METABOLIC ABNORMALITIES PRECEDING NON-INSULIN DEPENDENT DIABETES

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Doctor of Medicine (MD)

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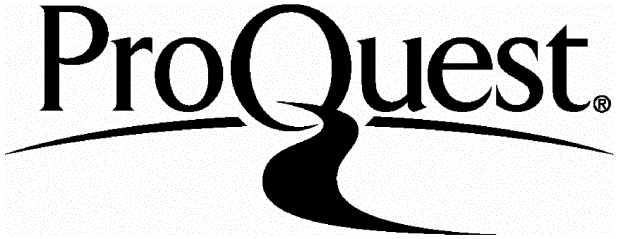

ABSTRACT

Non-insulin dependent diabetes (NIDDM) is characterised by disturbances in insulin action and insulin secretion with hyperproinsulinaemia, but the primary defect remains unknown. The pathogenesis has a strong genetic component and first-degree relatives of patients with NIDDM constitute a population at-risk. Metabolic abnormalities identified in this predisposed group, whilst glucose tolerance is still normal, may represent the primary cause of NIDDM. With this aim this thesis has investigated insulin secretion and insulin sensitivity in glucose-tolerant first-degree relatives from three ethnic groups.

In the progress of this work, three new methods for measuring insulin sensitivity were developed: the low dose short insulin tolerance test; glycerol turnover measured in response to low dose insulin using stable isotopic tracers and a glycerol clamp.

Relatives of patients of Asian (Indian-subcontinent) origin had raised fasting circulating immunoreactive insulin and glycerol levels and impaired suppression of glycerol and non-esterified fatty acid concentrations following oral glucose. This suggested insulin resistance, which was confirmed using the short insulin tolerance test. Relatives of European patients possessed more subtle abnormalities; when glycerol turnover was measured isotopically in response to low dose insulin infusion, insulin-induced suppression of lipolysis was impaired; these relatives also demonstrated increased levels of 32, 33 split proinsulin following intravenous glucose, indicating a defect in insulin processing. Afro-Caribbean relatives exhibited disturbed pancreatic ß cell processing as well with exaggerated intact and 32, 33 split proinsulin responses to intravenous glucose, but a coexistent defect in insulin sensitivity was also apparent. No abnormality in serum lipoprotein concentrations was identified in any ethnic group, suggesting that the dyslipidaemia of NIDDM is a secondary phenomenon.

Insulin insensitivity to lipolysis was present in relatives of all ethnic groups despite normal glucose tolerance, suggesting that this is one of the earliest metabolic abnormalities in the pathogenesis of NIDDM. Insulin processing defects identified in European and Afro-Caribbean subjects may also be of aetiological significance.

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Differential metabolic actions of biosynthetic insulin analogues in normal man assessed by stable isotopic tracers.
S.V. Gelding, N. Coldham, V. Anyaoku, K. Heslop, D. Halliday and D.G. Johnston. *Diabetic Medicine* 1993 10: 470-476.

Insulin sensitivity in non-diabetic relatives of patients with non-insulin dependent diabetes from two ethnic groups.
S.V. Gelding, R. Niththyananthan, S.P. Chan, E. Skinner, S. Robinson, I.P. Gray, H. Mather and D.G. Johnston. *Clinical Endocrinology* 1994; 40: 55-62.

Validation of the low dose short insulin tolerance test for evaluation of insulin sensitivity.
S.V. Gelding, S. Robinson, S. Lowe, R. Niththyananthan and D.G. Johnston. *Clinical Endocrinology* 1994; 40: 611-615.

Serum lipoprotein levels and plasma concentrations of insulin, intact and 32, 33 split proinsulin measured by specific immunoradiometric assay in normoglycaemic relatives of patients with Type 2 diabetes.
S.V. Gelding, C. Andres, R. Niththyananthan, W. Richmond, I.P. Gray and D.G. Johnston. *Diabetic Medicine* 1994 11: 748-754.

Insulin resistance with respect to lipolysis in non-diabetic relatives of European patients with non-insulin dependent diabetes.
S.V. Gelding, N. Coldham, R. Niththyananthan, V. Anyaoku and D.G. Johnston. *Diabetic Medicine* 1994 (in press).

Increased secretion of 32, 33 split proinsulin after intravenous glucose in glucose-tolerant first-degree relatives of patients with non-insulin dependent diabetes of European, but not Asian, origin.
S.V. Gelding, C. Andres, R. Niththyananthan, I.P. Gray, H. Mather and D.G. Johnston. *Clinical Endocrinology* 1994 (in press).

Disproportionate secretion of insulin, intact and 32, 33 split proinsulin in glucose-tolerant Afro-Caribbean subjects predisposed to later non-insulin dependent diabetes.
S.V. Gelding, C. Andres, R. Niththyananthan, I.P. Gray and D.G. Johnston. (submitted)

ABBREVIATIONS

ADA	adenosine deaminase
ADP	adenosine diphosphate
αKIC	alpha-ketoisocaproic acid
AMP	adenosine monophosphate
APE	atoms percent excess
Arg	arginine
Asp	aspartic acid
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BMI	body mass index
^{o}C	degrees Centigrade
Ca^{2+}	calcium ions
cAMP	cyclic adenosine monophosphate
cDNA	complimentary deoxyribonucleic acid
CO_2	carbon dioxide
CoA	Coenzyme A
cv	coefficient of variation
DNA	deoxyribonucleic acid
ECF	extracellular fluid
Ei	isotopic enrichment of infusate
Ep	isotopic enrichment of plasma
FFM	fat free mass
FM	fat mass
FPG	fasting plasma glucose
FPI	fasting plasma insulin
g	grammes
G6PDH	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCMS	gas chromatography-mass spectrometry
Glu	glutamic acid
Gly	glycine
GPI	glycosyl phosphatidylinositol

GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HA	high affinity insulin analogue
HbA_1	glycosylated haemoglobin
HDL	high density lipoprotein
HGP	hepatic glucose production
HLA	human leucocyte antigen
HMG CoA	hydroxy methylglutaryl Coenzyme A
IAPP	islet amyloid polypeptide
IDDM	insulin-dependent diabetes mellitus
IGF-1	insulin-like growth factor-1
IGT	impaired glucose tolerance
IRI	immunoreactive insulin
IRMA	immunoradiometric assay
IRS-1	insulin receptor substrate-1
IS	insulin sensitivity
ITT	insulin tolerance test
IVGTT	intravenous glucose tolerance test
K_G	glucose disappearance constant
Kg	kilogram
LA	low affinity insulin analogue
LDL	low density lipoprotein
Lys	lysine
m:f	male:female
M	amount of glucose metabolised
met	methionine
M_f	insulin sensitivity of lipolysis per unit fat mass
$MgCl_2$	magnesium chloride
ml	millilitres
MODY	maturity onset diabetes of the young
mRNA	messenger ribonucleic acid

µl	microlitres
µmol	micromoles
Na^+	sodium ions
NAD(H)	nicotinamide adenine dinucleotide (reduced form)
NEFA	non-esterified fatty acids
NH_4^+	ammonium ions
NIDDM	non-insulin dependent diabetes mellitus
nm	nanometres
NS	not significant
OGTT	oral glucose tolerance test
Ω	ohms
p	probability
PDH	pyruvate dehydrogenase
PEP	phosphoenol pyruvate
PEPCK	phosphoenol pyruvate carboxykinase
pmol	picomoles
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PtdIns	phosphatidylinositol
ρ	impedance
Ra	rate of appearance
revs/min	revolutions per minute
RNA	ribonucleic acid
se	standard error
SH_2	*src* homology
TBW	total body water
Tg	triglyceride
tRNA	transfer ribonucleic acid
Tyr	tyrosine
vs	versus
W	weight
WHO	World Health Organisation

DECLARATION

The work presented in this thesis was carried out at the Unit of Metabolic Medicine, St Mary's Hospital Medical School, London under the supervision of Professor D.G. Johnston. I have been the principal investigator for all the studies and have personally devised the study protocols, recruited subjects and controls and performed the clinical studies. I have carried out most of the laboratory analyses myself with assistance from laboratory technicians, and I prepared the samples for mass spectrometry. I have performed all the statistical analysis of the results. None of the work contained in this thesis has been submitted in support of a higher degree at any other university or higher educational institution.

ACKNOWLEDGEMENTS

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I thank Dr David Halliday of the Nutrition Research Group at the Clinical Research Centre, Harrow for his advice and helpful discussions concerning the stable isotope studies and for allowing use of the group's mass spectrometers for measurement of ^{13}C tracers. I also thank Dr Nick Coldham who performed many of the deuterium tracer measurements and developed glycerol tracer detection by mass spectrometry.

I wish to express my appreciation to Sister Elizabeth Skinner for her help with the clinical studies and to medical students Richard McManus and Sian Lowe who assisted with the clinical tests and laboratory assays while working for their intercalated BSc degrees in the department. Laboratory assistance was also provided by Miss Corla Murphy. I thank Dr Lindy Murphy for her pioneering work in developing the glycerol electrode and for her collaboration with the glycerol clamp study. I am grateful to Dr Siew-Pheng Chan, visiting lecturer, for her assistance with the metabolic studies during her sabbatical. Novo Industries kindly provided financial support for the insulin analogue study and donated the analogues used. Mr Luke Whittaker gave statistical advice with the analogue study. The lipid and glycosylated haemoglobin measurements were performed by Dr Bill Richmond's laboratory.

I thank Dr Hugh Mather, Consultant Physician at Ealing Hospital, London and Dr Robert Elkeles, Consultant Physician at St Mary's Hospital, London for allowing me to approach their patients as a source of relatives for study. In addition, I thank all those individuals who so kindly volunteered and donated their time and blood for the clinical studies.

Finally, I should like to thank all the members of the Unit of Metabolic Medicine at St Mary's, past and present, for their help and friendship and in making my time in the department so memorable.

"To the majority of cases who ask why they developed diabetes I have to answer that I don't know..... The influence of heredity is undoubted..... (but) our ignorance of the fundamental diabetic process and particularly its complications is great."

R. D. Lawrence

'The Diabetic Life' **1925**

CHAPTER 1:

INTRODUCTION

Non-insulin dependent diabetes is a common disease which affects about 5-7% of the global population and accounts for the major proportion of diabetes. Despite this high frequency and the substantial associated morbidity and mortality, the fundamental cause of the condition remains elusive.

Diabetes has been recognised since ancient times and was described as a "mysterious affection" by Aretaeus in AD 170. Its precise aetiology remains a mystery to this day. The combination of characteristic symptoms and sweet urine led to the early clinical diagnosis, but with the development of laboratory methods, the importance of hyperglycaemia was identified and chemical diagnosis supervened. Large scale population studies have since revealed the heterogeneous nature of the condition and the presence of many co-existing metabolic disturbances, making it impossible to distinguish primary cause from secondary effects. These factors complicate the study of patients with a view to determining the disease pathogenesis.

Recent advances in molecular genetics have enabled characterisation of a large number of genetic defects which may contribute to NIDDM, but this has also served to underline the diversity of the disease. It is clear that glucose and insulin physiology become deranged, but the exact sequence of events is unknown. In an attempt to identify the metabolic defect, it is necessary to study the condition in the very early stages before the secondary metabolic consequences are established. It is with this aim that this thesis proposes to examine the metabolic changes preceding NIDDM.

In this introduction a fairly detailed account of the mechanism of insulin action and glucose homeostasis is presented. This is followed by a discussion of the potential defects in these mechanisms which may lead to NIDDM, with particular reference to the relevance of the genetic background of the condition.

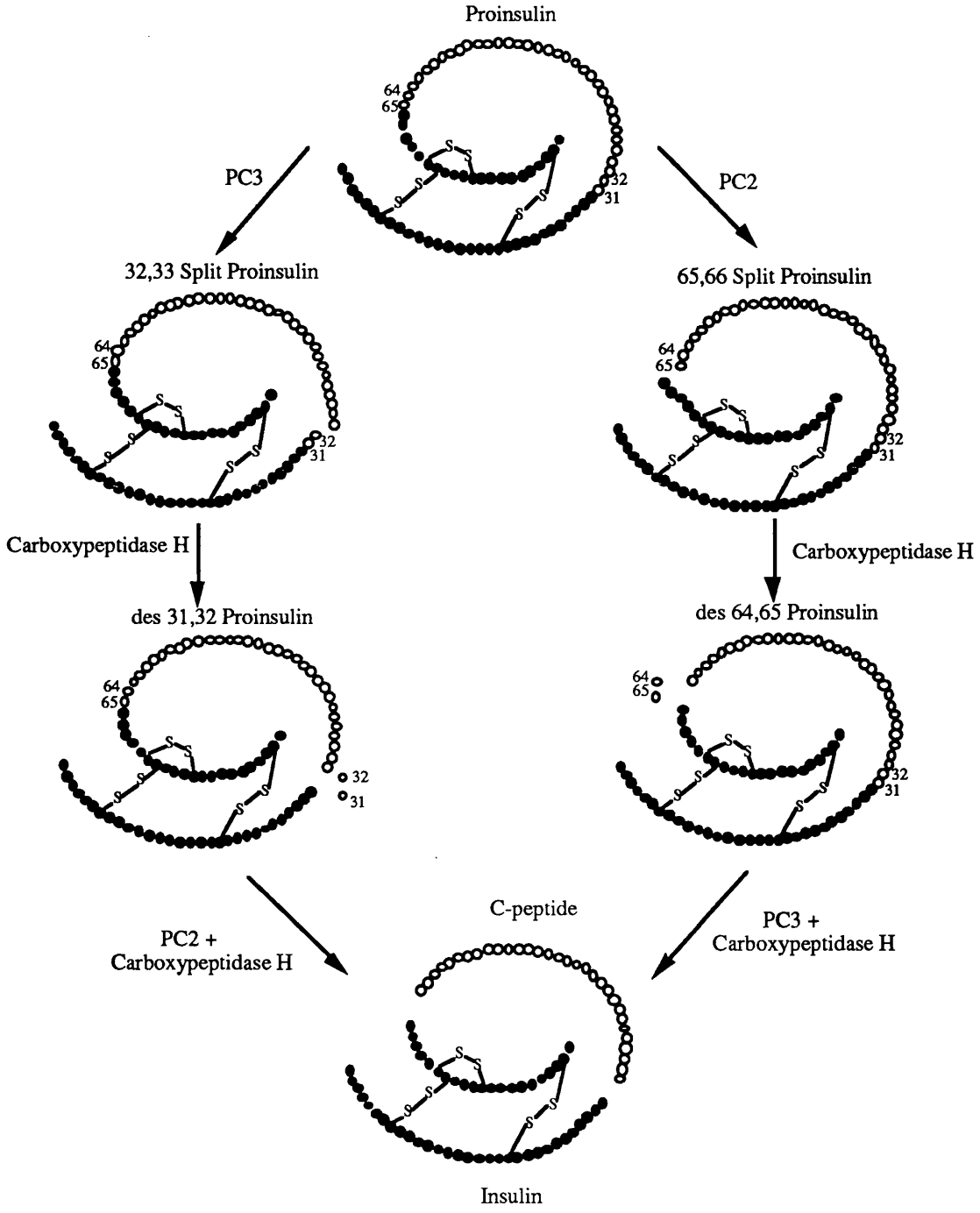
1.1. INSULIN PHYSIOLOGY

1.1.1. Insulin Synthesis

Insulin is synthesised in the pancreatic ß cell as the precursor preproinsulin. Synthesis is initiated by transcription of the insulin gene, which is located on the short arm of chromosome 11. Transcription, and the subsequent translation of insulin mRNA on the ß cell rough endoplasmic reticulum, are regulated by glucose and cyclic AMP. Cleavage of the amino terminal signal peptide in the lumen of the rough endoplasmic reticulum by signal peptidase produces proinsulin. This 86 amino acid polypeptide is transported via the Golgi complex to secretory granules, where it is converted to insulin. The conversion requires an acidic environment which is maintained by an ATP-dependent proton pump and is associated with loss of the granule clathrin coat. Proinsulin is cleaved at one of two possible basic sites, Arg-Arg and Lys-Arg, by the PC-2 and PC-3 endopeptidases, producing 32, 33 and 65, 66 split proinsulins respectively; this is followed by cleavage of residual C terminal basic amino acid residues by carboxypeptidase H, to form the desdiamino peptides. This series of reactions then occurs on the contralateral side to yield mature insulin and C peptide in equimolar amounts (Kemmler et al. 1971) (Figure 1.1).

