating lipolysis via end product inhibition (Burns *et al*, 1978) and also the fact that adipose tissue blood flow appears abnormal in human obesity, and that we can account for the known trophic effects of the SNS without direct adipocyte innervation (Nishimura *et al*, 2007), we would advance the hypothesis that the SNS mediates its effects on lipolysis predominantly via the vasculature.

We did not find evidence that adipocytes adjacent to SNS axons formed a distinct morphological subtype by virtue of, for example, size as would be expected if its main function were limited to stimulating and co-ordinating lipolysis. We suggest that this concords well with physiological studies showing that the SNS exerts regulatory influence on pre-adipocyte recruitment and differentiation and that, given common lineage with vascular progenitor cells lying in close proximity to blood vessels, the SNS is anatomically placed to fulfil this function without exclusive (non vascular) innervation. We were not able to determine with our methods whether or not SNS axons synapse in an *en passant* fashion with adipocytes whilst coursing along blood vessels although electron microscopic studies have previously suggested this to be the case.

Further studies are needed to resolve whether the SNS innervates decision points on the vascular tree which would suggest that vascular regulation is its main purpose or whether there is any evidence in man to support the electrochemical coupling of mature adipocytes which would suggest that direct adipose tissue regulation is possible despite the relatively sparse innervation of the tissue by the SNS.

CHAPTER VIII

GENERAL DISCUSSION and CONCLUSION

We have used both established and novel techniques to study the role of the sympathetic nervous system in adipose tissue anatomy and physiology in man.

The first study, reported in Chapter III, confirmed that our venous sampling techniques were able to demonstrate the metabolic characteristics of abdominal subcutaneous white adipose tissue as well as that of two other quantitatively and qualitatively different tissues, forearm skeletal muscle and skin. This study revealed differences in the metabolic characteristics of these tissues *in vivo* both in the post-absorptive state and in response to glucose infusion. We went on to demonstrate with the studies reported in Chapter IV that there are abnormalities of SNS outflow within this quantitatively important abdominal subcutaneous adipose tissue depot both in the fasting state and in response to meal ingestion. These abnormalities would tend to favour increased fat storage in this depot and, if not matched by changes in omental fat, relative redistribution of lipolysis towards central depots, a situation known to be associated with the development of insulin resistance and other features of the metabolic syndrome.

This finding of defective adipose tissue norepinephrine spillover in the fasting state in obese subjects is vulnerable to the assertion that a variable (here, norepinephrine spillover) measured per unit mass of tissue may spuriously be changed in a condition in which adipocyte volume is known to be increased. In other words, it is possible that the fasting differences simply reflect the fact that there are fewer adipocytes per unit volume (and therefore, per unit mass) of adipose tissue in obesity, each potentially receiving the same autonomic nerve traffic. The failure of the norepinephrine spillover to respond normally to mixed meal ingestion in obesity is, however, fully consistent with our hypothesis of defective innervation as the denominator of adipose tissue mass remains constant. Tissue heterogeneity was evident in the apparently normal responses of forearm muscular tissue to the same change in metabolic conditions. There has been much debate over whether the SNS is overactive in obesity leading to hypertension (the 'Landsberg' hypothesis (Reaven *et al*, 1996)) or whether its under-activity might

be responsible for the increased tendency for lipid deposition in adipose tissue in obesity (the 'MONA LISA' hypothesis (Bray & York 1998)). Our studies demonstrate how regional heterogeneity in SNS function may reconcile these two seemingly opposed positions. Whilst SNS outflow to adipose tissue appears defective and demonstrates 'metabolic inflexibility' in terms of transition to the fed state in obesity, whole body SNS activity is increased in both metabolic states, consistent with the well known association between obesity and hypertension. Further studies are underway to examine regional SNS outflow in post obese individuals. A number of the obese subjects who participated in the studies reported in Chapter IV have subsequently undergone bariatric surgery and some (currently 5) have been restudied at a lower, stable body mass. Our reasoning for these studies is that abnormalities of SNS function that persist in the post-obese state have the potential to have had a causative role in the development or maintenance of the obesity. Conversely, if the apparent abnormalities of SNS outflow normalise after weight loss, it may be that they simply reflected the obese state itself and were not responsible for its development. Either way, the relevance of our current findings to understanding important elements of the pathophysiology of established obesity (abnormal regional lipolysis, hypertension etc.) would remain.

We report in Chapter V that NEFA export from adipose tissue appears to be pulsatile. Attenuation of insulin pulses using a euglycaemic-hyperinsulinaemic clamp technique reduces the amplitude but does not reduce the frequency of NEFA pulses from the subcutaneous abdominal adipose tissue depot studied. This suggests a co-ordination of lipolysis which depends, at least in part, on factors other than insulin pulses. Further studies using different techniques will be needed to establish whether or not the SNS acts as the external oscillator. Our methods for measuring ATBF did not permit time resolution of less than around 20-30 minutes for SNS outflow and therefore could not address this question directly. Our positive and novel finding in respect of the pulsatility of NEFA export from adipose tissue is in a way strengthened by the negative results in

relation to leptin release. By developing and using control datasets, we were able to show that commercially available pulse detection algorithms may detect apparent pulsatility in datasets characterised by biological or other inherent variability. Our conclusions about NEFA export were robust to this evaluation and less tentatively advanced as a result.

We have shown for the first time (Chapter VII) that SNS axons do indeed innervate human white adipose tissue although TH immuno-reactivity appeared confined to axons coursing through it in association with blood vessels. The distribution of these fibres suggests that lipolytic regulation and/or co-ordination by the SNS is brought about indirectly either through changes in blood flow (and presumably therefore end product inhibition of lipolysis (Burns et al, 1978)) or, alternatively, through some kind of coupling of adipocytes allowing propagation of SNS impulses throughout the tissue from adipocytes lying in close proximity to vascular axons. An alternative explanation of these data is that the SNS may have a more important role in the recruitment and differentiation of adipocyte precursors in man than it does in the minute to minute quantitative regulation of net lipolysis and would tend to suggest that co-ordination of pulsatile secretion is likely to depend on neither the SNS nor insulin pulses. Nevertheless, such a conclusion would be consistent with the notion that the SNS primarily regulates fat cell number rather than fat cell volume (Youngstrom & Bartness 1998), at least in animals.

Many questions remain unanswered by our studies. Having developed histological techniques to study the SNS innervation of white adipose tissue in man, it will be important to determine whether the functional abnormalities we have found are reflected in structural changes in obese subjects as well as in other states of adipose tissue dysfunction (e.g. HIV adipose redistribution syndrome (HARS), lipodystrophies and PPAR- γ polymorphisms). Further work will be required to develop robust and reproducible histological methods of quantification for such studies.

Human studies are complex practically and ethically and, particularly in those studies where difficult cannulation techniques and very frequent sampling were combined, the numbers of subjects completing some of these studies declined as a result. Nevertheless, there are important differences between human and animal adipose tissue function which mean that conclusions derived in one species may not necessarily be directly transferable to the other. We have demonstrated that such studies can be done and their application to different experimental and pathological situations will, we hope, provide the basis for greater understanding of this tissue. Future studies to develop these concepts might include the following:

- a. Further investigation of what appears to be an incretin-like effect on adipose tissue blood flow which is increased by orally ingested but not intravenously injected glucose administration. This might include measuring ATBF in subjects before and after bariatric surgery, with and without diabetes and in response to incretin (e.g. exenatide or GLP-1) administration.
- b. Studies of adipose tissue NE spillover after bariatric surgery are already underway as discussed above
- c. The use of similar techniques to those used in Chapter IV to study individuals predisposed to the development of insulin resistance as a result of predominant visceral fat accumulation (e.g. Asian vs. Caucasian subjects and viscerally obese men vs. women with predominant gluteo-femoral lipid deposition). Visceral fat accumulation is known to be a risk factor for the development of insulin resistance. Defective lipolysis in the abdominal subcutaneous adipose tissue depot may lead to relative redistribution of lipolysis towards visceral depots. We would advance the hypothesis that subcutaneous abdominal NE spillover will be blunted in

subjects with, or at greater risk of, visceral adiposity in comparison to that of weight matched controls.

- d. Extension of the adipose tissue cannulation techniques to study different adipose tissue depots e.g. gluteal adipose tissue in lean and obese subjects with varying fat distribution. The hypothesis would be that, in subjects with gluteo-femoral rather than visceral adiposity, SNS outflow will not be reduced in the gluteo-femoral depot. This might have the consequence of redistributing lipolysis towards this depot rather than to visceral fat stores under circumstances of reduced SNS outflow to the subcutaneous abdominal depot.
- e. In order to strengthen our findings in relation to the pulsatile release of NEFA from adipose tissue, studies using constant rate infusion of stable isotope-labelled fatty acids could be undertaken. We would reason that any pulsatility encountered in the concentrations of infused stable isotopes either suggests that our methods and use of control datasets have not excluded spurious pulsatility or, alternatively, that oscillations in plasma NEFA clearance are a better explanation than co-ordinated regulation of NEFA production from adipose tissue.
- f. A somatostatin ‘pancreatic’ clamp technique with basal insulin replacement could be used to strengthen our conclusion that NEFA pulsatility does not depend on spontaneous insulin pulses.
- g. Beta-adrenoceptor blockade could be used to examine the effects this might have on NEFA pulsatility. The use of stable isotope techniques may help disentangle changes due to the inevitable cardiovascular effects of non-selective beta-adrenoceptor blockade from those due to blockade of adrenoceptors within adipose tissue itself.

