



**Unravelling the pathogenesis of
type 2 diabetes**

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ABSTRACT

Type 2 diabetes has become a worldwide epidemic. It is characterised by insulin resistance in major metabolic tissues, and failure of β -pancreatic cells to compensate for this abnormality. Insulin resistance is recognised as an early event in the pathogenesis of type 2 diabetes. Although the precise factors that lead to insulin resistance have not been elucidated fully, there is strong association between insulin resistance and lipid accumulation, in particular lipotoxic fatty acid metabolites in insulin-target tissues. Most recently, evidence has been presented to link abnormal fatty acid accumulation in muscle with reduced mitochondrial activity. However it was unclear if these aberrations are causally related to the development of insulin resistance and type 2 diabetes. The two major pathophysiological abnormalities that underlie type 2 diabetes have long been viewed to require two separate pathogenic processes. The resolution of type 2 diabetes after bariatric surgery has allowed the elucidation of the sequence of events that lead to the restoration of normal metabolism, paving the way for a new understanding of type 2 diabetes as a metabolic state precipitated by a single cause of chronic excess intra-organ lipid accumulation. Magnetic resonance technique provides a non-invasive way to evaluate metabolism in both normal and pathological states. Specifically, ^{31}P magnetic resonance spectroscopy allows the observation of real-time ATP synthesis as a direct measurement of mitochondrial activity. ^{13}C magnetic resonance spectroscopy can be applied to assess muscle glycogen concentration. Both ^1H magnetic resonance spectroscopy and magnetic resonance imaging can be used to evaluate intra-organ lipid concentration. Collectively, these innovative techniques offer safe and powerful approaches to study the role of skeletal muscle oxidative capacity and intra-organ fatty acid accumulation and metabolism in the pathogenesis of type 2 diabetes. This thesis presents data

which lead to a simplified understanding of the aetiology and pathogenesis of type 2 diabetes.

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DECLARATION

I conducted all the studies presented in this thesis from 2007-2010, at the Newcastle Magnetic Resonance Centre, Newcastle University, with the following assistance. All magnetic resonance examinations were carried out by senior MR radiographers, Louise Morris, Carol Smith and Tim Robson. ^{13}C MR spectroscopy analyses were performed by Dr Fiona Smith and Dr Peter Thelwall. ^{31}P MR spectroscopy and three-point Dixon MRI analyses for the liver, pancreas, subcutaneous and visceral fat areas were performed by Dr Kieren Hollingsworth and myself. Mrs Sandra Blackwood assisted in the analysis of the subcutaneous and visceral fat areas. Mrs Jean Gerrard and Ms Mei Jun Chen provided nursing assistance during the clinical studies. Metabolite and hormone assays were done by Mrs Heather Cook and Mrs Annette Lane. The latter also performed the quantification of plasma 6,6-dideuterated glucose and ^{13}C plasma glucose enrichment. Mathematical modelling of the insulin secretion rate data was carried out by Dr Benjamin Aribisala. The design and organisation of the clinical studies, recruitment of subjects, their care during the studies and all other aspects apart from those specifically mentioned above were undertaken by myself. The composition of this thesis is my own work. The research contained within this thesis has not previously been submitted elsewhere for the degree of Doctor of Philosophy.

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LIST of ORIGINAL PUBLICATIONS

- 1) Measuring the acute effect of insulin infusion on ATP turnover rate in human skeletal muscle using phosphorus-31 magnetic resonance saturation transfer spectroscopy. Lim EL, Hollingsworth KG, Thelwall P, Taylor R. *NMR Biomed* 2010 Oct; 23(8):952-7.

- 2) Inhibition of lipolysis in type 2 diabetes normalizes glucose disposal without change in muscle glycogen synthesis rates. Lim EL, Hollingsworth KG, Smith FE, Thelwall PE, Taylor R. *Clin Sci (Lond)* 2011 Aug; 121(4):169-77.

- 3) Reversal of type 2 diabetes: normalisation of β -cell function in association with decreased pancreas and liver triacylglycerol. Lim EL, Hollingsworth KG, Aribisala BS, Chen MJ, Mathers JC, Taylor R. *Diabetologia* 2011 Oct; 54(10):2506-14. Epub 2011 Jun 9.

- 4) Effects of raising muscle glycogen synthesis rate on skeletal muscle ATP turnover rate in type 2 diabetes. Lim EL, Hollingsworth KG, Smith FE, Thelwall PE, Taylor R. *Am J Physiol Endocrinol Metab* 2011 Dec; 301(6):E1155-62. Epub 2011 Sep 13.

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- 1) Is sub-normal insulin stimulation of muscle ATP synthesis in type 2 diabetes always a consequence of low rates of glycogen synthesis?
Lim EL, Trenell MI, Hollingsworth KG, Smith FE, Thelwall PE, Taylor R. *Diabetologia* 2008, 51: (Supplement 1), S306 – abstract from poster presentation at 44th Annual Meeting of EASD Sept 2008, Rome.

- 2) Insulin effect on muscle ATP turnover rates: extent and reproducibility.
Lim EL, Hollingsworth KG, Taylor R. *Diabetic Medicine* 2009, 26: (Supplement 1), 66 – abstract from poster presentation at Diabetes UK Annual Professional Conference March 2009, Glasgow.

- 3) Intramyocellular ATP flux is not dependent on insulin action in type 2 diabetes.
Lim EL, Trenell MI, Hollingsworth KG, Smith FE, Thelwall PE, Taylor R. *Diabetologia* 2009, 52: (Supplement 1), S99 – abstract from oral presentation at 45th Annual Meeting of EASD Oct 2009, Vienna.

- 4) Acute inhibition of NEFA on muscle mitochondrial function in type 2 diabetes.
Lim EL, Trenell MI, Hollingsworth KG, Smith FE, Thelwall PE, Taylor R. *Diabetic Medicine* 2010, 27: (Supplement 1), 9 – abstract from oral presentation at Diabetes UK Annual Professional Conference March 2010, Liverpool.

- 5) Non-surgical cure of type 2 diabetes: effect of change in liver and pancreas fat content.

Lim EL, Hollingsworth KG, Aribisala B, Chen MJ, Mathers JC, Taylor R. *Diabetic Medicine* 2011, 28: (Supplement 1), 15 – abstract from oral presentation at Diabetes UK Annual Professional Conference March 2011, London.

- 6) Reversal of type 2 diabetes is associated with decrease in pancreas and liver fat levels.

Lim EL, Hollingsworth KG, Aribisala B, Chen MJ, Mathers JC, Taylor R. *Diabetes* 2011, 60: (Supplement 1), A413 – abstract from poster presentation at the American Diabetes Association's 71st scientific sessions June 2011, San Diego.

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ABBREVIATIONS

3-BOH	3-hydroxy-butyrate
ADP	adenosine diphosphate
ALT	alanine transaminase
AMP	adenosine monophosphate
ANOVA	analysis of variance
APE	atom percent excess
ATP	adenosine triphosphate
BMI	body mass index
BSA	body surface area
CK	creatine kinase
CoA	coenzyme A
CV	coefficient of variation
DNA	deoxyribonucleic acid
EMCL	extramyocellular lipid
ETC	electron transport chain
FADH ₂	flavin adenine dinucleotide
ffm	fat free mass
FID	free induction decay
G6P	glucose 6-phosphate
GC-MS	gas chromatography-mass spectrometry
γ-GT	gamma-glutamyltransferase
GIR	glucose infusion rate
GLUT	glucose transporter
HGP	hepatic glucose production
HOMA	homeostasis model assessment
IGT	impaired glucose tolerance
IMCL	intramyocellular lipid

IRS	insulin receptor substrates
jMRUI	java-based magnetic resonance user interface
MET	metabolic equivalent of task
MR	magnetic resonance
NADH	nicotinamide adenine dinucleotide
NEFA	non-esterified fatty acid
NGT	normal glucose tolerance
OGTT	oral glucose tolerance test
PI-3	phosphatidylinositol-3
pCO ₂	partial pressure of carbon dioxide
PCr	phosphocreatine
PDE	phosphodiesterases
PGC	PPAR coactivator
P_i	inorganic phosphate
PKB	protein kinase B
<i>PNPLA3</i>	patatin-like phospholipase 3
pO ₂	partial pressure of oxygen
PPAR	peroxisomal proliferator activator receptor
ppm	parts per million
R _a	glucose appearance
R _d	glucose disappearance
ROI	region-of-interest
SAT	subcutaneous adipose tissue
T2D	type 2 diabetes
TE	echo time
TR	repetition time
UCP	uncoupling protein
VAT	visceral adipose tissue
VLCD	very-low-calorie diet
VLDL	very low-density lipoprotein

WHO World Health Organisation

Chapter 1

INTRODUCTION

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1.1 Natural History of Type 2 Diabetes

Type 2 diabetes is one of the most frequent metabolic disorders worldwide and the number of people with diabetes is rising rapidly due to increasing consumption of high-energy food and the increasing adoption of sedentary lifestyle. It is projected that by 2030 as many as 366 million of the population would have this metabolic disorder (Wild et al., 2004). In addition, the age of onset of type 2 diabetes is decreasing, giving rise to an increasing proportion of young people of working age being affected. This leaves individuals exposed to longer periods of hyperglycaemia and hence increased risks of developing diabetes-related complications. The global epidemic of type 2 diabetes is clearly a cause of serious health-related and socioeconomic burden on individuals and society alike.

Type 2 diabetes is a complex, heterogenous, polygenic disease characterised by two fundamental defects: impaired insulin action in skeletal muscle, liver and adipocytes and impaired β -cell function. The sequence with which these metabolic abnormalities develop and their relative contributions in the causation of type 2 diabetes remain much debated. It has long been regarded by some that insulin resistance is the primary abnormality and that β -cell dysfunction is a late occurrence arising from the prolonged increased demand placed on the β -cell by insulin resistance (DeFronzo et al., 1992). Insulin resistance itself has been associated with increased lipid depositions in skeletal muscle and liver, and has recently been linked to a functional impairment of mitochondria.

1.1.1 Observation of the earliest defect

The Pima Indians who live in the Gila River Indian Community in Arizona have participated in a longitudinal study of diabetes and its complications since 1965 (Bennett et al., 1971). The prevalence and incidence of type 2 diabetes in this community is the highest of any population in the world (Knowler et al., 1981). In the Pima Indians, the prevalence of type 2 diabetes is over 50% above

the age of 55 and peaks at almost 70% around the age of 60 years (Knowler et al., 1990, Pavkov et al., 2007). Amongst the youths (15- to 24-year olds), type 2 diabetes affects 6.4% of Pima Indians (a six-fold increase in prevalence over the last 40 years) (Dabelea et al., 1998, Pavkov et al., 2007). Diabetes was rare or largely unrecognised among the Pima Indians until the late 1930s when increasing settlers from the east led to disruption of their agriculture, curtailing subsistence farming and the ensuing change in lifestyle. Evidence suggests that the incidence of diabetes in this indigenous population did rise abruptly in the 1930s, and this rise was followed by relatively stable incidence since that time, but with a shift to a younger age at onset of diabetes as a consequence of increasing childhood obesity and increasing frequency of exposure to diabetes *in utero* (Pettitt et al., 1983, Dabelea et al., 1998, Pavkov et al., 2007).

Pima Indians with type 2 diabetes are characterised by obesity, insulin resistance, insulin secretory dysfunction and increased rates of endogenous glucose production, which are the characteristics that define type 2 diabetes across most populations (Bogardus et al., 1984). Compared with Caucasians, Pima Indians have, on average, lower insulin sensitivity and higher plasma insulin concentrations, both fasting and in response to intravenous and oral glucose loads (Bogardus et al., 1984). Prospective studies indicate that hyperinsulinaemia and abnormal insulin secretory function were both independent predictors of the development of type 2 diabetes in this population (Weyer et al., 1999a). In their study, Weyer and colleagues carried out repeated analyses on 17 Pima Indians who developed diabetes and compared these to a control group of 31 individuals who remained normal glucose tolerant over the same 5-year period of follow-up (Weyer et al., 1999a). Results demonstrated that both insulin secretion and insulin action significantly decrease early in the development of type 2 diabetes – during the transition from normal glucose tolerance (NGT) to impaired glucose tolerance (IGT). With the progression from IGT to diabetes, the abnormalities in insulin

secretion and insulin action worsen in parallel with an increase in endogenous glucose output.

IGT is well recognised as a risk factor for diabetes and studies have estimated that between 2% and 14% of individuals with IGT progress to type 2 diabetes each year (Yudkin et al., 1990, Knowler et al., 2002). The progression rate is clearly influenced by age, ethnicity and the degree of glucose intolerance at baseline. Individuals with IGT have been found, on average, to be more obese and more insulin-resistant than those with NGT (Ferrannini and Camastra, 1998, Weyer et al., 1999b). Insulin sensitivity as measured with hyperinsulinaemic-euglycaemic clamp has been shown to be consistently lower in individuals with IGT compared with NGT across a variety of ethnic groups studied: 27% reduction in Pima Indians (Weyer et al., 1999b), 32% in Hispanics (Abdul-Ghani et al., 2006), and 21% in Japanese (Wasada et al., 2004). This magnitude of decline in insulin sensitivity in IGT subjects is comparable to that in normoglycaemic first-degree relatives of diabetic subjects (Gulli et al., 1992, Vauhkonen et al., 1998, Kashyap et al., 2004, Arslanian et al., 2005). In addition, defects in insulin secretory function have been reported to be present before overt diabetes developed. Abdul-Ghani and co-workers demonstrated that subjects with IGT have significant impairment in early insulin secretory response to ingested glucose (Abdul-Ghani et al., 2006). Studies by Weyer et al. (Weyer et al., 1999b) and Festa et al. (Festa et al., 2004) reported reductions between 8-18% in acute insulin response in IGT subjects compared with NGT individuals. Ahren and colleagues found a 32% decrease in acute insulin response in Caucasian postmenopausal women with IGT (Ahren and Pacini, 1997).

Normoglycaemic first-degree relatives of type 2 diabetes patients have an approximately 40% lifetime risk of developing diabetes. In addition to being more insulin-resistant than normoglycaemic individuals without family history of type 2 diabetes, they also exhibit impairment in insulin secretion. Eriksson and colleagues divided first degree relatives of type 2 diabetes patients into

those with normal glucose tolerance and those with impaired glucose tolerance according to a standard oral glucose tolerance test (OGTT) (Eriksson et al., 1989). Insulin-stimulated glucose metabolism was assessed by the euglycaemic clamp technique and a comparable defect in glucose uptake was observed in both groups compared to healthy controls with no family history of type 2 diabetes. However, the first-phase insulin response was normal in subjects with NGT whilst subjects with IGT had a decreased first-phase response. O’Rahilly et al. also reported very early defect in insulin secretion, with alterations in oscillations of insulin secretion, in first-degree relatives of patients with type 2 diabetes at time when their first-phase insulin response to intravenous glucose was still normal (O’Rahilly et al., 1988). Taken together, these studies suggest that both insulin resistance and impaired insulin secretion are necessary for the development of impaired glucose tolerance and type 2 diabetes. However, the temporal sequence with which these abnormalities change from NGT to IGT and to diabetes remains less clear. As yet, it is not possible to determine which of these characteristics is aetiologic and which is a secondary abnormality resulting from the disease process since some individuals with IGT will never go on to develop diabetes.

1.1.2 Insulin resistance

Insulin resistance, first described by Himsworth in 1930s, can be defined as the inability of target tissues to respond properly to circulating concentrations of insulin that is otherwise effective in normal subjects. Normally, insulin binds to insulin receptors on target organ cells, resulting in a series of cellular events that lead to intracellular glucose transport and metabolism. Skeletal muscle and liver are the two key insulin-responsive organs responsible for maintaining normal glucose homeostasis. Insulin resistance leads to impaired suppression of hepatic glucose production and to reduced peripheral uptake of glucose, both of which contribute to hyperglycaemia. In addition, insulin resistance in the adipose

tissue increases the flux of non-esterified fatty acid (NEFA) to the liver and skeletal muscle and consequently impairs the action of insulin on glucose metabolism in these tissues.

There is ample evidence indicating that insulin resistance is an important and early feature of type 2 diabetes. Cross sectional studies have shown that virtually all patients with type 2 diabetes are insulin-resistant, and prospective studies have shown that insulin resistance precedes the onset of diabetes by 10 to 20 years (Lillioja et al., 1988, DeFronzo et al., 1992). Insulin-resistance in young lean non-smoking offspring of parents with type 2 diabetes has been shown to be the best predictor for the later development of the disease (Warram et al., 1990, Martin et al., 1992). On the other hand, any perturbations that reduce insulin resistance such as exercise and weight loss prevented the development of type 2 diabetes (Tuomilehto et al., 2001, Knowler et al., 2002).

The cellular mechanism of insulin resistance involves abnormal activation of a complex network of signalling pathways which regulate intermediary metabolism within insulin-sensitive cells. In normal target cells for the hormone, insulin action is initiated by insulin binding to its receptor in the plasma membrane. This is followed by activation of receptor-associated tyrosine kinase, which in turn initiates a cascade of reactions leading to the translocation of the glucose transporters to the cell membrane where they allow the uptake of glucose into the cell (Figure 1.1).

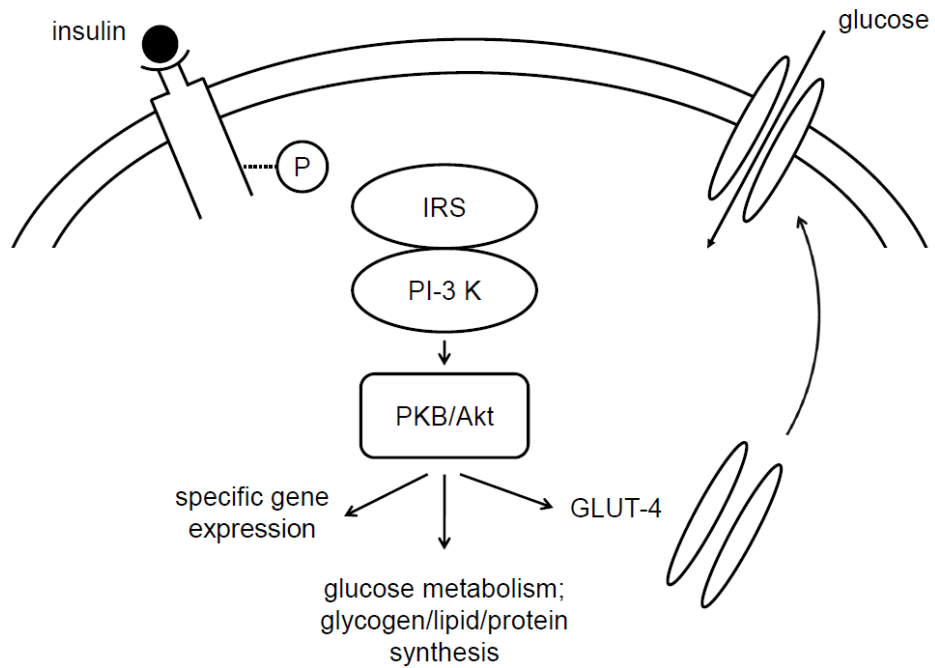


Figure 1.1 Schematic illustration of the insulin signalling pathway. Insulin binds to its receptor, resulting in autophosphorylation. This then catalyses the tyrosine phosphorylation of members of the insulin receptor substrates (IRS) family. Upon phosphorylation, these proteins interact with other signalling molecules which then activate several diverse pathways. One of these pathways is the activation of phosphatidylinositol-3 kinase (PI-3 kinase). This in turn activates protein kinase B (PKB) and Akt, which act on further pathways, resulting in glucose, lipid and protein metabolism, and specific gene expression. In muscle cells, Akt also promotes translocation of glucose transporter-4 (GLUT-4) to plasma membrane to allow glucose uptake into cell. Activation of PKB leads to the inactivation of glycogen synthase-3 kinase, thereby resulting in activation of glycogen synthase and therefore glycogen synthesis.

1.1.3 β -cell dysfunction

Glucose homeostasis is maintained, in large part, by pancreatic β -cells which secrete insulin in proportion to increasing concentrations of glucose. Insulin lowers blood glucose by stimulating glucose uptake into skeletal muscle and adipose tissue and by decreasing glucose production by the liver. β -cell function has been shown to decline before hyperglycaemia develops. The United Kingdom Prospective Diabetes Study, which evaluated β -cell function using the homeostasis model assessment (HOMA), indicated that at time when diabetes was diagnosed, β -cell function had already reduced by 50% (1995). Furthermore, the study confirmed that there was subsequent further decline in β -cell function and this was the same irrespective of treatment with dietary interventions alone, sulfonylurea, metformin or insulin (1995). Analysis of data from the Belfast Diet Study, a 10-year prospective evaluation of newly diagnosed patients with intensive dietary management, documented an inexorable increase in fasting plasma glucose as the years go by despite best possible clinic management (Hadden et al., 1986). Using the HOMA model, they observed that β -cell dysfunction began early on and reached a critical point between 3 and 5 years after diagnosis where the annual rate of β -cell decline accelerated abruptly (Levy et al., 1998).

The defects in β -cell function in type 2 diabetes include absent first-phase and diminished second-phase insulin secretion in response to hyperglycaemia in hyperglycaemic clamp experiments (Ferner et al., 1986, van Haefen et al., 1989). In addition, responses to non-glucose stimuli are delayed and/or blunted as well as decreased in maximal secretory capacity (Van Haefen et al., 1991, Dimitriadis et al., 1985). Insulin secretion normally decreases with age. The decline rate has been shown to be about 0.7-1.0% per year for basal and glucose-stimulated insulin secretion during adult human life in individuals with normal glucose tolerance (Chiu et al., 2000). The rate of decline is doubled in people with IGT (Szoke et al., 2008) and is approximately 6% per year in patients with type 2 diabetes (1995). β -cell mass has been shown to decrease

with increasing duration of diabetes (Weyer et al., 1999b, Leahy, 1990). Alterations in the timing and amount of insulin secreted have also been reported in relatives of patients with type 2 diabetes prior to the development of hyperglycaemia (Vauhkonen et al., 1998, O'Rahilly et al., 1988). There is ongoing debate as to whether insulin resistance is the primary defect that precedes β -cell failure in the evolution of hyperglycaemia in type 2 diabetes, or vice versa. It has been generally thought that insulin resistance preceded β -cell dysfunction and caused β -cell failure via the mechanism of β -cell exhaustion i.e. increased secretory demand due to peripheral insulin resistance resulting in continual β -cell hyperstimulation and eventual failure. In the absence of a defect in β -cell function, individuals can compensate indefinitely for insulin resistance with appropriate hyperinsulinaemia.

Knowledge of the molecular control of insulin secretion is important for understanding β -cell dysfunction of type 2 diabetes. Pancreatic β -cells sense glucose through its metabolism and the resulting increase in adenosine triphosphate (ATP) leads to the closure of the ATP-sensitive potassium K_{ATP} channel, causing plasma membrane depolarisation, influx of calcium ions, and finally insulin secretion (Figure 1.2) (Ashcroft and Gribble, 1999, Matschinsky et al., 1998).

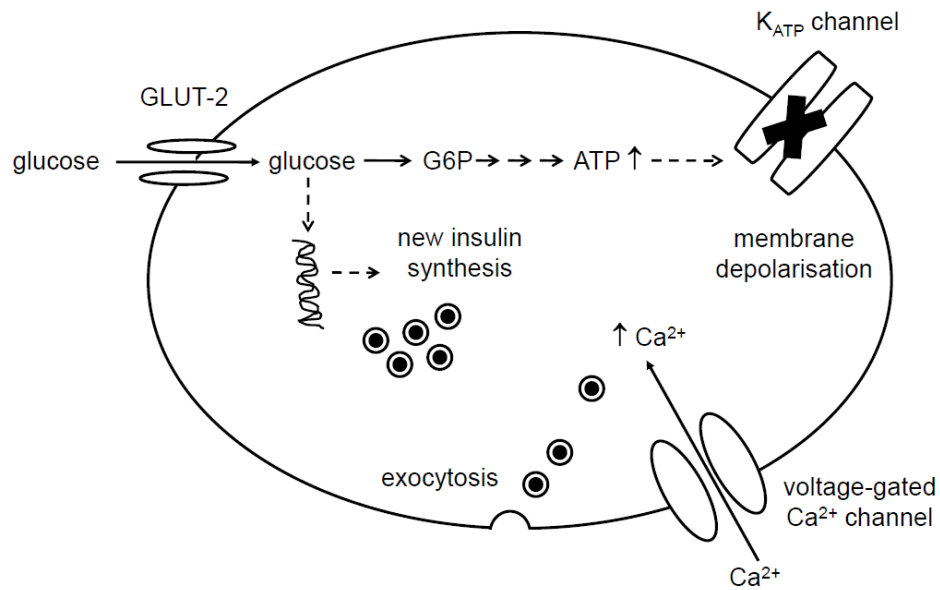


Figure 1.2 Intracellular mechanisms through which glucose stimulates insulin secretion. Glucose is rapidly taken up by the pancreatic β -cell via GLUT-2. Following its entry, glucose is phosphorylated to glucose 6-phosphate (G6P) by the enzyme glucokinase for utilization. This generates ATP which closes the ATP-sensitive potassium K_{ATP} channels in the cell membrane, preventing the efflux of potassium ions that normally maintains resting membrane potential. This causes membrane depolarization and opening of voltage-gated calcium channels in the membrane, leading to influx of calcium ions. The increase in cytosolic calcium initiates exocytosis of insulin-containing granules. Glucose also stimulates synthesis of new insulin via transcription factor PDX 1.

1.2 Glucose Metabolism in Type 2 Diabetes

Normal glucose homeostasis is maintained by 3 important mechanisms which occur in a coordinated manner: 1) secretion of insulin by pancreatic β -cells, 2) suppression of hepatic glucose production, and 3) stimulation of glucose uptake by the liver and muscle.

1.2.1 Skeletal muscle

Skeletal muscle is the largest metabolically active tissue in the human body (approximately 20 kg in a man of average weight). When glucose is taken up by the muscle, it can undergo one of three fates: 1) be oxidized to carbon dioxide and water, 2) converted to lactate which is then released into blood or 3) be stored as glycogen or to a lesser extent as fat (via *de novo* lipogenesis). Using indirect calorimetry in combination with femoral vein catheterization and the euglycaemic-hyperinsulinaemic clamp, it has been shown that most of the glucose taken up by muscle in normal subjects undergoes non-oxidative glucose metabolism (DeFronzo et al., 1981). Combining indirect calorimetry and ^{13}C magnetic resonance (MR) spectroscopy, Shulman and colleagues were able to show that during hyperglycaemic-hyperinsulinaemic conditions, skeletal muscle accounted for the vast majority of glucose uptake in normal subjects and that over 80% of this glucose was then stored as muscle glycogen (Shulman et al., 1990). However, in type 2 diabetes subjects, the rate of muscle glycogen synthesis was 50% lower than the rate in normal subjects during hyperglycaemic-hyperinsulinaemic clamps (Shulman et al., 1990). This study provided undisputed evidence that a defect in muscle glycogen synthesis is the major intracellular metabolic defect responsible for insulin resistance in type 2 diabetes. Further work by the same group has shown that the defect in insulin-stimulated glycogen synthesis in type 2 diabetes is attributable mostly to reduced insulin-stimulated glucose transport into skeletal muscle (Rothman et al., 1992, Cline et al., 1999). Similar observations were also made in lean insulin-

resistant offspring of type 2 diabetes patients (Rothman et al., 1995) and in non-diabetic obese adults (Petersen et al., 1998), suggesting that the defect in insulin-stimulated muscle glycogen synthesis precedes the development of type 2 diabetes.

1.2.2 Liver

The liver plays a pivotal role in maintaining normal glucose homeostasis especially during the fed-fasting transition. In the post-absorptive (i.e., fasting) state, at least 85% of endogenous glucose production is derived from the liver, and the remaining amount is produced by the kidney (Gerich et al., 2001). Both gluconeogenesis and glycogenolysis are responsible for maintaining normal rates of endogenous glucose production. The relative contributions of these two processes to glucose production have been shown by various studies to be dependent on duration of the fast (Rothman et al., 1991, Petersen et al., 1996, Landau et al., 1996). During the early stages of fasting (10-12 hour overnight), gluconeogenesis and glycogenolysis appear to contribute relatively equal proportion (55% : 45%) to whole-body glucose production, but with increasing duration of fast, the contribution from glycogenolysis decreases while that of gluconeogenesis increases (Roden et al., 2001). In the post-prandial state, whilst the peripheral tissues (predominantly skeletal muscle) account for the majority of insulin-stimulated glucose disposal, the liver plays a key role in buffering ingested carbohydrate by suppressing hepatic glucose output and stimulating glucose deposition as liver glycogen. Taylor and colleagues showed that hepatic glucose output is suppressed within 30 minutes after a liquid mixed meal and that approximately 19% of the carbohydrate content of the meal was deposited as glycogen in the liver (Taylor et al., 1996).

It is well established that fasting hyperglycaemia in type 2 diabetes is strongly related to increased rates of endogenous glucose production (Fery, 1994, Jeng et al., 1994). Tracer studies have shown that increased

gluconeogenesis is primarily responsible for the elevated fasting glucose levels in type 2 diabetes (Consoli et al., 1989, Magnusson et al., 1992, Gastaldelli et al., 2000). In the post-prandial state, the suppression of endogenous glucose production is incomplete both because of hepatic insulin resistance, deficient insulin and excessive glucagon secretion in type 2 diabetes, thereby causing post-meal hyperglycaemia (Mitrakou et al., 1990, Campbell et al., 1988, Singhal et al., 2002).

1.3 Lipid Abnormalities in Type 2 Diabetes

1.3.1 Plasma lipids

The importance of abnormal lipid metabolism in both the pathogenesis and aetiology of type 2 diabetes has long been recognised (McGarry, 2002). Increased plasma NEFA concentrations are typically associated with many insulin-resistant states, including obesity and type 2 diabetes (Boden, 1997). In a cross-sectional study of young, lean offspring of type 2 diabetic patients, Perseghin and colleagues found an inverse relationship between fasting NEFA concentration and insulin sensitivity suggesting that fasting NEFA concentration best predicts the risk of developing insulin resistance and type 2 diabetes (Perseghin et al., 1997). Similarly, in the Pima Indian population, individuals with the high fasting NEFA levels had a 2.4 relative risk of developing type 2 diabetes independent of sex, percent body fat, waist/hip ratio, insulin-mediated glucose uptake and fasting triglyceride concentration (Paolisso et al., 1995). Conversely, lowering of NEFA levels by acipimox, a nicotinic acid that inhibits lipolysis, improves insulin resistance in type 2 diabetic subjects (Vaag et al., 1991b, Santomauro et al., 1999, Bajaj et al., 2005).

Sir Philip Randle first recognised the importance of the role of fatty acid in glucose dysregulation back in 1960s. He demonstrated that fatty acids effectively compete with glucose for substrate oxidation in isolated rat heart

muscle and diaphragms (Randle et al., 1963). As a result, he proposed that increased fat oxidation led to the inhibition of glucose oxidation, decreased glycolytic flux and eventually inhibition of glucose transport in muscle which all lead to hyperglycaemia. This homeostatic mechanism became known as the glucose-fatty acid cycle or more commonly, the Randle cycle. He postulated that the increase in fatty acid oxidation causes elevation of the level of intramitochondrial acetyl coenzyme A (acetyl CoA), which subsequently inactivates pyruvate dehydrogenase. This in turn causes citrate concentration to increase, leading to the inhibition of phosphofructokinase and subsequent accumulation of intracellular glucose and glucose 6-phosphate. The accumulation of these latter two substrates results in the decrease in insulin-stimulated glucose uptake.

There is a wealth of evidence to support the causative association between increased plasma lipid availability and impaired insulin-mediated glucose metabolism (Boden et al., 1994, Boden and Chen, 1995). Most of this information has been gained from studies employing artificial lipid infusion and pharmaceutical interventions to manipulate NEFA supply. Roden and colleagues observed a significant reduction in insulin-stimulated rates of muscle glycogen synthesis and whole-body glucose oxidation in healthy individuals given lipid infusion to raise NEFA levels (Roden et al., 1996). These observations were only detected 4.5 hours after elevations in NEFA levels, and were similar to those observed in patients with type 2 diabetes (Rothman et al., 1992) and lean normoglycaemic insulin-resistant offspring of type 2 diabetic individuals (Rothman et al., 1995). Further work has shown that elevation of NEFA in healthy individuals resulted in significantly lower intracellular glucose concentrations during insulin stimulation, implying that the rate controlling step for NEFA-induced insulin resistance in humans is glucose transport (Dresner et al., 1999). In addition, lipid infusion studies have further demonstrated that increasing circulating NEFA enhances gluconeogenesis and impairs glycogenolysis in young insulin-sensitive individuals (Roden et al., 2000, Stingl et al., 2001). Augmented gluconeogenesis in turn is known to be primarily

responsible for the rise in fasting endogenous glucose production typically observed in type 2 diabetes (Hundal et al., 2000, Magnusson et al., 1992). The mechanisms by which NEFA induce gluconeogenesis remain poorly understood. It has been postulated that the activation of pyruvate carboxylase secondary to increases in acetyl CoA may be driving this (Bahl et al., 1997).