The secretory granules act as an insulin store and, when prompted by a secretagogue, discharge their contents by exocytosis. Any insulin which is not released is degraded when the granules fuse with lysosomes. This sequence of proinsulin packaging into granules, conversion to insulin, insulin storage and release represents the '*regulated*' secretory pathway and accounts for the processing of the majority of proinsulin. Less than 1% of proinsulin is processed by the alternative '*constitutive*' pathway; this comprises the rapid delivery of proinsulin from the Golgi to the plasma membrane, and lacks the facilities for storage, degradation or control by secretagogues of the regulated pathway (Halban 1991). In the ß cell granule insulin asssociates into dimers and then hexamers which are stored as a stable crystalline structure.

Figure 1.1. The conversion of proinsulin to insulin.



The amino acid sequence of insulin is represented by the closed circles and C-peptide by the open circles. Amino acid 64 is lysine and 31, 32 and 65 are arginine.

1.1.2. Insulin Secretion

Insulin secretion is pulsatile, with rapid oscillations every 11-13 minutes and slower large amplitude oscillations mainly after meals ((Lang et al. 1979; Polonsky et al. 1988b). The major regulator of insulin secretion is the plasma glucose concentration. Fluctuations in blood glucose level are detected by the ß cell glucose sensor, glucokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) which catalyses glucose utilization, and thus regulates insulin secretion (Matschinsky 1990). In response to glucose, insulin is secreted in a biphasic manner, with an intial rapid phase of 5-10 minutes and a slower second phase. Both phases are calcium-dependent and represent the release of prestored insulin, but the second phase also involves the release of newly synthesised hormone (Curry et al. 1968; Porte Jr and Pupo 1969).

Glucagon secreted by pancreatic A cells stimulates insulin release and somatostatin from pancreatic D cells inhibits insulin secretion. These actions may result from paracrine regulation of pancreas ß cell insulin release. Insulin secretion may also be stimulated by fatty acids, amino acids, of which arginine is the most potent stimulus, and gastrointestinal hormones, such as gastrointestinal inhibitory peptide (GIP), cholecystokinin (CCK) and secretin. The insulin response to these non-glucose secretagogues is dependent on the ambient glucose concentration; this is called *glucose potentiation.* Insulin secretion is also under neural control. The parasympathetic system triggers insulin release via vagal stimulation. Alpha adrenergic activation inhibits insulin secretion, but ß adrenergic activation stimulates insulin secretion. These autonomic factors account for the insulin release which occurs at the thought of food (cephalic phase of insulin secretion).

In normal man insulin and C peptide are secreted in equimolar quantities (Polonsky and Rubenstein 1984). Insulin has a half life of approximately 5 minutes (Horwitz et al. 1975)) and 50% of the insulin secreted is removed by the liver in its first pass. Insulin is also cleared by the kidney, where 40% of the insulin presented to it is extracted. C peptide has a half life of 30 minutes and has no biological activity. About half of the C peptide secreted is removed by the kidney, its extraction by the liver being negligible

(Horwitz et al. 1975). A small amount of proinsulin and its conversion intermediates normally escapes cleavage (3-10%) and is secreted into the portal circulation. Proinsulin has a half life of 17 minutes (Horwitz et al. 1975). The fractional hepatic extraction of proinsulin is less than 5%, with clearance predominantly occurring in the kidney. Proinsulin clearance is approximately 20-30% that of insulin. Intact proinsulin and its conversion intermediates can bind to the insulin receptor but with reduced affinities of 1-3% and 2-8% of that of insulin respectively. The concentration of proinsulin required to compete at the insulin receptor is ten times higher than that of insulin (Madsbad et al. 1992). The biological activity of proinsulin parallels its metabolic clearance rate and is only 8-20% that of insulin (Yudkin 1993).

1.1.3. Assessment of Insulin Secretion

Measurement of insulin secretion is complex due to the elaborate mechanisms controlling insulin secretory function outlined above. The technique selected depends, amongst other factors, on the type of subject to be tested and the information required from the study. The **oral glucose tolerance test** is widely used for investigation of insulin secretion, but information gained is limited by poor reproducibility (McDonald et al. 1965)) and the insulin response is complicated by variations in glucose absorption due to differences in gastric emptying and gastrointestinal motility, the gastrointestinal hormone response and glucose utilization (Groop et al. 1993b). Nevertheless, the test is simple to perform, is acceptable to subjects and can also be diagnostic and it is therefore a valuable method for assessing insulin secretion.

The **intravenous glucose tolerance test** is preferable for assessing glucose-regulated secretion and ß cell function. It enables investigation of the first (early) phase of insulin secretion reliably and with good reproducibility (Rayman et al. 1990). The second phase is also measured, but is more dependent on the glucose concentration achieved. This test may be combined with an injection of another secretagogue, such as arginine or glucagon, to maximally stimulate insulin secretion, though these additional secretagogues are sometimes poorly tolerated due to side effects and require multiple blood samples. The **hyperglycaemic clamp** provides a controlled glucose stimulus, providing an alternative method for studying the first and second phases of insulin

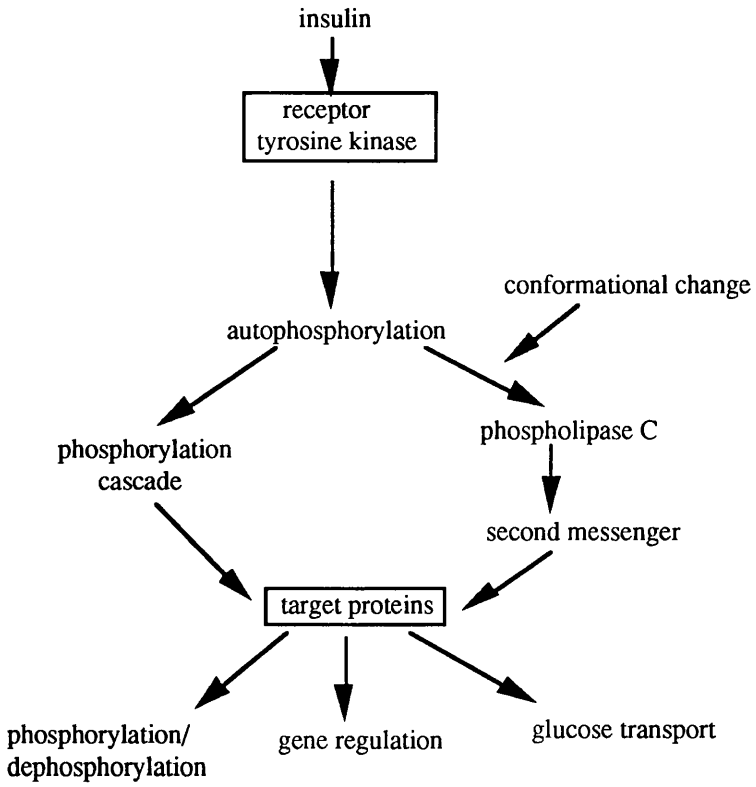
secretion, but the technique is complex to perform and again involves many blood samples.

In these tests peripheral plasma insulin levels are taken to represent pancreatic insulin secretion. However, as discussed above, in normal man fractional hepatic extraction of insulin is large and varies both between subjects and under different physiological circumstances in the same subject (Gibby and Hales 1983). This is particularly a problem when studying patients with insulin treated diabetes. As C peptide is produced in equimolar amounts with insulin but undergoes negligible hepatic extraction, measurement of peripheral C peptide concentrations is valuable for studying insulin secretion in a variety of clinical disorders, but results should be interpreted with caution since the kinetics and metabolism of C peptide under different conditions are not fully understood (Polonsky and Rubenstein 1984). Measurement of the C peptide response to intravenous glucagon is a useful test in insulin dependent diabetes, when injected insulin and insulin antibodies make it difficult to measure insulin itself. The peak C peptide response correlates with the response to a mixed meal and the fasting C peptide level (Faber and Binder 1977), but the glucagon bolus may induce unacceptable nausea and vomiting. Insulin secretion rate can also be assessed using mathematical modelling (Polonsky et al. 1988a). However, the intravenous glucose tolerance test is adequate for assessment of insulin secretion in healthy individuals with normal glucose tolerance.

1.2. MECHANISM OF INSULIN ACTION

Despite the diversity of actions produced by insulin, all known effects are initiated by insulin binding to cell surface receptors. This generates a series of changes resulting in transduction of insulin's signal to promote glucose transport and other aspects of cellular metabolism.

Figure 1.2. The mechanisms by which insulin interaction with its receptor results in target protein actions. Post-receptor events are speculative.



1.2.1. The Insulin Receptor

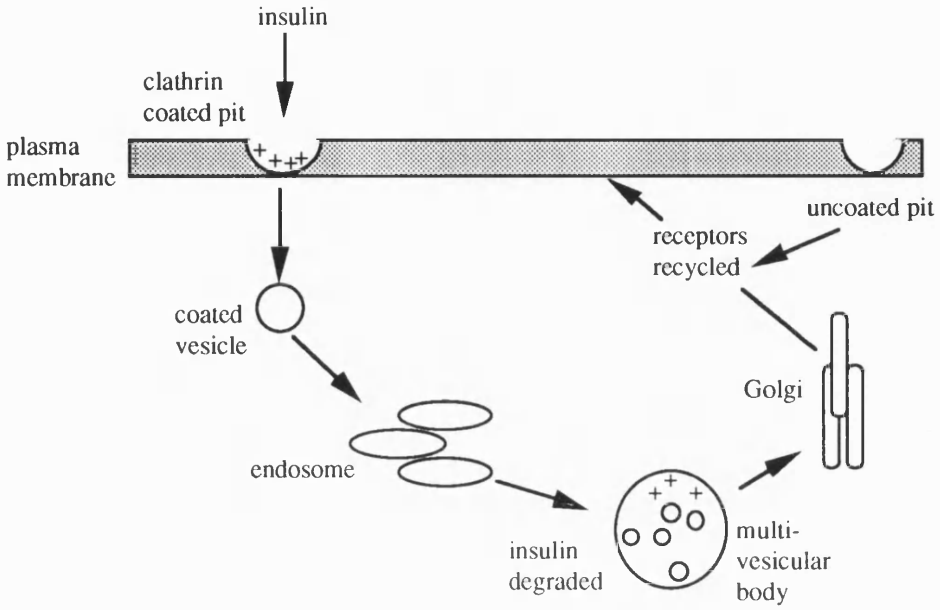
Insulin receptors are found on all tissues in varying concentrations. Each receptor has a glycoprotein structure arranged in four subunits covalently linked by disulphide bonds. The two α subunits are identical, have a molecular mass of 135 000 and are located on the extracellular surface of the cell membrane. The two ß subunits, each with a molecular mass of 95 000, traverse the plasma membrane and are involved in transmembrane signalling (Figure 1.4). The insulin receptor is synthesised from a proreceptor, which undergoes N-glycation and is cleaved into the individual

α and ß subunits in the Golgi apparatus. These are further modified with glucosamine, sialic acid and fatty acyls, before the formation of disulphide bonds produces the tetrameric receptor. The glycated cysteine-rich sections of the α subunit on the cell surface are thought to be the areas involved in insulin binding. The ß subunit extends from its amino terminus on the extracellular surface, through the plasma membrane as a transmembrane domain leading to an intracellular segment which possesses tyrosine kinase activity (Ullrich et al. 1985; Ebina et al. 1985).

The unoccupied insulin receptors are located on microvilli on the cell surface. Once insulin binds, the resulting hormone-receptor complexes migrate to the bases of the microvilli in pits lined by clathrin, a cytoplasmic protein. These coated pits become incorporated into the cell by endocytosis forming a coated vesicle. The clathrin coating is then lost and the vesicles combine with a network of tubules producing endosomes. These organelles have a proton pump to maintain an acidic environment which causes dissociation of the insulin-receptor complex. The insulin then passes to multivesicular bodies where it is degraded by lysosomal enzymes, whereas most of the receptors pass to the Golgi apparatus. Here they join newly synthesised receptors, are reprocessed and are then recycled to the cell membrane (Carpentier 1989).

A small proportion of the insulin-receptor complexes are endocytosed in uncoated pits; in this case, the dissociated receptors recycle directly back to the cell membrane by-passing the Golgi apparatus, and the insulin is partially degraded and extruded from the cell (Figure 1.3).

Figure 1.3. Insulin receptor recycling.



The insulin receptor serves not only to recognise its specific ligand and initiate the event leading to the hormone's action, but it also has a role in maintaining the balance between receptor number and hormone concentration. In situations of raised insulin concentration, receptor turnover and internalisation are increased, resulting in reduced receptor number (downregulation). In addition, as more receptors become occupied, the affinity of the adjacent unoccupied receptors decreases because of an increased rate of hormone:receptor dissociation, which is known as negative cooperativity.

1.2.2. Insulin Transmembrane Signalling

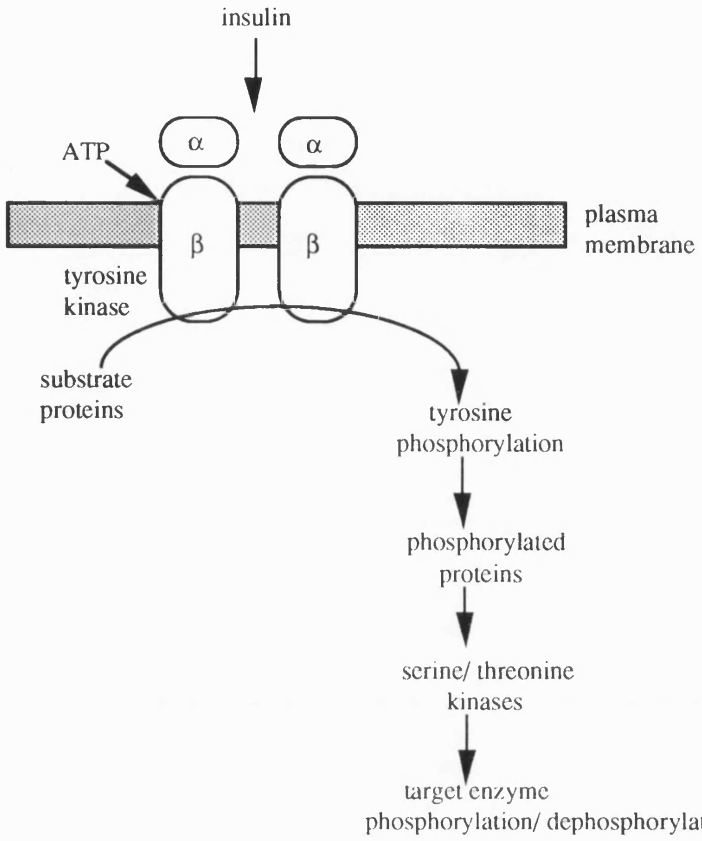
The ß subunit of the insulin receptor is a tyrosine-specific protein kinase. In the unoccupied state, the α subunit suppresses this kinase activity. Once insulin binds, it produces a conformational change which disinhibits the kinase (Ellis et al. 1987; Shoelson et al. 1988). In the activation of tyrosine kinase, ATP binds to the ß subunit near the transmembrane domain with a specific amino acid sequence which is essential for kinase activity. ATP binding is followed by phosphorylation of tyrosines on the

receptor itself (autophosphorylation). The ß subunit kinase activity is then directed towards other substrates in the cell (Lane et al. 1990). This kinase activity continues until the receptor is dephosphorylated and it is not dependent on insulin remaining bound to the α subunit (Yu and Czech 1984). The kinase may be inactivated by serine phosphorylation (Takayama et al. 1988). Autophosphorylation and kinase activity are vital to produce insulin's metabolic actions (Morgan and Roth 1987). However other effects of insulin, such as mitogenesis, may be mediated by alternative mechanisms. (Debant et al. 1988).