- h. Further development of quantitative techniques for our anatomical studies of human white adipose tissue may permit examination of its innervation in different pathological states including obesity and HARS and also develop our understanding of whether differences in the size and lipolytic behaviour of different adipose tissue depots is due predominantly to receptor/post receptor differences, differences in the release of NE in the tissue or instead to changes in the anatomical distribution of SNS axons within adipose tissue depots.

REFERENCES

Ahima RS, Qi Y and Singhal NS. Adipokines that link obesity and diabetes to the hypothalamus. *Prog Brain Res.* 2006, **153**: 155-74.

Ahmad AM, Guzder R, Wallace AM, Thomas J, Fraser WD and Vora JP. Circadian and ultradian rhythm and leptin pulsatility in adult GH deficiency: effects of GH replacement. *J Clin Endocrinol Metab.* 2001, **86** (8): 3499-506.

Anderson EA, Hoffman RP, Balon TW, Sinkey CA and Mark AL. Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans. *J.Clin.Invest.* 1991, **87**: 2246-52.

Andres R, Cader G and Zierler KL. The quantitatively minor role of carbohydrate in oxidative metabolism by skeletal muscle in intact man in the basal state. Measurements of oxygen and glucose uptake and carbon dioxide and lactate production in the forearm. *J Clin Invest.* 1956, **35**: 671-682.

Ardilouze J, Fielding B, Currie J, Frayn K and Karpe F. Nitric oxide and beta-adrenergic stimulation are major regulators of pre prandial and post prandial subcutaneous adipose tissue blood flow in humans. *Circulation* 2004, **109**: 47–52.

Arner P, Kriegholm E, Engfeldt P and Bolinder J. Adrenergic regulation of lipolysis in situ at rest and during exercise. *J Clin Invest.* 1990, **85**: 893– 898.

Arner P. Catecholamine-induced lipolysis in obesity. *Int.J.Obes.Relat Metab Disord.* 1999, **23** Suppl 1: 10-3.

Asano A, Morimatsu M, Nikami H, Yoshida T and Saito M. Adrenergic activation of vascular endothelial growth factor mRNA expression in rat brown adipose tissue: implication in cold-induced angiogenesis. *Biochem.J.* 1997, **328** (Pt 1): 179-83.

Astrup A, Andersen T, Christensen NJ, Bulow J, Madsen J, Breum L *et al.* Impaired glucose-induced thermogenesis and arterial norepinephrine response persist after weight reduction in obese humans. *American Journal of Clinical Nutrition* 1990, **51**: 331-7.

Astrup A and Macdonald IA. Sympathoadrenal system and metabolism. In Bray GA, Bouchard C, James WP, eds. *Handbook of Obesity*, pp 491-511. New York: Marcel Dekker Inc, 1998.

Bachman ES, Dhillon H, Zhang CY, Cinti S, Bianco AC, Kobilka BK and Lowell BB. Beta AR signaling required for diet-induced thermogenesis and obesity resistance. [see comments]. *Science* 2002, **297**: 843-845.

Bahnsen M, Burrin JM, Johnston DG, Pernet A, Walker M and Alberti KG. Mechanisms of catecholamine effects on ketogenesis. *Am J Physiol.* 1984, **247**(2 Pt 1): E173-80.

Ballard K, Malmfors T and Rosell S. Adrenergic innervation and vascular patterns in canine adipose tissue. *Microvasc Res.* 1974 Sep, **8**(2): 164-71.

Ballard K. Blood flow in canine adipose tissue during intravenous infusion of norepinephrine. *Am J Physiol.* 1973 Nov, **225**(5): 1026-31.

Ballard KW. Functional characteristics of the microcirculation in white adipose tissue. *Microvasc Res.* 1978 Jul, **16**(1): 1-18.

Baltzan MA, Andres R, Cader G and Zierler KL. Heterogeneity of forearm metabolism with special reference to free fatty acids. *J Clin Invest.* 1961, **41**: 116-125.

Barbe P, Millet L, Galitzky J, Lafontan M and Berlan M. In situ assessment of the role of the β 1-, β 2-, and β 3-adrenoceptors in the control of lipolysis and nutritive blood flow in human subcutaneous adipose tissue. *Br J Pharmacol.* 1996, **117**: 907-913.

Bartness TJ and Bamshad M. Innervation of mammalian white adipose tissue: implications for the regulation of total body fat. [Review] [141 refs]. *American Journal of Physiology* 1998, **275**: R1399-R1411.

Bartness TJ and Song CK. Thematic review series: adipocyte biology. Sympathetic and sensory innervation of white adipose tissue. *J Lipid Res.* 2007, **48**: 1655-1672.

Barzilai N, She L, Liu BQ, Vuguin P, Cohen P, Wang J et al. Surgical removal of visceral fat reverses hepatic insulin resistance. *Diabetes* 1999, **48**: 94-8.

Belfrage E, Hjemdahl P and Fredholm BB. Metabolic effects of blood flow restriction in adipose tissue. *Acta Physiol Scand.* 1979, **105**: 222-7.

Bergendahl M, Iranmanesh A, Evans WS and Veldhuis JD. Short-term fasting selectively suppresses leptin pulse mass and 24-hour rhythmic leptin release in healthy midluteal phase women without disturbing leptin pulse frequency or its entropy control (pattern orderliness). *J Clin Endocrinol Metab.* 2000, **85**: 207-13.

Berkowitz DE, Brown D, Lee KM, Emala C, Palmer D, An Y *et al.* Endotoxin-induced alteration in the expression of leptin and beta3-adrenergic receptor in adipose tissue. *Am.J.Physiol.* 1998, **274**: E992-E997.

Berraondo B, Marti A, Duncan JS, Trayhurn P and Martinez JA. Up-regulation of muscle UCP2 gene expression by a new beta3-adrenoceptor agonist, trecadrine, in obese (cafeteria) rodents, but down-regulation in lean animals.

Int.J.Obes.Relat Metab Disord. 2000, **24**: 156-63.

Blaak EE, Saris WH and Van Baak MA. Adrenoceptor subtypes mediating catecholamine-induced thermogenesis in man. *Int.J.Obes.Relat Metab Disord.* 1993, **17** Suppl 3: S78-S81.

Boden G, Chen X, Kolaczynski JW and Polansky M. Effects of prolonged hyperinsulinemia on serum leptin in normal human subjects. *J Clin Invest.* 1997 Sep 1, **100**(5): 1107-13.

Bonadonna RC, Groop LC, Zych K, Shank M and DeFronzo RA. Dose dependent effect of insulin on plasma free fatty acid turnover and oxidation in humans. *Am J Physiol.* 1990, **259**: E736-E750.

Borsheim E, Lonnroth P, Knardahl S and Jansson PA. No difference in the lipolytic response to beta-adrenoceptor stimulation in situ but a delayed increase in adipose tissue blood flow in moderately obese compared with lean men in the postexercise period. *Metabolism* 2000, **49**: 579-87.

Boyardjian R and Steil GM. Diurnal and ultradian rhythmicity of plasma leptin: effects of gender and adiposity. *J Clin Endocrinol Metab.* 1998 Feb, **83**(2): 453-9.

Bray GA, York DA and Fisler JS. Experimental obesity: a homeostatic failure due to defective nutrient stimulation of the sympathetic nervous system. [Review] [770 refs]. *Vitamins & Hormones* 1989, **45**: 1-125.

Bray GA and York DA. The MONA LISA hypothesis in the time of leptin. [Review] [122 refs]. *Recent Progress in Hormone Research* 1998, **53**: 95-117.

Bray GA. Obesity, a disorder of nutrient partitioning: the MONA LISA hypothesis. *J.Nutr.* 1991, **121**: 1146-62.

Bray GA. Obesity-a state of reduced sympathetic activity and normal or high adrenal activity (the autonomic and adrenal hypothesis revisited). [Review] [91 refs]. *International Journal of Obesity* 1990, **14** Suppl 3: 77-91.

Bray GA. Reciprocal relation of food intake and sympathetic activity: experimental observations and clinical implications. *Int.J.Obes.Relat Metab Disord.* 2000, **24** Suppl 2: S8-17.

Breslow MJ, An Y and Berkowitz DE. Beta-3 adrenoceptor (beta-3AR) expression in leptin treated OB/OB mice. *Life Sci.* 1997, **61**: 59-64.