1.3.2 Ectopic lipid accumulation

The deposition of lipid in non-adipose tissue, also called ectopic lipid accumulation, is a central feature of type 2 diabetes. When excess lipid accumulates in organs such as skeletal muscle, liver, heart and pancreatic β -cells, the lipids are stored within the cytosol, and not generally released into the circulation, but exert an influence on cellular glucose uptake and utilisation directly. Lipid can accumulate in ectopic sites in three ways: 1) through increased uptake of fatty acids, 2) through increased synthesis within the tissue involved, and/or 3) reduced fatty acid oxidation or disposal (Shulman, 2000). The relative contribution of each of these factors to ectopic lipid accumulation may well vary in different tissue and in different pathological states.

There is greater than two-fold elevation of muscle lipid in people with type 2 diabetes compared to normal non-diabetic subjects (Goodpaster et al., 2000). Using ^1H magnetic resonance (MR) spectroscopy, lipid within muscle fibres, i.e. intramyocellular lipid (IMCL) and lipid located in adipocytes between muscle fibres, i.e. extramyocellular lipid (EMCL) can be distinguished (Boesch et al., 1997, Szczepaniak et al., 1999). Furthermore, IMCL was found to exhibit an even stronger relationship between muscle triglyceride content and insulin resistance. Excess IMCL accumulation has been shown to be inversely correlated with insulin-stimulated whole body glucose disposal in healthy adults (Krssak et al., 1999), in first degrees relatives of people with type 2 diabetes (Perseghin et al., 1999) and in children (Weiss et al., 2003).

Although findings from above studies strongly suggest that lipid accumulation within muscle fibres can be associated with insulin resistance, there is also a paradox that increased IMCL can be found within muscle of highly trained athletes (Goodpaster et al., 2001). The high content of muscle triglyceride provides a reservoir for high rates of lipid oxidation, an important fuel depot for sustained anaerobic exercise suggesting that the muscles of these highly trained athletes have the metabolic capacity for efficient lipid utilisation (Brechtel et al., 2001). On the contrary, these precepts do not appear to apply to skeletal muscle in sedentary and insulin-resistant individuals in whom the expanded intramyocellular energy store is out of proportion to physiological needs. These findings raise the question as to whether the increased IMCL levels observed in insulin-resistant individuals are the cause of insulin resistance or rather the result of impaired oxidative capacity of their skeletal muscle.

Hepatic triglyceride accumulation is frequently observed in obese individuals (Marceau et al., 1999) and is linked to insulin resistance and hypertriglyceridaemia (Marchesini et al., 2001, Seppala-Lindroos et al., 2002, Tiikkainen et al., 2002). The association between hepatocellular lipid content and features of insulin resistance has been examined in different populations. Hepatocellular lipid content was increased and associated with various measures of insulin resistance in type 2 diabetes (Anderwald et al., 2002, Ryysy et al., 2000, Mayerson et al., 2002, Kelley et al., 2003), in women with previous gestational diabetes (Tiikkainen et al., 2003), in obese (Seppala-Lindroos et al., 2002) and in insulin-resistant lipodystrophic patients (Petersen et al., 2002, Sutinen et al., 2002). Liver fat content has also been linked with hepatic insulin resistance as measured by suppression of endogenous glucose production during hyperinsulinaemic clamps in patients with non-alcoholic fatty liver disease (Marchesini et al., 2001). Treatments targeting obesity and insulin resistance reverse the associated accumulation of hepatic triglyceride, and weight loss reduces hepatic fat store and improves insulin resistance (Tiikkainen et al., 2003). Rosiglitazone treatment in patients with type 2 diabetes results in a

40% reduction in plasma free fatty acid levels and hepatic triglyceride content, which is accompanied by a marked improvement in insulin-stimulated glucose metabolism during insulin clamp studies (Mayerson et al., 2002, Neuschwander-Tetri et al., 2003).

Experimental and autopsy data indicate that infiltration of pancreatic islets by adipocytes could possibly contribute to β -cell dysfunction. In obese rodents observed during the development of diabetes, the onset of hyperglycaemia is preceded by a rapid increase in ectopic triglyceride overload in β -cells (Lee et al., 1994), which contributes to β -cell dysfunction through the process of lipoapoptosis (Shimabukuro et al., 1998). Tushuizen and colleagues were first to show that pancreas triglyceride content, measured by ^1H MR spectroscopy, is doubled in patients with type 2 diabetes compared with age- and body mass index (BMI)-matched controls (Tushuizen et al., 2007). Pancreatic triglyceride content was negatively correlated with β -cell glucose sensitivity even after correction for BMI, plasma glucose and triglycerides, but only in their obese normoglycaemic controls, suggesting that pancreatic triglyceride might contribute to β -cell dysfunction in addition to effects induced by glucotoxicity. However, islet cell mass comprises only approximately 2% of the total pancreas mass and measurement of triglyceride levels exclusively within the islets *in vivo* is presently unachievable. Despite this, the validity of the method has been established and shown to be sensitive to detect differences between groups of volunteers from lean to obese with progressive levels of glucose intolerance to established type 2 diabetes (Lingvay et al., 2009).

1.4 Mitochondrial Dysfunction and Type 2 Diabetes

Mitochondria are the primary location of fuel metabolism and ATP production. For this reason, there has been much recent interest in the role of the mitochondria in the development of type 2 diabetes.

1.4.1 Mitochondrion

Mitochondria are double-membrane organelles within all nucleated cells. Their primary function is to produce energy in the form of ATP from acetyl CoA derived from carbohydrates, fats and proteins. ATP is the universal currency of energy in the cell. It is an energy-rich molecule because it contains two phosphoanhydride bonds. Energy is released when these bonds are broken. ATP can be synthesized from adenosine diphosphate (ADP) by two processes: 1) substrate-level phosphorylation and 2) oxidative phosphorylation. Substrate-level phosphorylation is the process by which ATP is formed from the direct phosphorylation of ADP. This process does not require oxygen and is independent of the mitochondria, making it important for generating ATP when oxygen supply is limited. However, almost 90% of cellular ATP is generated by oxidative phosphorylation, a process by which ATP is formed as electrons are transferred from nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) to molecular oxygen, via a series of electron carriers that make up the electron transport chain (ETC) (Figure 1.3).

Carbohydrates are converted to pyruvate through glycolysis, which takes place in the cytosol. Pyruvate is subsequently actively transported across the inner mitochondrial membrane, and into the mitochondrial matrix where it is oxidised and converted to acetyl CoA by pyruvate dehydrogenase. Fatty acids are transported into the mitochondria through the carnitine shuttle and further degraded to acetyl CoA through β -oxidation. Both carbohydrate and fatty acid metabolism result in acetyl CoA, the primary substrate to enter the tricarboxylic acid cycle. This is a cyclical sequence of eight enzymatically driven chemical

reactions which oxidise acetyl CoA to carbon dioxide and in the process produces NADH and FADH₂ for the ETC, which consists of four protein complexes. Complex I, III and IV act as proton pumps. Complex II is, in addition to complex I, an acceptor of reducing equivalents. The complexes are linked by two freely-diffusible membrane proteins: ubiquinone (coenzyme Q) and cytochrome *c*. The ETC results in a stepwise transfer of electrons from NADH and FADH₂ to oxygen molecule to form water molecules. During this process, the energy that is released is harnessed into pumping of protons against their concentration gradient from the matrix of the mitochondria into the intermembrane space. The protons are allowed only to travel back into the mitochondrial matrix via an enzyme, F₁F₀ATP synthase, present in the inner mitochondrial membrane. The movement of the protons back activates the F₁F₀ATP synthase to catalyse the synthesis of ATP by combining ADP and P_i.

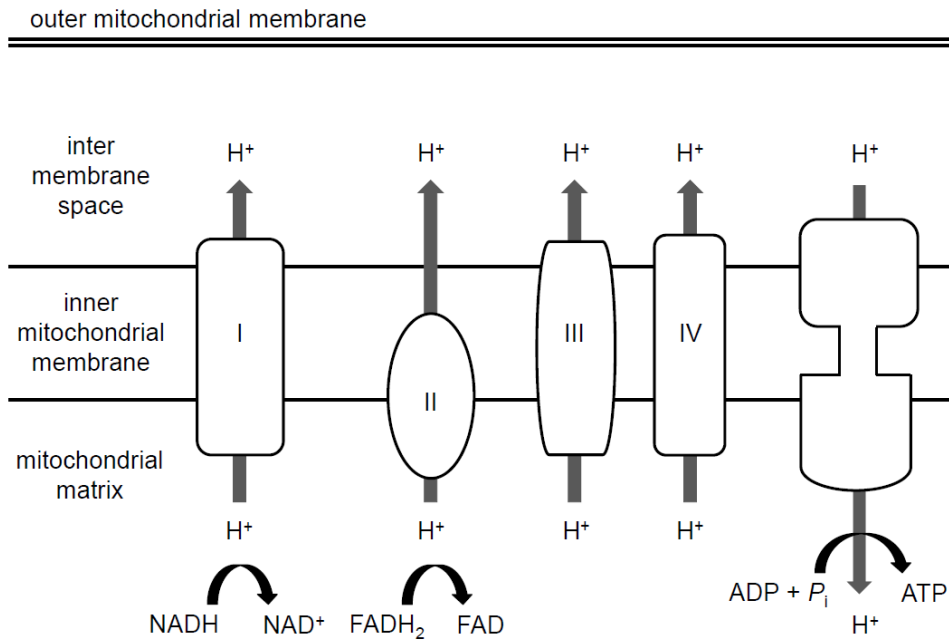


Figure 1.3 Diagrammatic illustration of oxidative phosphorylation, showing the different components of the electron transport chain and the F_1F_0 ATP synthase in the inner membrane of mitochondrion. Upon transferring the electrons of NADH and $FADH_2$ to complex I and II respectively, the electrons travel along the different complexes accompanied by the pumping of protons from the mitochondrial matrix into the intermembrane space. The protons in the intermembrane space subsequently travel back to the matrix passing through the F_1F_0 ATP synthase, which goes along with the formation of ATP.

1.4.2 Measuring mitochondrial function

As oxidative capacity depends on the presence of adequate mitochondrial content and/or individual mitochondrial functionality, mitochondrial aberrations could be due to lower mitochondrial content or true lower intrinsic functionality per mitochondrion itself (Phielix et al., 2011). Several markers for mitochondrial content are commonly used, such as mitochondrial deoxyribonucleic acid (DNA) copy numbers, citrate synthase activity or protein subunits of the ETC. These include biochemical measurement of oxidative enzyme activities such as succinate dehydrogenase and NADH-dehydrogenase subunit (both of these enzymes reflect ETC capacity) and measurement of mitochondrial oxygen consumption using high-resolution respirometry in either intact isolated mitochondria or in permeabilised muscle fibres. A method which permits mitochondrial function to be investigated *in vivo* is that of phosphorus MR spectroscopy. With this technique, phosphorus-containing metabolites in the muscle can be determined non-invasively and followed over time. Two different methods of ^{31}P MR spectroscopy have been used to measure skeletal muscle mitochondrial function. The first technique involves the determination of the rate of unidirectional flux through ATP synthase in the non-exercising state by using the magnetization (saturation) transfer method, in which the signal derived from ATP is temporarily suppressed and subsequent changes in the steady state MR signal of free phosphate are quantified to calculate unidirectional ATP synthesis rate (Lebon et al., 2001). The other method measures phosphocreatine (PCr) kinetics during recovery from exercise (Kemp and Radda, 1994). During exercise, PCr concentrations decrease and ADP concentrations increase, with both recovering rapidly after exercise. As recovery is driven mainly by oxidative phosphorylation, the recovery rate of PCr reflects mitochondrial capacity. In contrast to the magnetization saturation transfer method, the PCr recovery method assesses mitochondrial function under conditions of increased metabolic demand. These *in vivo* measures of mitochondrial oxidative activity and capacity can be influenced by several

factors such as blood perfusion, circulating hormones and metabolites, and do not allow the discrimination between changes in mitochondrial content or lower function per mitochondrion.

1.4.3 Mitochondria and insulin resistance in skeletal muscle

Mitochondrial abnormalities have been reported in insulin resistance and type 2 diabetes (Petersen et al., 2003, Petersen et al., 2004). More than a decade ago, Simonneau and colleagues first reported reduction in activity of oxidative enzymes in skeletal muscles from individuals with obesity and type 2 diabetes, raising the possibility of true mitochondrial aberrations in the pathogenesis of type 2 diabetes (Simoneau and Kelley, 1997). They showed that the ratio between maximal activities of glycolytic and oxidative enzymes in skeletal muscle of type 2 diabetes was different from that in skeletal muscle of obese or lean glucose-tolerant subjects and suggested that a mismatch between mitochondrial oxidative capacity and the capacity for glycolysis may be an important factor in the development of insulin resistance. Further work by Kelley and colleagues found that the overall capacity of the ETC (estimated using the activity of rotenone-sensitive NADH:O₂ oxidoreductase) was reduced by 40% in skeletal muscle of type 2 diabetes compared to that in healthy volunteers (Kelley et al., 2002). In the same study, electron microscopic imaging revealed that mitochondria in skeletal muscle of type 2 diabetes were ~55% smaller than those in the muscle of lean healthy controls, with the mitochondrial surface area positively correlating with insulin sensitivity. Furthermore, there was elevated number of damaged mitochondria in skeletal muscle of type 2 diabetes subjects.

The link between mitochondrial dysfunction and type 2 diabetes has been further extended by MR spectroscopy studies. Application of the saturation transfer method has shown that *in vivo* mitochondrial function in skeletal muscle of elderly people and insulin-resistant offspring of type 2 diabetes

patients was decreased under basal and insulin-stimulated conditions (Petersen et al., 2003, Petersen et al., 2004, Petersen et al., 2005b). Petersen and colleagues were first to report a reduction in mitochondrial oxidative phosphorylation in insulin-resistant offspring compared to insulin-sensitive controls, and this was associated with an increased IMCL content (Petersen et al., 2004). The authors suggested that inherited defects in mitochondrial phosphorylation in these young people, who had strong tendency to develop type 2 diabetes at later life, were responsible for causing lipid accumulation and insulin resistance. Using a similar method, Szendroedi and co-workers observed that type 2 diabetic subjects had lower fasting ATP turnover rate when compared to younger controls, but not when compared to sex-, age-, and weight-matched controls (Szendroedi et al., 2007). The observation of no abnormality in basal ATP synthesis in type 2 diabetes implies that abnormal mitochondrial function is unlikely a primary causative factor in type 2 diabetes. However, insulin stimulation was observed to increase ATP turnover rate in both groups of control subjects but failed to do so in the diabetic subjects. Using the PCr recovery method as an alternative method to investigate *in vivo* mitochondrial function, Schrauwen-Hinderling and colleagues found that mitochondrial function was compromised by ~45% in type 2 diabetic patients compared to matched healthy controls (Schrauwen-Hinderling et al., 2007). However, unlike Petersen et al., they found similar IMCL content between the groups suggesting that fatty acid metabolites, rather than lipid deposition are related to mitochondrial function.

Current hypotheses link inherited or acquired mitochondrial dysfunction to a reduced ability to oxidise lipid, thereby promoting the accumulation of fatty acids and their metabolites within skeletal muscle as a mechanism of inducing insulin resistance (Lowell and Shulman, 2005). Increased levels of intramyocellular metabolites such as fatty acyl CoA, ceramide and diacylglycerol have been shown to impede insulin signalling, providing an indirect mechanism from impaired mitochondrial function to the development of type 2 diabetes

(Morino et al., 2005). Brehm and colleagues examined the interference of increased lipid availability on skeletal muscle ATP synthase flux (Brehm et al., 2006). By elevating circulating lipids via lipid infusion, they found that glucose transport/phosphorylation was impaired in parallel with a reduction in the flux increase through ATP synthase upon insulin stimulation. This finding led the authors to conclude that physiologically elevated NEFA levels cause a reduction in insulin-stimulated muscle ATP production in parallel with the induction of insulin resistance.

To further examine if mitochondrial impairment was due to reduction in mitochondrial content and/or intrinsic defect in functionality, *ex vivo* studies are necessary. Few studies have been carried out whereby measurement of mitochondrial function was normalised for mitochondrial content, using either citrate synthase or mitochondrial copy DNA number. Studies by Mogensen et al. (Mogensen et al., 2007) and Phielix et al. (Phielix et al., 2008) revealed functional impairment in mitochondria of type 2 diabetic patients after correction for mitochondrial content. In contrast, Boushel and colleagues found no impairment in mitochondrial function from biopsy study of skeletal muscle of type 2 diabetes subjects (Boushel et al., 2007). Any apparent impairment of oxidative phosphorylation and electron transport capacity disappeared when corrected for mitochondrial density.

The finding of mitochondrial dysfunction in the pre-diabetic and diabetic states has led to an intensive search for target genes and proteins regulating mitochondrial biogenesis and function. DNA microarray studies have demonstrated decreased expression of peroxisomal proliferator activator receptor γ (PPAR γ) coactivator-1 α (PGC-1 α) in individuals with type 2 diabetes (Mootha et al., 2003) as well as in offspring of type 2 diabetes parents (Patti et al., 2003). Together, *in vivo* and *ex vivo* data appear to suggest an important role of mitochondrial function related to type 2 diabetes. However, whether the abnormality in mitochondria relates to mitochondrial number and/or functionality and whether such abnormality is the cause or consequence of type

2 diabetes remains to be addressed. Furthermore, there is now emerging evidence to suggest that mitochondrial dysfunction is not required for the development of type 2 diabetes.

1.5 Pathogenesis of Type 2 Diabetes

1.5.1 Mechanism of insulin resistance

The molecular mechanism of insulin resistance remains to be established. Many hypotheses have been proposed to explain insulin resistance including inflammation in adipocytes associated with increased adipocytokines (Hotamisligil, 2006, Shoelson et al., 2006), increased reactive oxygen species production (Evans et al., 2002) and the “lipocentric” hypothesis (Roden et al., 1996, Shulman, 2000). Lipid infusion studies combined with repetitive muscle biopsies showed that the fatty acid-induced insulin resistance was associated with alterations in insulin signalling (Dresner et al., 1999). Elevated NEFA levels inhibited the increase in IRS-1-associated PI-3 kinase. Subsequent studies suggested that this might be a consequence of reduction in insulin-stimulated IRS-1 tyrosine phosphorylation, which was associated with activation of protein kinase C (Griffin et al., 1999, Morino et al., 2005). Although increased skeletal muscle and liver triglycerides correlate closely with insulin resistance, triglycerides are generally perceived to be metabolically inert and do not directly cause insulin resistance. Triglycerides are in a constant state of turnover, leading to the production of several lipid metabolites which include long-chain fatty acyl CoA, diacylglycerol and ceramides.

It has been proposed that increased intracellular lipid in muscle and liver cells leads to an increase in these intracellular fatty acid metabolites which in turn lead to the activation of a serine/threonine phosphorylation cascade and subsequent decrease in insulin-stimulated IRS-1 tyrosine phosphorylation, PI-3 kinase activity and downstream insulin signalling (Figure 1.3) (Shulman, 2000).

In the muscle, this results in decreased muscle glycogen synthesis due to reduced GLUT-4 translocation to the plasma membrane and reduced glucose uptake into muscle. In the liver, this results in decreased hepatic glycogen synthesis due to decreased activation of glycogen synthase, and increased hepatic gluconeogenesis.

Diacylglycerol has been proposed to be the main candidate in mediating lipid-induced resistance compared to other fatty acid metabolites (Erion and Shulman, 2010). Yu and colleagues have been able to dissociate lipid-induced insulin resistance from any increases in intramuscular triglyceride or ceramide content, suggesting that these lipid metabolites are not the trigger in mediating fat-induced insulin resistance in muscle (Yu et al., 2002). Furthermore, Itani and co-workers observed that diacylglycerol accumulation in human muscle during lipid/heparin infusions was associated with increased protein kinase C activities (Itani et al., 2002). The use of transgenic and knockout mice, as well as antisense oligonucleotides to knock down specific pathways has allowed the validation of the diacylglycerol-mediated insulin hypothesis of insulin resistance through manipulation of key proteins in the pathway (Erion and Shulman, 2010).

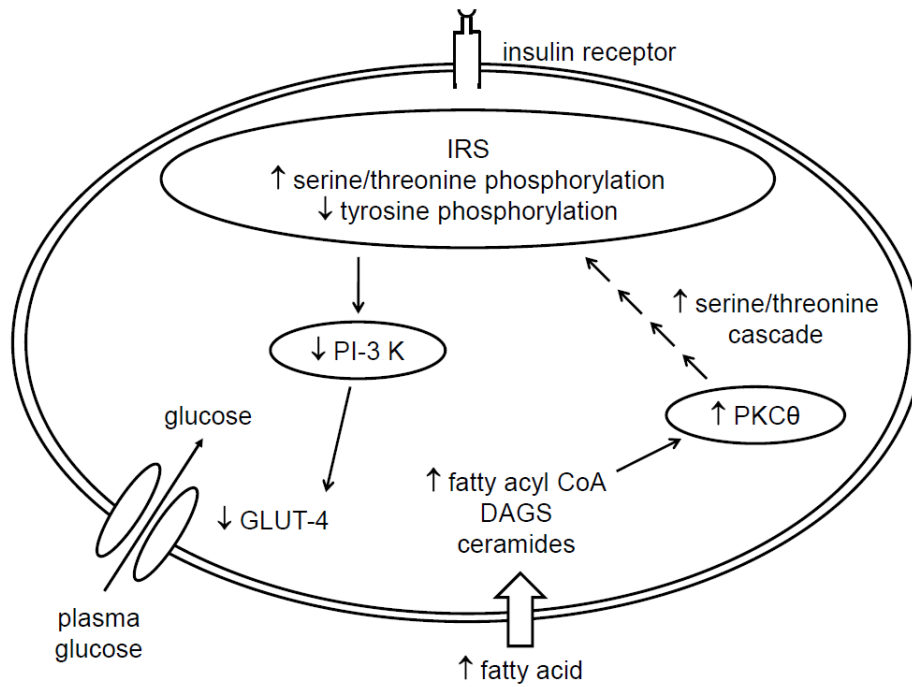


Figure 1.4 Proposed mechanism for fatty acid-induced insulin resistance in human skeletal muscle – adapted from Shulman et al. (Shulman, 2000). An increase in delivery of fatty acids and/or a decrease in intracellular fatty acid metabolism lead to an increase in intracellular fatty acid metabolites such as fatty acyl CoA, diacylglycerol and ceramides. These metabolites activate a serine/threonine kinase cascade, presumably mediated by protein kinase C (PKC)- θ , leading to phosphorylation of serine/threonine sites and inhibiting phosphorylation of tyrosine sites on IRS. Consequently, the decreased ability of IRS to activate PI-3 kinase leads to impaired insulin signalling and decreased GLUT-4 translocation and glucose transport.

1.5.2 Mechanism of β -cell dysfunction

Several inherited and acquired factors have been identified to affect β -cell function. Given that type 2 diabetes is very heterogeneous, genetic studies have so far yielded very diverse results. To date, only a few polymorphisms have been identified as risk factors with confidence. One of them is the Pro₁₂Ala polymorphism in the PPAR γ which is expressed in insulin target tissues and β -cells. This polymorphism conveys susceptibility to adverse effects of NEFA on insulin release (Stumvoll and Haring, 2002). The second involves the gene encoding calpain-10, a cysteine protease that modulates insulin release as well as insulin effects on muscle and adipose tissue (Horikawa et al., 2000). The third is the E23K variant of the Kir6.2 gene (potassium inwardly-rectifying channel J11 gene) which has been shown in a large association study to increase the risk of type 2 diabetes by 15%, presumably through its effect on β -cells' potassium channel and in turn on insulin secretion (Gloyn et al., 2003). Use of knockout mouse models has identified several elements of the insulin signalling cascade that might be potential sites where genetic polymorphisms may affect β -cell function but to date none of these has been found to occur in individuals with type 2 diabetes (Saltiel and Kahn, 2001).

Hyperglycaemia, both acute and chronic, has been shown to adversely affect β -cell function. Correction of hyperglycaemia with use of secretagogues, insulin sensitizers and even insulin improves β -cell function in individuals with type 2 diabetes (Garvey et al., 1985, Yki-Jarvinen, 2004). Hyperglycaemia has been proposed to lead to large amounts of reactive oxygen species in β -cells, with subsequent damage to cellular components (Robertson et al., 2003). However, glucotoxicity is unlikely to be the primary causative factor because β -cell dysfunction does not completely reverse despite optimization of glycaemic control. Lipotoxicity associated with elevated levels of NEFA is commonly observed in the obese, the insulin-resistant and individuals with type 2 diabetes and has been linked to decreased insulin synthesis and increased metabolic stress in β -cells (Standl, 2007, Poitout and Robertson, 2002). A number of *in*

vitro and animal studies have demonstrated that chronic exposure to elevated NEFA levels impairs β -cell function (Poitout and Robertson, 2002). Evidence suggests that elevated NEFAs inhibit glucose-stimulated insulin secretion, impair insulin gene expression and promote β -cell apoptosis (Poitout and Robertson, 2002). One proposed mechanism involves the uncoupling protein-2 (UCP-2), a mitochondrial carrier protein that uncouples substrate oxidation from ATP synthesis (Fleury and Sanchis, 1999). NEFA is said to increase UCP-2 activity in β -cells, which leads to impaired ATP generation from glucose metabolism and consequently decreased glucose-stimulated insulin secretion (Chan et al., 1999, Medvedev et al., 2002). Another mechanism might involve apoptosis of β -cells via fatty acid or triglyceride-induced ceramide synthesis or generation of nitric oxide (Robertson et al., 2004).

Amyloid deposition in β -cells is a characteristic histopathology feature of type 2 diabetes, and has been observed in up to 90% of individuals with type 2 diabetes at autopsy (Clark et al., 1990, Kahn et al., 1999). Human amyloid, composed of insoluble fibrils formed from a protein called amylin or islet amyloid polypeptide, has been reported to be cytotoxic to β -cells, possibly related to radical production (Butler et al., 2003b). However, it is unlikely to have a major role in the pathogenesis of type 2 diabetes as it is not present in all patients with type 2 diabetes and is actually found in up to 20% of islets in elderly individuals with normal glucose tolerance (Clark et al., 1988).

1.5.3 Reversibility of type 2 diabetes

Gastric bypass surgery has been known for 25 years to bring about major weight loss and improve glucose tolerance in morbidly obese individuals. In the original series by Pories et al., 86 of 88 obese people with diabetes reverted to normal glucose tolerance within 4 months, and this remained normal over 6 years of follow-up (Pories et al., 1987). A large prospective, case control study, the Swedish Obese Subjects Study, suggests that weight loss arising from

bariatric surgery reduces the incidence of type 2 diabetes by up to 97% (Sjostrom et al., 1999, Sjostrom et al., 2004). In a recent meta-analysis by Buchwald and colleagues, resolution of diabetes was achieved in 99% after bilio-pancreatic diversion, 84% for gastric bypass and 48% for gastric banding (Buchwald et al., 2004). Bilio-pancreatic diversion consists of distal gastric resection with closure of duodenal stump, transaction of the small intestine, proximal anastomosis to stomach and anastomosis of the remaining ileum to the distal ileum. At the other end of the scale, gastric banding involves placement of an adjustable cuff around the gastro-oesophageal junction, and may be done via a laparoscope. Simple gastric bypass involves ileo-gastric anastomosis so that food bypasses the duodenum and upper jejunum. Initially, the resolution of type 2 diabetes has been attributed to the weight loss imparted by these operations; however, there are observations that suggest other factors may play a role. Resolution of diabetes often occurred days following bariatric surgery, before any substantial weight loss (Guidone et al., 2006, Pories et al., 1995). It has therefore been suggested that diabetes resolution or improvement after bariatric surgery may be related to some of the changes in the gut-related hormones (Bose et al., 2009). One proposed hypothesis regarding the mechanism of increased incretins following bariatric surgery is the rapid exposure of the distal gut to nutrients – so called the ‘hindgut’ theory. Meanwhile, the ‘foregut’ theory proposes that glucose homeostasis is improved as a consequence of the exclusion of the foregut from nutrient exposure. However, both hypotheses overlook a crucial change that immediately follows all categories of bariatric surgery – an acute, profound decrease in calorie intake.

1.6 Magnetic Resonance Techniques in Metabolism

Magnetic resonance is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. This phenomenon forms the basis of magnetic resonance spectroscopy and magnetic resonance imaging, both of which are non-invasive techniques suitable for metabolic research into both normal physiology and disease in humans. Specifically, MR spectroscopy allows for the continuous monitoring of tissue concentrations of metabolites and metabolic fluxes while MR imaging is well-suited for fat quantification due to its ability to provide sensitive mechanisms for differentiating fat from lean tissues based on relaxation time and chemical-shift properties.

1.6.1 Basic principles

Certain atomic nuclei possess magnetic properties known as magnetic moment or spin. In the earth's weak magnetic field (50 μ Tesla), such nuclei are usually randomly aligned. However in the presence of a strong magnetic field, the nuclei will precess around their own axes with a characteristic frequency so as to align with or against the magnetic field. The frequency of the precession is directly proportional to the strength of the magnetic field and is known as the resonant frequency. By applying a transient radio signal at the resonant frequency of the nuclei, energy is absorbed by the nuclei causing them to move out of alignment, usually at 90 degrees to the direction of the magnetic flux. Once the signal has ceased, the nuclei will swing back into their original alignment and emit the energy they have absorbed. This energy is released in the form of an oscillating magnetic field which induces an electric current in a receiver coil. Under standard experimental conditions, resonant signals from nuclei incorporated into different chemical species are superimposed, generating a picture of oscillating amplitudes in an intensity vs. time display (known as free induction decay, FID). Fourier transformation, a mathematical

transformation is used to convert the FID display into a display of signal intensities vs. frequencies, thereby enabling one to distinguish compounds with characteristic peak frequencies (Figure 1.4).

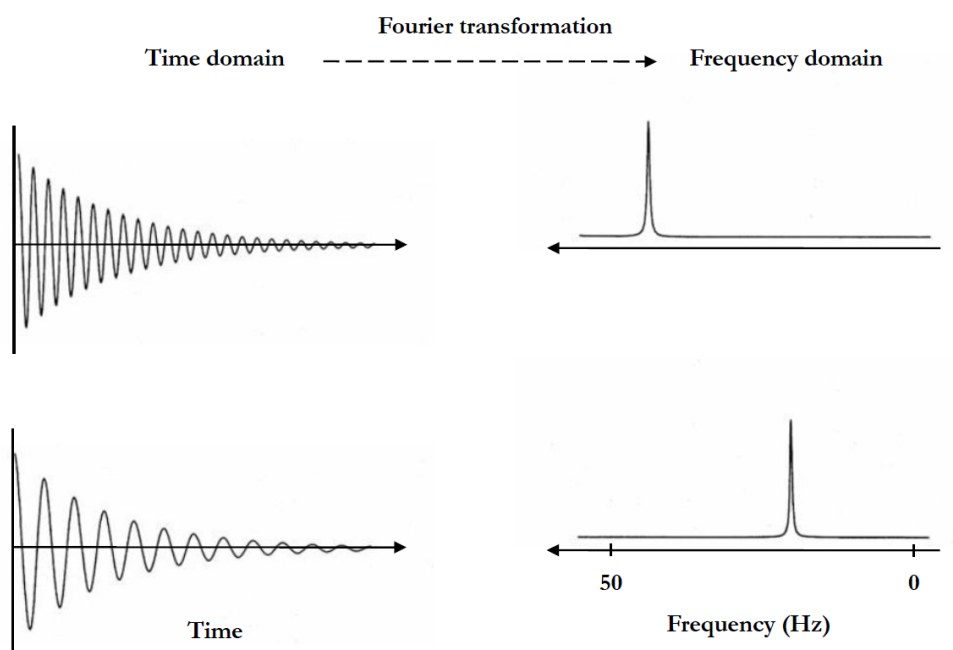


Figure 1.5 Diagrammatic representation of the signal emitted by the excited nuclei in its “raw” form, known as free induction decay (FID) which displays the signal in the time domain (left). Before an FID can be usefully analysed, it must first be transformed to the frequency domain (right) by a mathematical operation known as Fourier transformation.