1.2.3. Insulin Post-Receptor Events

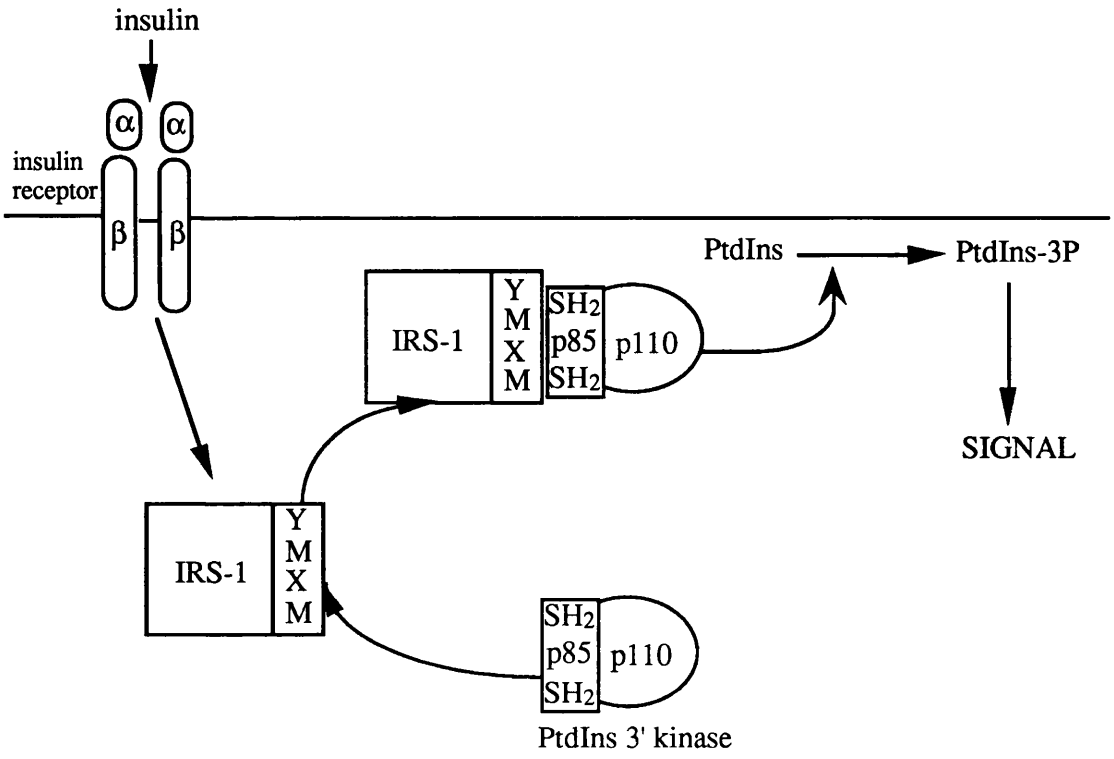
The events which follow insulin binding and transmembrane signalling and culminate in the metabolic actions of insulin are less well understood. Many actions involve the phosphorylation and dephosphorylation of enzymes and this has led to the theory of a phosphorylation cascade initiated by the receptor tyrosine kinase. These phosphorylation/dephosphorylation processes are mediated by serine/threonine kinases (Czech et al. 1988), with insulin-induced receptor autophosphorylation as the initiating event in a cascade of phosphorylation of cellular substrates. This results in amplification of the signal and alteration of the phosphorylation state, and hence the activity, of target enzymes (Lane et al. 1990) (Figure 1.4). Many proteins have been proposed as substrates in this phosphorylation cascade (Kasuga et al. 1990).

Figure 1.4. Post-receptor phosphorylation cascade



The first endogenous cellular substrate of the insulin receptor, **insulin receptor substrate 1 (IRS-1)**, has recently been identified (Sun et al. 1991). This cytosolic protein of molecular weight 131 000, contains over 30 potential serine/threonine phosphorylation sites. Insulin stimulates the association of IRS-1 with phosphatidylinositol (PtdIns) 3'-kinase, an enzyme which phosphorylates the PtdIns of the myoinositol ring by an alternative route to the classical phospholipase C pathway discussed below. PtdIns 3'-kinase is composed of 2 subunits, a 110 000 molecular weight catalytic subunit and a regulatory protein of molecular weight 85 000 (p85α). The latter contains two SH (src homology) 2 domains which effect protein-protein interactions by binding to Tyr-phosphorylated protein motifs, and in particular, those with the sequence YMXM (Tyr-Met-Xaa-Met).

Figure 1.5. Insulin signalling initiated by insulin receptor substrate 1.



PtdIns-3P = phosphatidylinositol 3'-phosphate

When the insulin receptor is activated, the receptor kinase phosphorylates specific tyrosine residues with the sequence YMXM in IRS-1 and these phosphorylated sites associate with the SH2 domains of p85α, thus activating PtdIns 3'-kinase. IRS-1 contains six YMXM motifs, so it may potentially bind multiple PtdIns 3'-kinase molecules, or simultaneously bind several different proteins containing an SH2 domain. Subunit p85 exists as two isoforms, α and ß. Only isoform p85α associates with p110 subunit to form the PtdIns 3'-kinase enzyme. The p85ß does not, but may act as an adaptor molecule linking IRS-1 to a different signalling enzyme. An increasing number of different proteins containing SH2 domains are currently being identified which can interact with IRS-1 after insulin stimulation (Myers and White 1993). Although IRS-1 has been studied in relation to insulin stimulation of cell proliferation, it may also be involved in insulin-mediated metabolic events, with the particular pattern of IRS-1 phosphorylation determining the specific metabolic actions in individual tissues.

Alternatively, insulin's metabolic actions may be the result of the production of a second messenger which conveys the signal to the target enzyme. Autophosphorylation of the insulin receptor results in a conformational change and this may allow its non-covalent interaction with cellular proteins that can act as signal transducers (Perlman et al. 1989). The addition of insulin to myocytes stimulates the hydrolysis of an inositol glycophospholipid, designated glycosylphosphatidylinositol (GPI), thereby generating diacylglycerol and the polar headgroup of the molecule. Glycosylphosphatidylinositol is present in many other tissues and its structure is similar to that of a membrane bound glycosyl phosphatidylinositol. This membrane-linked phosphoinositol is hydrolysed by a specific phospholipase C releasing diacylglycerol, inositol phosphate glycan and over 40 membrane proteins with diverse functions (Saltiel 1990). The link between insulin receptor activity and stimulation of this phospholipase C is still speculative. Phospholipase C may be activated as part of the tyrosine kinase-induced phosphorylation cascade or it may result from a receptor conformational change, possibly following GTP binding (Goren et al. 1985; Korn et al. 1987).

Amongst the numerous proteins yielded by phosphoinositol hydrolysis is lipoprotein lipase (Saltiel 1990). The observation that both insulin and phospholipase C release lipoprotein lipase with identical kinetics *in vitro* is of significance as insulin causes lipoprotein lipase release from adipocytes *in vivo* (Chan et al. 1988). Phospholipase C hydrolysis also yields diacylglycerol which may itself act as a second messenger by promoting protein kinase C activity. Activation of protein kinase C by other means, such as by phorbol esters, has the effect in some cases of mimicking insulin action and in others, of opposing insulin's action (Van de Werve et al. 1985). This apparent paradox may be resolved by the existence of distinct chemical forms of diacylglycerol possibly deriving from different sources; each chemical form of diacylglycerol may selectively activate different isoforms of protein kinase C (Pelosin et al. 1987) with different substrate affinities and tissue distributions.

Finally it may be that the diversity of insulin's actions lends support to the hypothesis that no single mechanism accounts for the transmission of insulin's signal to target enzymes but rather, a combination of phosphorylation cascade, conformational change

and second messengers act synergistically.

1.2.4. Glucose Transport into Cells

The uptake of glucose by cells across the plasma membrane is achieved by means of two gene families of glucose transporters. The Na^+ glucose cotransporter or *symporter* is an active transport mechanism located on the luminal epithelial surface of cells of the small intestine and proximal convoluted tubule of the kidney. Glucose is transported against its concentration gradient and is coupled to the transport of Na^+ down its concentration gradient, maintained by Na^+/K^+ ATPase. The gene coding for the Na^+ glucose transporter has been localised to the q11.2 region of chromosome 22 (Bell et al. 1990).

The second family of glucose transporters comprises the *facilitative transporters*, a group of glycoproteins which transport glucose down its concentration gradient in an energy independent manner. The concentration gradient exists as a result of the more rapid rate of phosphorylation of glucose inside the cell compared to the rate of glucose entry into the cell. The facilitative glucose transporters have been designated GLUT 1-5 according to the chronological order in which their cDNA was described and they have also been named according to the tissue in which they are most plentiful. This facilitative transport is saturable, stereospecific for D-glucose and it is inhibited by cytochalasin B.

GLUT 1, the erythrocyte-type glucose transporter, is expressed in most tissues as well as in cultured cells, but is found in highest concentration in the brain microvessels, kidney, colon and placenta. The human Glut 1 gene has been located on the short arm of chromosome 1 and comprises 10 exons. Human Glut 1 is composed of 492 amino acids, has a molecular weight of 45 000 - 55 000 and is arranged in a structure which weaves in and out of the plasma membrane lipid bilayer 12 times. The resulting peptide transmembrane loops are described in a proposed structural model as M1-12. The segment between M1 and M2, which contains an oligosaccharide, resides

extracellularly and that between M6 and M7 is a highly hydrophilic intracellular domain. The amino and carboxy terminals both lie on the cytoplasmic side of the plasma membrane (Mueckler et al. 1985).

Glut 1 exhibits considerable structural similarities, not only with the other four human facilitative transporters, but also with those in the rat, with bacterial H^+/xylose and H^+/arabinose symporters and with yeast transporters. The marked sequence conservation between Glut 1-5 in certain transmembrane regions suggests that these domains, particularly M7 and M8, may function as the channel for glucose migration. The glycine/proline rich sections may be implicated in the conformational change accompanying glucose transport. Other highly conserved segments, including charged amino acids, may form ionic interactions with intracellular proteins involved in glucose metabolism, such as hexokinase/glucokinase, or with different segments of the transporter itself (Gould and Bell 1990). The Glut 1 transporter is responsible for the provision of the cell's basal glucose requirement.

GLUT 2, the liver-type glucose transporter, is predominantly distributed in the liver, kidney, small intestine and pancreatic ß cells. Glut 2 is composed of 524 amino acids and the Glut 2 gene has been located on chromosome 3. In the intestine and the kidney Glut 2 is located on the basolateral cell membrane and it is employed in the transepithelial transport of glucose into the blood. Glucose accumulates in the cells by the Na^+ glucose cotransporter on the apical membrane, which creates a glucose concentration gradient between the cell contents and the blood. The expression of Glut 2 in pancreatic islets may be important in the control of insulin secretion from ß cells (Mueckler 1990).

The cells of the liver, kidney and intestine are capable of releasing as well as taking up glucose, and to this end the Glut 2 transporter functions such that glucose flux across the cell changes almost linearly with the extracellular glucose concentration. This prevents glucose transport becoming rate limiting for intracellular glucose metabolism

when the glucose concentration is high, and also enables glucose efflux to continue when the intracellular glucose concentration is raised.

GLUT 3 is widely distributed in all tissues but is predominantly expressed in brain, kidney and placenta. It is coded for by a gene on chromosome 12 (Gould and Bell 1990). **GLUT 4**, the muscle/adipocyte transporter, is the major glucose transporter in the insulin sensitive tissues and is expressed in skeletal muscle, white and brown fat and in the heart (James et al. 1989). It is composed of 509 amino acids and the Glut 4 gene has been localised to chromosome 17. **GLUT 5** is expressed mainly in the small intestine. It is probable that other transporters exist and have yet to be characterised.

1.2.5. Insulin-Stimulated Glucose Transport

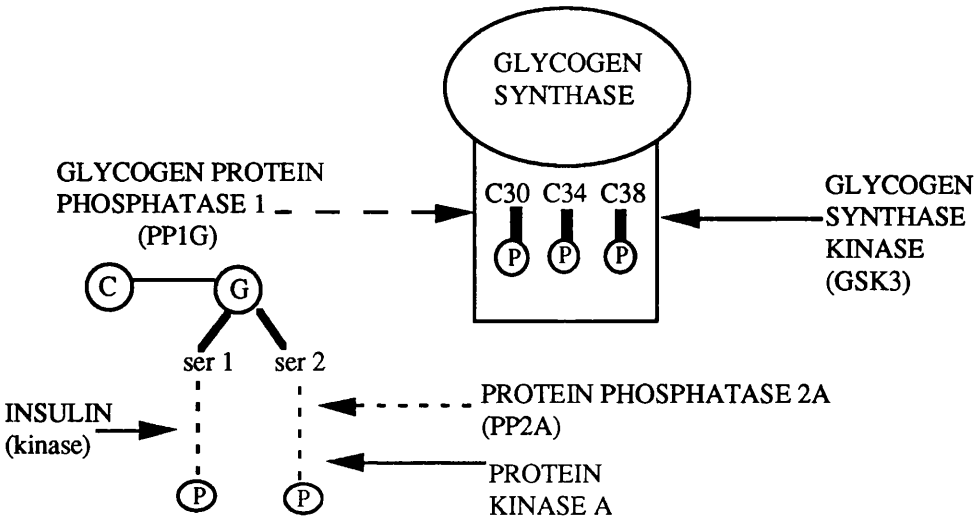
In the fed state, skeletal muscle is the main site of insulin-stimulated glucose disposal, with adipose tissue having a minor direct role (less than five per cent). Glucose transport in muscle and adipocytes can increase more than 30 fold when stimulated by insulin. In the basal state most Glut 1 transporters are located in the plasma membrane, whereas Glut 4 transporters are situated chiefly intracellularly in low density microsomes. When exposed to insulin the intracellular transporters are recruited to the plasma membrane (Cushman and Wardzala 1980). This translocation involves predominantly the Glut 4 transporter and can produce a 10-fold increase in glucose transport over the basal rate. This mechanism alone does not account for the massive increase in glucose transport observed after insulin exposure. Another mechanism which has been proposed is an increase in the intrinsic activity of the Glut 4 transporter. The molecular mechanism may be through alteration of the phosphorylation state of the transporter (Glut 4 is phosphorylated by ß adrenoceptor agonists which also decrease the intrinsic activity of this transporter (Joost et al. 1986)). Another possible explanation for the discrepancy is stimulation of glucose transport by diacylglycerol (Stralfors 1988). Other mechanisms are certainly involved, with a possible role for cAMP.