Briscini L, Tonello C, Dioni L, Carruba MO and Nisoli E. Bcl-2 and Bax are involved in the sympathetic protection of brown adipocytes from obesity-linked apoptosis. *FEBS Letters* 1998, **431**: 80-4.

Buchalter SE, Crain MR and Kreisberg RA. Regulation of lactate metabolism in vivo. *Diabetes Metab Rev.* 1989, **5**: 379-392.

Buettner R, Schaffler A, Arndt H, Rogler G, Nusser J, Zietz B *et al.* The Trp64Arg polymorphism of the beta 3-adrenergic receptor gene is not associated with obesity or type 2 diabetes mellitus in a large population-based Caucasian cohort. *J.Clin.Endocrinol.Metab.* 1998, **83**: 2892-7.

Burns TW, Langley PE, Terry BE, and Robinson GA. The role of free fatty acids in the regulation of lipolysis by human adipose tissue cells. *Metabolism* 1978, **27**: 1755–1762.

Campos H, Blijlevens E, McNamara JR *et al.* LDL particle size distribution. Results from the Framingham Offspring Study. *Arterioscler Thromb.* 1992, **12**: 1410-1419.

Carey G.B. Mechanisms regulating adipocyte lipolysis. *Adv.Exp.Med.Biol.* 1998, **441**: 157-170.

Carlson LA, Liljedahl SO, Verdy M and Wirsén C. Unresponsiveness to the lipid mobilising action of catecholamines in vivo and vitro in the domestic fowl. *Metabolism* 1964 Mar, **13**: 227-31.

Carlson SH, Shelton J, White CR and Wyss JM. Elevated sympathetic activity contributes to hypertension and salt sensitivity in diabetic obese Zucker rats. *Hypertension* 2000, **35**: 403-408.

Carmen GY and Víctor SM. Signalling mechanisms regulating lipolysis. *Cell Signal.* 2006 Apr, **18**(4): 401-8.

Chang PC, Kriek E, van der Krogt JA and van Brummelen, P. Does regional norepinephrine spillover represent local sympathetic activity? *Hypertension* 1991, **18**: 56-66.

Christin L, O'Connell M, Bogardus C, Danforth E, Jr., Ravussin E. Norepinephrine turnover and energy expenditure in Pima Indian and white men. *Metabolism: Clinical & Experimental* 1993, **42**: 723-9.

Christin L, Ravussin E, Bogardus C and Howard BV. The effect of propranolol on free fatty acid mobilization and resting metabolic rate. *Metabolism* 1989, **38**: 439-444.

Cinti S. Transdifferentiation properties of adipocytes in the Adipose Organ. *Am J Physiol Endocrinol Metab*. 2009 May 19. [Epub ahead of print]

Clark ML, Humphreys SM, Frayn KN. Stability of plasma glucose during storage. *Ann Clin Biochem*. 1990, **27**: 373-377.

Clasey JL, Kanaley JA, Wideman L *et al*. Validity of methods of body composition assessment in young and older men and women. *J Appl Physiol*. 1999, **86**: 1728-1738.

Coles DR, Cooper KE, Mottram RF and Occleshaw JV. The source of blood samples withdrawn from deep forearm veins via catheters passed upstream from the median cubital vein. *J Physiol Lond* 1958, **142**: 323-328.

Coll AP, Farooqi IS, O'Rahilly S. The hormonal control of food intake. *Cell* 2007 Apr 20, **129**(2): 251-62.

Collins S and Surwit RS. The beta-adrenergic receptors and the control of adipose tissue metabolism and thermogenesis. *Recent Prog Horm Res*. 2001, **56**: 309-328.

Cooper K, Edholm O and Mottram R. The blood flow in skin and muscle of the human forearm. *J Physiol Lond*. 1955, **128**: 258-267.

Coppack SW, Fisher RM, Gibbons GF *et al.* Postprandial substrate deposition in human forearm and adipose tissues in vivo. *Clin Sci.* 1990, **79**: 339-348.

Coppack SW, Fisher RM, Humphreys SM, Clark ML, Pointon JJ and Frayn KN. Carbohydrate metabolism in insulin resistance: glucose uptake and lactate production by adipose tissue and forearm tissues in vivo before and after a mixed meal. *Clin Sci.* 1996, **90**: 409-415.

Coppack SW, Frayn KN, Humphreys SM, Dhar H and Hockaday TDR. Effects of insulin on human adipose tissue metabolism in vivo. *Clin Sci.* 1989, **77**: 663-670.

Coppack SW, Frayn KN, Humphreys SM, Whyte PL, Hockaday TDR. Arteriovenous differences across human adipose and forearm tissues after overnight fast. *Metabolism* 1990, **39**: 384-390.

Coppack SW, Horowitz JF, Paramore DS, Cryer PE, Royal, HD *et al.* Whole body, adipose tissue, and forearm norepinephrine kinetics in lean and obese women. *American Journal of Physiology* 1998, **275**: E830-E834.

Coppack SW, Persson M, Judd RL and Miles JM. Glycerol and non-esterified fatty acid metabolism in human muscle and adipose tissue in vivo. *Am J Physiol Endocrinol Metab.* 1999, **276**: E233-E240.

Correll JW. Adipose tissue: ability to respond to nerve stimulation in vitro. *Science* 1963, **140**: 387-388.

Corry DB and Tuck A. Obesity, Hypertension, and Sympathetic Nervous System Activity. *Curr.Hypertens.Rep.* 1999, **1**: 119-26.

Cox HS, Kaye DM, Thompson JM, Turner AG, Jennings GL, Itsiopoulos C and Esler MD. Regional sympathetic nervous activation after a large meal in humans. *Clin.Sci.(Colch.)* 1995, **89**: 145-154.

Crandall DL, Fried SK, Francendese AA, Nickel M and DiGirolamo M. Lactate release from isolated rat adipocytes: influence of cell size, glucose concentration, insulin and epinephrine. *Horm Metab Res* 1983, **15**: 326-329.

Crandall DL, Hausman GJ and Kral JG. A review of the microcirculation of adipose tissue: anatomic, metabolic, and angiogenic perspectives. *Microcirculation* 1997, **4**: 211-32.

Cumin F, Baum HP and Levens N. Leptin is cleared from the circulation primarily by the kidney. *Int J Obes Relat Metab Disord.* 1996 Dec, **20**(12): 1120-6.

Cunningham BA, Deeney JT, Bliss CR, Corkey BE and Tornheim K. Glucose induced oscillatory insulin secretion in perfused rat pancreatic islets and clonal beta-cells (HIT). *Am J Physiol.* 1996, **271**: E702-E710.

Daly PA and Landsberg L. Hypertension in obesity and NIDDM. Role of insulin and sympathetic nervous system. [Review] [101 refs]. *Diabetes Care* 1991, **14**: 240-248.

Dano S, Sorensen PG and Hynne F. Sustained oscillations in living cells. *Nature* 1999, **402**: 320 -322.

DeFronzo RA, Tobin J and Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol.* 1979, **237**: E214-E223.

Demas GE and Bartness TJ. Direct innervation of white fat and adrenal medullary catecholamines mediate photoperiodic changes in body fat. *Am. J. Physiol.* 2001, **281**: R1499–R1505.

Denjean F, Lachuer J, Geloen A, Cohen-Adad F, Moulin C, Barre H *et al.* Differential regulation of uncoupling protein-1, -2 and -3 gene expression by sympathetic innervation in brown adipose tissue of thermoneutral or cold-exposed rats. *FEBS Lett.* 1999, **444**: 181-5.

Diculescu I and Stoica M. Fluorescence histochemical investigation on the adrenergic innervation of the white adipose tissue in the rat. *J Neurovisc Relat.* 1970, **32**(1): 25-36.

Dodt C, Lonnroth P, Fehm HL and Elam M. Intraneural stimulation elicits an increase in subcutaneous interstitial glycerol levels in humans. *J Physiol.* 1999 Dec 1, **521** Pt 2: 545-52.

Dodt C, Lonnroth P, Fehm H and Elam M. The subcutaneous lipolytic response to regional neural stimulation is reduced in obese women. *Diabetes* 2000, **49**: 1875-1879.

Dogiel AS. Die sensiblen Nervenendigungen im Herzen und in den Blutgefassen der Saugethiere. *Arch. Mikr. Anat.* 1898, **52**: 44–70.

Donahoo WT, Jensen DR, Yost TJ, Eckel RH. Isoproterenol and somatostatin decrease plasma leptin in humans: a novel mechanism regulating leptin secretion. *J.Clin.Endocrinol.Metab.* 1997, **82**: 4139-43.

Eikeli N and Esler M. The neurobiology of human obesity. *Exp Physiol.* 2005 Sep, **90**(5): 673-82.

Eikelis N, Lambert G, Wiesner G, Kaye D, Schlaich M, Morris M, Hastings J, Socratous F and Esler M. Extra-adipocyte leptin release in human obesity and its relation to sympathoadrenal function. *Am J Physiol Endocrinol Metab.* 2004 May, **286**(5): E744-52.