The intensity of each signal in the spectrum is proportional to the amount of the originating atomic nuclei from the tissue under study, while the radiofrequency range where the radio signal could appear depends on the atomic nuclei and the strength of the magnetic field. The exact position of the signal in its possible radiofrequency range depends mainly on the electronic environment of the molecule in which the nucleus is located and on the interaction with neighbouring nuclei, although in some instances other microenvironment conditions (i.e. pH, solvent, binding to ions, temperature) could affect this position as well. Each peak of the spectrum is characterised by its resonance frequency, height, width and area. The area under the peak may be calculated to give a relative measure of the concentration of the nuclei being studied. This result can be converted into molar terms by comparison with data obtained from a phantom containing a known amount of that compound. It can also be compared with the area under the peak of an intrinsic compound with a known concentration e.g. water peak in muscle. The ability to distinguish between different molecules containing the same nucleus relies upon what is known as “chemical shift”. This is a displacement of the spectral peak by a unique amount which is measured in parts per million (ppm). The electrons surrounding each nucleus have a shielding effect and the nuclei of different molecules thereby experience an altered static magnetic field and in turn resonate at an altered frequency, i.e. chemical shift, which is typical for the respective molecule. In order to examine a defined small volume of tissue, a surface coil which receives the MR signal is placed over the region of interest to ensure homogenous tissue filling in that region.

1.6.2 Magnetic resonance spectroscopy

This is a technique for acquiring metabolic information from a volume of tissue. It relies on the inherent differences in resonance frequency or chemical shift that exist due to different chemical environments experienced by the

nuclei. It allows continuous, non-invasive monitoring of tissue concentrations of metabolites and metabolic fluxes *in vivo*. The most common nuclei exploited in MR spectroscopy studies applied to metabolic organs are ^1H , ^{31}P and ^{13}C . The suitability of a nucleus for MR spectroscopy depends on its relative magnetic sensitivity, the tissue concentration range of the studied metabolites, and the chemical shift range. The ^1H nucleus is the most sensitive of the resonant nuclei, its natural abundance is almost 100% of the available pool of hydrogen containing molecules, and almost all biological molecules contain hydrogen. ^1H MR spectroscopy application in metabolic studies lies mainly in its evaluation of intra-organ lipid metabolism. The very large ^1H signals of water and lipids in living samples, so useful for magnetic resonance imaging, obscure direct visualization of other signals from metabolites of much lower concentration in MR spectroscopy. ^{31}P has 100% natural abundance, but its spectroscopic sensitivity is much lower than ^1H . The molecules that are most commonly visualised by ^{31}P MR spectroscopy are linked to skeletal muscle bioenergetics and the metabolism of carbohydrates. In contrast to ^1H and ^{31}P , ^{13}C has a natural abundance of 1.1% and therefore a relatively low sensitivity. Nevertheless, this nucleus can be used to study glycogen and fatty acid metabolism. When necessary, ^{13}C -enriched isotopes can be used to enhance the ^{13}C signal strength by a factor of up to 100.

1.6.2.1 ^{31}P MR spectroscopy

^{31}P MR spectroscopy is well suited for study of skeletal muscle bioenergetics. In muscle, the phosphorus atoms detected by MR spectroscopy reside predominantly in ATP, PCr and P_i . Other ^{31}P -containing compounds present in much lower concentrations are ADP and AMP; their concentrations are often below 1 mmol/l and therefore are too small to be visible on the spectra. These phosphorus-containing molecules (phosphagens) are of interest because of their central role in the energy flux of the cells. As previously mentioned, the

two different ^{31}P MR spectroscopy techniques applied are the ATP saturation transfer experiment and the measurement of post-exercise PCr recovery. The ATP saturation transfer experiment yields the rate of unidirectional flux from P_i towards ATP through ATP synthase at rest. Using a specific ^{31}P pulse sequence, the ATP signal is temporarily suppressed (saturated) and the subsequent changes of the steady state signal derived from P_i are monitored to calculate ATP synthesis rate. This process is illustrated by the spectra in Figure 1.5. The saturation transfer experiment can also be applied to study the creatine kinase (CK) reaction, in which $P_i + \text{creatine} \leftrightarrow \text{PCr}$ buffers the $\text{ATP} \leftrightarrow \text{ADP} + P_i$ reaction. Due to the relatively fast exchange of the reactants of the CK reaction, saturation of the γ -ATP peak results in large differences in the magnetization of PCr.

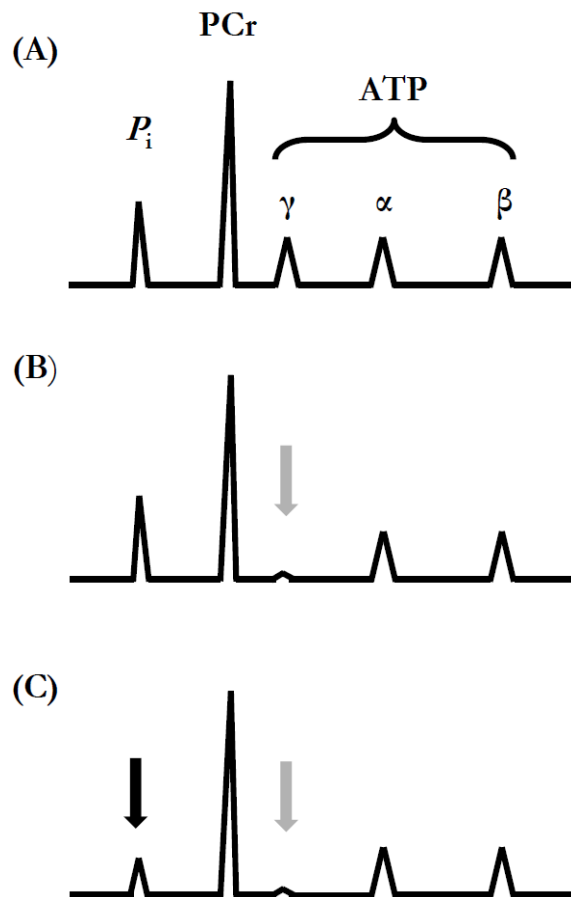


Figure 1.6 Diagrammatic representation of a saturation transfer experiment between P_i and ATP from calf muscle. (A) Control spectrum with characteristic signals from P_i , PCr and the three phosphate molecules of ATP; (B) spectrum obtained with the γ -phosphate spin of ATP selectively irradiated to make its signal invisible to MR (grey arrow); (C) the difference spectrum showing a clear effect that part of the P_i signal has disappeared as well (black arrow), which is because this pool is in rapid exchange with the γ -phosphate of ATP in the ATP synthase reaction.

1.6.2.2 ^{13}C MR spectroscopy

The measurement of human skeletal muscle glycogen concentration using ^{13}C MR spectroscopy has been validated against the more widely used method of muscle biopsy and direct biochemical assay for glycogen concentration (Taylor et al., 1992). There was a strong correlation between the two methods of measurement over a broad concentration range ($r = 0.95$; $p < 0.0001$). Moreover, the ^{13}C MR spectroscopy method was found to be more precise than the biopsy measurements; the coefficient of variation being $4.3 \pm 2.1\%$ for MR spectroscopy compared with $9.3 \pm 5.9\%$ for the biopsy method despite the optimisation of the latter technique. Therefore, *in vivo* ^{13}C MR spectroscopy is not only precise but also accurate as judged against biochemical assessment from biopsy samples.

1.6.2.3 ^1H MR spectroscopy

The use of ^1H MR spectroscopy to detect and quantify the different cellular compartments for stored lipids has been well validated. In animal models, hepatic triglyceride content measured by this method has been shown to correlate very closely to that measured chemically by liver biopsy ($r = 0.934$; $p < 0.0001$) (Szczepaniak et al., 1999). When applied to human calf muscle, based on the observation of two distinct lipid resonances, it is possible to differentiate between fat stored within cytoplasm of muscle fibres i.e. IMCL and that stored in adipocytes between muscle fibres i.e. EMCL (Schick et al., 1993). The coefficient of variation of the technique in measuring the IMCL content was 11.8% in non-obese subjects and 7.9% in obese subjects and of EMCL content was 22.6% and 52.5%, respectively. Hence, non-invasive *in vivo* ^1H MR spectroscopy measurement of intramyocellular triglyceride is feasible and comparable in accuracy to other conventional methods such as electron microscopic morphometry and biochemical analysis from biopsy samples (Schick et al., 1993, Boesch et al., 1997).

1.6.3 Three-point Dixon magnetic resonance imaging

The degree of fatty infiltration within an organ can be accurately estimated by either MR spectroscopy or chemical shift imaging. The difference between the precession frequencies of fat and water protons enables the use of chemical shift techniques to accurately detect and quantify fatty infiltration. In the original Dixon method, water and fat are separated using two image acquisitions (a two-point technique) (Dixon, 1984). Simple summation and subtraction of the two image sets yielded water-only and fat-only images that allowed direct image-based fat quantification. The main limitation of this method is that of magnetic field inhomogeneity (for example, homogeneity introduced from the presence of the patient within the MR scanner or at interfaces between air and soft tissue) which yields a phase error that leads to an incorrect solution for the water and fat images. The phase error can be corrected by using the information from a third data acquisition, thus termed the three-point Dixon method (Glover and Schneider, 1991). The chemical shift difference between water and fat resonances at 3 Tesla is 435 Hz, and therefore the proton spins of these two moieties are out of phase with each other at an echo time of $1000/(2 \times 435) = 1.15$ ms and all odd multiples of this echo time (3.45 ms, 5.75 ms): in short, the signals from fat and water moieties subtract. At even multiples of this echo time (2.3 ms, 4.6 ms, 6.9 ms) the water and fat proton signals add constructively. By acquiring three echoes (two out-of-phase and one in-phase) at adjacent echo times, separate intensities of fat (F) and water (W) within the MR signal can be extracted while also making allowance for the magnetic field (B_0) inhomogeneity. Additionally, the introduction of fast gradient-echo sequences with shorter echo times (TEs) and repetition times (TRs) with the three-point Dixon method has helped reduced acquisition time and also made breath-hold acquisitions possible. A comprehensive study of liver fat content in 110 subjects (including many with abnormally high liver fat content), compared various forms of multi-point Dixon method with proton MR spectroscopy in the liver and demonstrated

that the three-point Dixon technique with T_2^* correction was highly correlated with spectroscopy and had a slope very close to the line of identity (0.94) (Yokoo et al., 2009). However, in the pancreas, the correlation between methods of fat-water MR imaging and spectroscopic measurement has been observed to be weaker (Hu et al., 2009). The coefficient of variation in the repeatability of pancreatic proton MR spectroscopy itself varied widely from 4.5 to 15.2% in comparison to liver proton MR spectroscopy (4.7%) (Tushuizen et al., 2007, Lingvay et al., 2009). These discrepancies are most likely due to the varying degree of MR signal contamination from surrounding visceral fat presumably during respiratory motion. The three-point Dixon method has several advantages over MR spectroscopy in that it can be easily performed within a breath-hold, thus not requiring respiratory gating and in subsequent post-processing where manual segmentation of the organ allowed careful avoidance of blood vessels and non-tissue structures.

1.6.4 Advantages and disadvantages

MR methods are attractive for studies of human physiology and/or pathophysiology because they are non-invasive, non-radioactive and infinitely repeatable, thus allowing for longitudinal monitoring. Major metabolic organs such as liver, skeletal muscle, pancreas, adipose tissue and brain are suitable for either MR spectroscopy or imaging studies or even both. On the other hand, these techniques are limited by practical difficulties. MR methods in general are very susceptible to movement of the observed region. Subjects must lie still within the confined space of the magnet for long periods of time, for example, 20 minutes for ^{13}C spectrum acquisition. Movement during the acquisition of MR images and spectra can lead to smearing effects in phase direction of images and spectral line distortion, resulting in inaccurate quantification. In order to record enough signal for reliable calculation, signal to noise ratio must be optimal. For example, when ^{13}C MR spectroscopy is performed on natural

abundance ^{13}C metabolites at physiological concentrations, sensitivity is poor due to low signal to noise ratio. In order to derive quantitative information from MR spectroscopy, it is necessary to convert the obtained spectra into meaningful metabolic measurements. The quantitative evaluation of the spectra is performed using special software programmes which allow the incorporation of previous knowledge for improved spectral fitting. Although the AMARES method within java-based magnetic resonance user interface (jMRUI) is well validated for use in spectral analyses, two commonly encountered problems are the need to adjust for baseline correction to broad peaks and for overlapping spectral peaks. In addition subjects with metal implants must be excluded for safety reasons.

1.7 Aims of the Studies

The aims of this thesis are to address the concept of skeletal muscle mitochondrial dysfunction in relation to insulin resistance and type 2 diabetes, and to investigate the reversibility of the twin defects of β -cell dysfunction and insulin resistance in type 2 diabetes so as to unravel the basic pathogenetic mechanism of the disease. Non-invasive MR techniques play a crucial role in being the methodologies that were predominantly applied in the presented studies.

The studies described in Chapter 3 and 4 relate to the study of *in vivo* skeletal muscle mitochondrial function in type 2 diabetes and healthy subjects. I postulated that the observations of impaired capacity of the mitochondria to produce ATP in muscle of type 2 diabetes may be a consequence of reduced insulin action. This in turn is reflected by reduced glucose transport and muscle glycogen synthesis. Inhibition of lipolysis is known to improve insulin sensitivity and evidence suggests that it does so by improving glucose metabolism, specifically by increasing muscle glycogen synthesis. Consequently,

this would be expected to also improve muscle ATP turnover rate. The study, described in Chapter 3, was therefore designed to test this hypothesis using acipimox, a nicotinic acid derivative that reduces lipid availability. In addition, by enhancing muscle glycogen synthesis through mass action of raising blood glucose level, I was able to examine the relationship between muscle glycogen synthesis rate and ATP synthesis rate.

Chapter 4 deals with the sub-study that arose as a consequence of the initial observations of the study described in Chapter 3. Given the findings in the literature presented earlier, I expected to observe an acute effect of insulin on muscle ATP turnover rate in the healthy control subjects. When I did not observe this in the first three healthy control subjects (one male, two females; age 46 ± 3 years; and BMI 27 ± 1) in Chapter 3, I set up this sub-study to examine the relative time course of insulin action in stimulating muscle ATP turnover rates and glucose uptake into skeletal muscle in a group of young, lean normoglycaemic subjects. I purposefully selected this cohort so as to maximise any measurable effect. Measurements of muscle ATP turnover rate were performed continuously over 150 min period during a standard euglycaemic-hyperinsulinaemic clamp.

The third study, described in Chapter 5, was designed to test the hypothesis that acute negative energy balance alone can reverse type 2 diabetes by normalising β -cell function and insulin sensitivity. Specifically, this work aimed to test whether the reduction of intracellular fatty acid concentrations in the pancreas and liver would result in the reversal of the twin defects of β -cell dysfunction and insulin resistance respectively.

Chapter 2

METHODS

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2.1 Research Subjects

2.1.1 Recruitment

Subjects with an established diagnosis of type 2 diabetes, with or without metformin and/or sulphonylurea therapy were recruited. Subjects on other oral hypoglycaemic agents or insulin treatment were excluded. Subjects were also excluded if they had evidence of renal dysfunction (defined by serum creatinine > 150 $\mu\text{mol/l}$) or liver dysfunction (defined by serum alanine transaminase > 2.5 fold above upper limit of reference range) or if they were on thiazide diuretics or steroids as these would have an effect on metabolism (Lithell, 1991, Pagano et al., 1983).

The subjects were recruited from the Newcastle Diabetes Centre and General Practices within the North and South of Tyne Primary Care Trusts. Suitable individuals were identified from Newcastle Diabetes Centre databases, or by their General Practitioner. Once identified, a letter was sent giving a brief outline of the study and asking whether or not they would be interested in participating. Those who then responded positively were sent the information sheet detailing the purpose, nature and potential risks of the study.

Healthy volunteers were identified and subsequently recruited by advertising for research participants in the Newcastle Hospitals, Newcastle University, and Newcastle City Council. The advertisement provided brief outline of the study and details of how to volunteer. Those who responded to the advertisement were then sent the information sheet giving further details of the study. Again subjects were excluded if they were taking any of the medications listed above. No subjects in the control group had a family history of diabetes. Normal glucose metabolism was confirmed by a standard 75g OGTT (Lucozade Energy; GlaxoSmithKline Ltd., Middlesex, UK) following an overnight 12 hour fast. A total of 22 healthy volunteers were screened; 17 had normal glucose tolerance test while four had impaired glucose tolerance and one had impaired fasting glycaemia according to WHO criteria. None of the control or the type 2 diabetic

subjects performed intense exercise, heavy physical work or physical training on a regular basis.

Individuals who expressed an interest in participating in the studies were contacted by telephone. The study concerned was explained to them in detail, and any queries they had were answered. It was made clear to the subjects that agreeing to participate in the study was not binding, and that they were free to withdraw from the study at any time. On the day of the study, subjects were again informed of the procedure and the purpose, nature and potential risks of the study. Written, informed, voluntary consent was then obtained prior to their participation. All study protocols were reviewed and approved by the Newcastle upon Tyne and North Tyneside Local Research Ethics Committee 2.

2.1.2 Anthropometric and body composition

2.1.2.1 Body mass index

Body weight was measured to the nearest 0.1 kg with subjects in light indoor clothing (shoes removed) on an upright pedestal digital scale (Seca Ltd., Birmingham, UK). Height was measured to the nearest 0.5 cm (shoes removed) using a stadiometer (Seca Ltd., Birmingham, UK). Body mass index was calculated from weight and height measurements (BMI = weight in kilograms divided by height in meters squared).

2.1.2.2 Waist-hip ratio

A non-sticking plastic tape measure was used for all measurements, which were taken with the subjects in a relaxed posture. The subject's waist circumference was taken as the mid-point between the anterior superior iliac spine and the lower edge of their ribcage. Hip circumference was taken from the level of the subject's greater trochanter.

2.1.2.3 *Air displacement plethysmography*

Body composition was determined after an overnight fast by whole-body air displacement plethysmography using the BodPod Body Composition System (Life Measurement Inc., Concord, USA). This system is based on a two-compartment model of body composition (fat mass and fat-free mass), and uses the inverse relationship between pressure and volume (Boyle's law) to derive body volume (l) for each subject. Once body volume is determined, the principles of densitometry are used to determine body composition from body density. Percent body fat is then estimated using the Siri equation [% body fat = (495 divided by body density) – 450] (Siri, 1961). Prior measurement of the volume of the chamber is calibrated with a known standard (49.550 l) and the weighing scales calibrated against a known weight (20 kg). Subjects were asked to wear minimal clothing (swimsuit and swim cap) as hair and clothing had negative volume effects that alter body volume measurements. Before entering the BodPod chamber, subject's body weight was measured on the calibrated scale attached to the BodPod system. Whilst in the chamber, the subject was instructed to sit quietly in a relaxed manner with normal respirations. The subject's age, height and sex were entered in the computer system and two estimates of body volume were then obtained using predicted lung volume to calculate percent body fat. The intra-device repeatability, indicated by within-subject coefficient of variation (CV) for repeated measurements within a day was 0.9%.

2.1.3 **Physical activity level**

Physical activity was assessed over 3 days using the Body Monitoring System and SenseWear Armband (BodyMedia®, Pittsburgh, USA). The SenseWear Armband is a multi-sensor body monitor, worn on the back of the upper dominant arm, that allows calculation of energy expenditure and quantification of metabolic physical activity in free-living individuals (Fruin and Rankin, 2004,

Mignault et al., 2005). Validation of resting and activity related energy expenditure against doubly labelled water show high degrees of correlation and correspondence (Mignault et al., 2005). The SenseWear Armband employs four physiological sensors to continuously record an array of physiological data allowing overall measurement of energy expenditure. A two-axis accelerometer tracks the movement of the upper arm and provides information about body position. A heat-flux sensor measures the rate at which heat is being dissipated from the body. Surface skin temperature changes are also measured by sensitive thermistors. The device also measures galvanic skin response (the conductivity of the wearer's skin due to sweat and dilation of the sweat glands) which varies due to physical and emotional stimuli. Data are analysed using the InnerView® Professional software which contains activity detection and lifestyle algorithms to apply appropriate formula to estimate energy expenditure from the sensor data. Parameters calculated and reported include total energy expenditure, active energy expenditure, resting energy expenditure, metabolic equivalent of task (MET), total number of steps, physical activity duration, sleep duration and time spent lying down.

2.2 Magnetic Resonance Methods

Magnetic resonance data were acquired using a 3 Tesla Philips Intera Achieva scanner (Philips, Best, The Netherlands) with an in-built body coil used for imaging.

2.2.1 Magnetic resonance spectroscopy

A 14-cm diameter surface coil was used for phosphorus spectroscopy and a 6-cm diameter ^{13}C coil with an integral quad ^1H decoupling coil (PulseTeq, Wotton under Edge, UK) was used for ^{13}C spectroscopy. Subjects remained supine inside the magnetic resonance spectrometer with each coil positioned

beneath the widest part of the left calf during each investigation. The coil position was marked on the leg with indelible ink. Scout images were acquired to ensure identical coil positioning on repeat scans. To prevent movement during each study, the coil was secured in place using fabric straps around the calf. Analysis of all spectra was performed with jMRUI (version 3.0) (Naressi et al., 2001) using the AMARES fitting algorithm (Vanhamme et al., 1999).

2.2.1.1 ³¹P MR spectroscopy

Resting ATP turnover rate was measured using saturation transfer sequence between γ -ATP and P_i (Lebon et al., 2001). For each measurement, the P_i concentration was estimated from a 1D-ISIS spectrum centered on the gastrocnemius and soleus muscles (TR = 25 s, 8 averages, duration 3.3 min). Broadband ¹H decoupling was applied to data acquisition in all experiments. The γ -ATP resonances were assumed to have concentration 8.2 mM in line with previous studies (Harris et al., 1974, Arnold et al., 1984): the resonance frequency was placed mid-way between the γ -ATP and P_i resonances to ensure as even an excitation as possible at 3 Tesla. The pseudo-first order rate constant of the ATP synthase reaction was then estimated by a saturation transfer experiment as follows. The steady state-magnetization (M_z) of P_i was measured during selective irradiation of γ -ATP and compared with the equilibrium P_i magnetization (M_0) measured with the irradiation placed symmetrically down-field from the P_i frequency (TR = 25 s, bandwidth = 3000 Hz, 2048 points, 16 averages each, duration 13.3 min). The resonant frequency was set at the P_i frequency to allow symmetrical saturation pulses about that resonance. The fractional reduction of P_i magnetization upon saturation of γ -ATP, $(M_0 - M_z)/M_0$, was used to calculate the pseudo-first order rate constant using the Forsen-Hoffman equation: $k_1 = [(M_0 - M_z)/M_0](1/T_1^*)$, where T_1^* is the spin-lattice relaxation time for the phosphorus nucleus of P_i when ATP is saturated (Forsen and Hoffman, 1963). T_1^* was measured using an inversion

recovery experiment ($\tau_1 - 180^\circ - \tau_2 - 90^\circ - \text{acquire}$, TR = 25 s, four averages), while saturation of γ -ATP was performed during the delay times τ_1 and τ_2 . Eight variable τ_2 time delays were used ranging from 635 to 9,035 ms. Therefore the time required for a complete saturation transfer measurement was 26.7 mins. Unidirectional turnover of ATP synthesis was then calculated by multiplying the constant k_1 by the P_i concentration. The P_i concentration was measured from the spectrum acquired without irradiation. Prior knowledge was used to solve the dataset as follows: single Lorentzian peaks were modeled for P_i , phosphodiester (PDE) and PCr. The α - and γ -ATP resonances were modeled as Lorentzian duplets of equal magnitude separated by 18Hz. Due to the bandwidth of the pulses used and tuning P_i to be on resonance, the β -ATP resonance is only weakly detected and is not modeled. To fit the saturation transfer and inversion recovery experiments the zero and first order phasing and the P_i linewidth was determined from the control saturation scan. Phase values were then applied using the previous prior knowledge to fit the saturation, control and inversion recovery data. This provides sufficient stability to fit inversion recovery spectra with peaks of different T_1^* value using the AMARES algorithm. Sample spectra are given in Figures 2.1 and 2.2. In preparatory work using implementation of the saturation transfer technique, three healthy subjects were studied three times on one day without repositioning of the radiofrequency coil, yielding an intra-day variability of 6.5% in ATP turnover rate, and were studied twice more on two further separate days to examine the effect of coil repositioning, yielding an inter-day reproducibility of 8.0% in ATP turnover rate (Trenell et al., 2008).

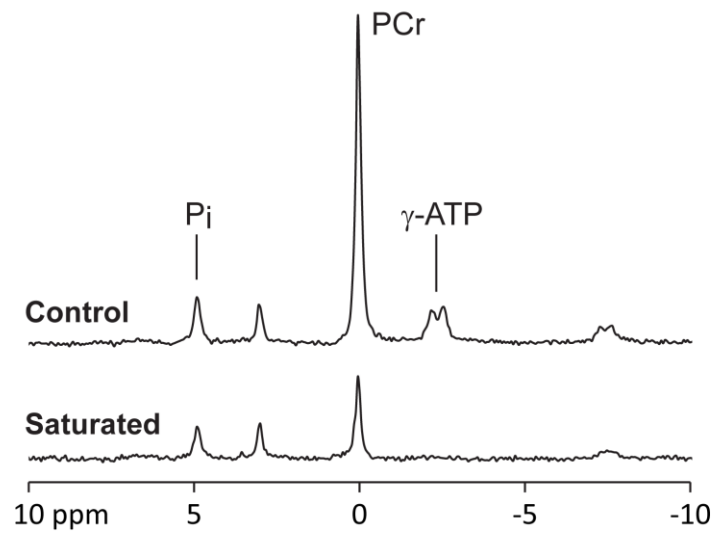


Figure 2.1 MR spectra from a study subject acquired using the saturation transfer measurement showing (bottom) the saturation of γ -ATP at -2.38 ppm and (top) the control saturation at 2.38 ppm. The differences in the amplitudes of PCr and P_i show the saturation transfer effect.

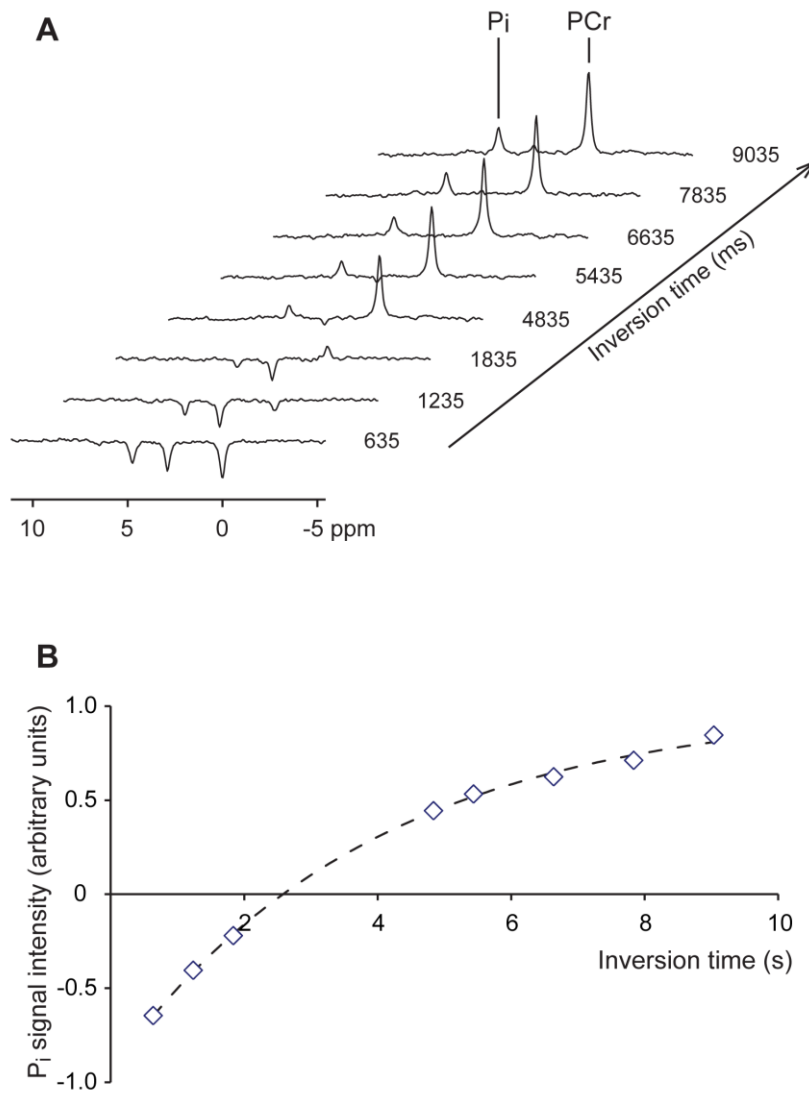


Figure 2.2 (A) Inversion recovery data showing P_i , PDE and PCr during inversion recovery and saturation. 12 Hz line broadening has been applied though fitting is in time domain. (B) Inversion recovery curve for P_i plotted through the eight inversion recovery data points. In this case, $T_1^* = 3.90$ s.

2.2.1.2 ^{13}C MR spectroscopy

The distance between the $^{13}\text{C}/^1\text{H}$ decoupling coil surface and the muscle was recorded from scout images. The ^{13}C pulse power was calibrated to a nominal value of 80° in the tissue of interest by observing the power-dependent variation in signal from a fiducial marker located in the coil housing, containing a sample exhibiting a ^{13}C signal with short T_1 (213 mM [2- ^{13}C]-acetone and 25 mM GdCl_3 in water). Spectra showing the glycogen [1- ^{13}C] resonance were acquired using a non-localized ^1H -decoupled ^{13}C pulse-acquire sequence (TR = 200 ms, spectral width = 8 kHz, 3000 averages, WALTZ decoupling, nominal tip angle = 80°) over a 15-min acquisition time. Calibration of ^{13}C spectra was performed by comparison of *in vivo* glycogen [1- ^{13}C] signal amplitudes with that of a standard glycogen solution [100 mM oyster glycogen (Sigma Aldrich Ltd., Gillingham, UK), 70 mM KCl and 0.05% sodium azide]. Quantitation was performed by comparison of signal amplitude to those from a leg-shaped phantom acquired at a range of separations between coil and phantom, representative of the separation between coil and muscle due to skin and subcutaneous fat. The same coils, pulse sequences, and tip angles as employed for *in vivo* spectra were used for acquisition of these calibration data. The concentration of muscle glycogen, $[\text{Glyc}]_{\text{muscle}}$, was calculated using the formula:

$$[\text{Glyc}]_{\text{muscle}} = \frac{S_{\text{muscle}} \times [\text{Glyc}]_{\text{phantom}}}{S_{\text{phantom}}}$$

where S_{phantom} and S_{muscle} are the signal intensities arising from glycogen in the phantom and muscle, respectively, and $[\text{Glyc}]_{\text{phantom}}$ is the concentration of glycogen in the phantom (100 mmol/l). The coefficient of variation for muscle glycogen measurement using this method has been reported at 4.3% (Taylor et al., 1992). The increments in muscle glycogen concentration at x min and y min

of the clamp, $[\Delta\text{Glyc}_x]$ and $[\Delta\text{Glyc}_y]$ respectively, were calculated from the published equation (Jue et al., 1989):

$$[\Delta\text{Glyc}_x] = \frac{(S_x - S_0) \times [\text{Glyc}_0] \times f_0}{S_0 \times f_x}$$

$$[\Delta\text{Glyc}_y] = \frac{(S_y - S_x) \times [\text{Glyc}_0] \times f_0}{S_0 \times f_y}$$

where S_0 , S_x and S_y represent the signal intensity of glycogen at 0, x and y min, and $[\text{Glyc}_0]$ is the basal glycogen concentration in mmol/l. f_0 corresponds to the natural abundance enrichment of ^{13}C -glycogen at baseline (1.1%), and f_x and f_y correspond to the mean percentage ^{13}C enrichment of plasma glucose at x and y min respectively. Each increment was then added to the previous concentration, and the slope calculated by linear regression to yield the rate of muscle glycogen synthesis (Shulman et al., 1990).

2.2.1.3 ^1H MR spectroscopy

Proton spectroscopy was acquired using a PRESS sequence localised to a 15 x 15 x 20 mm voxel of tissue located in the soleus muscle with water suppression (TR = 3000 ms, bandwidth = 2000 Hz, 2048 points and 32 averages). Sixteen averages were collected without suppression for reference. Proton spectra were analysed for IMCL from the water suppressed spectra and water from the non-water suppressed spectra. IMCL was then expressed relative to the water reference.