1.2.6. Insulin Action on Target Proteins

Many of the intracellular effects of insulin are associated with an alteration in the phosphorylation state of target proteins, as discussed above. The proteins may be phosphorylated on serine and threonine residues or they may be dephosphorylated. Two examples are given below.

a) mechanism of insulin action on muscle glycogen synthase (Figure 1.6). Glycogen synthase contains nine serine residues capable of being phosphorylated. In the inactive state, three of these residues (C30, C34 and C38) are phosphorylated by glycogen synthase kinase-3 (GSK 3). When activated by insulin these residues undergo dephosphorylation by the enzyme protein phosphatase 1 (PP1), which is also involved in the dephosphorylation of glycogen phosphorylase and phosphorylase kinase. The PP1 associated with glycogen ($PP1_G$) is composed of a catalytic subunit (C), relative molecular mass 37 000, linked to a glycogen-binding subunit (G), relative molecular mass 160 000 (Cohen and Cohen 1989). The G subunit is responsible for guiding PP1 to glycogen and it contains two serine (ser) residues as potential phosphorylation sites. Phosphorylation by cAMP-dependent protein kinase A occurs at serine site 2 and results in dissociation of the G and C subunits; the released C subunit is approximately five fold less effective in dephosphorylating, and hence activating, glycogen synthase than the intact $PP1_G$. This mechanism accounts for the inhibitory action of adrenaline on glycogen synthase. Recombination of the subunits is achieved by protein phosphatase 2A (PP2A), which dephosphorylates site 2. Adrenaline not only causes site 2 phosphorylation via cAMP dependent protein kinase A, inactivating $PP1_G$, but also phosphorylates site 1; in this way $PP1_G$ is prepared for the rapid resynthesis of glycogen once the effect of adrenaline has ceased. Insulin promotes glycogen synthase activity by phosphorylating serine site 1 on the G subunit of $PP1_G$ which activates PP1. This may occur via a specific kinase (Dent et al. 1990). This insulin-stimulated protein kinase itself requires phosphorylation to be active and may be related to the ribosomal protein S6 kinase II (Denton 1990).

Figure 1.6. The action of insulin on glycogen synthase.



b) mechanisms of insulin effects on fatty acid metabolism

In adipose tissue, phosphorylation/ dephosphorylation reactions are also implicated in insulin's action as glucose is directed into the synthesis of fatty acids and triglyceride. Pyruvate dehydrogenase is active when dephosphorylated; acetyl Co A carboxylase is active in the phosphorylated form. Pyruvate dehydrogenase (PDH) is activated by PDH phosphatase and inactivated by PDH kinase. Acetyl CoA carboxylase activation by insulin involves phosphorylation on serine residues, two of which have been characterised as I-peptide and T4 A peptide. The serine residue in the latter (serine-29) is also phosphorylated *in vitro* by casein kinase 2 (Haystead et al. 1988). The kinase responsible for acetyl CoA carboxylase phosphorylation by insulin requires the presence of a low molecular weight component, which inhibits acetyl CoA carboxylase (Borthwick et al. 1990). Phosphorylation of acetyl CoA carboxylase kinase reverses the inhibition. Acetyl CoA carboxylase is inactivated by phosphorylation of another serine residue, serine-79, by an AMP-activated protein kinase (Hardie et al. 1989).

c) insulin action via control of gene expression

Insulin can also exert its effects on target proteins by producing alterations in mRNA translation or gene transcription. This regulation is accomplished by means of DNA elements which do not code for a protein. Transcription of the gene for

phosphoenolpyruvate carboxykinase (PEPCK), the rate limiting enzyme in gluconeogenesis, is inhibited by insulin in a rapid, specific and reversible manner. Insulin inhibits transcript initiation, and also, to a lesser extent, inhibits transcript elongation (O'Brien and Granner 1990). In contrast, insulin stimulates transcription of gene for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Alexander et al. 1988).

1.3. GLUCOSE HOMEOSTASIS

In normal man blood glucose concentration is maintained within a narrow range despite variations in dietary intake and activity. This is achieved by means of an integrated series of metabolic processes which can adapt to permit continued fuel supply during periods of starvation and allow storage of substrate in times of excess.

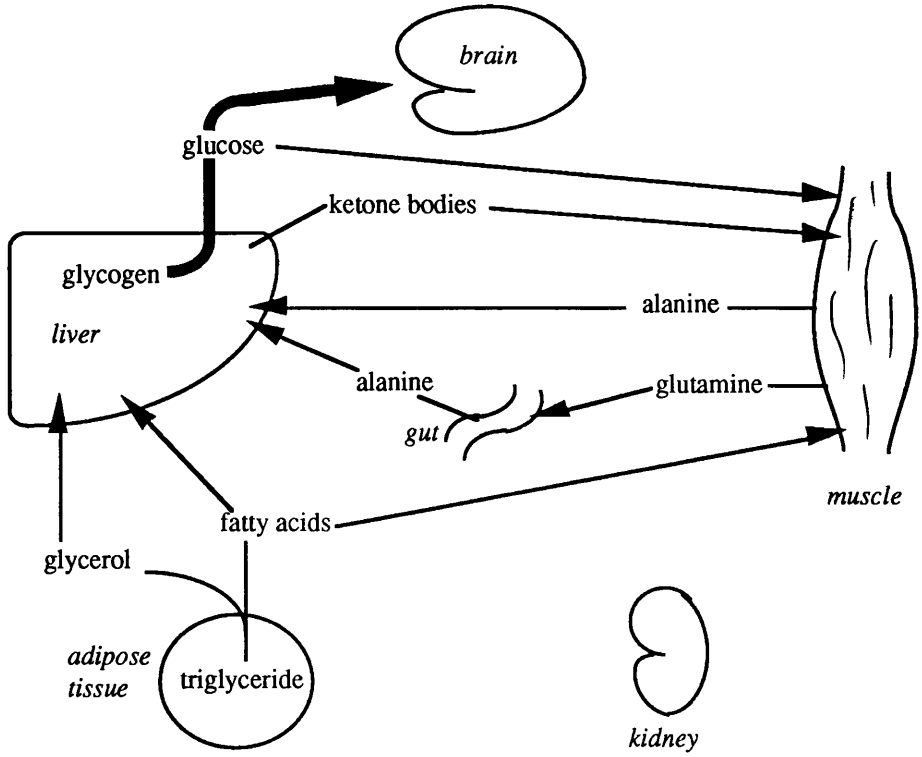
1.3.1. Starvation

In the postabsorptive period hepatic glycogen is the main source of glucose (60-80%) with the contribution from gluconeogenesis increasing as fasting continues. The glucose produced (5-6g/hour) is utilized mostly by the central nervous system, which has an obligatory requirement for glucose, with only 1-2g/hour being taken up by tissues such as muscle, which can utilize other fuels. The major substrates for glucose synthesis *de novo* are glycerol and the amino acids, alanine and glutamine (Figure 1.7).

Glycerol is released following triglyceride hydrolysis in adipose tissue together with non-esterified fatty acids (NEFA). Glycerol cannot be used for re-esterification as glycerokinase is not present in adipose tissue, but new glycerol 3-phosphate must be produced from glucose. In the fasting state glycerol 3-phosphate production is decreased. The majority of the NEFA released from lipolysis are therefore oxidised and only 25% are re-esterified to triglyceride. A small proportion of the NEFA oxidised are first converted in the liver to ketone bodies, prior to release for oxidation by muscle. In the hepatocyte NEFA enter the mitochondria via the carnitine shuttle. They are oxidised to acetyl CoA and then converted to ketone bodies through the hydroxy methylglutaryl CoA (HMG CoA) cycle.

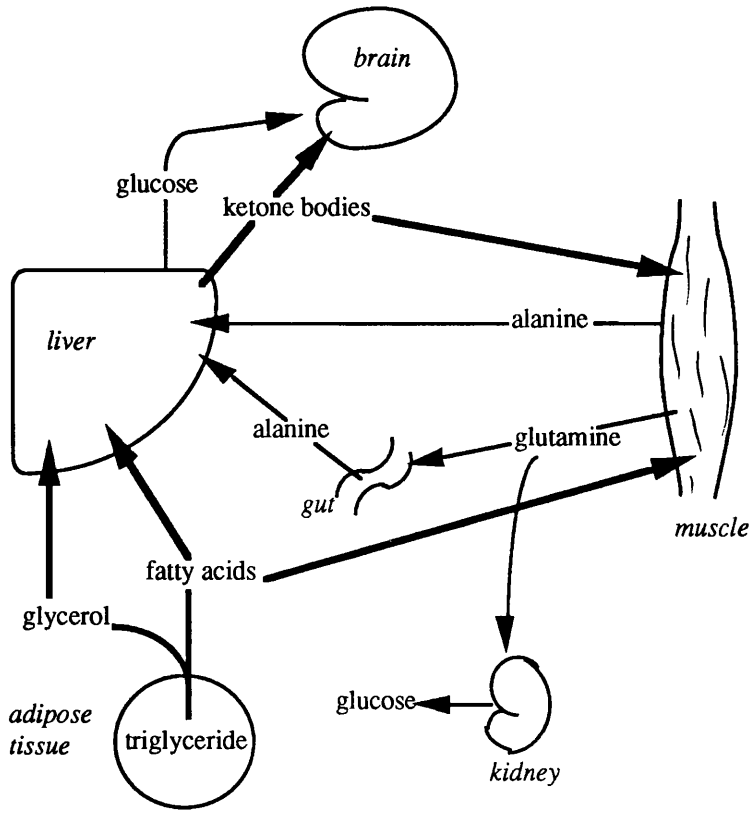
The amino acids destined for gluconeogenesis are released following protein degradation. Some of the glutamine released from muscle is taken up first by the gut and converted to alanine, before being released into the portal system for uptake by the liver. An additional amount of glucose is synthesised by gluconeogenesis from lactate derived mainly from anaerobic metabolism of glucose in peripheral tissues such as muscle (the Cori cycle).

Figure 1.7. Fuel production and utilization after an overnight fast.



In prolonged fasting hepatic glycogen stores are depleted after 24 hours and gluconeogenesis becomes the only source of glucose (Figure 1.8). The brain adapts to utilize ketone bodies as fuel and reduces its glucose requirement from 100g/day to 35g/day. As circulating ketone levels rise, their utilization increases and the ketones inhibit alanine release from muscle. These adaptive mechanisms spare further muscle protein breakdown which is important as loss of more than half of the body's protein is incompatible with survival. The kidney becomes an important gluconeogenic organ as it uses glutamine to produce ammonia and buffer protons from fatty acids and ketones (Newsholme and Leech 1983).

Figure 1.8. Fuel production and utilization after starvation for several days

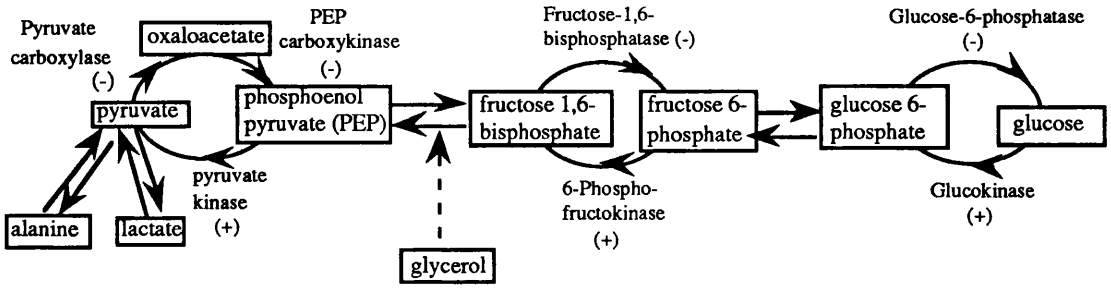


1.3.2. Regulation of Glucose Homeostasis During Fasting

The metabolic response to fasting is regulated by hormones and by substrate supply. In early fasting the high levels of glucagon and glucocorticoids and low levels of insulin increase **glycogenolysis**. Hepatic glycogenolysis is stimulated by glucagon through a cyclic AMP dependent mechanism which is antagonised by insulin. Glucocorticoids have a permissive effect and catecholamines also stimulate glycogenolysis.

Gluconeogenesis is stimulated by glucagon which acts at a number of sites. It increases amino acid uptake by the liver. It also acts via cyclic AMP to increase pyruvate carboxylase activity and decrease pyruvate kinase activity. It decreases 6-phosphofructokinase activity and increases that of fructose 1-6 bisphosphatase. Insulin antagonises these intrahepatic effects and also inhibits synthesis of phosphoenolpyruvate carboxykinase.

Figure 1.9. Regulatory steps in gluconeogenesis.



Stimulatory (+) and inhibitory (-) effects of insulin occur at the sites indicated.

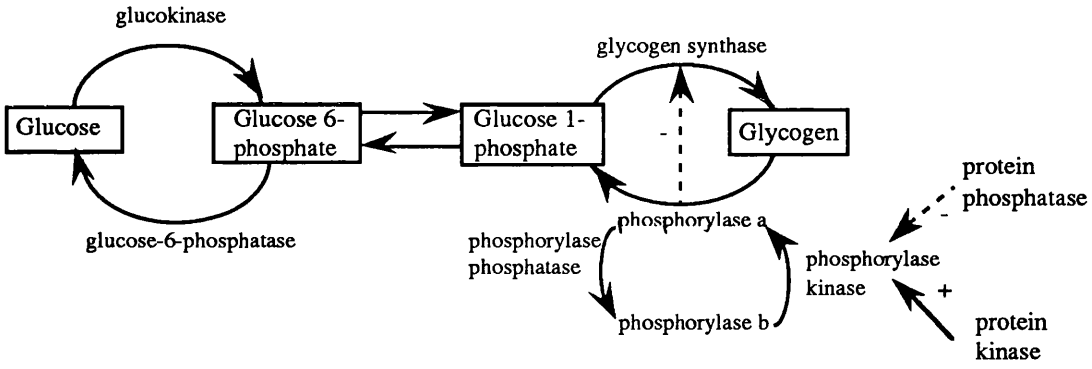
Glucocorticoids stimulate gluconeogenesis by increasing gluconeogenic substrates from the periphery and inducing phosphoenol pyruvate carboxykinase, fructose 1-6 bisphosphatase and glucose 6-phosphatase in the liver. The high rates of fatty acid oxidation stimulate gluconeogenesis through a hepatic mechanism (Blumenthal 1983). Insulin decreases the release of glycerol from adipose tissue and the low plasma insulin of starvation increases glycerol supply for gluconeogenesis.