Eikelis N, Wiesner G, Lambert G and Esler M. Brain leptin resistance in human obesity revisited. *Regul Pept.* 2007 Mar 1, **139**(1-3): 45-51.

Eisenhofer G, Goldstein DS, Stull R, Keiser HR, Sunderland T, Murphy DL and Kopin IJ. Simultaneous liquid-chromatographic determination of 3,4-dihydroxyphenylglycol, catecholamines, and 3,4-dihydroxyphenylalanine in plasma, and their responses to inhibition of monoamine oxidase. *Clin. Chem.* 1986, **32**: 2030-2033.

Elia M, Folmer P, Schlatmann A, Goren A and Austin S. Carbohydrate, fat, and protein metabolism in muscle and in the whole body after mixed meal ingestion. *Metabolism* 1988, **37**: 542-551.

Emdin M, Gastaldelli A, Muscelli E, Macerata A, Natali A, Camastra S et al. Hyperinsulinemia and Autonomic Nervous System Dysfunction in Obesity : Effects of Weight Loss. *Circulation* 2001, **103**: 513-9.

Engfeldt P, Hellmer J, Wahrenberg H and Arner P. Effects of insulin on adrenoceptor binding and the rate of catecholamine-induced lipolysis in isolated human fat cells. *J.Biol.Chem.* 1988, **263**: 15553-60.

Erickson JC, Clegg KE and Palmiter RD. Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. *Nature* 1996, **381**: 415–421.

Esler M, Jennings G, Korner P, Blomberg P, Sacharias N and Leonard P. Measurement of total and organ-specific norepinephrine kinetics in humans. *Am.J.Physiol.* 1984, **247**: E21-E28.

Esler M. The sympathetic system and hypertension. *Am.J.Hypertens.* 2000, **13**: 99S-105S.

Esler M, Jennings G, Lambert G, Meredith I, Horne M and Eisenhofer G. Overflow of catecholamine neurotransmitters to the circulation: source, fate, and functions. [Review] [219 refs]. *Physiological Reviews* 1990, **70**: 963-85.

Fagius J and Berne C. Increase in muscle nerve sympathetic activity in humans after food intake. *Clin.Sci.(Colch.)* 1994, **86**: 159-167.

Fanelli A, Grollman EF, Wang D and Philp NJ. MCT1 and its accessory protein CD147 are differentially regulated by TSH in rat thyroid cells. *Am J Physiol Endocrinol Metab.* 2003 Dec, **285**(6): E1223-9.

Farooqi IS, Jebb SA, Langmack G, Lawrence E, Cheetham CH, Prentice AM, Hughes IA, McCamish MA and O'Rahilly S. Effects of recombinant leptin therapy in a child with congenital leptin deficiency. *N Engl J Med.* 1999 Sep 6, **341**(12): 879-84.

Farooqi IS, Keogh JM, Yeo GS, Lank EJ, Cheetham T, O'Rahilly S. Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *N Engl J Med.* 2003, **348** : 1085 –1095.

Farooqi S and O'Rahilly S. Genetics of obesity in humans. *Endocr Rev.* 2006 Dec, **27**(7): 710-18.

Ferrannini E and DeFronzo RA. Insulin actions in vivo: glucose metabolism. In: Alberti KGMM, Zimmet P, DeFronzo RA, Keen H, editors. *International Textbook of Diabetes*. Chichester: John Wiley & Sons Ltd., 1997: 505-530.

Ferrannini E and Groop LC. Hepatic glucose production in insulin-resistant states. *Diabetes Metab Rev*. 1989, **5**: 711-725.

Fogelholm M. and van Marken LW. Comparison of body composition methods: a literature analysis. *Eur.J.Clin.Nutr*. 1997, **51**: 495-503.

Forbes S, Robinson S, Dungu J, Anyaoku V, Bannister P, Forster D, Dissanayake S, McCarthy MI, MacDonald IA, Venkatesan S and Johnston DG. Sustained endogenous glucose production, diminished lipolysis and non-esterified fatty acid appearance and oxidation in non-obese women at high risk of type 2 diabetes. *Eur J Endocrinol*. 2006 Sep, **155**(3): 469-76.

Forster CD and Macdonald IA. The assay of the catecholamine content of small volumes of human plasma. *Biomed Chromatogr*. 1999, **13**: 209 –215.

Frayn KN, Coppack SW, Humphreys SM and Whyte PL. Metabolic characteristics of human adipose tissue in vivo. *Clin Sci* 1989, **76**: 509-516.

Frayn KN, Coppack SW, Walsh PE, Butterworth HC, Humphreys SM and Pedrosa HC. Metabolic responses of forearm and adipose tissues to acute ethanol ingestion. *Metabolism* 1990, **39**: 958-966.

Frayn KN and Coppack SW. Assessment of white adipose tissue metabolism by measurement of arteriovenous differences. *Methods Mol Biol*. 2001, **155**: 269–279.

Frayn KN, Shadid S, Hamlani R *et al.* Regulation of fatty acid movement in human adipose tissue in the postabsorptive-to-postprandial transition. *Am J Physiol.* 1994, **266**: E308-E317.

Frayn KN, Coppock SW and Humphreys SM. Subcutaneous adipose tissue metabolism studied by local catheterization. [Review] [26 refs]. *International Journal of Obesity & Related Metabolic Disorders* 1993, **17** Suppl 3: S18-S21.

Fried SK, Leibel RL, Edens NK and Kral JG. Lipolysis in intraabdominal adipose tissues of obese women and men. *Obes. Res.* 1993, **1**: 443–448.

Gavrila A, Peng CK, Chan JL, Mietus JE, Goldberger AL and Mantzoros CS. Diurnal and ultradian dynamics of serum adiponectin in healthy men: comparison with leptin, circulating soluble leptin receptor, and cortisol patterns. *J Clin Endocrinol Metab.* 2003 Jun, **88**(6): 2838-43.

Gelfand RA and Barrett EJ. Effect of physiologic hyperinsulinemia on skeletal muscle protein synthesis and breakdown in man. *J Clin Invest.* 1987, **80**: 1-6.

Getty L, Pantaleon AE, Mittelman SD, Dea MK and Bergman RN. Rapid oscillations in omental lipolysis are independent of changing insulin levels in vivo. *J Clin Invest.* 2000, **106**: 421– 430.

Gilon P, Ravier MA, Jonas JC and Henquin JC. Control mechanisms of the oscillations of insulin secretion in vitro and in vivo. *Diabetes* 2002, **51** (Suppl.1): S144 –S151.

Giordano A, Morroni M, Carle F, Gesuita R, Marchesi GF and Cinti S. Sensory nerves affect the recruitment and differentiation of rat periovarian brown adipocytes during cold acclimation. *J Cell Sci.* 1998 Sep, **111** (Pt 17): 2587-94.

Giordano A, Morroni M, Santone G, Marchesi GF and Cinti S. Tyrosine hydroxylase, neuropeptide Y, substance P, calcitonin gene-related peptide and vasoactive intestinal peptide in nerves of rat periovarian adipose tissue: an immunohistochemical and ultrastructural investigation. *J Neurocytol.* 1996 Feb, **25**(2): 125-36.

Gohler L, Hahnemann T, Michael N, Oehme P, Steglich HD, Conradi E, Grune T and Siems WG. Reduction of plasma catecholamines in humans during clinically controlled severe underfeeding. *Prev.Med.* 2000, **30**: 95-102.

Grassi G and Esler M. How to assess sympathetic activity in humans. [Review] [170 refs]. *Journal of Hypertension* 1999, **17**: 719-34.

Grassi G. Debating sympathetic overactivity as a hallmark of human obesity: a pro's position [see comments]. *Journal of Hypertension* 1999, **17**: 1059-60.

Grassi G, Seravalle G, Colombo M, Bolla G, Cattaneo B M, Cavagnini F and Mancia G. Body weight reduction, sympathetic nerve traffic, and arterial baroreflex in obese normotensive humans. *Circulation* 1998, **97**: 2037-2042.

Green A and Newsholme EA. Sensitivity of glucose uptake and lipolysis of white adipocytes of the rat to insulin and effects of some metabolites. *Biochem J.* 1979, **180**: 365-370.

Greenfield ADM, Whitney RJ and Mowbray JF. Methods for the investigation of peripheral blood flow. *Br.Med.Bull.* 1963, **19**: 101-9.

Guo Z, Hensrud DD, Johnson CM and Jensen MD. Regional postprandial fatty acid metabolism in different obesity phenotypes. *Diabetes* 1999, **48**: 1586 –1592.

Hagström E, Arner P, Ungerstedt U and Bolinder J. Subcutaneous adipose tissue: a source of lactate production after glucose ingestion in humans. *Am J Physiol* 1990, **258**: E888-E893.

Halestrap AP and Meredith D. The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch.* 2004 Feb, **447**(5): 619-28.

Hammond VA and Johnston DG. Substrate cycling between triglyceride and fatty acid in human adipocytes. *Metabolism* 1987 Apr, **36**(4): 308-13.