2.2.2 Three-point Dixon magnetic resonance imaging

Three gradient echo scans were acquired with adjacent out-of-phase, in-phase and out-of-phase echoes. For human studies, the fat-water frequency difference at 3 Tesla is 435 Hz, and echo times of 3.45 ms, 4.60 ms and 5.75 ms were used to acquire adjacent out-of phase, in-phase and out-of phase images. A breath hold time of less than 17 seconds was used. The real and imaginary data (not magnitude corrected data) was then uploaded to a custom MATLAB (Mathworks, Cambridge, UK) script, following the algorithm outlined in Glover et al. (Glover and Schneider, 1991) to produce separate fat and water images (Figure 2.3). The fat content of the image (F) was then expressed as a percentage of the total signal in the original signal (F+W), leading to a “fat percentage in the visible MR signal” or what is henceforth referred as “fat content”. This excludes components which are invisible to MR at these echo times (chiefly structural macromolecules). Region-of-interest (ROI) definition was performed with ImageJ 1.43 (Abramoff et al., 2004), a freely available software, using the polygon ROI tool.

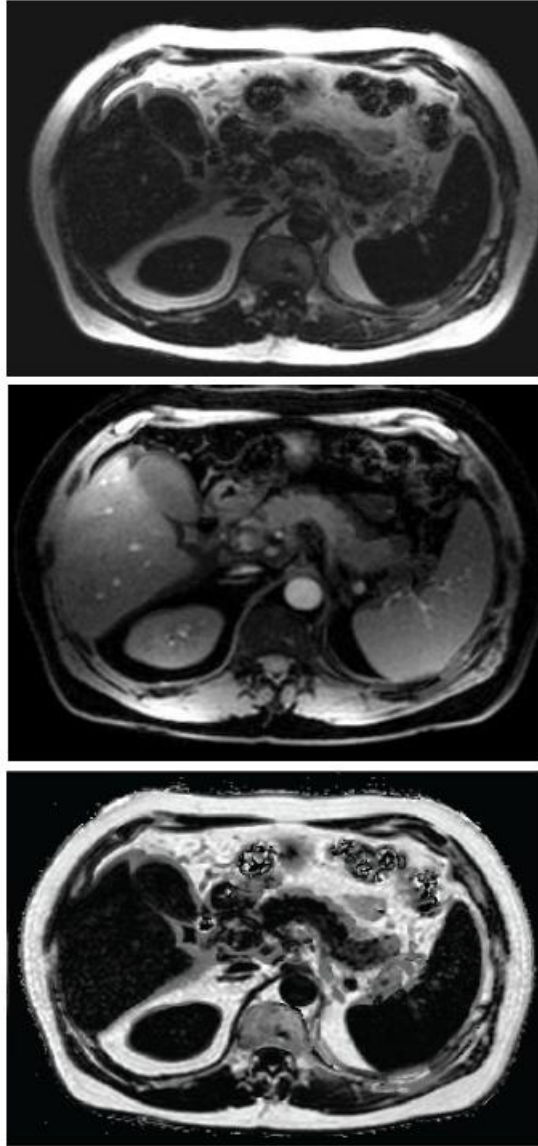


Figure 2.3 Three-point Dixon technique of the liver and pancreas. Water (top panel), fat (middle panel) and combined water and fat images (bottom panel) of the liver and pancreas.

2.2.2.1 Hepatic and pancreatic fat

The anatomical position of the subject's liver and pancreas was first ascertained by axial sections acquired by a single shot balanced turbo-field echo (bTFE or true-FISP) sequence (TR = 2.8 ms, TE = 1.4 ms, number of averages = 1, flip angle = 40°, matrix of 172 x 192, median field of view = 440 mm, range 400 – 480 mm to suit subject size with 70% phase field of view, turbo factor 101, 18 slices, slice thickness 5 mm and scan duration of 12.1 s). A SENSE factor (parallel imaging) of two was used in the phase direction. After completion of the automated reconstruction of the three-point Dixon images, the images were then independently analysed. The signal intensity in the images was calculated with investigator-defined ROIs (Figure 2.4). A total of five ROIs for the liver and two ROIs for the pancreas were acquired and averaged, with care given to placement of the ROIs to avoid contamination from blood vessels, gallbladder and visceral fat. The smaller number of ROIs was acquired for the pancreas due to its small size and elongated shape. The analysis was carried out in a blinded fashion by a single trained investigator (Dr Kieren Hollingsworth). To assess the reproducibility of this process of image analysis, one-quarter of the images were independently analysed by another trained investigator (myself). There was close agreement between the two investigators' analyses of hepatic and pancreas fat content, with the inter-observer CV at 1.9% and 2.5% respectively. Validation of the three-point Dixon method of measuring hepatic and pancreatic fat content by this method has been established and the inter-scan repeatability coefficients were found to be 0.5% for the liver and 0.9% for the pancreas.

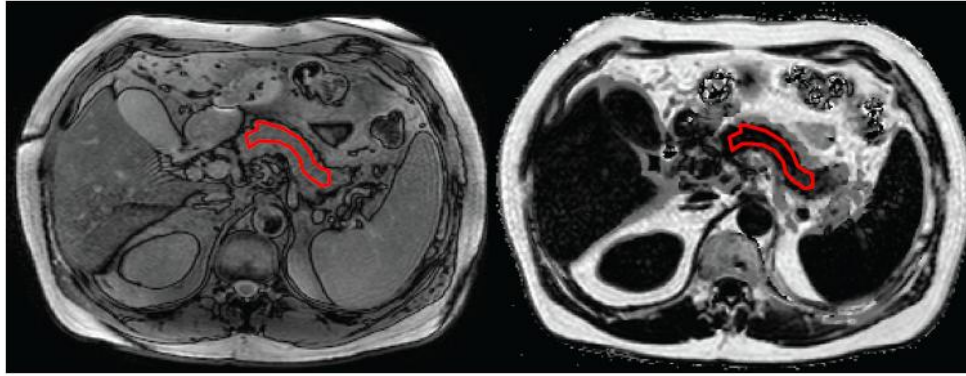


Figure 2.4 Polygonal ROI manually drawn at the same location in both water (left-side) and fat (right-side) images of the pancreas to avoid confounding anatomy such as large vessels and also to avoid the edges of the organ due to problems of partial averaging. A total of 2 contiguous slices of the pancreas were selected for drawing ROIs.

2.2.2.2 Visceral and subcutaneous adipose tissue areas

Visceral and subcutaneous fat measurements were performed by acquiring images at the L4/L5 junction using a three-point Dixon sequence (TR = 50 ms, TE = 3.45, 4.60 and 5.75 ms, number of averages = 1, flip angle = 30°, matrix of 160 x 109, median field of view = 440 mm, range 400 – 480 mm to suit subject size with 70% phase field of view). The slice was acquired during a breath-hold of 16-18 seconds and with slice thickness of 10 mm (Donnelly et al., 2003, Shen et al., 2004). Fat and water signals were separated, and binary gating applied to produce a map of structures containing more than 50% fat, identified as subcutaneous and visceral fat. A watershed algorithm was used to divide the binary image into distinct areas and allowed easy separation of the subcutaneous and visceral fat. Total adipose tissue area was calculated using image analysis software ImageJ 1.43 (Abramoff et al., 2004). The subcutaneous adipose tissue (SAT) area was then segmented and measured. The visceral

adipose tissue (VAT) area was then calculated as total adipose tissue area minus SAT area. This is illustrated in Figure 2.5. The inter-observer CV for SAT and VAT measurements were 2.8% and 2.2% respectively.

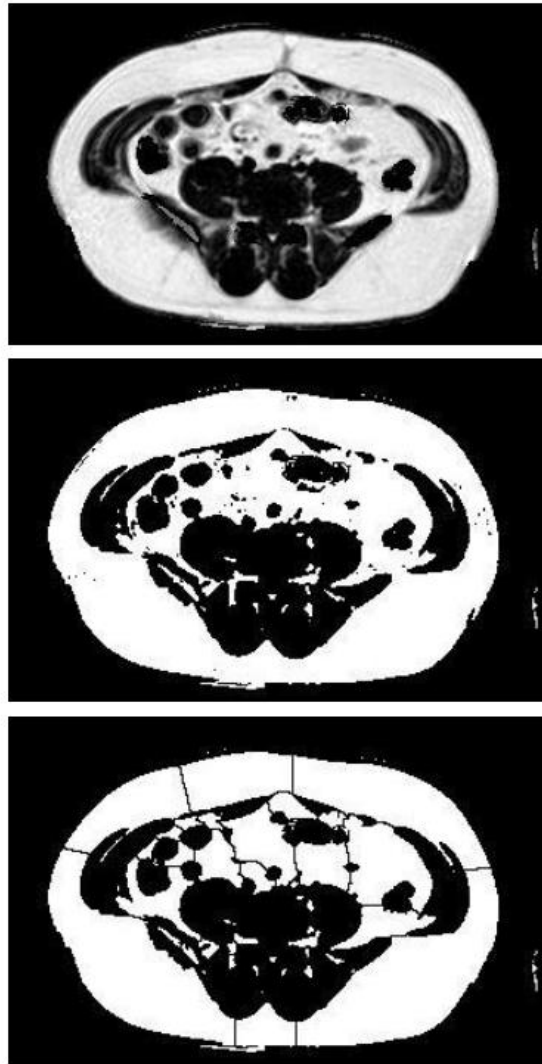


Figure 2.5 Top panel: The three-point Dixon fat percentage map acquired at the L4/L5 junction. Middle panel: Segmentation of structures with more than 50% fat (visceral and subcutaneous fat). Total area is calculated. Bottom panel: Application of thresholding algorithm divides segmented image into chunks and separates visceral and subcutaneous fat around muscle boundaries. Selection of subcutaneous fat allows measurement of this area and subtraction from the total yields visceral fat area.

2.3 Metabolic Studies

2.3.1 Venous cannulation and blood sampling

An 18 gauge cannula (Vasofix®; B. Braun Medical Ltd., Sheffield, UK) was inserted into a large antecubital vein for administration of intravenous glucose and insulin. A second cannula was inserted into the contralateral wrist vein for the purpose of blood sampling. Both lines were flushed with 0.9% sodium chloride. The hand with the wrist vein cannulation was kept warm for sampling of arterialised blood by placing it in between two hot water bottles which were changed regularly at 30 minutes interval to ensure continuous vasodilatation. Pilot blood samples were collected into a heparinised syringe and put on ice for transport to the Biochemistry Department at Royal Victoria Infirmary where pH, pO₂, O₂ saturation and pCO₂ were measured using the 800 Blood Gas System (Bayer Corporation, Norwood, MA, USA) to confirm complete arterialisation. After 10 minutes of placement of the hot water bottles, oxygen saturation of ≥95% was maintained throughout (Figure 2.6). The intravenous line for the sampling of arterialised blood was flushed with 0.9% sodium chloride after each blood sample was taken to keep the line patent. In order to avoid dilution of blood samples, the initial 2 ml of blood withdrawn was discarded and a fresh syringe was used to collect the blood sample. Blood samples were spun down and separated before being frozen at -40°C at the Newcastle Magnetic Resonance Centre before being transported in dry ice to the Diabetes Research Laboratory for assaying.

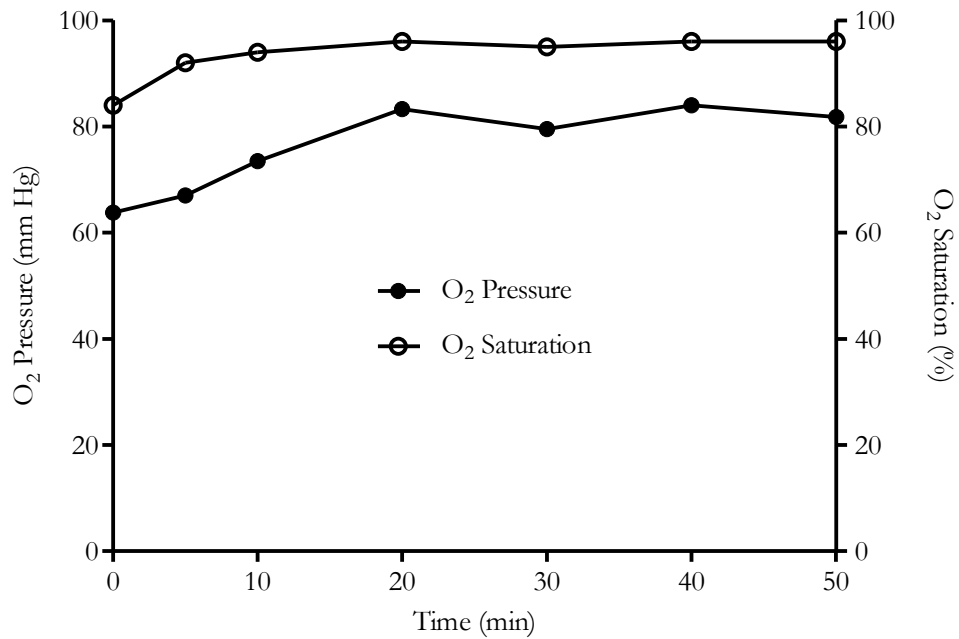


Figure 2.6 Measurement of oxygen content of ‘arterialised’ venous blood taken from a wrist vein of one subject as a function of time. The hand was placed between two hot water bottles at time zero. At 30 minutes, the hot water bottles were replaced with new ones.

2.3.2 Hepatic glucose production

2.3.2.1 6,6-dideuterated glucose

The 6,6-dideuterated glucose was made up into 10% solutions using sterile technique. All subjects were given a priming dose of 6,6-dideuterated glucose intravenously adjusted according to fasting plasma glucose level to allow early establishment of steady-state isotope enrichment (Hother-Nielsen and Beck-Nielsen, 1990, Ravikumar et al., 2008). This was followed by a continuous infusion for 270 min. The prime dose for a fasting plasma glucose level of 5 mmol was 300 mg and the resulting equation for other fasting plasma glucose level was:

$$\text{Bolus} = \frac{\text{Fasting plasma glucose (mmol/l)} \times 300 \text{ mg}}{5}$$

To achieve equilibrium of 6,6-dideuterated glucose, a continuous infusion of 6,6-dideuterated glucose (0.04 mg/kg/min) was commenced after the bolus injection. The 6,6-dideuterated glucose was infused at a rate of 0.12 ml/kg/hr, at a concentration of 20 mg/ml. For each study, a 1 ml sample (labelled NINF) was taken from the prepared 6,6-dideuterated glucose infusate (2% strength) for later analysis. In order to prevent any fall in atom percent excess (APE) of 6,6-dideuterated glucose during the insulin-glucose clamp, the 6,6-dideuterated glucose infusate was continued for the duration of the clamp. In addition, the variable glucose infusion used in order to maintain glycaemia during the clamp was enriched with 2% of 6,6-dideuterated glucose. For each enrichment, a 1 ml sample (labelled GINF) was taken for later analysis.

Plasma samples for determination of 6,6-dideuterated glucose APE were taken as follows:

Time	Plasma glucose	Plasma ² H ₂ APE
-160	Y	Y
-150	Y	Y
-90	Y	Y
-30	Y	Y
-20	Y	Y
-10	Y	Y
0	Y	Y
5	Y	
10	Y	
15	Y	
20	Y	
25	Y	
30	Y	Y
35	Y	
40	Y	
45	Y	
50	Y	

55	Y	
60	Y	Y
65	Y	
70	Y	
75	Y	
80	Y	
85	Y	
90	Y	Y
95	Y	
100	Y	Y
105	Y	
110	Y	Y
115	Y	
120	Y	Y

Table 2.1 Blood sampling schedule for assessing hepatic glucose production.

2.3.2.2 Gas chromatography-mass spectrometry analysis

Frozen samples were allowed to thaw and approximately 60 μ l plasma was transferred to an Eppendorf tube before 200 μ l acetonitrile:ethanol (2:1) was added. Following a vortex mix, the sample was spun in a micro-centrifuge at 13000 rpm for 5 min. The supernatant was then transferred to a 1 ml v-vial and evaporated to dryness at 90 °C under a gentle stream of dry air. 60 μ l pyridine and 20 μ l acetic anhydride were added to dry the residue. The vial was capped and heated on a dry block heater at 90 °C for 20 min. The samples were then cooled and evaporated to dryness at 90 °C under a gentle stream of dry air. The pentaacetate derivative of glucose was then dissolved in 80 μ l acetonitrile. The samples were then analysed using gas chromatography-mass spectrometry (GC-MS) analysis.

GC-MS analysis was carried out on a Thermo ‘Voyager’ single quadrupole mass spectrometer interfaced to a Thermo ‘Trace’ GC, with automated injection

via a Thermo 'AS2000' autosampler (Thermo Scientific, Waltham, MA, USA). Samples for the glucose analysis were analysed under EI-SIM conditions with separation of the pentaacetate derivatives carried out on a Zebron 30 m x 0.25 mm x 0.25 μ m ZB-5 ms capillary column (Phenomenex, Torrance, CA, USA). Injections of 1 μ l were made in the split mode with a 30:1 split and the injection port was maintained at a constant 260 °C. The carrier gas was Helium at a constant flow of 1 ml/min. GC conditions were: 100 °C ramping to 275 °C at a rate of 20 °C/min with a terminal hold of 5 min; MS: the mass detected were 200 and 206 (6,6 di-deuterated glucose). The CV for the precision of plasma $^2\text{H}_2$ APE measurements was 4.1%. The APE of the $^2\text{H}_2$ infusate was measured similarly and the CV was 3.4%.

2.3.2.3 Calculation of hepatic glucose production

Hepatic glucose production (HGP) can be estimated during steady-state conditions. HGP can be measured in the basal state and under conditions of hyperinsulinaemia – giving an index of hepatic insulin action. HGP is estimated indirectly using the model proposed by Steele et al (Steele et al., 1965), which are based on a single-compartment model for glucose and non-steady-state conditions. This approach assumes that the liver is the only source of endogenous glucose production during steady-state glucose infusion, and thus that HGP represents the difference between the rates of glucose appearance in circulation (R_a) and its disappearance (R_d). Although gluconeogenesis does occur in the kidneys, it is at a low rate and accounts for a very small portion of HGP (Ekberg et al., 1999, Gerich et al., 2001).

Basal rates of R_a or HGP were calculated during the last 30 min of the 150 min basal period by dividing the tracer infusion rate times tracer enrichment by the tracer enrichment in plasma and subtracting the tracer infusion rate (Inzucchi et al., 1998). During the insulin-glucose clamp, calculations were made

over the last 30 min of the 120 min clamp and HGP was calculated by the following formula:

$$\text{Clamp HGP} = \text{GIR}_{\text{mean}} \times [(\text{enrichment}_{\text{inf}}/\text{enrichment}_{\text{plasma}}) - 1]$$

where GIR_{mean} is the mean glucose infusion rate during the last 30 min of the clamp, $\text{enrichment}_{\text{inf}}$ is the APE of glucose in infusate, and $\text{enrichment}_{\text{plasma}}$ is the APE of plasma glucose during the steady-state conditions of the clamp.

2.3.3 Peripheral insulin sensitivity

2.3.3.1 Preparation of [1-¹³C] glucose

In order to increase sensitivity of measurement of muscle glycogen synthesis by ¹³C MR spectroscopy, the variable glucose infusion used during the insulin-glucose clamps was enriched with 20% of [1-¹³C] glucose. The [1-¹³C] glucose (Cambridge Isotope Laboratories, Andover, MA, USA) was made up into 20% solutions in either 10 ml or 50 ml vials by the Pharmacy Production Unit at the Pharmacy Department of the Royal Victoria Infirmary.

2.3.3.2 Isoglycaemic-hyperinsulinaemic clamp

Isoglycaemic hyperinsulinaemia was induced with the insulin-glucose clamp technique (DeFronzo et al., 1979). Isoglycaemia rather than euglycaemia was maintained in order to ensure that the true basal condition of each subject could be observed. Insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was administered as a prime-continuous infusion (40 mU/m²/min) for 150 mins. To inhibit endogenous insulin secretion, somatostatin (Somatostatin-UCB; UCB Pharma, Netherlands) was infused at 0.06 µg/kg/min from 5 min before the start of the insulin infusion and continued for the duration of the clamp. Fasting isoglycaemia was maintained by a variable glucose infusion containing 20% [1-

¹³C] glucose based on plasma glucose measurements performed at 5 min intervals in studies employing ¹³C MR spectroscopy. For studies using 6,6-dideuterated glucose to determine hepatic glucose production, a 10% dextrose infusion labelled with 6,6, di-deuterated glucose (2% enriched) according to the hot glucose infusion (hot GINF) protocol was periodically adjusted to maintain isoglycaemia during clamps.

The amount of insulin required for each clamp was individually calculated according to body surface area (BSA) at 40 mU/m²/min to achieve plasma insulin concentration ~400 pmol/l as follows:

$$\text{BSA (m}^2\text{)} = 0.007184 \times \text{Height}^{0.725} \times \text{Weight}^{0.425}$$

where the height is in centimetres and the weight is in kilograms (DuBois and DuBois, 1916).

$$\begin{aligned} &\text{Units of insulin needed to add to 50 ml 0.9\% sodium chloride} \\ &= \frac{\text{BSA (m}^2\text{)} \times 0.04 \text{ (U)} \times 60 \text{ (min)} \times \text{syringe volume (50 ml)}}{\text{constant infusion rate (15 ml/h)}} \end{aligned}$$

Example, for an 80 kg man of 175 cm in height;

the amount of insulin required = (1.956 x 0.04 x 60 x 50) ÷ 15 = 15.6 Units

A 7-min priming insulin infusion was then started as follows:

4 x constant infusion rate from time 0 – 4 min

2 x constant infusion rate from time 4 – 7 min

This was followed by a continuous infusion of:

1x constant infusion rate from time 7 – end of clamp

where constant infusion rate was set at 15 ml/hr.

The mean CV of plasma glucose level in the last 30 min of the clamps was 2.0%, while the mean CV of glucose infusion rate in the last 30 min of the clamps was 4.5%.

2.3.3.3 Hyperglycaemic-hyperinsulinaemic clamp

The hyperglycaemic-hyperinsulinaemic clamps were carried out in the same way as the isoglycaemic-hyperinsulinaemic clamps except that blood glucose was clamped at 5 mmol/l above fasting concentration. The mean CVs of plasma glucose level and of glucose infusion rate in the last 30 min of the clamps were 2.1% and 6.5% respectively.

2.3.3.4 Calculation of whole-body glucose metabolism

Whole-body insulin sensitivity was determined during the last 30 min of the hyperinsulinaemic glucose clamp as whole-body glucose disposal corrected for glucose space and urinary loss (DeFronzo et al., 1979). Whole-body glucose disposal was calculated from the glucose infusion rate (Rizza et al., 1981). Glucose metabolic clearance rates during steady-state conditions were calculated by dividing whole-body insulin sensitivity expressed as $\text{mg}/\text{kg}_{\text{ffm}}/\text{min}$ (where ffm is fat free mass) by steady-state plasma glucose. To assess rate of oxidation of infused glucose, breath samples for $^{13}\text{CO}_2$ measurements were obtained as described in the next sub-section. As this measure would be affected by differences in plasma ^{13}C glucose enrichment, the index of whole-body glucose oxidation was calculated as the ratio of breath to plasma ^{13}C APE ($[\text{breath APE}/\text{plasma APE}] \times 100$).

2.3.3.5 *Breath ¹³C analysis*

Breath samples for ¹³C enrichments were collected by asking subjects to exhale fully through a short straw into a glass tube (Labco Exetainer; Labco Ltd., Buckinghamshire, UK). The tube was immediately stoppered. ¹³C enrichments of breath samples were determined by continuous flow isotope ratio mass spectrometry (ABCA System; PDZ Europa Ltd., Crewe, UK). Breath samples were obtained just before the start of the insulin-glucose clamp (0 min), at 15 min, 45 min, 90 min and at the end of the clamp (150 min). In one of the control subject (randomly selected), eight breath samples were obtained consecutively over a 5 min period at the end of the insulin-glucose clamp. Data from this collection was used to calculate the CV for the analysis of ¹³C enrichment of expired air and for the collection of the breath sample, which were 0.07% and 0.3% respectively. All results of ¹³C enrichment of expired air measurements are expressed as APE.

2.3.4 **β-cell sensitivity to glucose**

2.3.4.1 *Stepped insulin secretion test with arginine*

To fully characterize first phase and maximal insulin secretory responses to consecutive steps of hyperglycaemia, a modified protocol from Toschi et al. (Toschi et al., 2002) was carried out. This consisted of two consecutive 30-min square-wave steps of hyperglycaemia (2.8 and 5.6 mmol/l above baseline) followed by an intravenous bolus of 5 g arginine. The priming dose of glucose for each step was calculated by multiplying the desired increment in plasma glucose concentration by the body glucose space (estimated to be 150 ml per kg of body weight). The 20% dextrose volume thus calculated was administered over 1 min. After the glucose bolus, plasma glucose concentrations were maintained at the desired plateau by means of a variable 20% glucose infusion according to the glucose clamp technique (DeFronzo et al., 1979). Blood samples for the determination of plasma glucose, plasma insulin and C-peptide

concentrations were obtained every 2 min for the first 6 min, at 10 min and every 5 min for the other 20 min of each step. After the arginine bolus, blood was sampled every 2 min for 10 min.

2.3.4.2 Calculation of insulin secretion rate

Insulin secretion rate was calculated using ISEC (Hovorka et al., 1996). ISEC is a computer program which implements a regularisation method of deconvolution (Twomey, 1965) constrained to non-negative values and uses a population model of C-peptide kinetics (Van Cauter et al., 1992). Each subject's age, weight, height, gender and classification of diabetes status together with C-peptide concentrations were supplied to ISEC as input and it produced the insulin secretion rate as output. A 1 min step-size was used and the coefficient of variation was individually and experimentally selected for each subject (range of CV = 5-9%). The computer programming and analysis were carried out by Dr Benjamin Aribisala. All insulin secretion values were expressed per square meter of body surface area.

2.3.5 Metabolites and hormone assays

Plasma glucose levels were measured by the glucose oxidase method (YSI glucose analyser; Yellow Springs Inc., Ohio, USA) (CV for measurement [control (10 mmol/l)]: 2.8%). Plasma insulin and C-peptide levels were both measured using ELISA kits (DAKO; Ely, Cambridgeshire, UK) (CV for measurement [insulin range 400 – 500 pmol/l]: 5.5% and [C-peptide range 1.20 – 2.00 nmol/l]: 7.1%). Plasma NEFA concentration was measured on a Roche Cobas centrifugal analyser using an enzymatic colorimetric Wako kit (Wako Chemicals, Neuss, Germany) (CV for measurement [range 1.02 – 1.25 μ mol/l]: 3.2%). Plasma triglyceride levels were hydrolysed by lipase, and the released glycerol was measured on a Roche Cobas centrifugal analyser using a colorimetric assay (ABX Diagnostics, Montpellier, France) (CV for

measurement [range 1.07 – 1.39 mmol/l]: 6.2%). Plasma 3-hydroxy-butyrate levels were measured on perchloric acid extracts using a Cobas biocentrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK). HbA_{1c} was measured by Biorad HPLC (TOSOH Corporation, Tokyo, Japan). The HbA_{1c} assay was standardised upon that used in the diabetes complications and control trial central laboratory at the University of Minnesota, with an upper limit of normal of 6.1% (Gibb et al., 1997).

The ¹³C enrichment in plasma glucose was determined by GC-MS of the pentaacetate derivatives of plasma glucose after deproteinization and deionization as described in *sub-section 2.3.3.2*. The glucose pentaacetate was analysed on a Thermo Finnigan Trace 2000 GC interfaced to a Voyager MS and AS2000 autosampler. Selective ion monitoring was used to determine tracer enrichments in the molecular mass ion fragment of glucose. The masses detected were 331 and 332 molecular weight (MW). The CV for the precision of ¹³C enrichment in plasma glucose was 5.8% at an average enrichment of 14.4 APE.

Chapter 3

EFFECTS OF CHANGES IN SUBSTRATE SUPPLY ON SKELETAL MUSCLE ATP TURNOVER RATES IN TYPE 2 DIABETES

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3.1 Introduction

Skeletal muscle mitochondrial dysfunction has been hypothesised to contribute to the development of insulin resistance and type 2 diabetes. Muscle biopsy studies of type 2 diabetes subjects demonstrated smaller mitochondria and reduced activities of oxidative enzymes compared with glucose-tolerant subjects (He et al., 2001, Kelley et al., 2002). Using ^{31}P MR spectroscopy to determine *in vivo* skeletal muscle mitochondrial function, Petersen and colleagues were the first to observed impairment in ATP turnover rates in skeletal muscle of insulin-resistant offspring of type 2 diabetic patients, both in the basal overnight-fasted condition and after stimulation by insulin (Petersen et al., 2004, Petersen et al., 2005b). This was associated with an increase in intramyocellular lipid content. These findings led the authors to suggest that insulin resistance in these young offspring was due to dysregulation of intramyocellular fatty acid metabolism, which may be caused by an inherited defect in mitochondrial oxidative phosphorylation. A similar impairment in muscle ATP turnover rates have been observed in individuals with type 2 diabetes (Szendroedi et al., 2007). Decreased expression of nuclear-encoded genes that regulate mitochondrial biogenesis such as PGC-1 α has also been reported in non-diabetic individuals with impaired glucose tolerance and in healthy first-degree relatives to type 2 diabetes patients (Mootha et al., 2003, Patti et al., 2003). These studies provide further support that abnormalities in oxidative metabolism may contribute to the development of insulin resistance and hence type 2 diabetes (Chanseume and Morio, 2009, Pagel-Langenickel et al., 2010).

In humans, the causal relationship between dyslipidaemia and insulin resistance has been examined in detail over the past several decades (McGarry, 1994, Boden, 1997). Elevated plasma NEFA levels are associated with many insulin-resistant states including type 2 diabetes and obesity (Reaven et al., 1988). Mitochondrial dysfunction has been suggested to be a key factor linking

increased NEFA levels to insulin resistance. Indeed, elevation of plasma NEFA levels in young healthy individuals via the use of lipid infusion induces hepatic and peripheral insulin resistance by a similar mechanism to that described in subjects with type 2 diabetes, i.e. increased intracellular fatty acid metabolites and impaired insulin signaling (Roden et al., 1996, Dresner et al., 1999, Griffin et al., 1999, Itani et al., 2002, Belfort et al., 2005). Employing *in vivo* measurement of mitochondrial function, Brehm and colleagues showed that short-term elevation of circulating lipids via lipid infusion was associated with impaired insulin-stimulated ATP synthesis in skeletal muscle of insulin-sensitive individuals in parallel with induction of insulin resistance as seen in type 2 diabetes (Brehm et al., 2006). Physiological elevation of NEFA levels in insulin-sensitive individuals increased intramyocellular fatty acyl CoA levels and down-regulated the expression of multiple genes encoding mitochondrial proteins including those involved in oxidative phosphorylation (Richardson et al., 2005), suggesting that elevated concentrations of fatty acid metabolites cause an acquired mitochondrial defect that leads to impaired substrate oxidation in skeletal muscle (Abdul-Ghani et al., 2008).

However, a substantial number of recent studies both in humans and rodents directly challenge the view that a defect in mitochondrial function, whether inherited or acquired, is responsible for the development of insulin resistance. In obese humans, muscle biopsy studies have observed improvement in mitochondrial function, reflected by an increase in electron transport activity without changes in mitochondrial content, after programs of weight loss and physical exercise (Menshikova et al., 2005). Furthermore, improvement in insulin sensitivity through calorie restriction has been demonstrated in overweight and obese subjects in the absence of any measurable change in mitochondrial DNA content and NADH-oxidase activity (Toledo et al., 2008). Several studies of genetically modified mice have failed to demonstrate a clear effect of altering mitochondrial function on insulin action (Handschin et al., 2007, Calvo et al., 2008). In light of all the evidence

presented, the reduced capacity of the mitochondria to produce ATP in muscle of type 2 diabetes could be secondary to the metabolic state itself, and a feature of insulin resistance rather than its cause (Phielix and Mensink, 2008, Szendroedi and Roden, 2008).