Glucose utilization is low in the fasting state. Peripheral glucose disposal is influenced indirectly by alterations in NEFA levels. Insulin is a powerful inhibitor of lipolysis, and this effect occurs at one tenth of the insulin level required to stimulate glucose uptake. The low concentrations of insulin favour lipolysis, which is also enhanced by the increased circulating growth hormone concentrations. The NEFA produced from lipolysis undergo oxidation. This results in an increase in intracellular acetyl CoA which inhibits pyruvate dehydrogenase; the increased NADH/NAD ratio is associated with citrate accumulation and citrate inhibits 6-phosphofructokinase. This leads to the accumulation of glucose 6-phosphate which inhibits hexokinase. The consequences are a reduction in muscle glucose transport with a decrease in glycogen synthesis and in glucose oxidation (Randle et al. 1963) (Figure 1.11). The low insulin:high glucagon levels of starvation stimulate ketogenesis by increasing fatty acyl CoA entry into the mitochondria. Circulating tri-iodothyronine (T_3) concentrations are reduced with an increase in reverse T_3 and these are important for limiting protein degradation and reducing total energy expenditure.

1.3.3. Postprandial Glucose Metabolism

Following a carbohydrate-rich meal glucose entry into the circulation exceeds immediate body fuel requirements and glucose storage occurs. The sight of food produces a reflex release of insulin which antagonises the gluconeogenic and glycogenolytic actions of glucagon. This primes the liver for when portal blood concentrations are high. Glucose uptake occurs to replenish hepatic glycogen stores used during earlier fasting. The proportion of glucose extracted in its first pass ranges from 10-60%.

Figure 1.10. Hepatic glucose metabolism.



Phosphorylase activity is stimulated (+) by kinases and inhibited by phoshatases (-)

The control of hepatic glucose uptake relies on the existence of enzyme substrate cycles (Figure 1.10). The regulatory step is the phosphorylation of glucose to glucose 6-phosphate by glucokinase. The activity of hepatic glucokinase is dependent on the concentration of glucose in the hepatic portal vein. This is discussed in more detail in section 1.3.4 below. After a meal, the glucose concentration in the hepatic portal vein is high and the activity of glucokinase increases accordingly, resulting in enhanced glucose uptake by the liver. When the blood glucose concentration falls, glucokinase activity decreases below that of glucose-6-phosphatase, resulting in a net release of glucose. Glucose itself stimulates glycogen synthesis. It combines with phosphorylase a and the resulting complex acts as a substrate for phosphorylase phosphatase. This inactivates phophorylase and disinhibits glycogen synthase. Insulin also increases synthase activity. Hepatic glycogen may be synthesised indirectly following the conversion of absorbed glucose to lactate in the liver. This may occur as a consequence of hepatocyte

specialization, with certain cells producing lactate while others synthesise glycogen (Dinneen et al. 1992). At postprandial insulin concentrations hepatic glucose production is suppressed by 50-75%.

In peripheral tissues glucose is taken up to replenish muscle glycogen. This occurs partly because of insulin-induced suppression of lipolysis, with the resultant decrease in circulating NEFA permitting uptake and metabolism of glucose by muscle by the glucose-fatty acid cycle (Randle et al. 1963). At higher insulin concentrations muscle glucose uptake is stimulated directly, by producing both an increase in glucose transport and an increase in glycogen synthase activity. This direct effect is maximal at plasma insulin concentrations of approximately 250mU/l. This insulin-induced increase in muscle glucose disposal is caused primarily by increased glucose storage, with a small increase in glucose oxidation at high insulin concentrations (DeFronzo et al. 1981).

In adipose tissue the high insulin levels of the fed state inhibit lipoprotein lipase with a reduction in NEFA levels; glucose transport is stimulated and this provides glycerol 3-phosphate for re-esterification. Dietary lipid is absorbed more slowly than carbohydrate in a mixed meal and chylomicrons are synthesised. In contrast to muscle, lipoprotein lipase in capillary endothelium is stimulated by insulin with release of fatty acids, which are then available for re-esterification. Dietary protein is digested and absorbed as amino acids or di- or tri-peptides. The high postprandial insulin levels favour amino acid transport into and protein synthesis in many tissues.

1.3.4. Glucokinase

Glucokinase is a member of the hexokinase family but differs from the other members by its low affinity and greater specificity for glucose and by its lack of product inhibition by glucose 6-phosphate. Thus, the rate of glucose phosphorylation is proportional to the plasma glucose concentration (Randle 1993). Glucokinase is found only in the liver and the pancreatic ß cell and is regulated differently in these two sites. In the liver, glucokinase is stimulated by insulin at the level of transcription and inhibited by glucagon. It enhances hepatic uptake and phosphorylation of glucose, maintaining a gradient for glucose uptake into the liver. In the ß cell, glucokinase

activity is controlled by glucose (Bedoya et al. 1986) and this is the basis for its proposed role as 'pancreatic glucose sensor' (Magnuson 1990). By regulating the rate of glucose phosphorylation and glycolysis, glucokinase determines the ATP:ADP concentration ratio. Enhanced glucose phosphorylation increases this ratio, resulting in closure of the ADP-dependent K^+ channels in the ß cell, causing membrane depolarisation. This opens the voltage-dependent Ca^{2+} channels and the increase in ß cell Ca^{2+} triggers insulin release.

The glucokinase gene has 10 exons and is located on chromosome 7. Exons 2-10 encode the bulk of the mRNA and there are 3 alternative isoforms of exon 1. The hepatic and ß cell glucokinase genes have different transcription control regions (Magnuson and Shelton 1989) which give rise to tissue specific glucokinase isoforms. This may explain how the glucokinase gene is chiefly regulated by insulin in liver, but by glucose in the ß cell. These different regulatory mechanisms may form the basis of a feedback loop; an increase in plasma glucose concentration induces pancreatic glucokinase expression, causing increased glucose phosphorylation and glycolysis and stimulating insulin secretion. This induces hepatic glucokinase and increases glucose utilization, resulting in a lowering of the plasma glucose concentration. Liver glucokinase may also influence hepatic intermediary metabolism coupled to fluctuations in blood glucose concentration (Magnuson 1990).

1.4. INSULIN ANALOGUES

In normal subjects, the concentration of insulin in plasma is maintained at a low basal level. In response to meals, insulin secretion rapidly increases to peak within 30-60 minutes and returns to basal levels after 2-3 hours. The aim of insulin therapy in the diabetic patient is to reproduce this physiological diurnal profile of plasma insulin and hence normalise blood glucose levels. Typically a combination of an intermediate acting insulin, to provide constant basal levels, with boluses of soluble insulin with meals is administered subcutaneously. However, absorption from the subcutaneous depot is slow with peak plasma concentrations of soluble insulin not attained for 90-120 minutes after injection, and plasma insulin levels remain inappropriately raised for the subsequent 3-5 hours. This results in postprandial hyperglycaemia and runs the risk of late hypoglycaemia (Brange et al. 1990; Kang et al. 1991).

In an attempt to more closely mimic the physiological pattern of insulin secretion biosynthetic insulin analogues have been developed. These are absorbed more rapidly than human insulin and have a shorter duration of action (Brange et al. 1988; Vora et al. 1988) and thus have potential therapeutic benefit (Kang et al. 1990).

1.5. ASSESSMENT OF INSULIN ACTION

The significance of defective insulin action in diabetes and other pathological states has led to the development of a variety of methods of assessment of insulin action *in vivo*. The majority of studies have focused on the effect of insulin on blood glucose concentration.

The measurement of insulin sensitivity in man was pioneered by Himsworth (1936), who studied subjects by **oral glucose tolerance testing** on two occasions, once with and once without an intravenous insulin injection. The difference in the rate of change of blood glucose between the two tests was utilized to classify diabetic individuals as insulin sensitive or insulin insensitive. The development of a radioimmunoassay for plasma insulin (Yalow and Berson 1960a) permitted quantitation of the plasma insulin response, which could then be related to the change in blood glucose. This revealed that obese individuals and subjects with non-insulin dependent diabetes (NIDDM) exhibited similar or elevated immunoreactive insulin responses to oral glucose compared to normal subjects (Yalow and Berson 1960b; Karam et al. 1963). This inappropriate hyperinsulinaemia in the presence of normal or supranormal blood glucose concentrations was interpreted as evidence for insulin insensitivity. However, use of the oral glucose tolerance test as a method of assessing insulin sensitivity is limited due to the persistent interaction between glucose and insulin. In such a closed loop system it is difficult to separate the individual effects of insulin sensitivity and ß cell function.

In an attempt to break this feedback loop, Shen et al. (1970) introduced the **insulin suppression test,** which consisted of the pharmacological suppression of endogenous insulin secretion by adrenaline and propranolol infusion, with simultaneous glucose and insulin infusions. The resulting plasma glucose concentration achieved at steady state was considered an index of total body sensitivity to insulin-mediated glucose disposal. The technique was subsequently modified, first by Harano et al. (1977) who employed somatostatin to inhibit endogenous insulin release to ensure suppression of glucagon and growth hormone in diabetics, and again by Nagulesparan et al. (1979) who administered insulin in a dose adjusted to body weight to produce

similar steady state plasma insulin levels in different subjects. The limitations of this technique are that it assumes complete suppression of hepatic glucose production occurs, it does not provide a quantitative estimation of glucose disposal and the possibility exists that the pharmacological agents employed may themselves influence insulin sensitivity.

The **hyperinsulinaemic glucose clamp** devised by DeFronzo (1979) overcame these problems and provided an alternative approach for assessment of insulin sensitivity. Insulin is infused at a constant rate in a dose adjusted to body weight or surface area and the plasma glucose level is kept constant by a variable glucose infusion. The technique was made possible with the development of rapid blood glucose analysers, whereby the glucose infusion rate is adjusted depending on blood glucose measurements made at 5 minute intervals. A steady state is reached when the rate of glucose infusion and the plasma glucose concentration are constant. The glucose infusion rate (M) is then equal to total body glucose disposal. However, the expression of insulin sensitivity by M values has a number of problems. Variations in insulin clearance between subjects and incomplete suppression of endogenous insulin secretion result in different steady state plasma insulin concentrations in different individuals (Bergman et al. 1985). To account for these factors, the M value may be divided by the steady state plasma insulin concentration (I).

A further complication is the contribution of insulin independent glucose uptake to total body glucose disposal, since glucose itself can enhance its own uptake by the law of mass action. In normal subjects this contribution is small, but it may be considerable in hyperglycaemic individuals. To allow for this, insulin sensitivity has been compared at different levels of glycaemia using the ratio of glucose utilization rate to the plasma glucose level or metabolic clearance rate (MCR). However, this assumes that the plasma glucose concentration and the glucose utilization rate are directly proportional. Yet insulin independent glucose uptake is saturated at euglycaemia and glucose clearance decreases as the plasma glucose rises. Hence use of MCR may be inappropriate. Ader and Bergman (1987) therefore, developed the insulin sensitivity index (Si). This is calculated from the increment in glucose utilization during a clamp (ΔRd) at plasma

glucose (G) and the increment in insulin concentration (ΔI) where $Si = \Delta Rd/G.\Delta I$ and is independent of the prevailing glycaemia and insulin concentration when the clamp is performed. This method also requires measurement of hepatic glucose output by prior infusion of a glucose tracer.

The **minimal model approach** to assessing insulin sensitivity uses a frequently sampled intravenous glucose tolerance test and obviates the requirement to achieve a steady state (Bergman 1989). Following the glucose bolus, a biphasic insulin response is triggered which stimulates glucose uptake by peripheral tissues, inhibits hepatic glucose output and also augments the ability of glucose itself to enhance its own uptake (glucose effectiveness S_G). By computer analysis, the simplest model is fitted to the observed glucose and insulin dynamics to produce an insulin sensitivity index (S_I), representing the incremental glucose disappearance rate per unit increase in plasma insulin and a measure of the insulin independent glucose disappearance (S_G) (Bergman et al. 1979). The model makes the assumption that the effect of insulin to promote plasma glucose disappearance depends on the insulin concentration in a compartment remote from plasma. The technique has been modified by addition of a tolbutamide or somatostatin injection to ensure a greater insulin response occurs after glucose has mixed in the extracellular fluid. The insulin sensitivity index reflects both hepatic glucose production and peripheral glucose disposal. Use of labelled glucose for the IVGTT has enabled measurement of glucose disposal alone, but has also prompted revision of the monocompartmental computer model to account for the variation in the volume of glucose distribution (Caumo et al. 1991).

Computer modelling has been employed in other methods of assessing insulin action, such as the continuous infusion of glucose with model assessment **(CIGMA)** (Hosker et al. 1985). This involves infusion of glucose at a constant rate, with measurement of steady state plasma insulin and glucose concentrations and provides a measure of endogenous insulin secretion.

More recently the requirement for a simple, minimally invasive method for measuring insulin sensitivity has been fulfilled by returning to the plasma glucose response to

intravenous insulin. In the past, such **insulin tolerance tests** have been complicated by the counter-regulatory response triggered by the fall in plasma glucose and the risk of dangerous hypoglycaemia (Rizza et al. 1979). However, Bonora et al (1989) have modified the test to examine the plasma glucose fall in the initial 15 minutes after insulin, before a counter-regulatory response is mounted. The insulin sensitivity measurement reflects both stimulation of peripheral glucose disposal and inhibition of hepatic glucose production. The method assumes that endogenous glucose output is completely suppressed with the supraphysiological insulin doses employed. This provides a valuable method of assessment of insulin action which does not require complicated laboratory or computer facilities. The further application of this method is discussed in chapters 2 and 3.

1.6. PATHOGENESIS OF NIDDM

The pathogenesis of non-insulin dependent diabetes (NIDDM) is characterised by both tissue insensitivity to insulin and by deficient insulin secretion. These features are manifest clinically by hyperglycaemia, as the normal mechanisms for maintaining glucose homeostasis are disrupted (DeFronzo 1988).

1.6.1. Insulin Action in NIDDM

1. a) Hepatic glucose production

In the fasting state hepatic glucose production (HGP) is increased and this is the main cause of fasting hyperglycaemia. The elevated HGP is of the order 0.5mg/kg/min and closely correlates with the plasma glucose level (DeFronzo 1988). Hepatic glucose production is increased in the presence of raised plasma insulin levels and this suggests that the liver is resistant to the suppressive effects of insulin (DeFronzo 1992). However, studies on the suppression of HGP by insulin have been inconsistent, with greater than 90% inhibition reported by workers using high insulin levels (DeFronzo et al. 1982; Firth et al. 1987), but inappropriately elevated HGP documented with the use of lower insulin levels in the physiological range (Firth et al. 1987). These discrepancies may have arisen from the different doses of insulin employed; due to the steep dose response curve, at supraphysiological insulin concentrations an effect on hepatic glucose output may have been obscured. The degree of suppression of HGP by insulin is dependent on the severity of diabetes. In addition, the use of non steady state tracer methods to quantitate HGP underestimates total body glucose disposal and this may have masked a defect in the suppression of HGP (Cobelli et al. 1983). Using techniques which reduce these negative errors, impaired suppression of HGP has been confirmed in NIDDM (Levy et al. 1989).

The majority of the excess glucose produced by the liver is derived from enhanced gluconeogenesis, up to three fold normal (Consoli et al. 1989). This is made possible by the increase in circulating gluconeogenic precursors, especially lactate, alanine and glycerol and by the stimulation of gluconeogenesis by the elevated glucagon levels and accelerated fatty acid oxidation (Baron et al. 1987; Groop et al. 1989). As a consequence of enhanced HGP and the resulting hyperglycaemia, muscle glucose

uptake is increased by a mass action effect.

b) Glucose uptake

Defronzo et al. using clamp studies in combination with hepatic and femoral venous catheterisation demonstrated that the principal site of insulin-stimulated glucose uptake in normal man is skeletal muscle. In NIDDM this action of insulin was delayed and the absolute rate of insulin-stimulated glucose uptake was reduced by up to 50% (DeFronzo et al. 1985). This confirmed that muscle is the primary site of insulin resistance in established NIDDM. In response to an oral glucose load, reduced suppression of HGP as well as impaired muscle glucose uptake, inappropriate for the level of plasma glucose, both contribute to the postprandial hyperglycaemia (Dinneen et al. 1992).