Hansen BC, Jen KC, Belbez Pek S and Wolfe RA. Rapid oscillations in plasma insulin, glucagon, and glucose in obese and normal weight humans. *J Clin Endocr Metab.* 1982, **54**: 785-792.

Haynes WG. Interaction between leptin and sympathetic nervous system in hypertension. *Curr. Hypertens. Rep.* 2000, **2**: 311-8.

Heaf DJ, Kaijser L, Eklund B and Carlson LA. Differences in heparin-released lipolytic activity in the superficial and deep veins of the human forearm. *Eur J Clin Invest.* 1977, **7**: 195-199.

Himms-Hagen J, Melnyk A, Zingaretti MC, Ceresi E, Barbatelli G and Cinti S. Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. *Am J Physiol Cell Physiol.* 2000 Sep, **279**(3): C670-81.

Himms-Hagen J. Physiological roles of the leptin endocrine system: differences between mice and humans. *Crit Rev Clin Lab Sci.* 1999 Dec, **36**(6): 575-655.

Hjöllund E, Beck-Nielsen H, Pedersen O, Richelsen B and Sorensen N. Defective non-insulin-mediated and insulin mediated glucose transport and metabolism in adipocytes from obese and lean patients with untreated Type 2 diabetes mellitus. *Diabetic Med.* 1985, **2**: 468-473.

Hoffstedt J, Arner P, Hellers G and Lonnqvist F. Variation in adrenergic regulation of lipolysis between omental and subcutaneous adipocytes from obese and non-obese men. *J.Lipid Res.* 1997, **38**: 795-804.

Horowitz JF, Coppack SW and Klein S. Whole-body and adipose tissue glucose metabolism in response to short-term fasting in lean and obese women. *Am J Clin Nutr.* 2001, **73**: 517-522.

Horowitz JF and Klein S. Whole body and abdominal lipolytic sensitivity to epinephrine is suppressed in upper body obese women. *Am.J.Physiol Endocrinol.Metab.* 2000, **278**: E1144-E1152.

Hsia SL. Potentials in exploring the biochemistry of human skin. In: Campbell PN, Dickens F, editors. *Essays in Biochemistry*, Volume 7. London: Academic, 1971.

Hücking K, Hamilton-Wessler M, Ellmerer M and Bergman RN. Burst-like control of lipolysis by the sympathetic nervous system in vivo. *J Clin Invest.* 2003, **111**: 257-264.

Jackson RA, Hamling JB, Sim BM, Hawa MI, Blix PM and Nabarro JDN. Peripheral lactate and oxygen metabolism in man: the influence of oral glucose handling. *Metabolism* 1987, **36**: 144-150.

Jackson RA, Roshania RD, Hawa MI, Sim BM and DiSilvio L. Impact of glucose ingestion on hepatic and peripheral glucose metabolism in man: an analysis based on simultaneous use of the forearm and double isotope techniques. *J Clin Endocrinol Metab.* 1986, **63**: 541-549.

Jansson PA, Gudbjornsdottir HS, Andersson OK and Lonnroth PN. The effect of metformin on adipose tissue metabolism and peripheral blood flow in subjects with NIDDM. *Diabetes Care* 1996, **19**: 160-4.

Jansson PA, Larsson A and Lonnroth PN. Relationship between blood pressure, metabolic variables and blood flow in obese subjects with or without non-insulin-dependent diabetes mellitus. *Eur.J.Clin.Invest.* 1998, **28**: 813-8.

Jansson P-AE, Larsson A, Smith U and Lönnroth P. Glycerol production in subcutaneous adipose tissue in lean and obese humans. *J Clin Invest.* 1992, **89**: 1610-1617.

Jansson P-AE, Smith U and Lönnroth P. Evidence for lactate production by human adipose tissue in vivo. *Diabetologia* 1990, **33**: 253-256.

Jansson PA and Lonnroth P. Comparison of two methods to assess the tissue/blood partition coefficient for xenon in subcutaneous adipose tissue in man. *Clin.Physiol.* 1995, **15**: 47-55.

Jensen MD. Regulation of forearm lipolysis in different types of obesity. In vivo evidence for adipocyte heterogeneity. *J Clin Invest.* 1991, **87**: 187-193.

Johnson D, Prud'homme D, Després JP, Nadeau A, Tremblay A and Bouchard C. Relation of abdominal obesity to hyperinsulinemia and high blood pressure in men. *Int J Obes Relat Metab Disord.* 1992 Nov, **16**(11): 881-90.

Johnson JA and Fusaro RM. The role of the skin in carbohydrate metabolism. In: Levine R, Luft R, editors. *Advances in metabolic disorders*. vol. 6. New York: Academic Press, 1972: 1-55.

Kaijser L and Rössner S. Removal of exogenous triglycerides in human forearm muscle and subcutaneous tissue. *Acta Med Scand*. 1975, **197**: 289-294.

Karlsson A-K, Elam M, Friberg P, Biering-Sörensen F, Sullivan L and Lönnroth P. Regulation of lipolysis by the sympathetic nervous system: a microdialysis study in normal and spinal cord-injured subjects. *Metabolism* 1997, **46**: 388-394.

Karpe F, Fielding BA, Ardilouze J-L, Ilic V, Macdonald IA and Frayn KN. Effects of insulin on adipose tissue blood flow in man. *J Physiol*. 2002, **540**: 1087-1093.

Kennedy RT, Kauri LM, Dahlgren GM and Jung SK. Metabolic oscillations in beta-cells (Review). *Diabetes* 2002, **51** (Suppl. 1): S152-S161.

Kerckhoffs DAJM, Arner P and Bolinder J. Lipolysis and lactate production in human skeletal muscle and adipose tissue following glucose ingestion. *Clin Sci*. 1998, **94**: 71-77.

Klein S, Coppack SW, Mohamed-Ali V, Landt M. Adipose tissue leptin production and plasma leptin kinetics in humans. *Diabetes*. 1996 Jul; **45**(7):984-7.

Klein S, Young VR, Blackburn GL, Bistrian BR and Wolfe RR. The impact of body composition on the regulation of lipolysis during short-term fasting. *J Am Coll Nutr*. 1988, **7**: 77-84.

Kopelman PG. Obesity as a medical problem. *Nature* 2000, **404**: 635-43.

Kousta E, Chrisoulidou A, Lawrence NJ, al-Shoumer KA, Parker KH, McCarthy MI and Johnston DG. The circadian rhythm of leptin is preserved in growth hormone deficient hypopituitary adults. *Clin Endocrinol (Oxf)*. 1998 Jun, **48**(6): 685-90.

Kousta E, Parker KH, Lawrence NJ, Penny A, Millauer BA, Anyaoku V, Mulnier H, Forster DC, MacDonald IA, Robinson S, McCarthy MI and Johnston DG. Delayed metabolic and thermogenic response to a mixed meal in normoglycemic European women with previous gestational diabetes. *J Clin Endocrinol Metab*. 2002 Jul, **87**(7): 3407-12.

Koutkia P, Canavan B, Johnson ML, DePaoli A and Grinspoon S. Characterization of leptin pulse dynamics and relationship to fat mass, growth hormone, cortisol, and insulin. *Am J Physiol Endocrinol Metab*. 2003 Aug, **285**(2): E372-9.

Kreisberg RA. Glucose-lactate inter-relations in man. *N Engl J Med*. 1972, **287**: 132-137.

Kunz I, Schorr U, Klaus S and Sharma AM. Resting metabolic rate and substrate use in obesity hypertension. *Hypertension* 2000, **36**: 26-32.

Kurabayashi T, Carey DG and Morrison NA. The beta 3-adrenergic receptor gene Trp64Arg mutation is overrepresented in obese women. Effects on weight, BMI, abdominal fat, blood pressure, and reproductive history in an elderly Australian population. *Diabetes* 1996, **45**: 1358-63.

Lafontan M, Barbe P, Galitzky J, Tavernier G, Langin D, Carpene C *et al.* Adrenergic regulation of adipocyte metabolism. *Hum Reprod*. 1997, **12** Suppl 1: 6-20.

Lafontan M and Berlan M. Fat cell alpha 2-adrenoceptors: the regulation of fat cell function and lipolysis. *Endocr Rev.* 1995 Dec, **16**(6): 716-38.

Lafontan M and Berlan M. Evidence for the alpha 2 nature of the alpha-adrenergic receptor inhibiting lipolysis in human fat cells. *Eur.J.Pharmacol.* 1980, **66**: 87-93.

Landsberg L. Insulin-mediated sympathetic stimulation: role in the pathogenesis of obesity-related hypertension (or, how insulin affects blood pressure, and why). [Review] [43 refs]. *Journal of Hypertension* 2001, **19**: 523-528.

Lang DA, Matthews DR, Peto J and Turner RC. Cyclic oscillations of basal plasma glucose and insulin concentrations in human beings. *N Engl J Med.* 1979, **301**: 1023-1027.