Increased lipid availability could have a deleterious effect on mitochondrial respiratory capacity via down-regulation of the expression of the genes involved in biogenesis of mitochondria (Richardson et al., 2005, Hoeks et al., 2006), fatty acid peroxidation (Schrauwen and Hesselink, 2004), and incomplete β -oxidation leading to intra-mitochondrial accumulation of acyl CoA (Koves et al., 2008). The suppression of lipolysis by acipimox or nicotinic acid has long been known to improve meal tolerance and insulin sensitivity (Fulcher et al., 1992, Vaag et al., 1991b, Johnson et al., 1992). Sustained suppression of NEFA is associated with a decrease in intra-muscular long-chain fatty acyl CoAs and it is believed that this enhances glucose metabolism via substrate competition (Bajaj et al., 2005). The mechanism for this interaction is well described, involving control of cellular glucose uptake in muscle (Roden et al., 1996). Both glucose oxidation and non-oxidative metabolism are involved, and as one of the original descriptions reported increase in the active form of glycogen synthase it has been assumed that increased rates of glucose storage as glycogen underlie the phenomenon (Vaag et al., 1991b). However, there is no direct *in vivo* evidence that glycogen synthesis rate is enhanced by NEFA suppression. This mechanism is important in fully understanding the insulin resistance of type 2 diabetes and evaluating potential interventions.

Application of ^{13}C MR spectroscopy has demonstrated that insulin-stimulated muscle glycogen synthesis is the major metabolic pathway of glucose disposal under post-prandial conditions, and that a defect in muscle glycogen synthesis was responsible for the decrease in insulin sensitivity in type 2 diabetes (Shulman et al., 1990). Further study showed that reduced muscle glucose transport is responsible for the decreased rate of insulin-stimulated glycogen synthesis in muscle of type 2 diabetes (Cline et al., 1999). Therefore,

the hypothesis that acute suppression of lipolysis by acipimox in type 2 diabetes would improve the rate of glucose metabolism specifically by increasing muscle glycogen synthesis and thus muscle mitochondrial ATP turnover rates was tested in this study. In addition, it was hypothesised that impairment in insulin-stimulated ATP turnover rates in type 2 diabetes would reflect the lower rates of muscle glucose uptake and glycogen synthesis, rather than causing it. If this is true, muscle ATP turnover rates in type 2 diabetes could be increased if glycogen synthesis rates were normalized by the mass action effect of hyperglycaemia. To test this second hypothesis, MR spectroscopy was used to quantify muscle ATP turnover rates and glycogen synthesis rates during isoglycaemic- and hyperglycaemic-hyperinsulinaemia in type 2 diabetic and non-diabetic control subjects.

3.2 Study Design

3.2.1 Subjects

Ten subjects with well-controlled type 2 diabetes (3 female, 7 male) and eight physical activity-, age- and BMI-matched normal glucose tolerant subjects (2 female, 6 male) were studied (Table 3.1). All subjects were recruited by means of advertisement and underwent a complete medical history, clinical examination and lab tests to exclude hepatic and renal diseases. Patients with diabetes on insulin treatment or any oral hypoglycaemic medications apart from metformin were excluded. The individuals in the control group had neither a family history of diabetes nor were taking any medication known to affect glucose tolerance or insulin sensitivity. All subjects with diabetes and no control subjects were taking statins. Normal glucose metabolism was confirmed by a standard 75g OGTT. None of the subjects performed moderate or intense exercise on a regular basis. The research was carried out in accordance with the

Declaration of Helsinki (2000) and the study protocol was approved by the Newcastle upon Tyne Ethics Committee No. 2.

Table 3.1 Clinical characteristic of study subjects

	T2D (7M, 3F)	Control (6M, 2F)	<i>p value</i>
Age (years)	57 ± 5	53 ± 7	0.3
BMI (kg/m ²)	28.7 ± 3.8	28.1 ± 3.1	0.8
Fat mass (kg)	26.1 ± 6.5	26.4 ± 4.8	0.9
Fat free mass (kg)	54.7 ± 11.9	58.5 ± 10.1	0.5
Fasting glucose (mmol/l)	7.7 ± 1.1	5.1 ± 0.3	0.001
Fasting insulin (pmol/l)	93 ± 44	49 ± 18	0.026
HbA _{1c} (%)	6.5 ± 0.5	5.4 ± 0.2	0.001
HbA _{1c} (mmol/mol)	48 ± 5	36 ± 3	0.001
Fasting triglyceride (mmol/l)	1.6 ± 0.7	1.4 ± 0.6	0.5
Fasting total cholesterol (mmol/l)	4.0 ± 0.9	5.6 ± 1.1	0.003
Fasting LDL-cholesterol (mmol/l)	2.1 ± 0.8	3.7 ± 0.9	0.001
Fasting HDL-cholesterol (mmol/l)	1.2 ± 0.2	1.4 ± 0.4	0.3
Average daily energy expenditure (cal)	2455 ± 561	2248 ± 215	0.4
Average daily steps taken	6160 ± 1090	5701 ± 815	0.4

Values are mean ± SD. T2D, type 2 diabetes

3.2.2 Experimental protocol

All subjects refrained from any physical exertion during the 3 days preceding the studies and fasted overnight for 12 hours before the experiments. Metformin was withdrawn 3 days before each experiment. For all experiments, subjects travelled to the Newcastle Magnetic Resonance Centre by taxi and were transported within the centre by wheelchair. At 0830 hours (-270 min), one cannula was inserted into an antecubital vein for administration of glucose and insulin. A second cannula was inserted into the contralateral wrist vein for blood sampling. Hand warming device was used to ensure arterialisation of venous blood (Copeland et al., 1992).

3.2.2.1 *Isoglycaemic-hyperinsulinaemic clamp tests*

Basal blood samples were taken and muscle spectroscopy to measure ATP turnover rate, glycogen and IMCL were performed (-260 to -180 min). Subjects were then given either acipimox 250mg or identical placebo tablets in a double blind fashion. Further ^{31}P and ^{13}C MR spectroscopy were acquired (-60 to -10 min) before the start of the isoglycaemic-hyperglycaemic clamp (DeFronzo et al., 1979). Isoglycaemia was maintained in order to ensure that the true basal condition of each participant could be observed. Insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was administered as a primed-continuous infusion (40 mU/m^2 body surface area/min) from 0 to 150 mins. To inhibit pancreatic hormone secretion, somatostatin was infused at $0.06 \mu\text{g/kg/min}$ (Somatostation-UCB; UCB Pharma, Netherlands) from 5 min before the start of the insulin infusion and continued for the duration of the clamp. In order to increase sensitivity of measurement of the glycogen synthesis rate by ^{13}C MR spectroscopy, the variable glucose infusion contained 20% [$1\text{-}^{13}\text{C}$]-glucose (Cambridge Isotope Laboratories, Andover, MA). A further dose of acipimox 250mg or placebo was given before the start of the clamp. Two further sets of ^{31}P and ^{13}C MR spectroscopy measurements were acquired during the clamp.

The protocol is illustrated diagrammatically in Figure 3.1. Control subjects were studied with the administration of placebo tablets only. To permit the frequent MR measurements, subjects were studied lying in the scanner for the entire duration of each study (~6 hours). Subjects lay in a supine position and had their arm with the wrist vein cannula outstretched in a 90° angle to allow for frequent blood sampling.

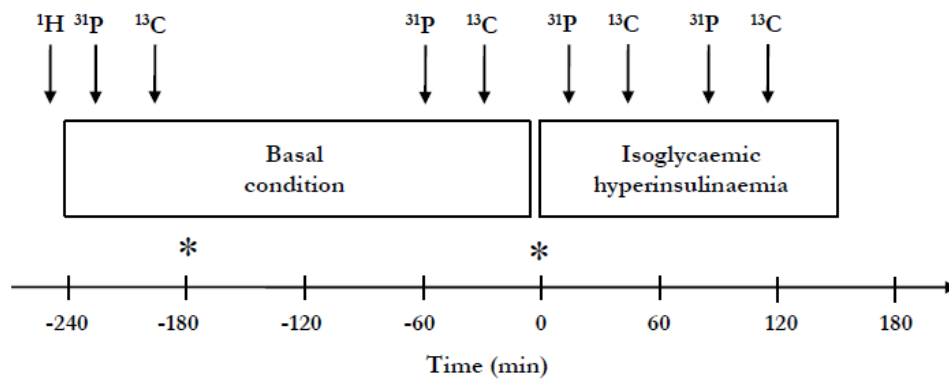


Figure 3.1 Schematic representation of experimental protocol where * denotes either doses of acipimox (250 mg) or identical placebo tablets were administered.

3.2.2.2 Hyperglycaemic-hyperinsulinaemic clamp tests

A hyperglycaemic-hyperinsulinaemic clamp was performed in all subjects on a separate day to examine the combined effect of insulin and glucose. The experimental protocol was the same as that for the isoglycaemic-hyperinsulinaemic clamp except that plasma glucose was clamped at a stable level 5 mmol/l above fasting concentrations. The protocol is illustrated diagrammatically in Figure 3.2. Studies for each subject were carried out not

more than 4-8 weeks apart, during which their body weight and lifestyle remained unchanged during the period of the study.

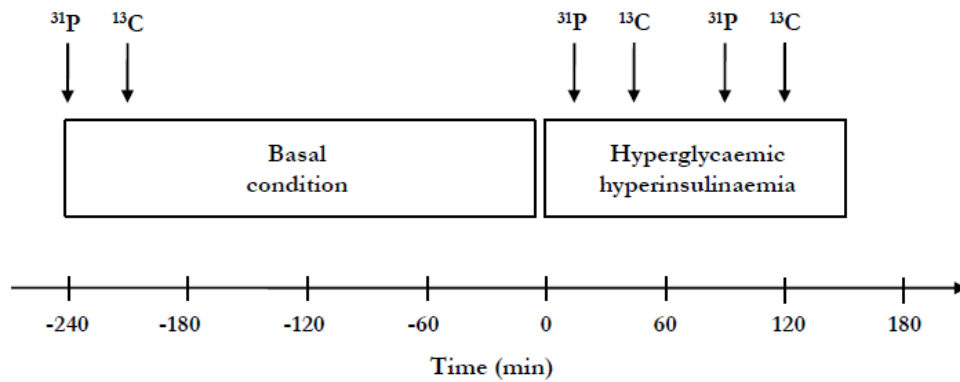


Figure 3.2 Schematic representation of the hyperglycaemic-hyperinsulinaemic clamp test.

3.2.3 Statistical analysis

Data are presented as mean \pm SD, unless otherwise stated. Statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Prior to any comparisons, the data was tested for normality using Shapiro-Wilk test. Statistical comparisons between diabetic and control groups were performed using Student's *t* test while within group differences were determined using paired *t* test where appropriate. Changes of sequential data within experiments were evaluated by repeated measures analysis of variance (ANOVA) with post hoc Tukey correction where appropriate. Non-parametric tests were used to determine differences for non-normally distributed data. Correlations were examined using Spearman rank test. Statistical significance was accepted at $p < 0.05$.

3.3 Results

3.3.1 Plasma glucose, insulin and NEFA

The experimental conditions necessary to test the hypothesis were achieved. During the 3 hour basal period, plasma glucose concentration remained steady in the control subjects (5.1 ± 0.3 to 5.0 ± 0.3 mmol/l) and gradually fell as expected in the diabetic subjects (7.7 ± 1.1 to 6.5 ± 1.0 mmol/l; $p = 0.002$). As per protocol, plasma glucose concentration was clamped at 5.0 ± 0.3 mmol/l in control group, 6.6 ± 0.8 mmol/l in diabetes/placebo group and 6.4 ± 0.6 mmol/l in diabetes/acipimox group respectively ($p < 0.001$ diabetes vs. control; Figure 3.3A). Average clamp plasma insulin concentration for all three experimental conditions was stable at 573 ± 61 pmol/l (Figure 3.3B). Plasma NEFA concentrations rapidly suppressed below 0.2 mmol/l in the diabetes/acipimox study ($p = 0.005$) and were stable; plasma NEFA concentrations were similar in diabetes/placebo and control groups until commencement of insulin infusion (Figure 3.3C). Plasma enrichment of ^{13}C -glucose increased steadily upon the [$1\text{-}^{13}\text{C}$] glucose infusion in all clamps (Table 3.2).

Table 3.2 Plasma enrichment of ^{13}C -glucose (APE) during the studies

Time (min)	0	15	45	90	150
<i>Isoglycaemic clamp:</i>					
Control	0	3.8 ± 1.1	7.5 ± 2.1	12.3 ± 1.9	15.2 ± 2.0
Diabetes/P	0	3.0 ± 1.4	6.3 ± 1.6	9.7 ± 1.9	12.2 ± 1.8
Diabetes/A	0	3.8 ± 1.3	8.0 ± 1.4	11.3 ± 1.9	14.2 ± 2.0
<i>Hyperglycaemic clamp:</i>					
Control	0	7.5 ± 2.0	11.2 ± 2.5	13.3 ± 2.1	15.0 ± 1.8
Diabetes	0	4.8 ± 1.4	9.7 ± 1.6	13.1 ± 2.3	15.4 ± 2.4

Values are mean \pm SD. A, acipimox; P, placebo

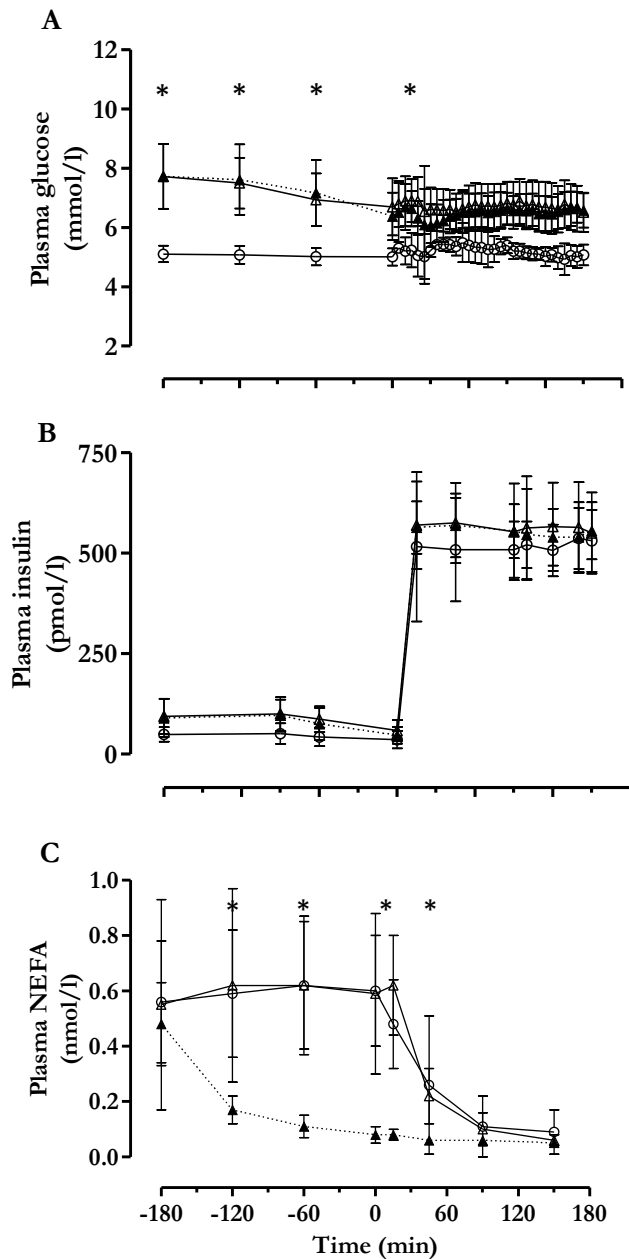


Figure 3.3 Time course of (A) plasma glucose, (B) insulin and (C) NEFA concentrations during the isoglycaemic-hyperinsulinaemic clamps in control (○), diabetes/placebo (△) and diabetes/acipimox (▲) studies. Values are mean \pm SD. * $p < 0.05$ diabetes vs. control.

3.3.2 IMCL content

The ratio of IMCL to water was not different between the diabetes subjects and controls (0.022 ± 0.012 vs. 0.018 ± 0.009 , $p = 0.452$).

3.3.3 Glucose disposal rates and muscle glycogen

During the clamp, glucose disposal rate was lower in the diabetes/placebo group compared with the control group (4.8 ± 2.0 vs. 6.6 ± 1.4 mg/kg_{ffm}/min, $p = 0.04$; Figure 3.4A). Prior suppression of plasma NEFA by acipimox brought about a 23% increase of glucose disposal rate in the type 2 diabetic group (6.2 ± 2.6 vs. 4.8 ± 2.0 mg/kg_{ffm}/min, acipimox vs. placebo respectively, $p = 0.005$; Figure 3.4A) so that it approached that of the control group (6.6 ± 1.4 mg/kg_{ffm}/min; $p = 0.72$). Fasting muscle glycogen concentration was similar in all three groups (diabetes/placebo: 67.5 ± 14.3 mmol/l, diabetes/acipimox: 69.0 ± 6.8 mmol/l and control: 71.1 ± 7.3 mmol/l) and remained unchanged during the basal period. The increment in muscle glycogen concentration after 70 min of insulin stimulation was small and not significantly different between the three groups. By the end of the clamp, insulin-stimulated muscle glycogen concentration increased by 3.8 ± 2.1 mmol/l in the control group and 1.9 ± 3.3 mmol/l in the diabetes/placebo group ($p < 0.05$; Figure 3.4B). Prior administration of acipimox did not change the increment in muscle glycogen (2.0 ± 2.3 mmol/l; Figure 3.4B). The mean rate of muscle glycogen synthesis from 70 to 150 min was 40 ± 19 μ mol/l/min in the control subjects, 19 ± 27 μ mol/l/min in the diabetes/placebo and 22 ± 24 μ mol/l/min in the diabetes/acipimox groups ($p < 0.05$ for control vs. both diabetes groups).

3.3.4 Breath ¹³C enrichments

¹³C APE in expired breath increased during the clamp in all groups (control 0.23 ± 0.10 and 0.44 ± 0.15; diabetes/placebo 0.14 ± 0.04 and 0.31 ± 0.08; diabetes/acipimox 0.22 ± 0.05 and 0.44 ± 0.10 at 90 and 150 min respectively). In order to compare rates of glucose oxidation corrected for plasma glucose enrichment the ratio of breath to plasma ¹³C APE was examined. At the end of the clamp period this index of glucose oxidation was 2.53 ± 0.53 vs. 3.16 ± 0.67 for diabetes/placebo and diabetes/acipimox respectively ($p < 0.005$) and 2.87 ± 0.69 for controls (Figure 3.4C).

3.3.5 Muscle ATP turnover rate

Muscle ATP turnover rates are shown in Table 3.3 and Figure 3.5. Baseline ATP turnover rate in the diabetes/placebo group (8.6 ± 2.6 μmol/g/min of muscle) was similar to that of the control subjects (8.6 ± 2.0 μmol/g/min of muscle). Muscle ATP turnover rate in the control group did not change during the basal period or during the insulin infusion (end of clamp: 8.6 ± 3.6 μmol/g/min of muscle). Although not statistically significant, there was a suggestion of a declining trend in ATP turnover rate during insulin infusion in the diabetes/placebo group (end of clamp: 7.1 ± 1.6 μmol/g/min of muscle, $p = 0.09$). This suggestion of a decline was not seen in the diabetes/acipimox group such that by the end of the clamp, ATP turnover rate was significantly higher than in the diabetes/placebo group (8.4 ± 2.1 vs. 7.1 ± 1.6 μmol/g/min of muscle; $p = 0.02$).

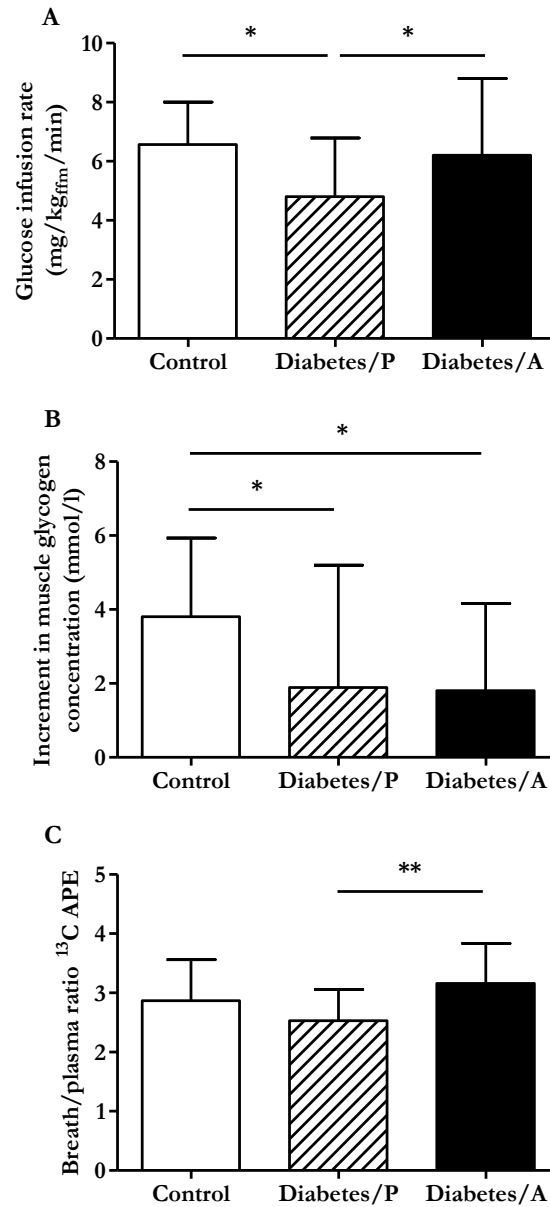


Figure 3.4 (A) Glucose infusion rates during the final 30 minutes in the isoglycaemic-hyperinsulinaemic clamps: control, diabetic with placebo and diabetic with acipimox. (B) Incremental changes in muscle glycogen concentration in the three studies. (C) Ratio of breath ¹³CO₂ to plasma ¹³C-glucose APE at the end of the each study. Values are mean ± SD. **p* < 0.05; ***p* < 0.005.

Table 3.3 Muscle ATP turnover rates ($\mu\text{mol/g/min}$) during the studies

Time	-180 to -150	-60 to -30	15 to 45	90 to 120
	min	min	min	min
<i>Isoglycaemic clamp:</i>				
Control	8.6 ± 2.0	8.3 ± 2.6	9.3 ± 3.1	8.6 ± 3.6
Diabetes/P	8.6 ± 2.6	7.4 ± 1.6	7.7 ± 2.1	7.1 ± 1.6
Diabetes/A	9.0 ± 2.1	8.4 ± 1.8	9.8 ± 3.0	8.4 ± 2.1
<i>Hyperglycaemic clamp:</i>				
Control	8.1 ± 2.4	-	8.9 ± 2.0	$10.0 \pm 2.6^*$
Diabetes	8.7 ± 2.2	-	8.1 ± 1.9	8.6 ± 2.3

Values are mean \pm SD. A, acipimox; P, placebo

* $p < 0.05$ vs. baseline.

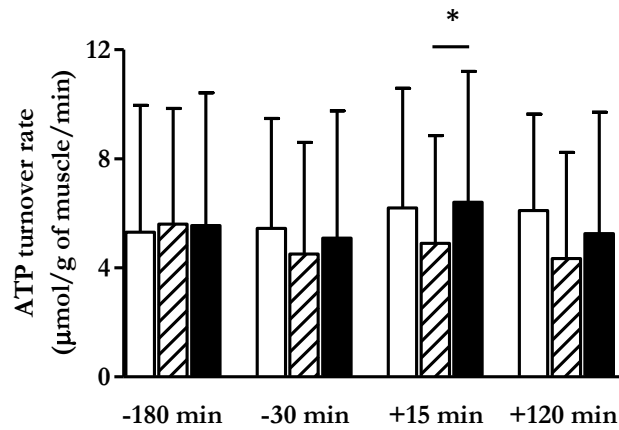


Figure 3.5 Muscle ATP turnover rate in control (open bar), diabetic with placebo (hatched bar) and diabetic with acipimox (filled bar) during basal period and after 120 min of isoglycaemic hyperinsulinaemia. Values are mean \pm SD. * $p < 0.05$.

3.3.6 Whole-body metabolism during hyperglycaemic-hyperinsulinaemic clamps

The steady state plasma glucose concentrations necessary to test the second hypothesis were achieved. During the basal period, plasma glucose concentration decreased steadily in the diabetic group (7.7 ± 1.1 to 6.5 ± 1.0 mmol/l, $p = 0.002$) and remained steady in the control group (5.1 ± 0.3 to 5.0 ± 0.3 mmol/l, $p = 0.186$). After observation of the basal state (-240 min to 0 min), the clamp period was characterized by stable plasma glucose and plasma insulin concentrations (Figure 3.6). During this protocol, plasma glucose concentration was clamped at 10.6 ± 0.5 and 13.0 ± 1.1 mmol/l for control and diabetic subjects respectively ($p < 0.01$). Plasma enrichment of ^{13}C -glucose increased steadily upon the $[1-^{13}\text{C}]$ glucose infusion during the studies (Table 3.2).

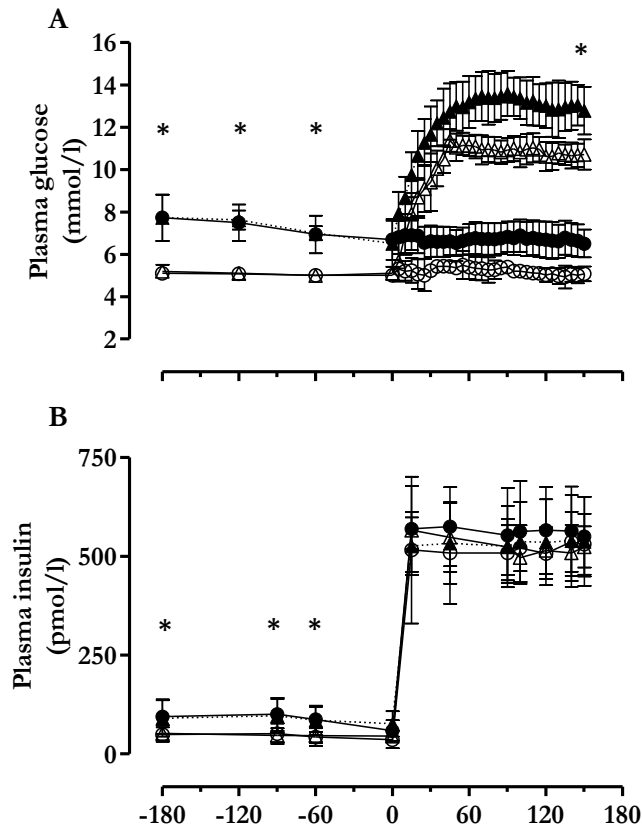


Figure 3.6 Time course of (A) plasma glucose and (B) plasma insulin concentrations during the two experimental conditions: isoglycaemia in control (○) and in diabetic (●) subjects; hyperglycaemia in control (△) and in diabetic (▲) subjects. Values are mean \pm SD. * $p < 0.01$ control vs. diabetes.

Data comparing glucose disposal rate, muscle glycogen synthesis and muscle ATP turnover rate during isoglycaemic hyperinsulinaemia and hyperglycaemic hyperinsulinaemia in the control and diabetes subjects is shown in Figure 3.7. Glucose disposal rate in the diabetes group increased 1.6-fold compared with the isoglycaemic clamp (7.7 ± 2.8 vs. 4.8 ± 2.0 mg/kg_{ffm}/min; $p = 0.005$). Hence, the glucose disposal rate became similar to that of the control subjects at isoglycaemic hyperinsulinaemia (7.7 ± 2.8 vs. 6.6 ± 1.4

mg/kg_{ffm}/min; $p = 0.573$). During hyperglycaemic clamps in the control group, glucose disposal rate also increased 1.6-fold compared with isoglycaemic clamps (10.5 ± 3.1 vs. 6.6 ± 1.4 mg/kg_{ffm}/min; $p = 0.01$).

Fasting muscle glycogen concentrations were similar on the isoglycaemic and hyperglycaemic clamp days (67.5 ± 14.3 and 67.0 ± 12.0 mmol/l respectively for diabetic subjects; and 71.1 ± 7.3 and 72.3 ± 17.6 mmol/l respectively for control subjects). The mean rate of muscle glycogen synthesis from 70 to 150 min was lower in the diabetic group compared to controls during isoglycaemia (19 ± 27 vs. 40 ± 19 μ mol/l/min; $p = 0.012$). This subnormal rate in the diabetic subjects at isoglycaemia was increased 2.2-fold by hyperglycaemia (19 ± 27 to 41 ± 38 μ mol/l/min ($p = 0.013$), making the glycogen synthesis rate almost identical to that of the controls at isoglycaemia (40 ± 19 μ mol/l/min; $p = 0.460$). In control subjects, the mean rate of muscle glycogen synthesis between 70 and 150 min was increased 2.5-fold by hyperglycaemia compared with isoglycaemia (100 ± 67 vs. 40 ± 19 μ mol/l/min; $p = 0.028$). The absolute increment in glycogen concentration in the diabetic subjects during hyperglycaemia was similar to that seen during isoglycaemic clamp conditions in the control group (3.8 ± 4.2 vs. 3.8 ± 2.1 mmol/l, $p = 0.515$). In the control subjects during hyperglycaemia, the increment in glycogen concentration was three-fold higher compared to during isoglycaemia (11.1 ± 7.6 vs. 3.8 ± 2.1 mmol/l, $p = 0.012$).

Muscle ATP turnover rates are shown in Table 3.3 and Figure 3.7. In diabetic subjects, during isoglycaemia, muscle ATP turnover tended to decline (8.6 ± 2.6 to 7.1 ± 1.6 μ mol/g/min; $p = 0.09$), whereas hyperglycaemia prevented this decline in insulin-stimulated muscle ATP turnover rates (7.1 ± 1.6 and 8.6 ± 2.3 μ mol/g/min for isoglycaemia and hyperglycaemia respectively; $p = 0.04$). In control subjects, during the hyperglycaemic-hyperinsulinaemic clamps, insulin increased muscle ATP turnover rate by 23% (8.1 ± 2.4 vs. 10.0 ± 2.6 μ mol/g/min; $p = 0.025$). Overall, muscle ATP

turnover rates correlated positively with muscle glycogen synthesis rates ($r_s = 0.46, p = 0.005$; Figure 3.8).

^{13}C APE in expired breath increased steadily during the hyperglycaemic-hyperinsulinaemic clamps in both the diabetic and control groups (diabetes: 0.28 ± 0.08 and 0.50 ± 0.10 ; control: 0.44 ± 0.20 and 0.72 ± 0.22 at 90 and 150 min respectively). Whole-body glucose oxidation index was higher during the hyperglycaemic clamps compared to the isoglycaemic clamps in both diabetic (3.32 ± 0.67 vs. $2.53 \pm 0.53, p = 0.001$) and control subjects (5.06 ± 2.39 vs. $2.87 \pm 0.69, p = 0.008$).

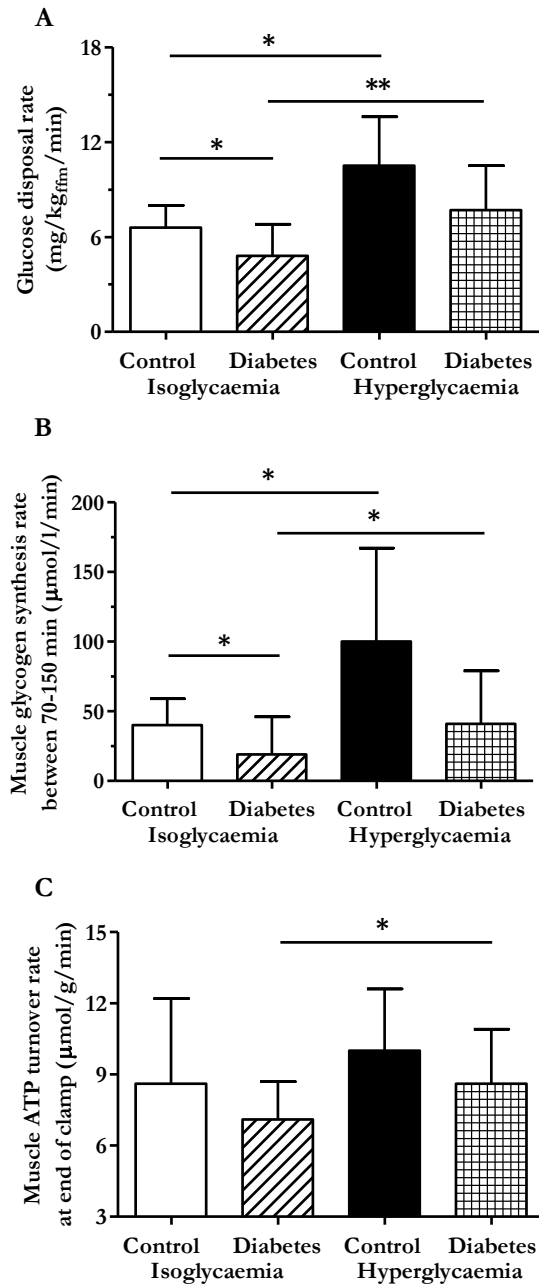


Figure 3.7 (A) Glucose disposal rate during the final 30 mins of the clamps, (B) muscle glycogen synthesis rate between 70-150 min and (C) muscle ATP turnover rate between 90-120 min in the isoglycaemic- and hyperglycaemic-hyperinsulinaemic clamps. Values are mean \pm SD. * $p < 0.05$ and ** $p < 0.01$.