Once taken up by muscle, glucose may be oxidised to carbon dioxide and water, converted to lactate or stored as glycogen by non-oxidative metabolism. Studies which have combined indirect calorimetry to assess glucose oxidative metabolism with the hyperinsulinaemic clamp technique to measure total glucose uptake, have found that the main fate of glucose was non-oxidative metabolism and that this was assumed to represent glycogen synthesis (DeFronzo et al. 1981). Direct confirmation for this came from nuclear magnetic resonance spectroscopy during hyperglycaemic hyperinsulinaemic clamp studies. These demonstrated that muscle glycogen synthesis is the major pathway of glucose disposal and that it is impaired glycogen synthesis which accounts for the defect in glucose metabolism in NIDDM (Shulman et al. 1990).

2. Mechanism of impaired insulin action

a) The molecular mechanism for this defect in insulin sensitivity in NIDDM has yet to be fully elucidated. At the level of the insulin receptor, reduced insulin binding has been documented with a shift of the dose response curve to the right. With mild hyperglycaemia, high levels of insulin can elicit a normal maximal response. The reduced binding may result from receptor down-regulation due to persistent hyperinsulinaemia. With more severe hyperglycaemia, there is also a decrease in maximal response, suggestive of a post binding defect (Kolterman et al. 1981).

A number of postbinding defects have been reported. There is evidence for an impairment in insulin receptor tyrosine kinase activity in skeletal muscle in NIDDM, though this may be secondary to the metabolic disturbance (Häring 1991). Investigation of glucose transport in NIDDM has revealed reduced numbers of GLUT 4 transporters in adipocytes (Garvey et al. 1988), but in skeletal muscle numbers are normal (Pedersen et al. 1990). It is possible that glucose transport is impaired secondary to the hyperglycaemia (Moller and Flier 1991).

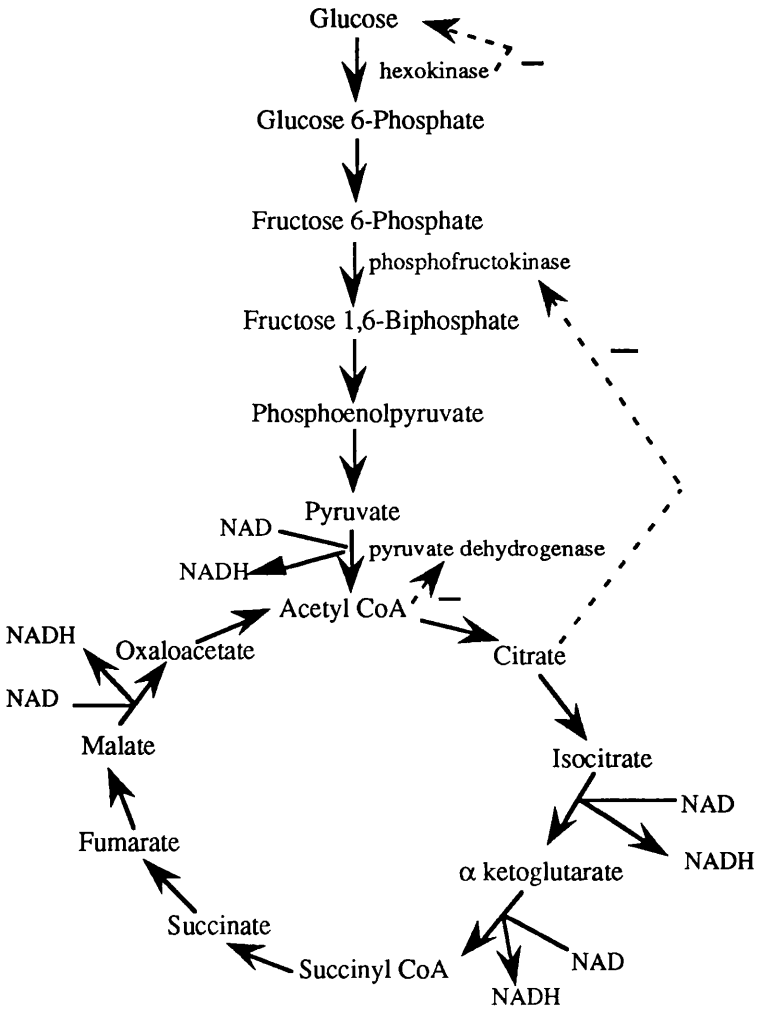
Defects have also been reported in NIDDM in the activity of enzymes involved in the metabolic pathways of glucose disposal. In the pathway of glucose oxidation, insulin activation of pyruvate dehydrogenase is reduced in muscle (Mandarino et al. 1986). Decreased muscle glycogen synthase activity correlating with the impairment in non oxidative glucose disposal has been demonstrated by Damsbo et al (1991). In addition, the insulin stimulation of glycogen synthase phosphatase, the enzyme which maintains glycogen synthase in an active form, is impaired in NIDDM (Kida et al. 1990).

b) lipid oxidation

Another potential mechanism for the defective insulin action in NIDDM is the role of fatty acid metabolism. Non-esterified fatty acids (NEFA) are released into the circulation from the hydrolysis of adipose tissue triglyceride by the action of hormone sensitive lipase, an enzyme which is inhibited by insulin. The NEFA may then either be oxidised to carbon dioxide and water or be re-esterified to triglyceride (non-oxidative metabolism). In a study of NIDDM patients using ^{14}C palmitate to quantitate fatty acid turnover, indirect calorimetry to measure glucose and lipid oxidation and graded insulin euglycaemic clamps, there was impaired suppression of NEFA turnover, oxidation and re-esterification by insulin compared to control subjects. This enhanced NEFA metabolism was associated with decreased oxidative and non-oxidative glucose metabolism. In addition, the rate of fatty acid oxidation correlated with the rate of HGP (Groop et al. 1989). These results demonstrate that the decreased insulin sensitivity in NIDDM extends to lipid, as well as glucose metabolism. Lipid oxidation and glucose metabolism are inversely related (Felber et al. 1987). A rise in NEFA levels in plasma results in increased fatty acid oxidation. Gluconeogenesis is stimulated by the enhanced

supply of ATP and by the accumulating acetyl CoA, which activates pyruvate carboxylase and inhibits pyruvate dehydrogenase, thereby increasing the availability of pyruvate as substrate. Glucose metabolism by muscle is impaired by the Randle cycle (Randle et al. 1963) (Figure 1.11) as described in section 1.3.2.

Figure 1.11. The glucose-fatty acid cycle.



c) glucose toxicity

Chronic hyperglycaemia may itself cause impaired insulin action and this has led to the theory of "glucose toxicity". This mechanism appears to involve reduced glucose transport and a defect in non-oxidative glucose metabolism (Del Prato et al. 1986; Kahn et al. 1991). Hyperglycaemia can also lead to defective ß cell secretion, as discussed in the next section.

1.6.2. Insulin Secretion in NIDDM

In established NIDDM insulin secretion is deficient and although it remains controversial whether this is the primary event in the development of NIDDM, the nature of the defect in insulin secretion appears complex.

1. Insulin levels

Early studies demonstrated that subjects with maturity onset diabetes have similar insulin levels to normal individuals (Yalow and Berson 1960a). However, these subjects and controls were not matched for the degree of obesity, which is now known to be an independent factor affecting insulin secretion (Bagdade et al. 1967).

Evaluation of fasting and glucose-stimulated plasma insulin concentrations in subjects with a range of fasting glucose concentrations has revealed an inverted U relationship (DeFronzo 1988). Progression from normal to impaired glucose tolerance was associated with a gradual increase in insulin levels and development of insulin insensitivity. As glucose tolerance deteriorated to frank diabetes, there was a further increase in fasting and glucose-stimulated insulin levels, but with severe hyperglycaemia insulin levels fell back to the control range. However, the insulin response is influenced by a number of other factors which also require consideration.

2. Dynamics of insulin secretion

The insulin response to intravenous glucose is delayed in NIDDM with loss of the initial first phase (Brunzell et al. 1976). Yet the response to other secretagogues remains intact. In the presence of hyperglycaemia, insulin secretion to non glucose secretagogues should be exaggerated due to glucose potentiation. Thus, when matched for the level of glycaemia these insulin responses are also impaired. In overt NIDDM, the glucose potentiated maximum insulin response to other secretagogues is reduced by 90% (Leahy 1990; Porte Jr 1991). The normal pulsatile pattern of insulin secretion is disturbed in NIDDM. Pulse amplitude is reduced and the usual relationship to meals is lost (Polonsky et al. 1988a). The functional significance of these disturbances has yet to be established.

3. Proinsulin

The contribution of impaired ß cell function to the pathogenesis of NIDDM has been further complicated by the development of specific immunoradiometric assays for insulin and its precursors (Temple et al. 1989). Conventional radioimmunoassays for insulin cross react with proinsulin and its split peptides which are biologically inactive and consequently may have overestimated insulin levels.

Proinsulin is normally secreted in very small amounts (Horwitz et al. 1975) and is cleared relatively slowly by the kidney, so that it comprises less than 10% of circulating immunoreactive insulin in normal subjects. In patients with NIDDM, there is a disproportionate increase in circulating proinsulin (Ward et al. 1987; Davies et al. 1993a), even if the absolute proinsulin levels are normal (Levy et al. 1993). This could reflect the increased demand on the ß cell causing depletion of mature insulin granules with premature release of unprocessed proinsulin and intermediate peptides; alternatively, it may represent a defect in prohormone processing (Porte Jr 1991). The use of specific immunoradiometric assays has revealed that fasting and glucose-stimulated insulin levels are decreased in overt NIDDM (Temple et al. 1989; Leahy 1990; Levy et al. 1993) and the defect becomes more severe with increasing hyperglycaemia (Davies et al. 1993a). There are reports of patients with mild fasting hyperglycaemia having normal or elevated specific insulin levels, though these may still be inappropriate for the degree of glycaemia (Leahy 1990). In addition, subjects with impaired glucose tolerance have increased proinsulin and 32, 33 split proinsulin concentrations indicating some degree of ß cell dysfunction (Davies et al. 1993b).

4. Aetiology of insulin deficiency

a) ß cell mass

The origin of the ß cell dysfunction in NIDDM is uncertain. A modest reduction in ß cell mass of up to 50% has been demonstrated in autopsies of NIDDM patients (Maclean and Ogilvie 1955), but this would not be sufficient to account for the greater than 90% reduction in ß cell function which is required to produce diabetes. The morphology of the islets is normal.

b) Islet amyloid polypeptide

Recent interest has focused on the role of islet amyloid polypeptide (IAPP). Amyloid deposits are frequently identified in the islets of patients with NIDDM, but are also found in those of non-diabetic individuals, the elderly and in benign insulinomas (Steiner et al. 1991). The main constituent of this amyloid material is IAPP, a 37 amino acid peptide which shows considerable sequence homology with the calcitonin gene related peptide (CGRP). The gene for IAPP is located on the short arm of chromosome 12. IAPP is produced by the ß cell as an 89 amino acid precursor, pre-pro IAPP (Johnson et al. 1989) which undergoes proteolytic cleavage to produce the mature peptide. The ability of IAPP to form amyloid fibrils is related to the amyloidogenic sequence in the 25-28 region. IAPP is co-stored with insulin in the secretory granule and in response to glucose and other secretagogues, it is secreted together with insulin into the extracellular space (Westermark et al. 1992). In the fasting state the serum IAPP concentration is about 10% of the insulin level. There is little evidence for increased secretion of IAPP in NIDDM, but there may be abnormal regulation of IAPP secretion resulting in an altered IAPP: insulin ratio.

The biological function of IAPP is unknown. At pharmacological concentrations IAPP has been demonstrated to inhibit glucose-stimulated insulin release and to impair basal and insulin-stimulated glucose disposal by reducing glucose transport, inhibiting glycogen synthase and stimulating glycogen phosphorylase activities (Westermark et al. 1992). However, these effects are not observed at physiological concentrations. IAPP is a vasodilator and may cause increased islet blood flow when insulin is released and it also has an hypocalcaemic action. The presence of amyloid fibrils in ß cell cytoplasm in NIDDM and IAPP in lysosomes suggests that during degradation of secretory granules, the acidic environment of the lysosome may induce fibril deposition. The fibrils are then extruded from the cell (Steiner et al. 1991). It has been proposed that extracellular amyloid deposits hinder the passage of glucose and hormones between plasma and the ß cells (Johnson et al. 1989). Alternatively, IAPP may be deposited secondary to abnormal ß cell function. The precise role of IAPP in the pathogenesis of NIDDM has yet to be elucidated.

c) Nutrition in early life

The insulin deficiency characteristic of established NIDDM has been postulated to result from impaired nutrition in early life. This theory was prompted by the observation that an association exists between impaired glucose tolerance in men and low weight at birth and age 1 year (Hales et al. 1991; Robinson et al. 1992). Hales and Barker proposed that impaired nutrition and in particular protein malnutrition, in fetal life and early infancy lead to poor development of the ß cells, such that in later life in times of overnutrition, the islets are unable to meet the increased insulin demand and diabetes results (Hales and Barker 1992). This theory has been expanded to explain the association reported between birth weight and the development in later life of other facets of insulin resistance including hypertension, hypertriglyceridaemia and increased plasminogen activator inhibitor concentrations (Barker et al. 1993a), as well as mortality from cardiovascular disease (Barker et al. 1993b). It is suggested that the specific clinical manifestations reflect poor nutrition at different critical times of organ and blood vessel development (Barker et al. 1993a), although this remains an hypothesis. The significance of these associations and their potential contribution to the development of NIDDM require further exploration.

1.6.3. Environmental Factors Contributing to NIDDM

The high prevalence of NIDDM in the westernised world suggests that the accompanying sedentary lifestyle may have an aetiological role; however, the individual contributions of diet, obesity and physical inactivity are not easily separated.

Obesity is a major independent risk factor for the development of NIDDM (Björntorp 1991). Obese subjects are insulin resistant both at the level of the liver and at the periphery. The dose response curves for insulin-mediated suppression of hepatic glucose production and insulin-stimulated glucose utilization are shifted to the right (Kissebah et al. 1988), and in the severely obese, there is also a reduction in maximum response. These defects are more pronounced when obesity is centrally located. Such upper body male fat distribution (android) is associated with a more adverse metabolic profile compared to lower body peripheral (gynoid) obesity with hyperinsulinaemia, hypertriglyceridaemia and hypertension (Krotkiewski et al. 1983). It is also

characterised by increased cortisol secretion which stimulates hepatic gluconeogenesis and hyperandogenism, both of which promote adipose tissue lipolysis; the resulting increased NEFA flux contributes to the insulin resistance (Björntorp 1991). However, the risk of developing NIDDM in subjects of equivalent obesity remains greater where there is a positive family history and in certain ethnic groups, such as the Mexican Americans compared to American Whites (Haffner et al. 1986).