Larsen OA, Lassen NA and Quaade F. Blood flow through human adipose tissue determined with radioactive xenon. *Acta Physiol Scand.* 1966, **66**: 337-345.

Lawrence VJ and Coppack SW. The endocrine function of the fat cell-regulation by the sympathetic nervous system. *Horm Metab Res.* 2000 Nov-Dec, **32**(11-12): 453-67.

Lean ME, James WP, Jennings G and Trayhurn P. Brown adipose tissue in patients with phaeochromocytoma. *Int.J.Obes.* 1986, **10**: 219-227.

Leibel RL, Rosenbaum M and Hirsch J. Changes in energy expenditure resulting from altered body weight [see comments] [published erratum appears in *N Engl J Med.* 1995 Aug 10, **333**(6): 399]. *New Eng J Med.* 1995, **332**: 621-8.

Leibowitz S, Roossin P and Rosenn M. Chronic norepinephrine injection into the hypothalamic paraventricular nucleus produces hyperphagia and increased body weight in the rat. *Pharmacol.Biochem.Behav.* 1984, **21**: 801-8.

Licinio J, Mantzoros C, Negrao AB, Cizza G, Wong ML, Bongiorno PB *et al.* Human leptin levels are pulsatile and inversely related to pituitary-adrenal function. *Nat.Med.* 1997, **3**: 575-9.

Licinio J, Negrão AB, Mantzoros C, Kaklamani V, Wong ML, Bongiorno PB, Negro PP, Mulla A, Veldhuis JD, Cernal L, Flier JS and Gold PW. Sex differences in circulating human leptin pulse amplitude: clinical implications. *J Clin Endocrinol Metab.* 1998 Nov, **83**(11): 4140-7.

Loncar D. Convertible adipose tissue in mice. *Cell Tissue Res.* 1991, **266**: 149-161.

Lowell BB. Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature* 1993, **366**: 740-2.

Lu H, Duanmu Z, Houck C, Jen KL, Buisson A and Dunbar JC. Obesity due to high fat diet decreases the sympathetic nervous and cardiovascular responses to intracerebroventricular leptin in rats. *Brain Res.Bull.* 1998, **47**: 331-5.

Lukaski HC, Johnson PE, Bolonchuk WW and Lykken GI. Assessment of fat-free mass using bioelectrical impedance measurements of the human body. *Am.J.Clin.Nutr.* 1985, **41**: 810-817.

Lundberg JM, Martinsson A, Hemsen A, Theodorsson N, Svedenhag J *et al.* Co-release of neuropeptide Y and catecholamines during physical exercise in man. *Biochemical & Biophysical Research Communications* 1985, **133**: 30-6.

Macdonald IA. Advances in our understanding of the role of the sympathetic nervous system in obesity. [Review] [31 refs]. *International Journal of Obesity & Related Metabolic Disorders* 1995, **19** Suppl 7: S2-S7.

Mackintosh VS, Elsegood CL and Redgrave TG. Effects of adrenoreceptor antagonists and agonists on clearance of emulsion models of triacylglycerol-rich lipoproteins from plasma in rats. *Clin.Exp.Pharmacol.Physiol.* 1991, **18**: 775-88.

Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S. *et al.* Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat.Med.* 1995, **1**: 1155-1161.

Manoharan C, Wilson MC, Sessions RB and Halestrap AP. The role of charged residues in the transmembrane helices of monocarboxylate transporter 1 and its ancillary protein basigin in determining plasma membrane expression and catalytic activity. *Mol Membr Biol.* 2006 Nov-Dec, **23**(6): 486-98.

Mårin P, Hogh Kristiansen I, Jansson S, Krotkiewski M, Holm G and Björntorp P. Uptake of glucose carbon in muscle glycogen and adipose tissue triglycerides in vivo in humans. *Am J Physiol.* 1992, **263**: E473-E480.

Mårin P, Rebuffé-Scrive M, Smith U and Björntorp P. Glucose uptake in human adipose tissue. *Metabolism* 1987, **36**: 1154-1160.

Martin ML and Jensen MD. Effects of body fat distribution on regional lipolysis in obesity. *J Clin Invest* 1991, **88**: 609-613.

Masuo K, Mikami H, Ogihara T and Tuck ML. Weight gain-induced blood pressure elevation. *Hypertension* 2000, **35**: 1135-1140.

Matsumoto T, Miyawaki C, Ue H, Kanda T, Yoshitake Y and Moritani T. Comparison of thermogenic sympathetic response to food intake between obese and non-obese young women. *Obes.Res.* 2001, **9**: 78-85.

Matthews DR. Oscillatory insulin secretion: a variable phenotypic marker. *Diabet Med* 1996, **13**: S53-S58.

Mercer SW and Williamson DH. The influence of starvation and natural refeeding on the rate of triacylglycerol/fatty acid substrate cycling in brown adipose tissue and different white adipose sites of the rat in vivo. The role of insulin and the sympathetic nervous system. *Biosci.Rep.* 1988, **8**: 147-53.

Meyer C, Nadkarni V, Stumvoll M and Gerich J. Human kidney free fatty acid and glucose uptake: evidence for a renal glucose-fatty acid cycle. *Am J Physiol* 1997, **273**: E650-E654.

Mingrone G, Manco M, Granato L, Calvani M, Scarfone A, Mora EV, Greco AV, Vidal H, Castagneto M and Ferrannini E. Leptin pulsatility in formerly obese women. *FASEB J.* 2005 Aug, **19**(10): 1380-2.

Mohamed-Ali V, Pinkney JH and Coppack SW. Adipose tissue as an endocrine and paracrine organ. *Int J Obes Relat Metab Disord.* 1998 Dec, **22**(12): 1145-58.

Møller N, Butler PC, Antsiferov MA and Alberti KGMM. Effects of growth hormone on insulin sensitivity and forearm metabolism in normal man. *Diabetologia* 1989, **32**: 105-110.

Mottram RF and Butterfield WJH. The human forearm as a preparation for metabolic investigations. *Proc R Soc Med* 1961, **54**: 549-552.

Muntzel MS, Morgan DA, Mark AL and Johnson AK. Intra-cerebroventricular insulin caused non-uniform regional increases in sympathetic nerve activity. *Am J Physiol.* 1994, **267**: R1350-R1355.

Newby FD, Sykes MN and DiGirolamo M. Regional differences in adipocyte lactate production from glucose. *Am J Physiol.* 1988, **255**: E716-E722.

Newby FD, Wilson LK, Thacker SV and DiGirolamo M. Adipocyte lactate production remains elevated during refeeding after fasting. *Am J Physiol.* 1990, **259**: E865-E871.

Nielsen SL. Adipose tissue blood flow determined by the washout of locally injected ¹³³Xenon. *Scand J Clin Lab Invest.* 1972, **29**: 31-36.

Nijima A. Reflex effects from leptin sensors in the white adipose tissue of the epididymis to the efferent activity of the sympathetic and vagus nerve in the rat. *Neurosci Lett.* 1999, **262**: 125-8.

Nishimura S, Manabe I, Nagasaki M, Hosoya Y, Yamashita H, Fujita H, Ohsugi M, Tobe K, Kadokawa T, Nagai R and Sugiura S. Adipogenesis in obesity requires close interplay between differentiating adipocytes, stromal cells, and blood vessels. *Diabetes* 2007 Jun, **56**(6): 1517-26.

Nishizawa Y and Bray GA. Ventromedial hypothalamic lesions and the mobilization of fatty acids. *J. Clin. Invest.* 1978, **61**: 714-721.

O'Rahilly S, Turner RC and Matthews DR. Impaired pulsatile secretion of insulin in relatives of patients with non-insulin-dependent diabetes. *N Engl J Med.* 1988 May 12, **318**(19): 1225-30.

Orban Z, Remaley AT, Sampson M, Trajanoski Z and Chrousos GP. The differential effect of food intake and beta-adrenergic stimulation on adipose-derived hormones and cytokines in man. *J.Clin.Endocrinol.Metab.* 1999, **84**: 2126-2133.

Pages G, Girard A, Jeanneton O, Barbe P, Wolf C, Lafontan M *et al.* LPA as a paracrine mediator of adipocyte growth and function. *Ann.N.Y.Acad.Sci.* 2000, **905**: 159-64.

Pernet A, Walker M, Gill GV, Orskov H, Alberti KG and Johnston DG. Metabolic effects of adrenaline and noradrenaline in man: studies with somatostatin. *Diabetes Metab.* 1984 May, **10**(2): 98-105.

Patel JN, Eisenhofer G, Coppack SW and Miles JM. Norepinephrine spillover in forearm and subcutaneous adipose tissue before and after eating. *Journal of Clinical Endocrinology & Metabolism* 1999, **84**: 2815-9.

Pørksen N, Hollingdal M, Juhl C, Butler P, Veldhuis JD and Schmitz O. Pulsatile insulin secretion: detection, regulation, and role in diabetes. *Diabetes*. 2002 Feb, **51** Suppl 1: S245-54.