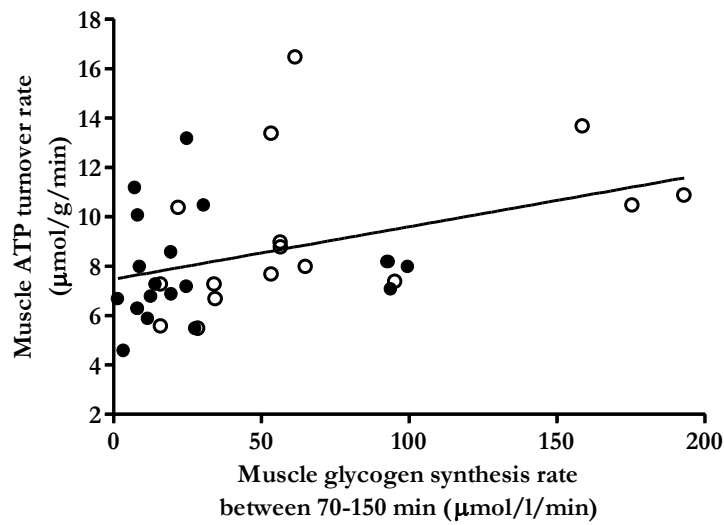


Figure 3.8 Positive correlation between muscle glycogen synthesis rate between 70-150 min and muscle ATP turnover rate in control (○) and diabetic (●) subjects ($r_s = 0.46, p = 0.005$).

3.4 Discussion

In the first part of this study, results showed that prior suppression of lipolysis effectively normalizes glucose infusion rate during hyperinsulinaemia in type 2 diabetes, but has no effect upon the rate of muscle glycogen synthesis. In contrast, the subnormal rate of whole-body glucose oxidation in type 2 diabetes was increased. The muscle ATP turnover rate was found to be normal in type 2 diabetes but could be modulated to a degree by suppression of lipolysis.

In type 2 diabetes, insulin-stimulated whole-body glucose uptake was observed to be associated with a 2-fold lower incorporation of ^{13}C glucose into glycogen. Acipimox brought about a 23% increase in glucose infusion rate during hyperinsulinaemia, similar to that reported by others (Vaag et al., 1991b, Bajaj et al., 2005). Previously observed increases in glucose disposal rates during

acipimox administration have been attributed to change in rate of glycogen synthesis (Vaag et al., 1991b, Lindegaard et al., 2007). Clearly this was not the case over the 6 hours of NEFA suppression in the present study, a period which has been shown to be sufficient for the effect upon glucose metabolism to be maximal (Roden et al., 1996). The concept that the synthesis rate of glycogen would be increased under such circumstances derives from the observation of an increase in the active form of muscle glycogen synthase, even though no change in muscle glycogen concentrations could be detected (Vaag et al., 1991b). Subsequently it has been demonstrated that a change in the activation state of glycogen synthase by suppression of lipolysis occurs in the absence of change in the proximal steps of insulin activation of glycogen synthase (GSK-3 β and Akt Thr308) (Lindegaard et al., 2007), and its relevance to substrate flux must be questioned.

Glucose infusion rate is a useful independent measure of overall glucose disposal, but does not take into account possible incomplete suppression of hepatic glucose production during the clamp. An estimate of endogenous glucose production can be obtained from the measured isotopic enrichments of infusate and plasma together with glucose infusion rate (Ravikumar et al., 2008). However, as this is affected by recycling of the ^{13}C , it will be a modest underestimation. Hepatic glucose production was 0.01 ± 0.79 , 1.13 ± 0.56 and 0.39 ± 0.75 mg/kg_{ffm}/min in control, diabetes/placebo and diabetes/acipimox groups. By adding this value to the glucose infusion rate, total glucose disposal rates can be estimated to be 6.61 ± 0.79 , 5.93 ± 1.97 and 6.59 ± 2.30 mg/kg_{ffm}/min respectively. Hence, acipimox brought about an 11% increase in total glucose disposal. Isoglycaemic-hyperinsulinaemic clamps were used in this study in order to observe the physiological mechanisms operating at ambient plasma glucose concentrations in the diabetic and control subjects, and in respect of the primary comparison between acipimox and placebo studies in the diabetic subjects clamp glucose levels were similar.

Whole-body glucose oxidation rate was increased approximately 25% by acipimox administration. This could not be quantified using indirect calorimetry in the present study as subjects remained in the 3 Tesla magnet throughout the test periods. However, the extent of acipimox-induced change in glucose oxidation has previously been shown to be approximately one third of the simultaneous change in rate of whole-body glucose uptake (Vaag et al., 1991b). It is likely that processes additional to glucose oxidation and muscle glycogen synthesis could explain the change in whole-body glucose uptake. Glycolysis with lactate release (Cori cycle activity) could account for some of the change. Approximately 15% of a glucose load is taken up by skeletal muscle then released as lactate in normal health (Kelley et al., 1988) and accounts for approximately 45% of systemic lactate appearance (Consoli et al., 1992). In type 2 diabetes, the proportion of glucose taken up by muscle then released as lactate is 2 to 3-fold higher than normal during hyperinsulinaemia (Mitrakou et al., 1990, Capaldo et al., 1990). This flux could be affected if early steps in glucose metabolism were rate limiting so that the increased transport of glucose into the muscle cell by low fatty acid availability caused increased export from muscle as lactate, and an 18% increase in plasma lactate has been observed to accompany acipimox-induced suppression of lipolysis during a clamp (Vaag et al., 1991a). Further work is required to examine specifically the acute changes in glycolysis and glucose oxidation in response to change in NEFA supply.

Although the change in plasma NEFA is a useful general indicator of rate of lipolysis, suppression of lipolysis would be expected to exert greatest effect upon muscle metabolism by preventing breakdown of intramyocellular triglyceride and decreasing intracellular availability of fatty acid and diacylglycerols. Evidence is accumulating for diacylglycerols to be major modulators of substrate level control of metabolism (Chibalin et al., 2008, Erion and Shulman, 2010). In the present study the overweight control subjects were not restudied with acipimox, and although it would appear likely that similar effect would be produced, this awaits experimental verification. As all

subjects with type 2 diabetes were taking statin therapy, reflecting current clinical practice, the possible effect of this on glucose metabolism must be considered. Statins do not affect glucose tolerance nor modulate 72 hour glucose profiles, but may bring about a very small beneficial effect on insulin sensitivity as shown in a randomized crossover study designed for this purpose (Huptas et al., 2006). No impact of statins would be expected on the primary comparison of acipimox or placebo exposure within the diabetic group.

In the second part of this study, the design was successful in using the mass action effect of acute hyperglycaemia to increase the rate of glycogen synthesis in muscle of type 2 diabetes subjects to that of the non-diabetic control group studied at isoglycaemic hyperinsulinaemia. Hyperglycaemia increased muscle glycogen synthesis rate by 2.2-fold in the diabetic subjects and by 2.5-fold in the control subjects with a simultaneous increase in muscle ATP turnover rates (1.2-fold for both). Muscle ATP turnover rates were found to be positively correlated with muscle glycogen synthesis rates. Hyperglycaemic clamp studies have previously demonstrated that acute hyperglycaemia, by the mass-action effect of glucose, can stimulate oxidative and non-oxidative glucose disposal (Yki-Jarvinen et al., 1987, Yki-Jarvinen, 1990). Vaag and colleagues showed that fasting and insulin-stimulated glucose oxidation, glucose storage and muscle glycogen synthase activation were all fully normalized during hyperglycemia in type 2 diabetic patients (Vaag et al., 1992). Rate of insulin-stimulated glucose disposal in type 2 diabetic subjects was shown to be 57% greater during hyperglycaemia compared with euglycaemia, and the major part (89%) of the increase in glucose metabolism during hyperglycaemia was due to an increase in non-oxidative glucose metabolism (Franssila-Kallunki et al., 1992).

Overall in this study, insulin had no effect upon muscle ATP turnover rates in neither diabetic nor exercise-, weight- and age-matched non diabetic subjects. This is in contrast with previous work which observed that acute elevation of insulin to post-prandial levels would increase muscle ATP turnover rate in healthy normoglycaemic controls but not in the diabetic subjects (Petersen et

al., 2004, Szendroedi et al., 2007, Petersen et al., 2005b). Hence, the present study was designed with this expectation. When it became clear that there was no stimulation of ATP turnover rates in the matched control subjects of the present study, a separate investigation of time course of stimulation of muscle ATP turnover rates was conducted. This study is presented in Chapter 4. There is also the considerable variation amongst ATP turnover rates at different glycogen synthesis rates observed during isoglycaemia and hyperglycaemia to consider (Figure 3.8). The reason for this is unclear, as is the proportion of ATP utilization related to glycogen synthesis *per se*. Bajpeyi and co-workers also reported a broad range of maximal ATP synthesis rate, determined from the rate of phosphocreatine recovery, within type 2 diabetes subjects whereby 52% had maximum ATP synthesis rates that were within the range observed in healthy sedentary controls and 24% had overlapped with the active control group (Bajpeyi et al., 2011).

In vivo skeletal muscle ATP turnover rate has typically been observed over considerably longer periods of time to achieve a summed single measurement (Petersen et al., 2004, Brehm et al., 2006, Szendroedi et al., 2007). These studies used measurements of ATP turnover rate averaged over 120-350 min of insulin stimulation, and made the assumption that an insulin-stimulated increase in ATP turnover rate was responsible for the changes in glucose metabolism. The time period over which ATP turnover rate is measured is crucial. Brehm and colleagues did not observe any change in muscle ATP turnover rate during 3 hours of elevated NEFA levels despite observing a marked reduction in whole-body glucose disposal (Brehm et al., 2010). The authors only observed a decrease in insulin-stimulated ATP turnover rate when NEFA levels were elevated for more than 4 hours (Brehm et al., 2006). In the present study the time resolution of the technique allowed acquisition of data over 30 minutes time periods. This revealed a gradual fall in muscle ATP turnover rate in the type 2 diabetic subjects during the isoglycaemia study. Although the reasons for this cannot be determined from the present study, it is postulated that the

downward trend occurred as a consequence of prolonged fasting (~15 hours) and a decrease in plasma glucose concentration during the basal period of the study protocol (7.7 ± 1.1 to 6.5 ± 1.0 mmol/l), and that increased mobilization of fatty acid from intramyocellular lipid as acipimox administration prevented the fall in ATP turnover rate and would have inhibited intracellular lipolysis. This phenomenon requires further study. During hyperglycaemic-hyperinsulinaemic clamp conditions, this fall in muscle ATP turnover in the diabetic subjects was prevented. The suppression of plasma NEFA in the diabetic subjects during the 5.5 hour protocol did modestly affect the rate of muscle ATP turnover, and the converse finding of elevation of plasma NEFA and suppression of muscle ATP turnover rate has been reported (Brehm et al., 2006). However, other recent work has demonstrated decreased glucose disposal during short-term elevation of plasma NEFA without change in muscle ATP turnover rate in healthy subjects (Brehm et al., 2010). The time course of change in fatty acid supply also requires further study. Taken together, these results further emphasizes the dependence of ATP turnover rate upon intracellular substrate supply and that ATP turnover rates are not limited by the fact of the type 2 diabetes but respond to metabolic conditions.

The present results together with other recent studies suggest that changes in metabolic state rather than diabetes *per se* can affect measured ATP turnover rates in muscle. Other recent studies support this interpretation. A high fat diet decreases ATP turnover rate in muscle in rodents (Laurent et al., 2007). Raising NEFA levels in young healthy individuals for more than 6 h decreases insulin-stimulated muscle ATP production (Brehm et al., 2006). Despite early reports of studies showing an association between mitochondrial dysfunction and insulin resistance in type 2 diabetic patients, there are increasing reports of a lack of association between the two. Recent *ex vivo* studies found no effect of short term elevation of NEFA levels on mitochondrial function in healthy individuals despite reduction in whole-body glucose metabolism (Chavez et al., 2010, Brands et al., 2011). Improvement in insulin sensitivity through either

calorie restriction (Toledo et al., 2008) or pharmacological agents such as thiazolidinedione (Schrauwen-Hinderling et al., 2008) in both insulin-resistant states and type 2 diabetic patients have been observed without any accompanying changes in mitochondrial function in muscle. Using post-exercise PCr re-synthesis rate as an alternative method to assess mitochondrial function, rosiglitazone and pioglitazone were found to have opposite effects on mitochondrial function although both improve insulin sensitivity (Rabøl et al., 2010). Using the same technique, no defect was detectable in either early or late stage type 2 diabetes compared with exercise-matched normoglycaemic controls (De Feyter et al., 2008). This is in contrast to other studies which have reported reduced phosphocreatine recovery in type 2 diabetes subjects compared with matched controls (Schrauwen-Hinderling et al., 2007, Phielix et al., 2008, Meex et al., 2010). First-degree relatives of type 2 diabetes patients also showed *ex vivo* decreased mitochondrial capacity, and it has been suggested that gradual changes in mitochondrial function may occur (Phielix et al., 2008). The importance of matching for physical activity must be noted, as the greatest deficits in ATP turnover rates have been reported in a study which explicitly compared extremes of insulin sensitivity with no matching for habitual physical activity (Petersen et al., 2004). It should also be noted that different exercise protocols used in the phosphocreatine recovery method may account for the differences reported (De Feyter et al., 2008). Furthermore, the observation by Schrauwen-Hinderling and colleagues that muscle ATP turnover rates are inversely proportional to fasting blood glucose is critical in that abnormal ATP turnover cannot be an early feature explaining the onset of type 2 diabetes (Schrauwen-Hinderling et al., 2007, Phielix et al., 2008).

Ex vivo studies of mitochondria have also shown discordance between mitochondrial function and insulin sensitivity in offspring of mothers with type 2 diabetes (Irving et al., 2011). In earlier observations by Kelley et al., obese and type 2 diabetic subjects were found to have less mitochondria in their muscles than age-matched insulin-sensitive individuals (Kelley et al., 2002). The

subsarcolemmal fraction of mitochondria content has also been found to be lower in type 2 diabetes (Ritov et al., 2005). In addition, mitochondrial surface area was positively correlated with insulin-stimulated glucose disposal (Kelley et al., 2002). On the other hand, intramyofibrillar mitochondrial content has been observed not to differ between type 2 diabetes and weight-matched control subjects even though it was lower compared to lean insulin-sensitive controls (Chomentowski et al., 2011). Although the present data relate to observations during established type 2 diabetes, overall the concept of mitochondrial dysfunction as a primary abnormality in type 2 diabetes is not well supported.

The present data demonstrate that lowering fatty acid availability increased whole-body glucose uptake without increasing the rate of glucose storage as glycogen but with increase in whole-body glucose oxidation rates. A change in fasting ATP turnover rate in muscle was observed after suppression of lipolysis in type 2 diabetes, but this change was associated with no effect on muscle glycogen synthesis. The study also demonstrated that normalizing the rate of glycogen synthesis by hyperglycaemia is associated with increase in muscle ATP turnover rate in type 2 diabetes. Therefore, ATP turnover rates reflect prevailing substrate availability and defects in mitochondrial function are unlikely to underlie and initiate the metabolic abnormalities of type 2 diabetes.

Chapter 4

ACUTE EFFECTS OF INSULIN ON MUSCLE ATP TURNOVER RATES

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4.1 Introduction

ATP is the universal energy currency in cells for supporting the energy demands of various cellular activities and functions. Healthy skeletal muscle is abundant with mitochondria and relies heavily on oxidative phosphorylation for regenerating ATP from carbohydrate- and fat-based fuels. In resting skeletal muscle, mitochondrial oxidative phosphorylation provides up to 90% of cellular ATP, with the balance coming from substrate-level phosphorylation such as glycolysis. Insulin plays a key role in regulating fuel metabolism. Its main metabolic action can be summarised as anabolic affecting not only glucose but also lipid and protein metabolism. In insulin-responsive tissues such as skeletal muscle, insulin stimulates the uptake, utilization and storage of glucose and the inhibition of lipid oxidation. Insulin maintains glucose homeostasis throughout the fed and fasted states by regulating these processes.

Mitochondrial dysfunction in skeletal muscle has been reported in skeletal muscle in type 2 diabetes (Kelley et al., 2002), insulin-resistant offspring of type 2 diabetes patients (Petersen et al., 2004), obesity (Ritov et al., 2005) and in age-related insulin resistance (Petersen et al., 2003, Short et al., 2005), and it has been proposed that there is a causative link between the impairment of oxidative capacity and pathogenesis of insulin resistance and type 2 diabetes in humans (Morino et al., 2005). Several research laboratories have applied ³¹P MR spectroscopy magnetization saturation transfer methods to calculate unidirectional ATP synthesis rate (Lebon et al., 2001, Brehm et al., 2006, Trenell et al., 2008). Using this methodology, some have reported that insulin increases ATP turnover rate in muscle of normal subjects but not in insulin resistant subjects (Petersen et al., 2005b, Szendroedi et al., 2007). This has led to a widespread acceptance that a defect in ATP generation could be a direct cause of insulin resistance (Petersen et al., 2004, Szendroedi et al., 2007, Pagel-Langenickel et al., 2010, Ritov et al., 2010, Schrauwen et al., 2010).

However, all previous studies measured ATP turnover rate in muscle after an insulin infusion of 2-8 hours duration, whereas insulin is a rapid-acting regulator of metabolism which brings about major metabolic changes within minutes. The time course of stimulation of ATP turnover rate during the rapid onset of insulin's action on metabolic processes has not been previously examined. Therefore, having observed a lack of insulin stimulation on ATP turnover rates in the matched control subjects of the previous study, a separate investigation to determine the time course of insulin stimulation on muscle ATP turnover rate was designed and conducted. In this study, the ^{31}P MR spectroscopy saturation transfer method was applied continuously during the first 150 min of insulin stimulation in young, lean and normoglycaemic individuals to examine whether the time course of ATP stimulation is consistent with a primary role in insulin's metabolic effects.

4.2 Study Design

4.2.1 Subjects

Seven healthy volunteers (3 male, 4 female) without family history of diabetes were studied. None were glucose intolerant or taking any medication known to affect glucose tolerance or insulin sensitivity (for example steroids, beta-blockers or diuretics). Anthropometric and metabolic characteristics of the subjects are summarised in Table 4.1. All participants refrained from any physical exercise during the 3 days preceding the studies and fasted overnight for 12 hours before the experiments. The study protocol was approved by the Newcastle upon Tyne Research Ethics Committee 2, and informed consent was obtained from all subjects.

Table 4.1 Clinical characteristics of study subjects.

Male: female	3:4
Age (years)	28 ± 5
Weight (kg)	66.6 ± 17.4
BMI (kg/m ²)	22.9 ± 4.4
Fat mass (kg)	14.9 ± 3.6
Fat free mass (kg)	51.7 ± 14.2
Estimated body fat (%)	22.6 ± 2.8
Fasting plasma glucose (mmol/l)	5.1 ± 0.3
Fasting plasma insulin (pmol/l)	49 ± 22
HbA _{1c} (%)	5.3 ± 0.6
HbA _{1c} (mmol/mol)	34 ± 6

Values are mean ± SD.

4.2.2 Experimental protocol

For all experiments, participants travelled to the magnetic resonance facility by taxi and were transported within the centre by wheelchair. At 0800 (-90 min), one cannula was inserted into a large antecubital vein for administration of intravenous glucose and insulin. A second cannula was inserted into the contralateral wrist vein for the purpose of blood sampling. Use of a hand warming device ensured arterialisation of venous blood. Conditions of fasting glycaemia and standardised hyperinsulinaemia were achieved for 150 min using the euglycaemic-hyperinsulinaemic clamp technique (DeFronzo et al., 1979).

Skeletal muscle ATP turnover rate was measured in the left calf muscle using the saturation transfer experiment applied to the exchange between P_i and ATP. Two consecutive baseline measurements were acquired from 0830 to 0930 (-60 to -30 min and -30 to 0 min respectively, relative to the start of

infusion). During the 150 min of the hyperinsulinaemic glucose clamp, four measurements were performed (15-45 min, 50-80 min, 85-115 min, and 120-150 min). Unidirectional rates of ATP synthesis from P_i are the product of the pseudo-first order rate constant of ATP synthesis, k_1 , and the concentration of intracellular P_i . Owing to the fact that it takes 27 minutes to acquire data to measure k_1 , and that P_i concentration can increase across this time, it was decided to take the average of two measurements of P_i concentration before and after the saturation transfer measurement of k_1 in every case for calculations of ATP turnover rate. All seven subjects were studied twice on identical study days and the fourteen sets of ATP turnover rate measurements obtained were averaged. Blood samples were drawn at timed intervals for measurement of hormones and metabolite concentrations. The experimental protocol is shown in Figure 4.1.

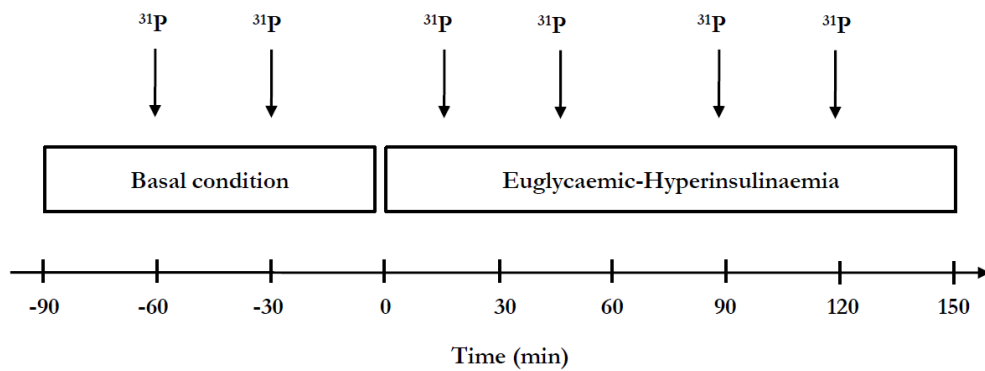


Figure 4.1 Schematic representation of the experimental protocol.

4.2.3 Statistical analysis

Statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, USA). Data are presented as mean \pm SD in the text and in the figures, unless otherwise stated. Prior to any comparisons, the data was tested for normality using Shapiro-Wilk test. Non-parametric Wilcoxon test was used to determine within group differences. Statistical significance was accepted at $p < 0.05$.

4.3 Results

4.3.1 Plasma glucose and insulin

Mean plasma glucose concentration during baseline measurement was 5.1 ± 0.3 mmol/l and was maintained at this concentration during the euglycaemic-hyperinsulinaemic period (5.2 ± 0.2 vs. 5.1 ± 0.2 mmol/l). Fasting plasma insulin concentration was 49 ± 22 pmol/l, and this increased approximately 10 fold within 15 min of the clamp. Plasma insulin concentration remained constant for the duration of the clamp (456 ± 71 pmol/l, $p < 0.001$ vs. baseline).

4.3.2 Glucose disposal rates

A rapid increase in whole body glucose disposal rate was observed via a rise of glucose infusion rate from 0 to 2.90 ± 0.42 mg/kg_{ffm}/min during the first 10 min and further to 6.17 ± 2.14 mg/kg_{ffm}/min between 15-45 min (Figure 4.2). There was a subsequent slower increase to 8.31 ± 2.52 mg/kg_{ffm}/min during the 120-150 min period.

4.3.3 Resting muscle ATP turnover rate

Resting muscle ATP turnover rates are shown in Figure 4.2. Mean baseline ATP turnover rate was $9.0 \pm 1.3 \mu\text{mol/g/min}$ of muscle. During the first 45 min of the clamp, there was no increase in ATP turnover rate to match the acute onset of the metabolic action of insulin as reflected by the steep increase in glucose utilisation. Between 50 and 80 minutes after the insulin infusion was commenced, ATP turnover rate increased by 8% and remained steady until the end of the clamp ($9.7 \pm 2.1 \mu\text{mol/g/min}$ of muscle, $p = 0.03$ vs. mean baseline).

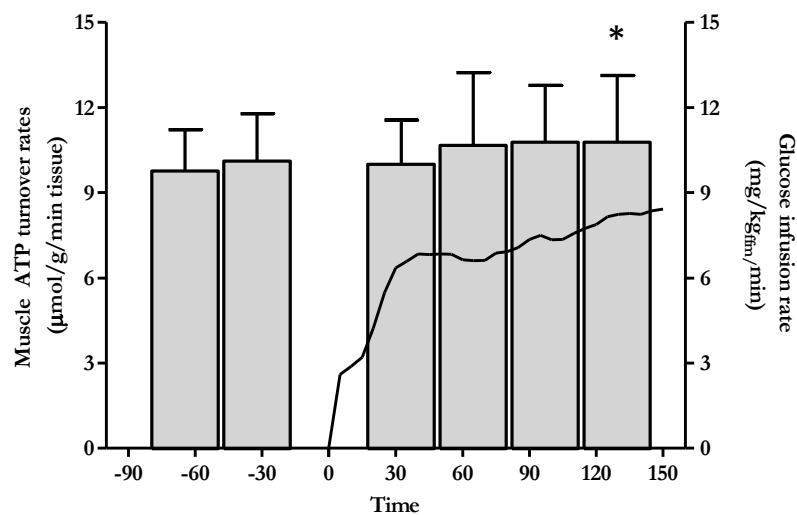


Figure 4.2 Simultaneous time course change in muscle ATP turnover rate. (represented by histogram bars) and glucose infusion rate (solid line) during the euglycaemic-hyperinsulinaemic clamp. Values are mean \pm SD. * $p < 0.05$ vs. mean baseline.

Components of the muscle ATP turnover rate measurement are presented in Table 4.2. The average of the two P_i concentration acquired at baseline was 3.62 ± 0.26 mmol/l. P_i concentration did not change during the early period of insulin infusion (15-45 min period: 3.58 ± 0.32 mmol/l, $p = 0.463$). Thereafter, P_i concentration increased to 3.98 ± 0.34 mmol/l between 50 and 80 min ($p < 0.05$ vs. baseline). This corresponded to a 14% increase compared to baseline. P_i concentration remained steady thereafter. There was no significant change in k_1 for ATP turnover rate between baseline and insulin-stimulated conditions. This was reflected by there being no significant change in either the T_1^* of P_i (the longitudinal relaxation time during γ -ATP saturation) or the fraction of P_i signal during saturation compared to control saturation (M_z/M_0) under insulin stimulation. As k_1 was unchanged, all change in ATP turnover rate was derived from change in P_i concentration.

Table 4.2 Components of the ATP turnover rate measurement at baseline and during insulin stimulation

	Baseline	During isoglycaemic hyperinsulinaemic clamp			
		15-45 min	50-80 min	85-115 min	120-150 min
k_1 (min^{-1})	4.09 ± 0.67	3.89 ± 0.77	3.91 ± 1.01	4.01 ± 0.96	4.08 ± 1.09
P_i (mmol/l)	3.62 ± 0.26	3.58 ± 0.32	$3.98 \pm 0.34^*$	$3.99 \pm 0.50^*$	$3.90 \pm 0.41^*$
T_1^* (s)	4.00 ± 0.38	4.30 ± 0.43	4.10 ± 0.54	4.25 ± 0.54	4.08 ± 0.47
M_z/M_0 (-)	0.73 ± 0.03	0.72 ± 0.04	0.73 ± 0.05	0.72 ± 0.05	0.73 ± 0.05

Values are mean \pm SD. * $p < 0.05$ vs. baseline.

4.4 Discussion

To my knowledge, this is the first study to investigate the acute effect of insulin on muscle ATP turnover rate, and to track the time course of ATP turnover rate during physiological hyperinsulinaemia. Insulin is responsible for increasing whole-body glucose disposal mainly by stimulating glycogen synthesis in the transition from fasted to fed state (Bonadonna et al., 1993). Carbon-13 MR spectroscopy studies have shown physiological hyperinsulinaemia to increase muscle glycogen concentration in as little as 15 min in healthy individuals (Shulman et al., 1990, Roden et al., 1996), which is reflected in the rapid increase in glucose disposal rate observed in this experiment.

The glucose infusion rate data shown in Figure 3.2 illustrate that the highest rate of change in glucose disposal occurs in the first 30-60 min of euglycaemic hyperinsulinaemia. The ATP turnover rate was unchanged during the first 20-50 min of the clamp, with an 8% increase after 50 min. There are notable differences with other reported measurements of ATP turnover rate during physiological hyperinsulinaemia. The single most important difference lies in the timing of the measurements. The original report of mitochondrial dysfunction associated with diabetes measured ATP turnover rate averaged over 30-150 min of euglycaemic hyperinsulinaemia (Petersen et al., 2005b) while subsequent studies compared the later effects (120-350 min) of insulin stimulation of ATP turnover rate (Szendroedi et al., 2007, Brehm et al., 2006). The long scan duration of previous studies limited the time resolution and prevented examination of time course. However, in order to determine the relationship between parameters it is important to measure ATP turnover rate during the onset of insulin action, as early as 15 min of insulin stimulation (Shulman et al., 1990). Other processes, separate from insulin's effect on muscle glycogen synthesis, such as insulin's effect on mitochondrial fusion and proliferation (Molina et al., 2009, Yang et al., 2009) and mitochondrial protein synthesis (Stump et al., 2003), may affect ATP turnover rate on a timescale of

4-8 hours of insulin stimulation. These processes are temporally dissociated from and not relevant to the early metabolic effects of insulin. They are likely to affect data on ATP turnover rates in longer studies of prolonged hyperinsulinaemia.

In the present study insulin stimulation for 150 minutes brought about an 8% increase in ATP turnover rate, less than that the 11-90% previously observed in normal control subjects (Petersen et al., 2004, Szendroedi et al., 2007). The report of a 90% increase in ATP turnover rate in insulin-sensitive subjects with insulin stimulation (Petersen et al., 2004) related to extreme phenotypes of insulin sensitivity. Additionally, the insulin resistant offspring were reported to have a low basal resting ATP turnover rate. This has not been confirmed in subsequent studies comparing subjects with and without type 2 diabetes when subjects have been matched for habitual physical activity (Szendroedi et al., 2007, Trenell et al., 2008). During a 240 minute period of hyperinsulinaemia, Szendroedi and colleagues reported a 10.5% increase in ATP turnover rates in the diabetic subjects, 11% in the age matched controls, and a 26% increase in ATP turnover rate in the young controls (Szendroedi et al., 2007). It has been widely postulated within the MR and diabetes communities that defects in mitochondrial function may underlie the reduced glucose disposal rate during hyperinsulinaemia in type 2 diabetes (Szendroedi et al., 2007, Pagel-Langenickel et al., 2010, Ritov et al., 2010, Schrauwen et al., 2010). Although the data suggest a relationship between insulin sensitivity and ATP turnover rate, this does not indicate cause and effect. Hence, the extrapolation of hypothesis from the original observations (Petersen et al., 2004) to the pathophysiology of type 2 diabetes itself has not been well justified.

In addition to the shorter period of insulin stimulation in the present study as explanation for a lesser increase of ATP turnover rate with insulin, other factors such as surface coil placement, coil diameter and acquisition sequences could affect the results in comparing between studies. The relative composition

of the radiofrequency coil's sensitive volume of gastrocnemius and soleus muscles requires consideration as soleus muscle, which has predominantly Type I muscle fibres, has been shown to have higher resting ATP turnover rate than gastrocnemius (Befroy et al., 2008). A larger coil was used in this study compared to Szendroedi et al. (Szendroedi et al., 2007) (14 cm diameter compared to 10 cm) and thus it would be expected that a higher percentage of contribution of signal from the soleus muscle.

ATP turnover rate is calculated from both the intramyocellular P_i concentration and the measured rate constant k_1 . To ensure that the estimates of ATP turnover rate were as accurate as possible during the rapidly changing P_i concentration in the first hour after insulin stimulation, the average of the P_i concentrations before and after the 27-minute saturation transfer measurement was used. Insulin-stimulated P_i concentration was observed to increase by 14% until the end of the euglycaemic hyperinsulinaemic clamp, in agreement with previous studies (Roden et al., 1999, Szendroedi et al., 2007). The concentration of P_i in the muscle tissue may be an important mechanism by which insulin regulates ATP synthesis (Bose et al., 2003): there was no change in measured reaction rate, k_1 across the two hour duration of this clamp, or in any of the parameters measured to calculate k_1 . Such changes were observed only in the early work in this field on young insulin sensitive volunteers (Petersen et al., 2004, Petersen et al., 2005b).