Physical activity is inversely related to the development of NIDDM and for every 500kcal increment in energy expenditure, the age adjusted risk of NIDDM declines by 6% (Helmrich et al. 1991). This protective effect of exercise is independent of obesity, age and parental history of diabetes. The inverse relationship between physical activity and glucose tolerance has also been demonstrated amongst the inbred Pima Indian population, which has an exceptionally high prevalence of NIDDM (Kriska et al. 1993). Thus, physical activity can prevent NIDDM in genetically predisposed subjects (Eriksson and Lindgärde 1991). There is little evidence to implicate specific **dietary factors** in the development of NIDDM, apart from promoting obesity. The reduction in insulin action observed with **age** is most likely attributed to the associated reduction in muscle mass and inactivity.

1.6.4. Evidence for a Genetic Susceptibility in NIDDM

It has long been recognised that NIDDM tends to cluster in families, but many of the early studies failed to distinguish between insulin-dependent (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) (Steinberg 1961; Gottlieb and Root 1968). Recent evidence from twin, family and population studies however, has confirmed a strong genetic component.

1.Twin studies

Barnett et al (1981a) studied 53 monozygotic twin pairs in whom one or both had NIDDM and found 48 (91%) were concordant for NIDDM. With follow up, concordance increased to almost 100%. This compared with 54% concordance for twins with IDDM. The high concordance rate in the NIDDM twin pairs is particularly notable as most twins were living apart and were of different body weight, underlining

the importance of genetic factors. It is possible that this study may have overestimated disease concordance as the twins were selected because one member was already known to have diabetes, increasing the likelihood that concordant rather than discordant twins would be identified.

To overcome this ascertainment bias, Newman (1987) used a population-based twin registry identifying male twins born between 1917 and 1927 from military records, regardless of disease status. Of the monozygotic twin pairs aged 52-65 years studied, 58% were concordant for NIDDM and at follow up 10 years later, only one of 15 originally discordant twin pairs remained discordant.

2. Family studies

The familial aggregation of NIDDM also extends to other family members. In the Barnett study cited above (Barnett et al. 1981a) 42% of probands had a first-degree relative with diabetes. Some (Alcolado and Alcolado 1991), but not all (Cook et al. 1993a), workers report an excess of maternal rather than paternal diabetes in affected pedigrees. Allowing for the late age of onset of NIDDM, Köbberling and Tillil (1982) calculated that 38% of siblings of patients with NIDDM would develop diabetes by age 80 years. Similar results were reported for siblings by O'Rahilly et al. (1987), but there is a lower risk to offspring of affected parents (Beaty et al. 1982; O'Rahilly et al. 1987). This may reflect the influence of environmental factors such as physical activity delaying the age of onset of the disease. However, where the proband developed NIDDM before the age of 40 years, O'Rahilly reported both parents were more likely to be affected and 69% of siblings were found to be glucose intolerant.

This increased prevalence associated with early onset diabetes has been attributed to the offspring inheriting diabetogenic genes from both parents, resulting in a higher gene dose and consequently, an earlier age of onset of diabetes than in the previous generation (O'Rahilly and Turner 1988). Viswananthan et al. (1985) have also reported a high prevalence of abnormal glucose tolerance (62%) in offspring of conjugal NIDDM parents in South India.

3. Population studies

Further evidence for the genetic contribution to NIDDM is provided by the large variation in the prevalence of the condition in different ethnic groups living in the same geographical region. In the USA the prevalence of NIDDM ranges from 1% in those of Japanese origin, 2% in subjects of European origin, 4-6% in Blacks and 35% in the Pima Indians of Arizona (Zimmet 1982). The Pimas are one of certain isolated inbred populations which have an exceptionally high prevalence of NIDDM; prevalence in those over 35 years reaches 50%.

Another such population are the inhabitants of the Pacific island of Nauru, which recently acquired great wealth from its phosphate industry. This was associated with a change to a westernised lifestyle, reduced levels of physical activity and obesity. Amongst the inhabitants over age 60 years, the prevalence of diabetes in full blooded Nauruans is nearly five times that in Nauruans with foreign genetic admixture (Serjeantson et al. 1983) and the age adjusted prevalence increases with the strength of parental history of diabetes.

In both the Pima and the Nauruan populations glucose tolerance assumes a bimodal distribution with age, suggesting a major gene effect (Rushforth et al. 1971; Zimmet and Whitehouse 1978). In the Pimas, euglycaemic hyperinsulinaemic clamp studies have confirmed that insulin-stimulated glucose uptake is a familial characteristic, with familial membership accounting for up to one third of the variance in insulin action (Lillioja et al. 1987). Longitudinal studies have demonstrated that the progression from normal glucose tolerance to NIDDM is preceded by reduced insulin action and elevated mean insulin levels (impaired glucose tolerance) followed by both reduced insulin-stimulated glucose uptake and relative insulin deficiency once diabetes supervenes (Lillioja et al. 1988).

In this population, increased fasting insulin concentrations were strong predictors of later development of NIDDM (Saad et al. 1989). The fasting insulin levels conform to a trimodal distribution, implying a single gene codominant mode of inheritance of insulin resistance (Bogardus et al. 1988). Similar findings have been reported in the high

prevalence Mexican Americans; Haffner et al. (1988) demonstrated a stepwise increase in fasting and postglucose insulin levels in normal subjects with progressive strength of parental history of NIDDM.

These population studies provide strong evidence for a genetic contribution to the aetiology of NIDDM. Such isolated inbred communities have a relatively restricted gene pool with little dilution by foreign genetic admixture. They are also characterised by a recent rise in the prevalence of obesity, which may have unmasked a latent diabetes susceptibility that is genetically determined. The reason why this genetic susceptibility to diabetes should exist has been explained by the 'thrifty genotype' hypothesis (Neel 1962). This proposes that in populations exposed to fluctuating periods of feast (when food was plentiful) and famine a 'thrifty genotype' would confer a selective advantage when food was scarce. Energy storage would be maximised in times of abundant food supply enabling the individual to survive periods of famine. Nowadays, with an assured regular food supply, this genotype becomes detrimental with the development of hyperinsulinaemia, obesity, insulin resistance and NIDDM. This may also explain the recently reported reduction in incidence of progression from normal to impaired glucose tolerance or NIDDM in the Nauruans as the number of individuals with the susceptible genotype is exhausted (Dowse and Zimmet 1989).

1.6.5. Candidate Genes in NIDDM

The search for the genes involved in NIDDM has proved elusive as the primary metabolic disturbance remains unknown, but a number of potential candidates have been examined.

1. The insulin gene

A defect in the insulin gene has been implicated as this could potentially result in relative or absolute insulin deficiency. The insulin gene is located on the short arm of chromosome 11 in close proximity to the tyrosine hydroxylase gene and the insulin-like growth factor II gene (Permutt and Elbein 1990). Mutations in the insulin gene structure causing abnormal cleavage of the A or B chains have been reported in cases of diabetes with hyperinsulinaemia (Gabbay 1980), but these are rare. Furthermore, the insulin

gene sequence is normal in the high prevalence Pima and Nauruan populations (Raben et al. 1991). Consequently, defects in the regulatory regions for glucose stimulation of insulin release have been sought.

The insulin gene is closely linked with a hypervariable region of DNA containing a variable number of tandem repeat sequences producing three classes of alleles. Population studies examine the allelic frequencies of such genetic markers in diabetic and non diabetic subjects and look for a marker in linkage disequilibrium with the gene causing diabetes. Initial studies suggested an association of the 5' flanking region with NIDDM, but these studies were in mixed ethnic groups (Rotwein et al. 1983). Later reports in ethnic-specific populations failed to confirm these findings (Serjeantson et al. 1983; Awata et al. 1985; Permutt and Elbein 1990).

The value of these population association studies is limited; a spuriously positive association may be found if the patients and controls are not carefully matched for ethnic background and although a positive association is suggestive, a negative association does not exclude involvement of the candidate gene. Linkage analyses in multiplex families is a more powerful technique and such pedigree analyses have failed to demonstrate linkage with the insulin gene (Hitman et al. 1984; Permutt and Elbein 1990). Thus, insulin gene mutations may contribute to diabetes susceptibility in a few rare instances, but do not have a major role in the development of NIDDM.

2. The insulin receptor gene

Mutations in the insulin receptor gene have been described in patients with the syndromes of extreme insulin resistance and in their unaffected relatives (Odawara et al. 1989; Seino et al. 1990; Shimada et al. 1990). These conditions however, are rare. A number of studies in different populations have failed to demonstrate an association between NIDDM and the insulin receptor gene (Hitman et al. 1987; Permutt 1990; Sten-Linder et al. 1991), although an association has been reported in NIDDM patients with a strong family history (Elbein et al. 1987). Pedigree analyses in families with NIDDM (Serjeantson and Zimmet 1989; Elbein et al. 1991) or maturity onset diabetes of the young (Elbein et al. 1987) have also failed to implicate the insulin receptor gene.

3. The glucose transporter gene family

Li et al (1988) have reported an association of markers for the GLUT 1 gene and NIDDM in North European, South European and the Japanese populations, although this has not been demonstrated in other ethnic groups (Cox et al. 1988) nor in family studies (O'Rahilly et al. 1989; Lesage et al. 1991). Investigation of other members of the glucose transporter gene family has revealed that the GLUT 4 sequence is normal in Caucasians and Mexican Americans (Kusari et al. 1991). Pedigree analysis has also failed to implicate GLUT 2 or GLUT 4 in the development of NIDDM (Lesage et al. 1991).

Other candidate genes include HLA loci, with minor associations reported of HLA Bw22 in Nauruans (Serjeantson et al. 1983) and in American Blacks (Banerji et al. 1993), the amylin gene and in view of the reported excess maternal transmission of NIDDM (Alcolado and Alcolado 1991), mitochondrial DNA, which is exclusively maternally inherited. Mutations in this genome, in particular at position 3243 in the tRNA leucine, have been associated with mitochondrial myopathy and in some cases glucose intolerance (Reardon et al. 1992; Oka et al. 1993; Alcolado et al. 1994b), where defects in insulin secretion have been reported (Alcolado et al. 1994a; Walker et al. 1994). These genetic defects however, are rare and are not likely to explain the vast majority of NIDDM.

1.6.6. Difficulties with the Search for the Gene in NIDDM

These candidate gene analyses demonstrate how, until recently, little progress had been made with the search for the genetic defect in NIDDM. The diseases most suited to genetic analysis have a clear mode of inheritance, a known inherited biochemical defect and an early age of onset. Regrettably, NIDDM does not fulfill these requirements.

Despite being a common condition, its mode of inheritance remains uncertain. Linkage analyses are based on a proposed model and may be subject to error if the specified model is incorrect. NIDDM may have a polygenic aetiology, possibly with a major gene conferring disease susceptibility and minor genes contributing to disease expression. Subjects may be clinically unaffected but still possess the susceptibility gene, reducing

the power of these analyses (Hitman and McCarthy 1991). Linkage studies in families are more powerful than population association studies, but suitable three generation pedigrees can be difficult to ascertain. The late age of onset of NIDDM means that one or both parents of a patient with NIDDM may be dead, while affected children may not yet be old enough to express the disease. Furthermore, the premature mortality of those with NIDDM make it less likely that affected parents will still be alive.

Co-operation of all family members with the project is essential but can be difficult as NIDDM is often subclinical or associated with only mild symptoms and may not be perceived as a life-threatening condition. The unknown mode of inheritance and variable penetrance of NIDDM make it essential that all members of the pedigree and their spouses are formally tested for glucose intolerance to exclude subclinical disease. It is possible that NIDDM may be genetically heterogenous and different loci may contribute to NIDDM in different pedigrees. Linkage may be missed in population studies. The underlying metabolic defect has yet to be defined and many of the biochemical abnormalities described may be secondary to the initial defect. Thus, for a number of reasons NIDDM is not ideally suited to genetic analysis.

To overcome these difficulties attention has focused on maturity-onset diabetes of the young (MODY), a subtype of NIDDM characterised by autosomal dominant inheritance and disease onset before age 25 years (Tattersall et al. 1975). Study of subjects with MODY enables large multigeneration pedigrees to be identified and so surmounts many of the problems previously encountered in the genetic study of NIDDM.

1.6.7. Recent Developments in Candidate Gene Analysis

1. MODY

a) Glucokinase

A number of exciting advances have been made in the last few years involving the study of large MODY pedigrees. The glucokinase gene is an attractive candidate as it is involved in both regulation of insulin secretion and in glucose utilization (see section 1.3.4). In 1992, Froguel et al. reported close linkage of the glucokinase gene on chromosome 7 with MODY overall in 16 French families. This was followed by the

identification of a nonsense mutation in the glucokinase gene in the affected individuals of one of these families (Vionnet et al. 1992). Subsequently, linkage between MODY and the glucokinase gene has been reported in one British (Hattersley et al. 1992) and one Japanese (Shimada et al. 1993) MODY pedigree, but not confirmed in other MODY families studied (Hattersley et al. 1992; Cassell et al. 1992; Froguel et al. 1993; Dow et al. 1994). In addition, a large number of different mutations in the glucokinase gene have been identified, including missense mutations each producing amino acid substitutions, nonsense mutations resulting in the expression of a truncated protein and splicing mutations which affect RNA processing (Froguel et al. 1993).

This illustrates the genetic heterogeneity of MODY, not only between families but also within pedigrees. Furthermore, these genetic characteristics may distinguish different clinical phenotypes. The affected individuals who display a mutation in the glucokinase gene have been demonstrated to have a reduced insulin secretory response to glucose infusion (Velho et al. 1992; Hattersley et al. 1992; Permutt et al. 1992). The clinical features of reduced insulin secretion and mild hyperglycaemia may be explained on the basis of the genetic defect. Since MODY exhibits autosomal dominant inheritance, an affected individual would possess one mutant and one normal allele. The mutant glucokinase would be inactive and so the cell would have only half the normal glucokinase activity. This would result in reduced insulin secretion for any given glucose concentration. Consequently, hepatic glucose output would increase and peripheral glucose disposal decline. The plasma glucose concentration would rise to a new steady state until ß cell glucose metabolism became normal and a higher set point for insulin secretion was established (Weir 1993).

b) The adenosine deaminase gene

Maturity-onset diabetes of the young has been linked to a DNA polymorphism in the adenosine deaminase gene (ADA) on the long arm of chromosome 20 in a single family (Bell et al. 1991). The identity of the gene involved is unknown but is unlikely to be ADA, as diabetes does not occur in adenosine deaminase deficiency (Bell et al. 1991). The finding has not been confirmed in other MODY pedigrees (Patel et al. 1992; Vaxillaire et al. 1992; Froguel et al. 1992 and 1993; Dow et al. 1994), nor in diabetes in

the Pima Indians (Cox et al. 1992). This provides further evidence of the genetic heterogeneity of MODY and with further investigation, other susceptibility genes may be identified. Several groups have excluded linkage of the GLUT 2 transporter gene with MODY (Patel et al. 1992; Froguel et al. 1992).

2. NIDDM

a) Glucokinase

The success in identification of glucokinase mutations in MODY has prompted investigation of this locus in classical NIDDM. A nonsense mutation of the glucokinase gene has been identified in a Japanese family with NIDDM. The affected subjects also had a reduced 30 minute insulin response to oral glucose, suggestive of ß cell dysfunction (Katagiri et al. 1992). Population studies have reported polymorphic markers at the glucokinase locus associated with NIDDM in American Blacks (Chiu et al. 1992b) and in Mauritian Creoles (Chiu et al. 1992a), but no association was found in Caucasians (Tanizawa et al. 1993; Saker et al. 1993) or Mauritian Indians (Chiu et al. 1992a). In the American Blacks there was no evidence of a structural mutation to explain the association, though a defect was not excluded in the regulatory region of the gene (Chiu et al. 1993). Furthermore, linkage could not be demonstrated in a number of Caucasian pedigrees (Cook et al. 1992b; Froguel et al. 1993; Hattersley et al. 1993; Elbein et al. 1993 and 1994; Dow et al. 1994;).