Potter EK. Neuropeptide Y as an autonomic neurotransmitter. *Pharmacol.Ther.* 1988, **37**: 251-73.

Prins JB and O'Rahilly S. Regulation of adipose cell number in man [editorial]. *Clin.Sci.(Colch.)* 1997, **92**: 3-11.

Prinz WA, West DB, Cohen A, Silverberg S and Greenwood MRC. Intra-arterial insulin infusion reduces adipose tissue blood flow. *Fed Proc* 1986, **45**, 601.

Quaade F, Larsen OA, Lassen NA and Nielsen SL. Observations on the influence of glucose upon subcutaneous adipose tissue blood flow. *Acta Med Scand Suppl* 1967, **476**: 85-90.

Raposinho PD, White RB and Aubert ML. The melanocortin agonist Melanotan-II reduces the orexigenic and adipogenic effects of neuropeptide Y (NPY) but does not affect the NPY-driven suppressive effects on the gonadotropic and somatotropic axes in the male rat. *J Neuroendocrinol*. 2003 Feb, **15**(2): 173-81.

Rayner DV. The sympathetic nervous system in white adipose tissue regulation. *Proceedings of the Nutrition Society* 2001, **60**: 357-364.

Reaven GM, Lithell H and Landsberg L. Hypertension and associated metabolic abnormalities-the role of insulin resistance and the sympathoadrenal system. *N Engl J Med*. 1996, **334**: 374-381.

Rebuffé-Scrive M. Neuroregulation of adipose tissue: molecular and hormonal mechanisms. *Int J Obes*. 1991 Sep, **15** Suppl 2: 83-6.

Reynisdottir S, Wahrenberg H, Carlstrom K, Rössner S and Arner P. Catecholamine resistance in fat cells of women with upper-body obesity due to decreased expression of beta 2-adrenoceptors [see comments]. *Diabetologia* 1994, **37**: 428-435.

Ricci MR, Lee MJ, Russell CD, Wang Y, Sullivan S, Schneider SH, Brolin RE and Fried SK. Isoproterenol decreases leptin release from rat and human adipose tissue through posttranscriptional mechanisms. *Am J Physiol Endocrinol Metab*. 2005 Apr, **288**(4): E798-804.

Richelsen B, Pedersen SB, Møller-Pedersen T and Bak JF. Regional differences in triglyceride breakdown in human adipose tissue: effects of catecholamines, insulin, and prostaglandin E2. *Metabolism* 1991, **40**: 990-6.

Ricquier D, Nechad M and Mory G. Ultrastructural and biochemical characterization of human brown adipose tissue in pheochromocytoma. *J.Clin.Endocrinol.Metab* 1982, **54**: 803-7.

Ricquier D. Fundamental mechanisms of thermogenesis. *C R Biol.* 2006 Aug, **329**(8):578-86.

Rongen GA, Lenders JW, Smits P and Floras JS. Comparison of two indices for forearm noradrenaline release in humans. *Clin.Sci.(Lond).* 2000, **99**: 363-369.

Rosell S. Nervous and pharmacological regulation of vascular reactions in adipose tissue. *Advances in Experimental and Medical Biology*. 1969, **4**: 25–34.

Rosell S. Sympathetic control of transcapillary transport in canine adipose tissue. *Bibl Anat.* 1975, **13**: 28-31.

Rössner S, Eklund B, Kaijser L, Olsson AG and Walldius G. Removal of exogenous plasma triglycerides in forearm muscle and subcutaneous tissue of hyper- and normotriglyceridaemic men. *Eur J Clin Invest.* 1976, **6**: 299-305.

Roust LR and Jensen MD. Postprandial free fatty acid kinetics are abnormal in upper body obesity. *Diabetes* 1993, **42**: 1567-1573.

Rumantir MS, Vaz M, Jennings GL, Collier G, Kaye DM, Seals DR *et al.* Neural mechanisms in human obesity-related hypertension. *J.Hypertens.* 1999, **17**: 1125-33.

Saad MF, Alger SA, Zurlo F, Young JB, Bogardus C and Ravussin E. Ethnic differences in sympathetic nervous system-mediated energy expenditure. *Am.J.Physiol.* 1991, **261**: E789-E794.

Saad MF, Khan A, Sharma A, Michael R, Riad-Gabriel MG, Boyadjian R, Jinagouda SD, Steil GM and Kamdar V. Physiological insulinemia acutely modulates plasma leptin. *Diabetes*. 1998 Apr, **47**(4): 544-9.

Saad MF, Riad-Gabriel MG, Khan A, Sharma A, Michael R, Jinagouda SD, Boyadjian R and Steil GM. Diurnal and ultradian rhythmicity of plasma leptin: effects of gender and adiposity. *J Clin Endocrinol Metab*. 1998 Feb, **83**(2): 453-9.

Sakane N, Yoshida T, Umekawa T, Kogure A, Takakura Y and Kondo M. Effects of Trp64Arg mutation in the beta 3-adrenergic receptor gene on weight loss, body fat distribution, glycemic control, and insulin resistance in obese type 2 diabetic patients. *Diabetes Care* 1997, **20**: 1887-90.

Samra JS, Frayn KN, Giddings JA, Clark ML and Macdonald IA. Modification and validation of a commercially available portable detector for measurement of adipose tissue blood flow. *Clin Physiol*. 1995, **15**: 241–248.

Samra JS, Simpson EJ, Clark ML, Forster CD, Humphreys SM, Macdonald IA et al. Effects of epinephrine infusion on adipose tissue: interactions between blood flow and lipid metabolism. *American Journal of Physiology* 1996, **271**: E834-E839.

Saris WH. Effects of energy restriction and exercise on the sympathetic nervous system. [Review] [35 refs]. *International Journal of Obesity & Related Metabolic Disorders* 1995, **19** Suppl 7: S17-S23.

Scarpace PJ and Zhang Y. Leptin resistance: a predisposing factor for diet-induced obesity. *Am J Physiol Regul Integr Comp Physiol.* 2009 Mar, **296**(3): R493-500.

Schiffelers SL, Saris WH, Boomsma F and Van Baak MA. Beta(1)- and beta(2)-Adrenoceptor-mediated thermogenesis and lipid utilization in obese and lean men. *J.Clin.Endocrinol.Metab* 2001, **86**: 2191-2199.

Schoeller DA, Cella LK, Sinha MK and Caro JF. Entrainment of the diurnal rhythm of plasma leptin to meal timing. *J. Clin. Invest.* 1997, **100**: 1882–1887.

Schwartz RS, Jaeger LF and Veith RC. Sympathetic nervous activity and the thermic effect of feeding in man. *Int.J.Obes.Relat Metab Disord.* 1987, **11**: 141-9.

Shah SD, Clutter WE and Cryer PE. External and internal standards in the single-isotope derivative (radioenzymatic) measurement of plasma norepinephrine and epinephrine. *J.Lab Clin.Med.* 1985, **106**: 624-9.

Silver K, Mitchell BD, Walston J, Sorkin JD, Stern MP, Roth J *et al.* TRP64ARG beta 3-adrenergic receptor and obesity in Mexican Americans. *Hum.Genet.* 1997, **101**: 306-11.

Silverberg AB, Shah SD, Haymond MW and Cryer PE. Norepinephrine: hormone and neurotransmitter in man. *Am J Physiol.* 1978, **234**: E252–E256.

Simonsen L, Bulow J, Astrup A, Madsen J and Christensen NJ. Diet-induced changes in subcutaneous adipose tissue blood flow in man: effect of beta-adrenoceptor inhibition. *Acta Physiol Scand.* 1990, **139**: 341-6.

Simonsen L, Bülow J, Madsen J and Christensen NJ. Thermogenic response to epinephrine in the forearm and abdominal subcutaneous adipose tissue.
Am J Physiol. 1992, **263**: E850-E855.

Simonsen L, Bülow J and Madsen J. Adipose tissue metabolism in humans determined by vein catheterization and microdialysis techniques. *Am J Physiol.* 1994, **266**: E357-E365.

Sinha MK, Sturis J, Ohannesian J, Magosin S, Stephens T, Heiman ML, Polonsky KS and Caro JF. Ultradian oscillations of leptin secretion in humans. *Biochem. Biophys. Res. Commun.* 1996, **228**: 733-738.

Slavin BG and Ballard KW. Morphological studies on the adrenergic innervation of white adipose tissue. *Anat Rec.* 1978 Jul, **191**(3): 377-89.

Snitker S, Macdonald IA, Ravussin E and Astrup A. The sympathetic nervous system and obesity: role in aetiology and treatment. *Obesity Reviews* 2000, **1**: 5-15.

Spiegelman BM and Flier JS. Obesity and the regulation of energy balance. *Cell* 2001 Feb 23, **104**(4): 531-43.

Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas A *et al.* The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* 1995, **377**: 530-532.

Summers LK, Samra JS and Frayn KN. Impaired postprandial tissue regulation of blood flow in insulin resistance: a determinant of cardiovascular risk? *Atherosclerosis* 1999, **147**: 11-15.