Measurements of ATP turnover rate by the ^{31}P saturation transfer method report flux of ATP through P_i incorporation into ATP. They are thus comprised of ATP generated by glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase reactions as well as by ATP synthase. This could result in a mismatch between oxygen consumption and ATP synthesis, as demonstrated in studies performed in aerobic yeast suspensions *Saccharomyces cerevisiae* (Brindle and Krikler, 1985) and perfused rat myocardium (Ugurbil et al., 1987). However, arterio-venous balance studies across the leg have shown that there is no net export of lactate from human muscle following

insulinisation in either type 2 diabetic subjects or leaner normal controls such as used in the present study (Dela et al., 1995). This net measurement does not exclude the possibility of a change in redundant cycling of 3-carbon compounds through the glycolytic exchange flux, but the extent to which this cycling contributes to the observed rate of overall ATP turnover still remains to be determined (Laurent, 2008).

A hallmark of muscle physiology is the ability to support a high energy demand for muscle function by phosphocreatine buffering of ATP, and by aerobic and anaerobic respiration. Thus postulation that muscle glycogen synthesis may be limited by ATP production, especially in resting muscle where ATP capacity is high, seems at odds with muscle function. In support of this, more recent reconsideration of the data supporting mitochondrial dysfunction as pathogenic for type 2 diabetes has concluded that the cause-and-effect relationship between mitochondrial dysfunction and insulin resistance is not supported by all data (Trenell et al., 2008, De Feyter et al., 2008, Turner and Heilbronn, 2008).

For the first time, the early time course of skeletal muscle ATP turnover in response to insulin has been quantified in young, healthy controls. There was no increase in ATP turnover rate during the first hour of exposure to a high physiological concentration of insulin, the timescale within which glycogen synthesis is initiated. ATP turnover rate was observed to increase only after 1 hour, indicating that glycogen synthesis is not limited by ATP availability in healthy controls. This provides further support that mitochondria have no direct role in the origins of defective insulin sensitivity in type 2 diabetes.

Chapter 5

EFFECT OF CHANGES IN INTRA- ORGAN FAT CONTENT ON GLUCOSE HOMEOSTASIS IN TYPE 2 DIABETES

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5.1 Introduction

The pathogenesis of type 2 diabetes is complex but it is clear that both defects in β -cell function and insulin sensitivity are necessary prerequisites. Type 2 diabetes has long been regarded as a chronic progressive condition, capable of amelioration but not cure. The striking findings of the United Kingdom Prospective Diabetes Study (UKPDS) affirmed the clinically recognised progressive nature of the disease (1998). A steady rise in plasma glucose occurred irrespective of the degree of control or type of treatment. In fact, only 25% of the subjects in the intensive arm of the study achieved target HbA_{1c} of less than 7% with monotherapy alone after 9 years (Turner et al., 1999). The UKPDS also demonstrated that β -cell function, assessed using the homeostasis model assessment (HOMA), declined linearly with time without a change in insulin sensitivity, and after 10 years over 50% of individuals required insulin therapy (1995). Similar observations were made in the Belfast diet intervention study in which progressive deterioration in glycaemic control was associated with a progressive deterioration in β -cell function (Levy et al., 1998). The underlying changes in β -cell function have been well described (Weyer et al., 1999a, Kahn, 2003), and β -cell mass decreases steadily during the course of type 2 diabetes (Butler et al., 2003a, Hanley et al., 2010) whereas insulin resistance in muscle and liver can be modified at least to some extent (Williams et al., 2003). Overall, type 2 diabetes is inexorably progressive, with a high likelihood of insulin therapy being eventually required to maintain good glycaemic control.

However, it has been recently shown that both defects of β -cell and insulin resistance can be reversed in type 2 diabetes subjects after weight loss surgery (Camastra et al., 2007). Mean fasting plasma glucose fell from 7.7 to 5.1 mmol/l and HbA_{1c} from 8.2 to 4.1 % following biliopancreatic diversion in a group of severely obese type 2 diabetes patients. Insulin sensitivity, as measured by glucose infusion rate during euglycaemic-hyperinsulinaemic clamp, doubled when assessed at 24 months (glucose disposal rate rose from 26.4 to 55.2

$\mu\text{mol}/\text{kg}_{\text{ffm}}/\text{min}$). Most striking was the return of β -cell sensitivity to the level of non-diabetic post-surgery control group (from 44 to 76 $\text{pmol}/\text{min}/\text{m}^2$ per mmol/l , compared to 65 $\text{pmol}/\text{min}/\text{m}^2$ per mmol/l in the non-diabetic controls).

The normalisation of plasma glucose concentration occurred within days of surgery, long before major weight loss has occurred, and it has become widely assumed that the protective effects of gastrointestinal surgery are mediated by altered secretion of incretin hormones (Rubino et al., 2006, Kashyap et al., 2010). Improved control of blood glucose in type 2 diabetes by moderate energy restriction has been demonstrated by others (Petersen et al., 2005a). Petersen and colleagues observed a marked fall in fasting plasma glucose after an 8% decrease in body weight achieved over 7 weeks (Petersen et al., 2005a). The authors reported improved insulin sensitivity and normalisation of insulin suppression of hepatic glucose production which were associated with a fall of 81% in hepatic fat content. It is established that hepatic fat is mobilised within days of onset of hypocaloric feeding (Hollingsworth et al., 2006). This early and significant decrease in hepatic fat occurred at the same time as normalisation of hepatic insulin sensitivity and fall in fasting blood glucose, thus reflecting the tight relationship between hepatic response to insulin and hepatic fat content. Direct observations on the pancreas in type 2 diabetes have shown that the triglyceride content is twice as great as in normal individuals and is negatively correlated to β -cell function (Tushuizen et al., 2007). In animal models of type 2 diabetes, ectopic pancreatic triglyceride has been shown to affect β -cell dysfunction through the process of lipoapoptosis (Lee et al., 1994, Shimabukuro et al., 1998).

It is therefore hypothesised that the profound effect of a sudden negative energy balance could explain the post-bariatric surgery effect (Taylor, 2008) and that the decrease in the intracellular fatty acid concentrations in the liver would lead to a lower export of lipoprotein triacylglycerol to the pancreas, with reduced chronic inhibitory effects of excess fatty acid exposure on the β -cells.

This provides a unifying aetiology for the twin defects of β -cell dysfunction and insulin resistance that characterises type 2 diabetes. In order to test this hypothesis, weight loss was induced by an 8 week hypocaloric diet protocol in a group of patients with type 2 diabetes of less than 4 years duration. Changes in insulin secretory capacity, hepatic and peripheral insulin sensitivity to glucose were studied after 1, 4 and 8 weeks to define the sequence of pathophysiological events involved in resolution of type 2 diabetes. Additionally, the simultaneous changes in triglyceride content of the pancreas and liver were quantified to examine the mechanistic basis of the observed outcomes.

5.2 Study Design

5.2.1 Subjects

Individuals with type 2 diabetes who fulfilled the following inclusion criteria were recruited: 1) age between 35 and 65 years, 2) a diagnosis of diabetes not more than 4 years in duration, 3) HbA_{1c} between 6.5 and 9.0% (48-75 mmol/mol), 4) on treatment with diet alone or metformin alone or metformin plus sulphonylurea, and 5) BMI of 25-45 kg/m² with stable body weight for the preceding 3 months. Subjects were excluded if they: 1) had evidence of renal dysfunction (defined by serum creatinine > 150 mmol/l) or liver dysfunction (defined by serum alanine transaminase > 2.5 fold above upper limit of the reference range), 2) had contraindications to MRI, and 3) were on treatment with thiazolidinediones, insulin, steroids or beta blockers. At the time of study enrolment, three subjects' diabetes were controlled with diet, nine others were taking metformin alone, while one subject was treated with sulphonylurea alone and the remaining two subjects were taking metformin and sulphonylureas to control their diabetes. Subjects discontinued sulphonylureas two months before the baseline study. Metformin was discontinued one week before the baseline

study. Statin therapy was continued. Dietary adherence was assessed using capillary ketone levels (Xceed Optium; Abbott Diabetes Care, Maidenhead, UK) to assist ongoing monitoring and counselling and not for the purpose of testing study outcome. Capillary ketone levels were measured following an overnight 12 hour fast at baseline, week 1, 4 and 8 of the dietary intervention. An increase in capillary ketone level occurs after the first week of a very-low-calorie diet (VLCD) subsequent to increased fat catabolism (Henry et al., 1986). Therefore, a doubling of capillary ketone level was considered to indicate net lipolysis and dietary adherence. Weight loss of $> 2.8\%$ of initial body weight after one week of the diet plus total weight loss of $> 8\%$ at the end of the diet were considered to have achieved the necessary weight loss to be included in the study analysis. Three subjects failed to comply with the diet (two during the first week and one during weeks 4-8), and one left the study for an unrelated medical reason. Hence, eleven subjects completed the study (nine male and two female, age 49.5 ± 8.4 years). Their baseline metabolic and anthropometric data are summarised in Table 5.1 and 5.2 respectively.

A group of nine subjects with normal glucose tolerance matched for sex, age and BMI were also studied (seven male and two female, age 49.7 ± 7.5 years). These subjects had neither a family history of diabetes nor were taking any medication known to affect glucose tolerance or insulin sensitivity. Normal glucose metabolism was confirmed by a standard oral 75g glucose tolerance test. Their baseline metabolic and anthropometric data are summarised in Table 5.1 and 5.2 respectively. All subjects were recruited by means of advertisement and underwent a complete medical history, clinical examination, and lab tests to exclude hepatic and renal diseases. It was made clear to the subjects that agreeing to participate in the study is not binding, and that they were free to withdraw from the study at any time. The study protocol was approved by the Newcastle upon Tyne and North Tyneside Local Research Ethics Committee No. 2, and informed consent was obtained from all subjects.

5.2.2 Experimental protocol

A full dietary assessment was carried out at the screening visit and subjects were asked to continue with their habitual pattern of eating until the start of the study. A combined assessment of β -cell function, insulin sensitivity, liver and pancreas fat content and body fat composition was carried out on 4 occasions in subjects with type 2 diabetes: immediately prior to the hypocaloric period (day -1), and after 1, 4 and 8 weeks of the VLCD. A group of matched non-diabetic controls were studied on one occasion only, without dietary intervention.

Each combined assessment was carried out over 2 days. On the morning of the first day, after an overnight 12 hour fast, a cannula was inserted into an antecubital vein for infusions and a second cannula was inserted into the contralateral wrist vein for blood sampling. Assessments of hepatic glucose production and insulin sensitivity were then carried out using isoglycaemic-hyperinsulinaemic clamp with stable isotope dilution technique (Figure 5.1). Isoglycaemia rather than euglycaemia was used to ensure that the true fasting condition of each subject could be observed at each study time point. Following the insulin clamp, subjects rested for 60 mins before undergoing a stepped insulin secretion test with arginine to assess β -cell sensitivity to glucose (Figure 5.2).

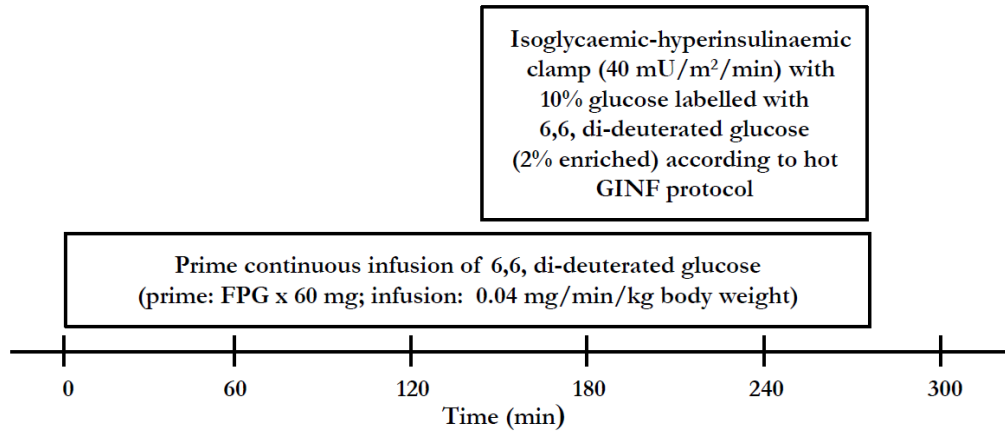


Figure 5.1 Isoglycaemic-hyperinsulinaemic clamp study with stable isotope dilution technique.

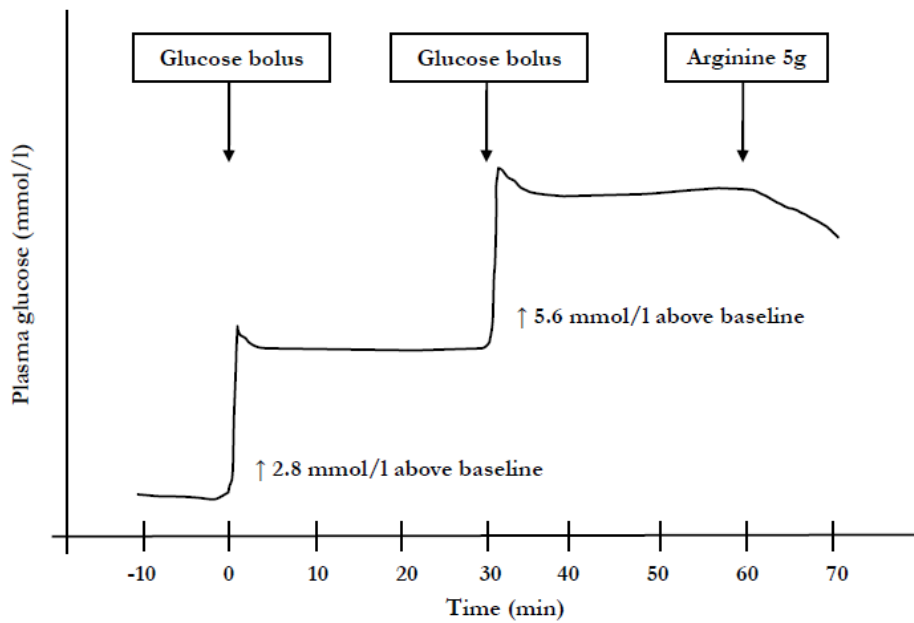


Figure 5.2 Stepped insulin secretion test with arginine.

The following morning, subjects attended the Newcastle Magnetic Resonance Centre following an overnight fast for tests to determine pancreas and liver triglyceride content and total body fat. Pancreas and liver triglyceride content were measured using the 3-point Dixon method on a 3 Tesla Achieva scanner (Phillips, Best, The Netherlands). Body composition was measured using air displacement plethysmography (BOD POD Express; Life Measurement, Concord, CA, USA). Waist and hip circumferences were measured using a non-sticking plastic tape measure with the subjects in a relaxed standing posture. All measurements were taken at baseline and throughout the study period by a single investigator (myself).

5.2.3 Weight loss diet

After completion of the baseline measurements, subjects with type 2 diabetes started a weight-loss regimen for 8 weeks. They were prescribed a very-low-calorie diet which consisted of a liquid diet formula (Optifast; Nestlé Nutrition, Croydon, UK) with 46.4% carbohydrate, 32.5% protein and 20.1% fat plus the recommended daily intake of vitamins, minerals and trace elements (2.1 MJ [600 kcal]/day). This was supplemented with 240g of non-starchy vegetables such that total energy intake was about 2.5 MJ (690 kcal)/day. Subjects were provided with suggestions of suitable vegetable recipes at the start of the programme to enhance compliance by varying daily eating (Appendix A). They were also encouraged to drink at least 2 L water and other calorie-free beverages each day, and asked to maintain their habitual level of physical activity. On-going support and encouragement during the dieting period was provided by means of regular telephone contact. At the end of the 8-week dietary intervention, subjects returned to normal eating but were provided with information about portion size and healthy eating (<http://www.bhf.org.uk/publications/publications-search-results.aspx?m=simple&q=weight>).

5.2.4 Statistical analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as mean \pm SD. Prior to any comparison, the data was tested for normality using Shapiro-Wilk test. Statistical comparisons between diabetes and control groups were performed using Student's *t* test, while within group differences were determined using paired *t* test. Changes of sequential data within experiments were evaluated by repeated measures one-way analysis of variance (ANOVA) with post hoc Bonferroni testing where appropriate. Non-normally distributed data was analysed using non-parametric tests. Correlations were examined using Spearman rank test. Statistical significance was accepted at $p < 0.05$.

5.3 Results

5.3.1 Plasma glucose, insulin and C-peptide

After 1 week of dietary intervention, fasting plasma glucose decreased from 9.2 ± 1.4 to 5.9 ± 1.2 mmol/l ($p = 0.003$; Figure 5.3A) and was not significantly different from that of the non-diabetic control group (5.3 ± 0.3 mmol/l; $p = 0.18$). It remained stable for the rest of the study (5.7 ± 1.6 mmol/l at weeks 4 and 8; $p = 0.52$ compared with control). HbA_{1c} decreased from $7.4 \pm 0.9\%$ (57 ± 10 mmol/mol) and at 8 weeks was not significantly different from non-diabetic control values (6.0 ± 0.7 vs. $5.7 \pm 0.3\%$ [42 ± 8 vs. 39 ± 3 mmol/mol]; $p = 0.27$). Fasting plasma insulin fell from 151 ± 104 to 73 ± 34 pmol/l after 1 week ($p = 0.03$) and to 57 ± 36 pmol/l by 8 weeks ($p = 0.03$ vs. baseline; $p = 0.04$ vs. control). Fasting plasma C-peptide decreased similarly (Table 5.1). During the isoglycaemic clamp, plasma glucose was clamped at 4.6 ± 0.3 mmol/l in the control group compared with 7.0 ± 1.1 mmol/l in the diabetes group at baseline ($p < 0.001$). At week 1, the achieved clamped plasma glucose level was 4.8 ± 0.6 mmol/l ($p < 0.001$ vs. baseline) in the diabetes individuals

and this was similar at weeks 4 and 8 (4.8 ± 1.1 and 4.8 ± 1.3 mmol/l respectively, $p < 0.001$ vs baseline).

5.3.2 Hepatic insulin sensitivity and hepatic triglyceride content

Basal hepatic glucose production decreased significantly during the first week of energy restriction (2.40 ± 0.92 to 1.59 ± 0.23 mg/kg_{ffim}/min; $p = 0.05$), remained decreased compared with baseline for the rest of the study, and at 8 weeks was not significantly different from that of the control group (1.77 ± 0.37 vs. 2.11 ± 0.65 mg/kg_{ffim}/min respectively; $p = 0.60$; Figure 5.3B). Hepatic insulin sensitivity, assessed by the suppression of hepatic glucose production by insulin infusion, was $43 \pm 14\%$ in the diabetic group compared with $68 \pm 16\%$ in the control group ($p = 0.001$) at baseline. During the first week of energy restriction, there was a marked improvement in hepatic insulin responsiveness, with insulin suppression of hepatic glucose production increasing to $74 \pm 17\%$ ($p = 0.003$ vs. baseline).

Hepatic triglyceride content decreased by $30 \pm 17\%$ during week 1 of intervention ($p < 0.001$), becoming similar to control values ($p = 0.75$). It continued to decline throughout the intervention period to reach the normal range for non-obese individuals (Szczepaniak et al., 2005) ($2.9 \pm 0.8\%$; $p = 0.003$; Figure 5.3C), i.e. a total reduction of $70 \pm 18\%$. At baseline, hepatic triglyceride content was $8.5 \pm 5.6\%$ in the control group compared with $12.8 \pm 7.9\%$ in the diabetic group ($p = 0.14$). Hepatic triglyceride at baseline correlated with BMI in the controls ($r_s = 0.71$; $p < 0.05$) but not in the diabetic group ($r_s = -0.50$; $p = 0.12$).

Table 5.1 Metabolic response to 8 weeks of dietary intervention in subjects with type 2 diabetes in comparison with controls

Fasting concentration	Controls	Baseline	Week 1	Week 4	Week 8
HbA _{1c} (%)	5.7 ± 0.3	7.4 ± 0.9 ^{††}	7.1 ± 0.9 ^{*††}	6.5 ± 0.8 ^{**†}	6.0 ± 0.7 ^{**}
HbA _{1c} (mmol/mol)	39 ± 3	57 ± 10 ^{††}	55 ± 10 ^{*††}	47 ± 9 ^{**†}	42 ± 8 ^{**}
Plasma glucose (mmol/l)	5.3 ± 0.3	9.2 ± 1.4	5.9 ± 1.2 ^{**}	5.7 ± 1.8 ^{**}	5.7 ± 1.6 ^{**}
Plasma insulin (pmol/l)	115 ± 81	151 ± 104	73 ± 34 [*]	71 ± 37 [*]	57 ± 36 [†]
Plasma C-peptide (nmol/l)	1.06 ± 0.36	1.21 ± 0.67	1.14 ± 0.53	1.19 ± 0.63	0.86 ± 0.36
Triglycerides (mmol/l)	1.8 ± 0.4	2.4 ± 1.7	1.2 ± 0.4 ^{*†}	1.0 ± 0.2 ^{*††}	1.3 ± 0.8 ^{*†}
NEFA (mmol/l)	0.57 ± 0.22	0.69 ± 0.20	0.93 ± 0.16 ^{*††}	0.81 ± 0.26 [†]	0.72 ± 0.20
Cholesterol (mmol/l)	5.1 ± 0.8	4.0 ± 0.8 [†]	3.3 ± 0.8 ^{*††}	2.8 ± 0.6 ^{*††}	3.2 ± 0.8 ^{††}
LDL-cholesterol (mmol/l)	3.2 ± 0.8	1.7 ± 0.6 ^{††}	1.8 ± 0.9 ^{††}	1.0 ± 0.6 ^{*††}	1.3 ± 0.7 ^{††}
HDL-cholesterol (mmol/l)	1.1 ± 0.2	1.1 ± 0.3	1.0 ± 0.2	1.1 ± 0.3	1.1 ± 0.2
ALT (U/l)	33 ± 8	46 ± 24	61 ± 34 ^{*†}	44 ± 10 [†]	33 ± 11
Gamma GT (U/l)	39 ± 15	62 ± 38	49 ± 28	25 ± 12 [†]	26 ± 17 ^{**}
Capillary ketone (mmol/l)	0.09 ± 0.06	0.08 ± 0.04	1.18 ± 0.92 ^{**}	1.17 ± 1.13 ^{**}	1.00 ± 1.57 ^{**}
3-BOH (μmol/l)	3.5 ± 5.7	3.6 ± 2.4	121.7 ± 87.4 ^{**}	122.1 ± 110.0 ^{**}	102.1 ± 154.3 ^{**}

Values are mean ± SD. 3-BOH, 3-hydroxy-butyrate; ALT, alanine transaminase; gamma GT, γ-glutamyltransferase

[†]*p* < 0.05 vs. controls, ^{††}*p* < 0.005 vs. controls, ^{*}*p* < 0.05 vs. type 2 diabetes baseline, ^{**}*p* < 0.005 vs. type 2 diabetes baseline.

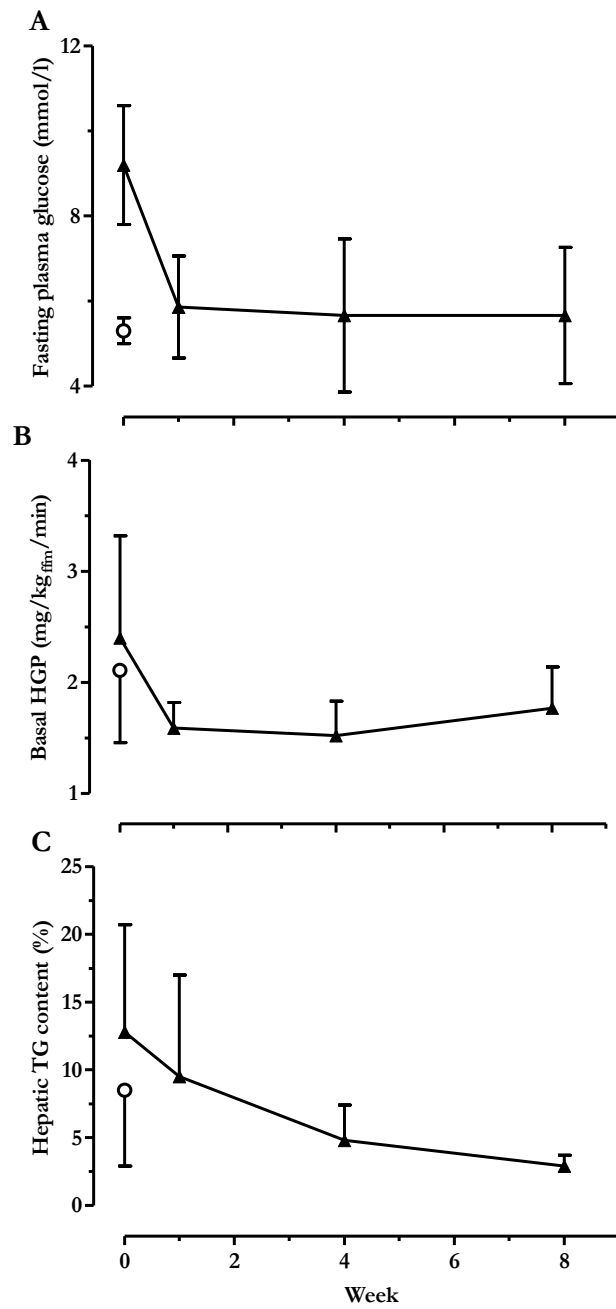


Figure 5.3 Effect of 8 weeks of dietary intervention on (A) plasma glucose, (B) HGP and (C) hepatic triglyceride (TG) content in diabetic subjects (black triangles). White circles indicate the mean for the weight-matched non-diabetic control group. Values are mean \pm SD.

5.3.3 β -cell sensitivity to glucose and pancreas triglyceride content

Fasting insulin secretion rate decreased from 0.10 ± 0.03 to 0.06 ± 0.03 nmol/min/m² during the first week ($p < 0.05$) and remained constant thereafter. The planned step increases in plasma glucose concentration of +2.8 and +5.6 mmol/l were achieved during the insulin secretion test (Figure 5.4A). Peak insulin secretion rate at 6 min was minimal at baseline in the diabetes individuals (0.19 ± 0.08 vs. control 0.62 ± 0.45 nmol/min/m²; $p < 0.001$; Figure 5.4B-C). The first-phase insulin response steadily increased and was significantly different from baseline by 8 weeks (0.29 ± 0.18 , 0.34 ± 0.19 and 0.46 ± 0.23 nmol/min/m² at 1, 4 and 8 weeks; $p = 0.20$, $p = 0.09$, $p = 0.006$, respectively). The insulin secretion rate in the individuals with type 2 diabetes at 8 weeks was not significantly different from the controls (0.46 ± 0.23 vs. 0.62 ± 0.45 nmol/min/m²; $p = 0.42$). There was an increase in arginine-induced insulin response after 1 week (from 0.73 ± 0.35 to 0.95 ± 0.62 nmol/min/m²; $p < 0.006$), and by 8 weeks it was completely normalised (1.37 ± 0.90 vs. 1.15 ± 0.55 ; $p = 0.77$ vs. control; $p < 0.03$ vs. baseline; Figure 5.4B-F). It was 38% lower in the participants with type 2 diabetes at baseline compared with the controls (0.73 ± 0.35 vs 1.15 ± 0.55 nmol/min/m²; $p = 0.04$).

Pancreatic triglyceride content in the diabetic group was $8.0 \pm 5.2\%$ and fell steadily to $6.2 \pm 3.7\%$ after 8 weeks ($p = 0.03$; Figure 5.5). In control individuals, pancreatic triglyceride content was $6.0 \pm 4.0\%$ ($p = 0.17$ compared with participants with type 2 diabetes at baseline). There was no correlation with BMI in either the control or the diabetic group ($r_s = 0.31$, $p = 0.36$; $r_s = 0.01$, $p = 0.98$, respectively).

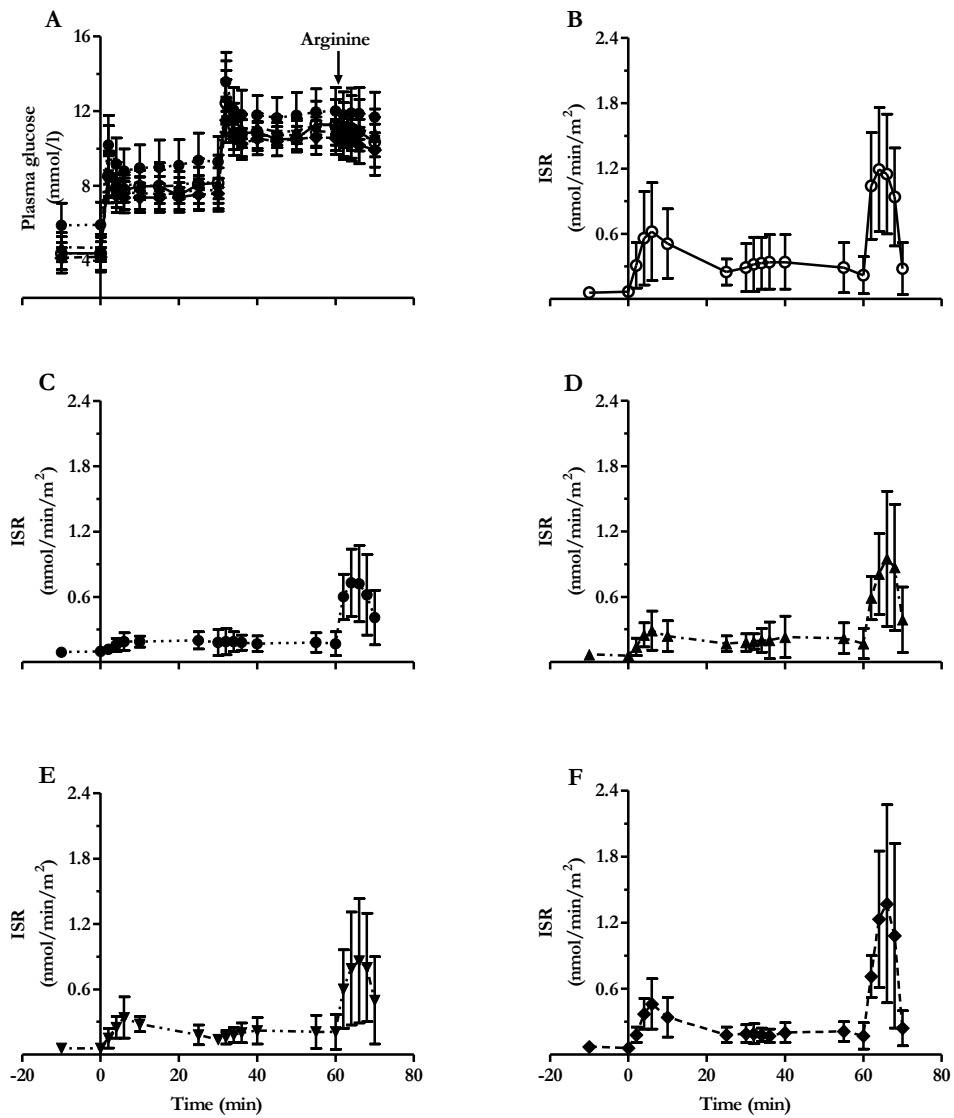


Figure 5.4 Insulin secretion test data in controls and in diabetic subjects at each time point. (A) Plasma glucose levels achieved in each group. Insulin secretion rate (ISR) obtained in the (B) non-diabetic group, (C) the diabetic group at baseline, (D) the diabetic group at 1 week of the diet, (E) the diabetic group at 4 weeks and (F) the diabetic group at 8 weeks. Values are mean \pm SD.