Summers LK, Samra JS, Humphreys SM, Morris RJ and Frayn KN. Subcutaneous abdominal adipose tissue blood flow: variation within and between subjects and relationship to obesity. *Clinical Science* 1996, **91**: 679-83.

Takahashi A and Shimazu T. Hypothalamic regulation of lipid metabolism in the rat: effect of hypothalamic stimulation on lipolysis. *J. Auton. Nerv. Syst.* 1981, **4**: 195–205.

Tataranni PA, Christin L, Snitker S, Paolisso G and Ravussin E. Pima Indian males have lower beta-adrenergic sensitivity than Caucasian males. *J.Clin.Endocrinol.Metab.* 1998, **83**: 1260-3.

Tataranni PA, Cizza G, Snitker S, Gucciardo F, Lotsikas A, Chrousos GP *et al.* Hypothalamic-pituitary-adrenal axis and sympathetic nervous system activities in Pima Indians and Caucasians. *Metabolism* 1999, **48**: 395-9.

Tataranni PA, Young JB, Bogardus C and Ravussin E. A low sympathoadrenal activity is associated with body weight gain and development of central adiposity in Pima Indian men. *Obes.Res.* 1997, **5**: 341-347.

Trayhurn P, Duncan JS, Hoggard N and Rayner DV. Regulation of leptin production: a dominant role for the sympathetic nervous system? *Proc.Nutr.Soc.* 1998, **57**: 413-419.

Trayhurn P, Duncan JS, Rayner DV and Hardie LJ. Rapid inhibition of ob gene expression and circulating leptin levels in lean mice by the beta 3-adrenoceptor agonists BRL 35135A and ZD2079. *Biochem.Biophys.Res.Commun.* 1996, **228**: 605-10.

Trayhurn P, Thomas ME, Duncan JS and Rayner DV. Effects of fasting and refeeding on ob gene expression in white adipose tissue of lean and obese (ob/ob) mice. *FEBS Lett.* 1995 Jul 24, **368**(3): 488-90.

Tsuji S and Bray GA. A beta-3 adrenergic agonist (BRL-37,344) decreases food intake. *Physiology & Behaviour* 1998, **63**: 723-728.

Turtzo LC and Lane MD. Completing the loop: neuron-adipocyte interactions and the control of energy homeostasis. *Horm. Metab. Res.* 2002, **34**: 607-615.

Unger RH. Lipotoxic diseases. *Annu. Rev. Med.* 2002, **53**: 319-336.

Valet P, Pages C, Jeanneton O, Daviaud D, Barbe P, Record M, Saulnier-Blache JS and Lafontan M. Alpha2-adrenergic receptor-mediated release of lysophosphatidic acid by adipocytes. A paracrine signal for preadipocyte growth. *J. Clin. Invest.* 1998, **101**: 1431-1438.

Vaz M, Cox HS, Kaye DM, Turner AG, Jennings GL and Esler MD. Fallibility of plasma noradrenaline measurements in studying postprandial sympathetic nervous responses. *J. Auton. Nerv. Syst.* 1995, **56**: 97-104.

Vaz M, Jennings G, Turner A, Cox H, Lambert G and Esler M. Regional sympathetic nervous activity and oxygen consumption in obese normotensive human subjects. *Circulation* 1997, **96**: 3423-3429.

Veldhuis JD and Johnson ML. Cluster analysis: a simple, versatile, and robust algorithm for endocrine pulse detection. *Am J Physiol.* 1986, **250**: E486-E493.

Wahren J. Quantitative aspects of blood flow and oxygen uptake in the human forearm during rhythmic exercise. *Acta Physiol Scand.* 1966, **67** (suppl 269): 5-93.

Wallin BG, Sundlof G, Eriksson BM, Dominiak P, Grobecker H and Lindblad LE. Plasma noradrenaline correlates to sympathetic muscle nerve activity in normotensive man. *Acta Physiol Scand.* 1981, **111**: 69 –73.

Webber J, Taylor J, Greathead H, Dawson J, Buttery PJ and Macdonald IA. A comparison of the thermogenic, metabolic and haemodynamic responses to infused adrenaline in lean and obese subjects. *International Journal of Obesity and Related Metabolic Disorders* 1994, **18**: 717–724.

Webber J and Macdonald I.A. Signalling in body-weight homeostasis: neuroendocrine efferent signals. *Proceedings of the Nutrition Society* 2000, **59**: 397-404.

Wellhoener P, Fruehwald-Schultes B, Kern W, Dantz D, Kerner W, Born J, Fehm HL and Peters A. Glucose metabolism rather than insulin is a main determinant of leptin secretion in humans. *J Clin Endocrinol Metab.* 2000 Mar, **85**(3): 1267-71.

Weyer C, Gautier JF and Danforth E. Development of beta 3-adrenoceptor agonists for the treatment of obesity and diabetes-an update. *Diabetes Metab.* 1999, **25**: 11-21.

Whitney RJ. The measurement of volume changes in human limbs. *J Physiol Lond.* 1953, **121**: 1-27.

Wirsén C. Adrenergic innervation of adipose tissue examined by fluorescence microscopy. *Nature* 1964 May 30, **202**: 913

Wirsén C. Studies in Lipid Mobilisation. *Acta Physiol Scand.* 1965, Suppl-46.

Xiang K, Jia W, Lu H, Zheng T, Lu J, Tang J *et al.* Effects of Trp64Arg mutation in the beta 3-adrenergic receptor gene on body fat, plasma glucose level, lipid profile, insulin secretion and action. *Chung Hua I.Hsueh I.Chuan Hsueh Tsa Chih* 1998, **15**: 337-40.

Yanagiya T, Tanabe A and Hotta K. Gap-junctional communication is required for mitotic clonal expansion during adipogenesis. *Obesity (Silver Spring)* 2007 Mar, **15**(3): 572-82.

Yoshitomi H, Yamazaki K, Abe S and Tanaka I. Differential regulation of mouse uncoupling proteins among brown adipose tissue, white adipose tissue, and skeletal muscle in chronic beta 3 adrenergic receptor agonist treatment. *Biochem.Biophys.Res.Commun.* 1998, **253**: 85-91.

Young JB, Rowe JW, Pallotta JA, Sparrow D and Landsberg L. Enhanced plasma norepinephrine response to upright posture and oral glucose administration in elderly human subjects. *Metabolism* 1980, **29**: 532–539.

Young JB and Macdonald IA. Sympathoadrenal activity in human obesity: heterogeneity of findings since 1980. [Review] [69 refs]. *International Journal of Obesity & Related Metabolic Disorders* 1992, **16**: 959-67.

Young JB and Landsberg L. Suppression of sympathetic nervous system during fasting. [Review] [17 refs]. *Obesity Research* 1997, **5**: 646-649.

Youngstrom TG and Bartness TJ. White adipose tissue sympathetic nervous system denervation increases fat pad mass and fat cell number. *Am.J.Physiol.* 1998, **275**: R1488-R1493.

Zeuzem S and Taylor R. Assessment of initial hexose uptake in adipocytes using the physiological substrate D-glucose. Particular relevance to human adipocytes. *Scand J Clin Lab Invest.* 1985, **45**: 545-551.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L and Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature.* 1994, **372**: 425–432.

Zierler KL. Theory of the use of arteriovenous concentration differences for measuring metabolism in steady and non-steady states. *J Clin Invest.* 1961, **40**: 2111-2115.

Zvonic S, Ptitsyn AA, Conrad SA, Scott LK, Floyd ZE, Kilroy G, Wu X, Goh BC, Mynatt RL and Gimble JM. Characterization of peripheral circadian clocks in adipose tissues. *Diabetes.* 2006 Apr, **55**(4):962-70.

APPENDIX

FUNDING, PUBLICATIONS ARISING FROM THE THESIS AND RESEARCH ETHICS COMMITTEE

APPROVALS

FUNDING

I was awarded a Wellcome Trust Research Training Fellowship which generously provided the funding for all the work I performed during these studies.

PUBLICATIONS ARISING FROM THE THESIS

Lawrence VJ, Coppack SW. The endocrine function of the fat cell-regulation by the sympathetic nervous system. Horm Metab Res. 2000 Nov-Dec, 32(11-12):453-67.

Karpe F, Fielding BA, Coppack SW, Lawrence VJ, Macdonald IA, Frayn KN. Oscillations of fatty acid and glycerol release from human subcutaneous adipose tissue in vivo. Diabetes. 2005 May, 54(5):1297-303

RESEARCH ETHICS COMMITTEE PERMISSIONS FOR THE WORK

All the studies I undertook as part of this thesis were given appropriate ethical permission by the East London and City Health Authority Research Ethics Committee under the following reference codes:

P99/228	Arterio-venous difference studies
P00/132	Leptin and NEFA pulsatility studies
P00/202	Adipose tissue biopsies and histological analysis

Work performed by those in collaboration with us was given appropriate local research ethics committee approval which is described in the text where relevant.

THE END