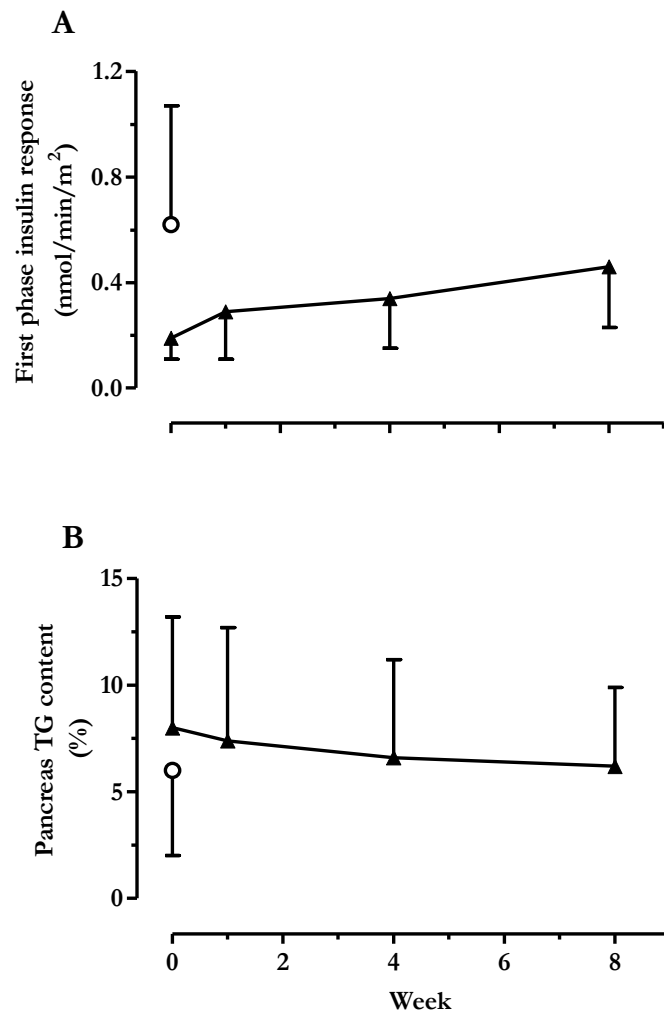


Figure 5.5 (A) Change in the first-phase insulin response, and (B) change in pancreas TG content during the 8 week dietary intervention in the diabetic individuals (black triangles). White circles indicate the mean for the weight-matched non-diabetic control group. Values are mean \pm SD.

5.3.4 Peripheral insulin sensitivity

There was no significant change in peripheral insulin sensitivity expressed as glucose disposal rates during the entire study. Insulin-stimulated glucose disposal was 3.83 ± 0.76 and 4.36 ± 1.20 mg/kg_{ffm}/min at baseline and 8 weeks respectively ($p = 0.21$). Change in glucose metabolic clearance rate was examined to correct for the difference in clamp glucose levels between study days. Fasting plasma glucose decreased between baseline and week 1. There was no significant effect of the dietary intervention on glucose metabolic clearance rate at either 1 or 4 weeks (3.14 ± 1.01 vs. 4.23 ± 1.13 and 4.21 ± 1.20 ml/kg_{ffm}/min, respectively), but improvement was demonstrable by week 8 (5.21 ± 1.64 ml/kg_{ffm}/min; $p = 0.003$ for baseline vs. 8 weeks respectively; control group 5.23 ± 1.32 ml/kg_{ffm}/min; $p = 0.98$).

5.3.5 Weight, body composition and fat distribution

Average weight loss during the 8 weeks of dietary intervention was 15.3 ± 4.0 kg, equivalent to $15 \pm 4\%$ of initial body weight (Table 5.2). Weight loss was greatest during the first week (3.9 ± 0.5 kg, 61% of which was fat loss), between weeks 1 and 4 was 5.7 ± 1.9 kg (86% as fat), and during the final 4 weeks was 5.7 ± 2.3 kg (94% as fat). Both waist and hip circumference decreased to the same extent and waist-hip ratio remained unchanged during the 8 weeks (Table 5.2). Changes in visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) areas over the study period are shown in Table 5.2. VAT and SAT areas decreased by 43 ± 11 and $27 \pm 13\%$ respectively. By the end of the study period, both VAT and SAT areas in the type 2 diabetes subjects were lower compared to the control subjects (121 ± 38 vs. 196 ± 52 cm² and 277 ± 109 vs. 390 ± 95 cm²; $p = 0.001$ and $p = 0.025$ respectively).

Table 5.2 Anthropometric data before and during the 8 weeks of dietary intervention in comparison with control individuals

Variable	Controls	Baseline	Week 1	Week 4	Week 8
Weight (kg)	101.5 ± 10.2	103.7 ± 15.0	99.7 ± 14.8**	94.1 ± 14.4**	88.4 ± 14.1**†
BMI (kg/m ²)	33.4 ± 2.7	33.6 ± 4.1	32.3 ± 4.1**	30.5 ± 4.1**	28.7 ± 4.2**†
Fat mass (kg)	36.2 ± 8.0	39.0 ± 11.6	36.6 ± 11.9**	31.7 ± 12.2**	26.3 ± 13.4**
Fat free mass (kg)	64.7 ± 11.2	64.7 ± 9.9	63.2 ± 10.4	62.4 ± 9.8**	62.1 ± 10.0**
Waist circumference (cm)	105.0 ± 4.4	107.4 ± 7.4	104.4 ± 7.4**	99.7 ± 8.1**	94.2 ± 8.1**†
Hip circumference (cm)	109.8 ± 7.3	109.5 ± 9.5	108.3 ± 8.9*	105.0 ± 8.6*	99.5 ± 8.7**†
Waist hip ratio	0.96 ± 0.07	0.98 ± 0.07	0.97 ± 0.05	0.95 ± 0.05	0.95 ± 0.04
VAT area (cm ²)	196 ± 52	214 ± 42	185 ± 48*	149 ± 33*†	121 ± 38*†
SAT area (cm ²)	390 ± 95	370 ± 104	361 ± 107	327 ± 112*	277 ± 109*†

Values are mean ± SD. SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue

†*p* < 0.05 vs. controls, ††*p* < 0.005 vs. controls, **p* < 0.05 vs. type 2 diabetes baseline, ***p* < 0.005 vs. type 2 diabetes baseline.

5.3.6 Plasma lipids and other metabolites

Plasma triglyceride levels halved during the first week of dietary energy restriction (2.4 ± 1.7 to 1.2 ± 0.4 mmol/l; $p < 0.02$) and remained constant thereafter (Table 5.1). Both total cholesterol and LDL-cholesterol decreased, while HDL-cholesterol remained unchanged during the study period (Table 5.1). Fasting plasma NEFA levels were modestly but not significantly higher in the diabetic participants compared with the matched controls at baseline (0.69 ± 0.20 vs 0.57 ± 0.22 mmol/l; $p = 0.24$). During the study period, fasting NEFA in the participants with diabetes increased significantly at week 1 (0.93 ± 0.16 mmol/l; $p = 0.03$ vs baseline). With continued hypoenergetic intake, plasma NEFA declined steadily towards baseline values (0.81 ± 0.26 and 0.72 ± 0.20 mmol/l at week 4 and 8, respectively).

Plasma alanine transaminase level tended to be higher in the type 2 diabetes subjects compared with control subjects (46 ± 24 vs. 33 ± 8 U/l; $p = 0.13$) and rose significantly after 1 week of dietary intervention (61 ± 34 U/l; $p = 0.006$) and decreased thereafter (44 ± 10 and 33 ± 11 U/l at week 4 and 8 respectively; $p = 0.04$ for week 8 vs. baseline). Plasma γ -glutamyl-transferase level tended to be higher in the type 2 diabetes subjects when compared with control subjects (62 ± 38 vs. 39 ± 15 ; $p = 0.09$) and decreased gradually over the 8 weeks, although did not reach statistical significance (Table 5.1).

After first week of the VLCD, capillary ketone levels increased by 15-fold and remained increased at that level throughout the 8 weeks of dieting (Table 5.1). Fasting plasma 3-hydroxybutyrate increased ~30-fold after 1 week of calorie restriction (3.6 ± 2.4 vs. 121.7 ± 87.4 μ mol/l; $p < 0.001$) and remained at that level throughout the dietary intervention (122.1 ± 110.0 and 102.1 ± 154.3 μ mol/l at weeks 4 and 8 respectively, $p < 0.001$ vs. baseline for both). There was strong positive correlation between capillary ketone and plasma 3-hydroxy-butyrates levels ($r_s = 0.65$, $p < 0.0001$). Both capillary ketone and

plasma 3-hydroxy-butyrate levels were positively correlated with the net percentage weight loss ($r_s = 0.37, p = 0.03$; $r_s = 0.39, p = 0.03$, respectively).

5.3.7 Post-intervention observation

At follow-up 12 weeks after completion of the dietary intervention, mean weight gain was 3.1 ± 3.3 kg. Hepatic triglyceride remained low and unchanged (2.9 ± 0.8 vs. $3.0 \pm 1.0\%$; $p = 0.80$), and pancreatic triglyceride decreased further to a small extent (6.2 ± 3.7 vs. $5.7 \pm 3.6\%$; $p = 0.005$). HbA_{1c} was unchanged (6.0 ± 0.7 vs. $6.2 \pm 0.3\%$; $p = 0.10$; 42 ± 8 vs. 44 ± 4 mmol/mol), and fasting plasma glucose increased modestly (5.7 ± 1.6 vs. 6.1 ± 0.5 mmol/l; $p < 0.01$), with a mean 2 h OGTT plasma glucose of 10.3 ± 3.2 mmol/l. Three participants had recurrence of diabetes as judged by a 2 h post-load plasma glucose of greater than 11.1 mmol/l. Fasting plasma insulin concentrations were unchanged (57 ± 36 vs. 65 ± 46 pmol/l), and fasting plasma NEFA decreased further (0.72 ± 0.20 vs. 0.54 ± 0.17 mmol/l; $p < 0.02$). One individual was unavailable for retesting, having had surgery for a non-malignant ovarian cyst.

5.4 Discussion

This study demonstrates that the twin defects of β -cell failure and insulin resistance that underlie type 2 diabetes can be reversed by acute negative energy balance alone. A hierarchy of response was observed, with a very early change in hepatic insulin sensitivity and a slower change in β -cell function. In the first 7 days of the reduced energy intake, fasting plasma glucose and hepatic insulin sensitivity fell to normal, and hepatocellular lipid decreased by 30%. Over the 8 weeks of dietary energy restriction, β -cell function increased towards normal and pancreatic fat decreased. Following the intervention, participants gained 3.1 ± 3.3 kg body weight over 12 weeks, but their HbA_{1c} remained steady while the

triglyceride content of both pancreas and liver did not increase. The data are consistent with the hypothesis that the abnormalities of insulin secretion and insulin resistance that underlie type 2 diabetes have a single, common aetiology, i.e. excess lipid accumulation in the liver and pancreas (Taylor, 2008). This provides a unified hypothesis to explain a common disease that previously appeared to require separate disease processes affecting the pancreas and insulin-sensitive tissues.

Absence of rapid insulin secretion in response to a rise in plasma glucose is the hallmark of type 2 diabetes (Weyer et al., 1999a, Pfeifer et al., 1981), and the decline in β -cell function determines the progression towards a need for insulin therapy (1995). However, conventional therapy, even with sulfonylurea, fails to produce more than a small increase in the first-phase insulin response. As a consequence, the rapidity and extent of return of β -cell function in response to dietary energy restriction in the present study is striking. Insulin secretion in non-diabetic individuals can be blunted by constant hyperglycaemia (Ferner et al., 1986) and the removal of “glucotoxicity” could be a partial explanation. Observation from this study supports the accumulating information on the inhibitory effect of fatty acids on insulin secretion *in vitro* and *in vivo* (Igoillo-Esteve et al., 2010, Cnop, 2008, Noushmehr et al., 2005) and is the first direct evidence in humans that the β -cell defect of type 2 diabetes is reversible by sustained negative energy balance. Prolonged elevation of plasma fatty acids in humans decreases insulin secretion (Carpentier et al., 2003, Kashyap et al., 2003), and it has previously been shown that there is an association between pancreatic fat content and type 2 diabetes (Tushuizen et al., 2007, Saisho et al., 2008, Saisho et al., 2007). Prior to the onset of spontaneous diabetes in rodents, both total pancreatic fat and islet triglyceride content increase sharply (Lee et al., 1994, Lee et al., 2010). *In vitro*, chronic saturated fatty acid exposure of β -cells inhibits the acute insulin response to glucose, and removal of fatty acids allows recovery of this response (Morgan et al., 2008).

The present data provide clear evidence that decreasing total pancreatic fat is associated with a return of β -cell function. However, it is probable that the negative effect on β -cell function is exerted by toxic intermediaries such as diacylglycerol and ceramides, which change rapidly in response to acute metabolic changes (Erion and Shulman, 2010), rather than by stored triacylglycerol per se, which acts as an index of fatty acid intermediary concentration. There was no correlation between indices of insulin secretion and pancreatic fat, which suggests that there are individual thresholds of tolerance for such toxic intermediaries rather than a simple dose–response relationship within the pancreas.

Fasting plasma glucose concentration is determined by the rate of hepatic glucose production, and hepatic insulin sensitivity is inversely proportional to intrahepatic lipid content (Ravikumar et al., 2008, Perseghin et al., 2006, Gastaldelli et al., 2007, D'Adamo et al., 2010). Moderate weight loss has previously been shown to be associated with a fall in intrahepatic fat content (Petersen et al., 2005a, Tiikkainen et al., 2003). Petersen and colleagues have previously reported improved liver insulin sensitivity and no significant change in muscle insulin sensitivity measured by euglycaemic-hyperinsulinaemic clamps after 8 weeks of moderate energy restriction in type 2 diabetes (Petersen et al., 2005a). A very low energy intake has been observed to lower liver fat content in healthy obese individuals within days (Kirk et al., 2009). The present study demonstrates for the first time the early time course with which both hepatic fat stores and hepatic glucose production fall in response to dietary restriction in type 2 diabetes. Change in peripheral insulin sensitivity played no part in the early return of normoglycaemia. It is possible that the sharp rise in plasma NEFA observed after 1 week of the hypocaloric diet could have prevented a change in peripheral insulin sensitivity even though this did not prevent the rapid improvement of hepatic insulin sensitivity.

It has been hypothesised that establishing the pathophysiological changes that accompany the reversal of type 2 diabetes will illuminate the sequence of

changes determining the onset of the disease (Taylor, 2008). The twin-cycle hypothesis was informed by observations following bariatric surgery (Taylor, 2008), especially with the demonstration of fasting plasma glucose concentrations falling within days after biliopancreatic diversion (Guidone et al., 2006). As these changes occur before substantial weight loss, it has become widely accepted that bariatric surgery exerts such rapid effects by changes in incretin hormones. However, the extent of change in incretins is modest, not always present in type 2 diabetes, and absent after gastric banding (Morinigo et al., 2006, Rodieux et al., 2008, Knop, 2009, Rubino et al., 2004). Little attention has been paid to the potential role of a sudden negative energy balance on glucose metabolism after bariatric surgery, although a recent small study suggested that the improvement in insulin resistance in the first week after surgery can be attributed to negative energy balance alone (Isbell et al., 2010). Increased glucagon-like peptide-1 secretion following bariatric surgery compared with a hypocaloric diet has been reported, but the oral glucose load used in the presence of a gastroenterostomy is likely to explain the early rapid rise in both plasma glucose and the incretin, occasionally with the symptoms of early dumping (Laferrere et al., 2008).

The limitations of this study must be considered. First, the sample size was necessarily small to allow the application of gold standard methods for metabolic investigation and examination by magnetic resonance techniques. However, the participants were drawn from the population with type 2 diabetes, and their clinical and anthropometric characteristics were typical for the condition. Second, pancreatic fat measurements included intra-organ adipocyte fat content because current methodology precludes the assessment of the more mechanistically important islet intracellular fatty acid content. Animal data suggest that the two variables are linked (Lee et al., 2010). Although pancreatic fat content was 30% higher in the diabetic group, the study was powered to demonstrate responses to the dietary intervention rather than to test differences from weight matched non-diabetic individuals. No correlation

was observed between pancreatic fat and BMI within the restricted range of BMI examined in this study.

Third, the participants were selected to have had a relatively short duration of type 2 diabetes (up to 4 years), and further studies must establish the extent of reversibility with longer duration type 2 diabetes. In addition, further studies are required to determine the long-term outcome in respect of glucose regulation as the observations made after 12 weeks of return to a normal diet were necessarily limited. Fourth, the raised liver fat levels despite metabolic normality in some controls can be considered in the light of the recent description of the patatin-like phospholipase 3 (*PNPLA3*) gene (He et al., 2010). The G allele of this gene determines high liver fat levels, but in a form that is not associated with metabolic abnormality. This provides a clear genetic basis for the observed individual variation in susceptibility to insulin resistance despite raised liver fat content, and offers a partial explanation of the overlapping hepatic fat levels in type 2 diabetic and control groups. It is likely that other genetic factors yet to be defined underlie the differing individual levels of susceptibility to pancreatic fat accumulation in terms of inhibition of glucose-dependent insulin secretion. Finally, the full impact of sudden negative energy balance on the incretin system could not be explored because oral glucose or meal testing was not performed.

This study demonstrates for the first time the time course of a return of normal β -cell function and hepatic glucose output by restricting dietary energy intake in individuals with type 2 diabetes over a short time course. The changes occurred in association with decreases in pancreatic and liver triglyceride concentrations. This new insight allows an understanding of the causality of type 2 diabetes in individuals as well as in populations. It carries major implications for information to be given to newly diagnosed patients, who should know that they have a potentially reversible condition and not one that is inevitably progressive.

Chapter 6

GENERAL DISCUSSION

Type 2 diabetes affects a very large number of people worldwide, yet, the precise causes of the disease have been elusive. Since the earliest predictor of the development of type 2 diabetes is insulin resistance in skeletal muscle, this tissue has been extensively studied. The role of oxidative capacity, which in essence is determined by the density and intrinsic properties of the mitochondria, has recently gained much attention. Data from *in vivo* MR spectroscopy measurements (Petersen et al., 2004, Szendroedi et al., 2007, Schrauwen-Hinderling et al., 2007), *ex vivo* studies of mitochondrial function (Mogensen et al., 2007, Phielix et al., 2008) as well as expression analysis of genes involved in oxidative metabolism (Mootha et al., 2003, Patti et al., 2003) all suggest that skeletal muscle mitochondrial dysfunction might play a role in insulin resistance and type 2 diabetes. The mechanism that has been proposed to mediate the insulin resistance is a decrease in capacity to oxidise fat, resulting in the accumulation of intracellular fatty acid metabolites (Lowell and Shulman, 2005).

On the other hand, several recent studies in both humans and rodents directly challenge the concept that mitochondrial dysfunction/deficiency causes muscle insulin resistance (Nair et al., 2008, De Feyter et al., 2008, Wredenberg et al., 2006, Toledo et al., 2007). In support of the latter, work from this thesis revealed no evidence of a mitochondrial defect in type 2 diabetes, as determined by measuring the rate of unidirectional flux through ATP synthase. Skeletal muscle ATP turnover rate was found to be normal in type 2 diabetes and could be modulated to a degree by suppression of lipolysis with acipimox. Muscle ATP turnover rates in type 2 diabetes reflect muscle glucose uptake and glycogen synthesis. In addition, data obtained from the cohort of young, lean and insulin-sensitive individuals suggest that there is not a relevant role for a subtle mitochondrial problem. These data are in keeping with findings of Trenell et al. (Trenell et al., 2008). Here, the authors found that both resting and maximum ATP turnover were similar between patients with well-controlled type 2 diabetes and controls matched for age, weight and physical activity.

Furthermore, the issue of whether a decrease in mitochondrial function as observed in insulin-resistant individuals would limit the ability of skeletal muscle to oxidise fatty acids and lead to lipid accumulation as proposed must be carefully considered. The rate of oxygen utilisation in muscle under normal resting conditions is usually low. However when energy demands are high such as during maximal exercise, skeletal muscle has enormous capacity to increase substrate oxidation over basal levels (Bangsbo, 2000). Considering there is such 'spare' capacity to increase substrate oxidation in muscle, it is therefore questionable whether mitochondrial deficiencies observed in insulin-resistant individuals would have any impact on the rate of fatty acid oxidation under normal, free-living conditions in which energy requirements would otherwise be low.

Insulin resistance and β -cell dysfunction are the two primary defects that are recognised to determine the pathogenesis of type 2 diabetes. The accepted view has been that β -cell dysfunction progresses inevitably, whereas insulin resistance in muscle and liver can be modified at least to some extent. Recent observations on resolution of type 2 diabetes after bariatric surgery raise the possibility that these underlying pathophysiological abnormalities can be reversed. Work from this thesis supports the notion and specifically shows that this is independent of the surgery itself. Within 7 days of hypocaloric diet, normal hepatic insulin sensitivity is regained as liver fat content falls to level similar of that of the age- and weight-matched non-diabetic control subjects. These changes resulted in normalisation of fasting plasma glucose. Insulin secretory capacity, which was tested by a stepped glucose infusion with subsequent arginine bolus, showed a steady increase over the 8 weeks of hypocaloric diet to become indistinguishable from that of the control subjects. Pancreas fat content decreased steadily during the 8 weeks to level similar to the control group. This occurred over a time course exactly matching that of the increase in insulin secretory capacity. Change in peripheral insulin sensitivity played no part in the early return of normoglycaemia. Although a cause and

effect relationship between raised intra-organ fat levels and metabolic effect has not yet been proven, the time course of the data is highly suggestive of a causal link.

Together with other evidence of normalisation of insulin secretion post-bariatric surgery (Camastra et al., 2007), work from this thesis supports the proposed 'twin cycle' hypothesis of the aetiology of type 2 diabetes (Figure 6.1) (Taylor, 2008). Chronic calorie excess in the circumstance of pre-existing muscle insulin resistance will lead to increased plasma insulin, which in turn accelerates fatty acid synthesis from glucose in the liver (Sidossis and Wolfe, 1996). The increase in liver fat will lead to resistance of insulin-suppression of hepatic glucose production and a modest increase in fasting plasma glucose level. Consequently, basal insulin secretion rates will rise to maintain euglycaemia. The resulting compensatory hyperinsulinaemia will further increase the conversion of excess calories into liver fat, leading to further rise in fasting plasma glucose due to hepatic insulin resistance. This vicious cycle of liver metabolism interacts with a second cycle involving pancreatic function. Fatty liver leads to increased export of very low-density lipoprotein (VLDL) triglyceride which will increase delivery of lipids to all tissues including the islets. Excess fatty acid derived from circulating and locally deposited triglyceride will suppress glucose-mediated insulin secretion, and at a certain level of fatty acid exposure, clinical diabetes will develop.

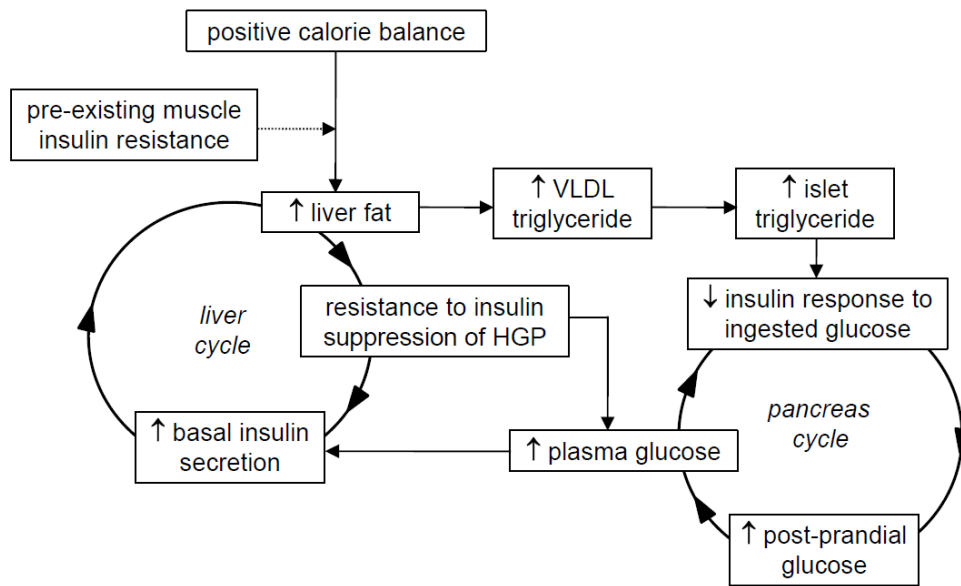


Figure 6.1 The twin cycles of type 2 diabetes – reproduced with permission from Taylor et al. (Taylor, 2008). *Liver cycle*: Fat will accumulate in the liver during chronic intake of more calories than are expended. As this process is promoted by insulin, individuals with a degree of insulin resistance (determined by familial trait or lifestyle factors) will accumulate liver fat more readily than others. As the liver fat increases, the process of glucose production by the liver becomes less sensitive to suppression by insulin, resulting in the rise of plasma glucose and basal insulin secretion rate. *Pancreas cycle*: The increased liver fat will cause increased hepatic VLDL triglyceride secretion. All tissues will be exposed to more triglyceride than required, with pancreatic islets particularly susceptible to local triglyceride accumulation. The post-meal response to glucose becomes blunted, and this is exacerbated by the modestly raised plasma glucose. The pancreas cycle causes impaired glucose regulation, and eventually the fatty acid and glucose inhibitory effects on the islet reach a threshold, leading to a relatively sudden onset of clinical diabetes.

Despite the strong association between obesity and type 2 diabetes, some obese individuals never develop type 2 diabetes and conversely some type 2 diabetic individuals are relatively lean. It is clear from this observation that individual susceptibility factors must therefore determine the onset of the condition. Given that diabetes cannot occur without a loss of acute insulin response to food, it would be expected that this critical step could relate either to the accumulation of fat in the pancreas, susceptibility to the adverse effect of excess fat in the pancreas, or to both of these factors. Tushuizen and colleagues observed that people with type 2 diabetes tended to have higher levels of pancreas fat than weight-matched controls (Tushuizen et al., 2007). However, they reported a wide scatter of pancreatic fat levels amongst the people with type 2 diabetes (range 13.4 – 43.6%) and there was a clear overlap between the diabetic and non-diabetic subjects (range 7.0 – 20.2%). This trend was also observed in my study despite using a more precise method of measuring pancreatic triglyceride content (diabetics: range 4.1 – 22.2% and non-diabetics: 2.9 – 15.4%). Why should it be that one person has normal β -cell function with a pancreas fat level of 10% whilst another has type 2 diabetes with a level of 5%? There must be varying degrees of liposusceptibility of the metabolic organs determining at which level of fat excess the adverse effects develop. Therefore, when an individual acquires excess fat stores then β -cell failure may or may not develop depending upon his/her individual degree of liposusceptibility. Such susceptibility may be genetically determined as seen in individuals with polymorphism in *PNPLA3*-(I148M) which confers high levels of liver fat without features of metabolic syndrome including obesity, hypertriglyceridaemia and insulin resistance (Speliotes et al., 2010, Sookoian and Pirola, 2011). This genetic variation allows build up of metabolically inert triglyceride, most likely without the excess of fatty acid intermediaries such as diacylglycerol which would normally be in equilibrium with the excess triglyceride deposit (Samuel et al., 2010).

Data from this thesis firmly support earlier observations that individuals with type 2 diabetes can return to normal metabolism by acute restriction of dietary energy intake and losing weight, at least in the early years after diagnosis. However, the extent of weight loss required is much greater than that conventionally advised. Dixon and colleagues showed in their randomised study that loss of 20% body weight allowed long-term remission in 73% of a bariatric surgery group, with weight change itself being the principal determinant of glucose control (Dixon et al., 2008). In my study, individuals with type 2 diabetes achieved a loss of 15% body weight over an eight-week period. The role of physical activity must also be considered. Increased levels of daily activity results in improved insulin sensitivity at the levels of skeletal muscle (Perseghin et al., 1996) and decreased liver fat (Perseghin et al., 2007). Although physical activity was not specifically examined in the work of this thesis, exercise intervention alone rarely achieves the amount of weight loss required to reverse diabetes. The initial major loss of body weight demands substantial reduction in calorie intake to achieve a target weight, followed by a continued modest calorie restriction in association with increased daily physical activity to maintain the newly acquired lower body weight. Further recommendation on details on how to reverse type 2 diabetes in clinical practice must however await further studies. As for now, it will be helpful for all individuals newly diagnosed with type 2 diabetes to know that they have developed a metabolic syndrome which is reversible.

Future work is also required to confirm a cause and effect relationship between raised intra-organ fat levels and metabolic effect in humans, especially that of islet triacylglycerol concentration and insulin secretion. As yet, only animal data demonstrate that blocking ectopic deposition of lipids by the daily administration of troglitazone to prediabetic *fa/fa* ZDF rats completely prevents diabetes from developing by reducing lipogenesis and lipoapoptosis in the β -cells (Higa et al., 1999). Furthermore, there is the need to examine the potential for reversibility of type 2 diabetes with increased duration of disease

process, as the patients that were studied here had diabetes diagnosed up to 4 years previously. It also remains to be proven whether long-term outcome in respect of glucose regulation can be maintained as well as achieving the anticipated impact of diabetes remission on macrovascular outcomes.

The work presented in this thesis demonstrates lack of a primary role for mitochondrial abnormalities in the aetiology of type 2 diabetes. However, complete reversal by dietary means has been demonstrated of abnormal β -cell insulin secretion and hepatic insulin sensitivity, in close temporal association with fall in pancreas and liver fat content. These observations allow a new understanding of type 2 diabetes and permit simple explanation of the observed pattern of onset of type 2 diabetes both in individuals and in populations.

Appendix A

Very Low Calorie Diet: Meal Plans and Ideas

Meal plan example:

Breakfast:	Optifast milkshake
Lunch:	Optifast soup or milkshake with salad
Evening meal:	Optifast soup or milkshake with one of the following (salad, roasted vegetables or homemade soup)
Snacks/supper:	Vegetable snacks

Type of vegetables allowed (at least 240g per day):

Cabbage	Tomatoes	Cauliflower
Broccoli	Celery	Cucumber
Onions	Bean Sprouts	Carrots
Lettuce	Leeks	Mushrooms
Peppers	Artichoke	Radish
Water chestnuts	Brussel sprouts	Mung bean sprouts

Boil, grill, dry fry, stir fry or wrap in tin foil and roast in the oven.

Herbs and spices:

You will need to use some flavouring to prevent your dishes tasting so bland.

Try to find some you like e.g.

Basil	Lemon juice	Parsley	Oregano
Vinegar	Tumeric	Tarragon	Chilli powder
Cinnamon	Rosemary	Cumin	Curry powder
Vanilla essence	Thyme	Sage	Dried chillies
Rum essence	Coriander	Ginger	Black pepper

Meal ideas:

- Salads
- Stir fry
- Soups
- Tomato based sauces

Some recipe ideas: SOUPS

Just a small bowl of soup (200 ml) can fill you up for a long time, also good to freeze or leave in the fridge for a couple of days.

French Onion Soup:

1 kg onions, chopped
2 tbsp thyme
1 litre beef stock

Mushroom Soup:

1 onion, chopped
1 garlic clove, crushed
fresh thyme
400g mushrooms
850ml vegetable stock

Tomato Soup:

3 x 400g tins of chopped tomatoes
850ml vegetable stock
1 clove garlic, crushed
basil leaves, chopped

Vegetable Soup:

2 onions, chopped
1 tsp mustard seeds
1 tsp cumin seeds
2 leeks, sliced
3 carrots, sliced
2-3 tsp curry powder
1-2 litre vegetable stock

Pea and watercress Soup:

1 onion, chopped
1 clove of garlic, crushed
500ml vegetable stock
300g fresh peas
100g watercress

Carrot Soup:

600g carrots, grated
2 tsp cumin seeds
pinch of chilli flakes
1 litre vegetable stock

Carrot and Coriander Soup:

1 onion, chopped
1 tsp ground coriander
450g carrots, chopped
1-2 litre vegetable stock
pinch of chilli flakes
pinch of cumin seeds

Other recipe ideas: SALADS

Try different lettuces such as rocket and crispy leaf, don't just stick to the iceberg lettuce. Try different types of fat free, low calorie dressings to add some different flavours. Using herbs and vinegars can help with flavour too.

Simple Salad:

80g of mixed lettuce/salad leaves (around 1 cereal bowl full)
1 medium tomato, sliced
3 mushrooms, sliced
2-3 sticks celery, chopped
3 sliced rings of green pepper
1 tablespoon fat free dressing

Red pepper and chilli soup:

6 red peppers, halved, cored and deseeded
2 red chillies, halved, cored and deseeded
1 bulb garlic, halved
6 tomatoes, halved
450ml vegetable stock

Tomato sauce:

1 onion, chopped

2 garlic cloves, crushed

1 red pepper

400g tinned chopped tomatoes

1 tbsp basil

1 tbsp parsley

1 tbsp chives

1 green pepper

Salsa sauce:

4 fresh plum tomatoes, finely chopped

1 bunch rocket, finely chopped

1 bunch flat leafed parsley, finely chopped

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