These inconsistent results suggest that glucokinase may have a major role in a minority of pedigrees, or may have a minor role in a polygenic model of inheritance, as recently proposed in South Indian pedigrees (McCarthy et al. 1993).

b) The glycogen synthase gene

This gene is a likely candidate to be implicated in NIDDM as glycogen synthase catalyses the rate limiting step in glycogen synthesis, which is the site of the major defect in established NIDDM (Shulman et al. 1990). Groop et al (1993a) reported a polymorphism in the glycogen synthase gene on chromosome 19 associated with NIDDM in subjects from Finland. Of the two alleles A_1 and A_2 identified, the A_2 allele was more frequent among NIDDM patients than controls and was associated with a

stronger family history, a higher prevalence of hypertension and a greater impairment in insulin-stimulated glucose disposal, although amounts of glycogen synthase protein were normal.These associations have not been confirmed in French (Zouali et al. 1993) Japanese (Kadowaki et al. 1993) nor in Swedish (Groop et al. 1993c) populations.

c) IRS-1 gene

Two missense mutations have been reported at higher prevalence in Caucasian NIDDM subjects than in healthy controls, and subjects with one mutation had lower fasting insulin levels (Almind et al. 1993). However, once again this has not been confirmed in a larger group of French NIDDM patients (Hager et al. 1993).

Thus, despite overwhelming evidence for a genetic aetiology in NIDDM, no consistent genetic defect has been identified. Important contributions have come from the investigation of MODY, but these have also served to emphasise the disease heterogeneity. Difficulties also arise with disease classification as some members of MODY families may only be diagnosed in later life. In many cases NIDDM may be a polygenic disease.

An alternative approach is to identify the underlying metabolic abnormality. However, in established NIDDM many of the overt biochemical disturbances may be secondary to the diabetic state, obscuring the primary defect. The study of individuals prior to the development of overt NIDDM may reveal which is the initial defect to arise. The challenge is to identify such predisposed subjects and in view of the strong genetic evidence, close relatives of affected patients constitute a group at risk. This thesis therefore, seeks to identify metabolic defects in first-degree relatives of NIDDM patients whilst glucose tolerance is still normal.

1.7. AIMS

1. To investigate insulin sensitivity in relatives of patients with NIDDM with normal glucose tolerance.

2. To assess intermediary metabolism and the sensitivity to insulin of lipolysis in glucose-tolerant relatives.

3. To evaluate the secretion of insulin and that of its precursor intermediates in glucose-tolerant relatives of patients with NIDDM.

4. To examine whether those subjects who are genetically predisposed to later NIDDM possess abnormal serum lipoproteins when glucose tolerance is still normal.

1.8. HYPOTHESIS

The hypothesis to be tested is that non-insulin dependent diabetes results from an abnormality in insulin secretion and/or in insulin action. These defects may reside in insulin-stimulated glucose suppression or in insulin-induced inhibition of lipolysis, or alternatively in the nature of the insulin secreted. The presence of such defects in first-degree relatives of patients, a group at increased risk of later diabetes, but whilst glucose tolerance is preserved, implies that these abnormalities have a primary role in the development of NIDDM.

CHAPTER 2:

METHODS

2.1 SUBJECTS AND ETHICAL APPROVAL

All studies involved human subjects who were either relatives or controls. Relatives were first-degree relatives of patients with NIDDM, who were attending the diabetic clinics of St Mary's, St Charles' or Ealing hospitals in London. To ensure the patients were not insulin-dependent they were only chosen if diabetes was diagnosed after age 40 years, and most were being treated with diet alone or oral hypoglycaemic agents. Patients treated with insulin were only selected as probands if insulin had not been initiated for weight loss, ketosis or within 2 years of diagnosis. Suitable patients were asked whether they had a healthy first-degree relative who would be willing and able to participate in these studies. These relatives were then contacted to inquire if they would like to participate. All relatives who volunteered underwent a 75g oral glucose tolerance test and only those with normal glucose tolerance by World Health Organisation criteria (WHO 1985) were included for study.

Control subjects were healthy individuals with no personal or family history of diabetes recruited from hospital and medical school staff and students, or from patients attending outpatient clinics. No subject had known cardiac, renal or hepatic disease and none was taking any regular medication. Each individual had a normal physical examination. All subjects and controls gave written informed consent after full explanation and the studies were approved by the Parkside Health Authority Ethical Committee.

2.2. CLINICAL STUDIES

2.2.1. Location

The clinical studies were performed in the Metabolic Day Ward at St Mary's Hospital, London.

2.2.2. Oral Glucose Tolerance Test

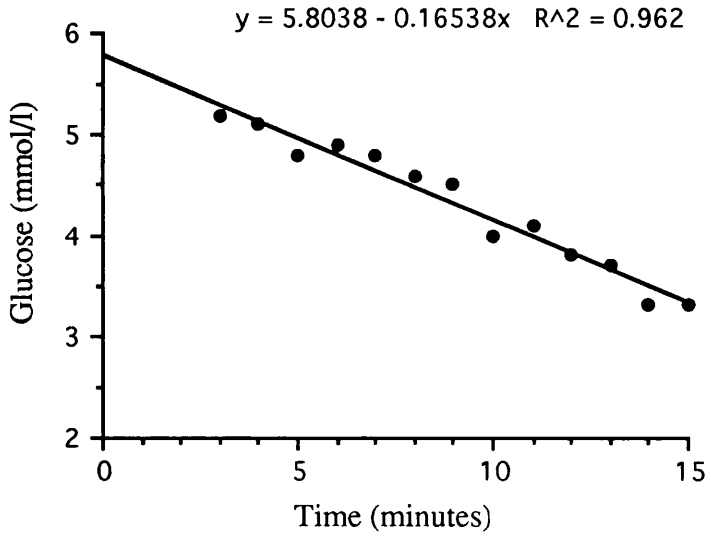
Subjects were asked to observe their usual diet for the three days prior to the study and after a 10-12 hour overnight fast they attended the ward at 08.00 hours. An 18G intravenous cannula (Venflon®, Viggo Spectramed, Helsingborg, Sweden) was positioned in a forearm vein under subcutaneous 1% lignocaine anaesthesia and the catheter was kept patent by flushing with normal saline intermittently. Baseline blood samples were taken for measurement of glucose, immunoreactive insulin, specific insulin, intact and 32, 33 split proinsulin and intermediary metabolites (NEFA, glycerol, 3-hydroxybutyrate, lactate, pyruvate and alanine). A 75g oral glucose load as 375ml Lucozade$_{TM}$ (Beecham, Greenford, UK) 19.2g/100ml was then administered and consumed within 5 minutes. Blood samples were taken every 30 minutes for 120 minutes for glucose, insulin and intermediary metabolites, and at 30 and 120 minutes after the glucose load for specific insulin, intact and 32-33 split proinsulin. Throughout the test subjects remained semirecumbent, fasted and did not smoke.

2.2.3. Insulin Tolerance Test

The short insulin tolerance test employed was a modification of the method of Bonora et al. (1989). Subjects attended the ward after a 10-12 hour overnight fast and having observed their usual diet for the preceding three days. An intravenous cannula was inserted into a dorsal hand vein under subcutaneous anaesthesia as above and was kept patent by flushing intermittently with normal saline.The cannula was attached via a 50cm catheter (Lectro-cath, Vygon, Ecouen, France) to a 3-way tap for sampling. The hand was placed in a perspex warming box heated to 50^{o}C to arterialise the blood samples (Abumrad et al. 1981). The subject remained semirecumbent during the test.

After resting for 20 minutes, two basal blood samples were taken over 15 minutes for measurement of glucose and insulin, followed by the administration of an intravenous bolus of soluble insulin (Human Actrapid, Novo Nordisk A/S, Bagsvaerd, Denmark) 0.05units/kg body weight, diluted to 1ml with normal saline. Blood was sampled for glucose at 1 minute intervals from 3 to 15 minutes after the insulin bolus, and for measurement of plasma immunoreactive insulin at times 4, 8 and 15 minutes. At 15 minutes after the insulin injection the test was terminated with oral Lucozade$_{TM}$ and the subject was given a light breakfast. Blood glucose was checked before departure. Insulin sensitivity was derived from the linear slope of the blood glucose concentration from 3-15 minutes and was expressed as μmol/l glucose fall per minute.

Figure 2.1. Plasma glucose response during the insulin tolerance test in one normal subject.



Insulin sensitivity = 165 μmol/l/min.

2.2.4. Intravenous Glucose Tolerance Test

Subjects were asked to observe their usual diet for the preceding three days and attended the Metabolic Day Ward at 08.00 hours after a 10-12 hour overnight fast. An intravenous cannula was inserted in a dorsal hand vein under lignocaine anaesthesia for sampling and kept patent by flushing with normal saline at intervals.The hand was placed in a warming chamber at 50°C for arterialisation. A second cannula was

positioned in an antecubital vein of the opposite arm for administration of glucose. After 20 minutes rest, two basal samples were taken over 15 minutes. A bolus of glucose (0.3g/kg body weight) as a 50% solution was then administered through the antecubital cannula over 1 minute and flushed well with normal saline. Taking zero time as the beginning of the glucose bolus, blood was sampled for measurement of glucose, immunoreactive insulin, specific insulin, intact and 32, 33 split proinsulin at the following times: -15, 0, 2, 4, 6, 8, 10, 20, 30, 40, 50 and 60 minutes. Blood for measurement of intermediary metabolites was sampled at times -15, 0, 4, 10, 30 and 60 minutes.

The glucose disappearance constant K_G was calculated from the slope of log glucose concentration over 10-60 minutes plotted against time. The first phase insulin or proinsulin response was calculated from the incremental area under the curve from 0-10 minutes using the trapezoidal rule (Matthews et al. 1990). The second phase response was calculated similarly from the area over 10-60 minutes.

2.2.5. Euglycaemic Hyperinsulinaemic Clamp

The euglycaemic clamp protocol was based on the method of DeFronzo et al. (1979). Subjects attended the Metabolic Day Ward at 08.00 hours after a 10-12 hour overnight fast. Two intravenous cannulae were inserted under subcutaneous lignocaine anaesthesia, one into an antecubital vein for infusion of insulin and glucose, and the other into a dorsal vein of the contralateral hand for sampling. The sampled blood was arterialised as previously. Subjects rested for 30 minutes, after which they received a primed continuous infusion of Human Actrapid insulin (priming dose 400 mU, infusion 40mU/m^2/min) for two hours. The insulin was diluted in Haemaccel® (Behringwerke AG, Marburg, Germany) and infused using a Braun infusion pump (Perfusor^R B.Braun, Melsungen AG., Germany). After 5 minutes an infusion of 10% d-glucose was commenced at 30ml/hr using a volumetric infusion pump (IMED, Abingdon, Oxon, UK) and the infusion rate was altered every 5 minutes in order to maintain the arterialised plasma glucose concentration at 0.3-0.5 mmol/l below the fasting level to prevent stimulation of endogenous insulin secretion.

Blood glucose was measured at the bedside. Time zero was taken as the start of the primed insulin infusion. Blood samples were collected for measurement of plasma insulin concentration at times -20, 0, 20, 40, 60, 80, 100 and 120 minutes. After 120 minutes the insulin infusion was terminated and the subject was a given a meal. The glucose infusion was discontinued after a further 30 minutes and the blood glucose level was checked before departure. Assuming that endogenous glucose production was suppressed, the mean glucose concentration over the final 30 minutes of the insulin infusion was calculated to represent the amount of glucose metabolised (M).The average plasma insulin level over the same period (I) was calculated and a measure of whole body insulin sensitivity was obtained as the ratio M/I.

2.2.6. Stable Isotope Tracer Studies

a) Stable Isotopes

Stable isotopes are substances which are chemically identical but differ in atomic weight due to changes in the number of neutrons in the nucleus. They are naturally-occurring and non radioactive. These properties enable stable isotopes to be safely used as metabolic tracers in children, young adults and in pregnancy. They also have the advantage that they may be used for repeated studies in the same subject and that multiple isotopes can be administered in a single study. The disadvantages of stable isotope tracers are the high natural background against which estimation of the label must be made, for example 1.1% for ^{13}C; in addition, they are expensive and require highly technical measuring equipment. In practice, these limitations are outweighed by their safety and wide applicability.

In this thesis the following stable isotopes have been used as metabolic tracers: [1,2,3 $^{2}H_5$] glycerol (99% ^{2}H), [6,6 ^{2}H] glucose (84% ^{2}H), L-[1-^{13}C] leucine (99% ^{13}C) and sodium ^{13}C bicarbonate (99% ^{13}C) purchased from Tracer Technologies Inc (Somerville, MA, USA). A constant infusion technique was employed. This has the advantages over a single bolus method that fewer blood samples are required, the turnover calculation is simple and the method is more accurate for rapidly metabolised substrates.

b) Theory of Isotope Dilution

The isotope was infused at a constant rate and the rate of appearance of unlabelled substrate which diluted the infused isotope was measured. The relative abundance of the isotope tracer is expressed as enrichment and is measured in atoms percent excess (APE). At isotopic equilibrium a plateau in enrichment is reached. To accelerate attainment of equilibrium a bolus priming dose of each tracer was given.

When isotopic equilibrium is attained, the relative concentration of isotope is equal in the infusion mixture and the extracellular fluid (ECF). For example, deuterated glucose tracer:

$$\frac{[^2H \text{ glucose}]}{[^2H + {}^1H \text{ glucose}]}_{\text{infusate}} = \frac{[^2H \text{ glucose}]}{[^2H + {}^1H \text{ glucose}]}_{\text{ECF}}$$

Multiplying the concentrations of ^2H glucose and total ($^2H + {}^1H$) glucose in the infusate by the rate of infusion, the rate of isotope infusion (I) and the rate of appearance of endogenous glucose (R_a) are obtained.

$$\frac{[^2H \text{ glucose}] \text{ mg/ml. ml/min}}{[^2H + {}^1H \text{ glucose}] \text{ mg/ml. ml/min}} = \frac{\text{isotope infusion rate (I) mg/min}}{\text{Ra}}$$

$$\text{Isotopic Enrichment} \quad E(\%) = \frac{[^2H \text{ glucose}]}{[^2H + {}^1H \text{ glucose}]} \times 100$$

$$\text{therefore} \quad \frac{E}{100} = \frac{I}{Ra}$$

$$\text{and} \quad Ra = \frac{I}{E \times 0.01}$$

To account for the contribution of isotope tracer to the total Ra, the rate of isotope infusion is subtracted to give the Ra of endogenous unlabelled glucose.

Thus

$$Ra = \frac{I}{E \times 0.01} - I$$

As the enrichment of the isotope infused is not 100%, some of the infusate will contain unlabelled glucose. This unlabelled glucose infused must be subtracted from the total Ra.

Therefore

$$Ra = \frac{I.Ei}{Ep \times 0.01} - I$$

$$= I\left(\frac{Ei}{Ep \times 0.01}\right) - 1$$

where Ei = isotopic enrichment of infusate

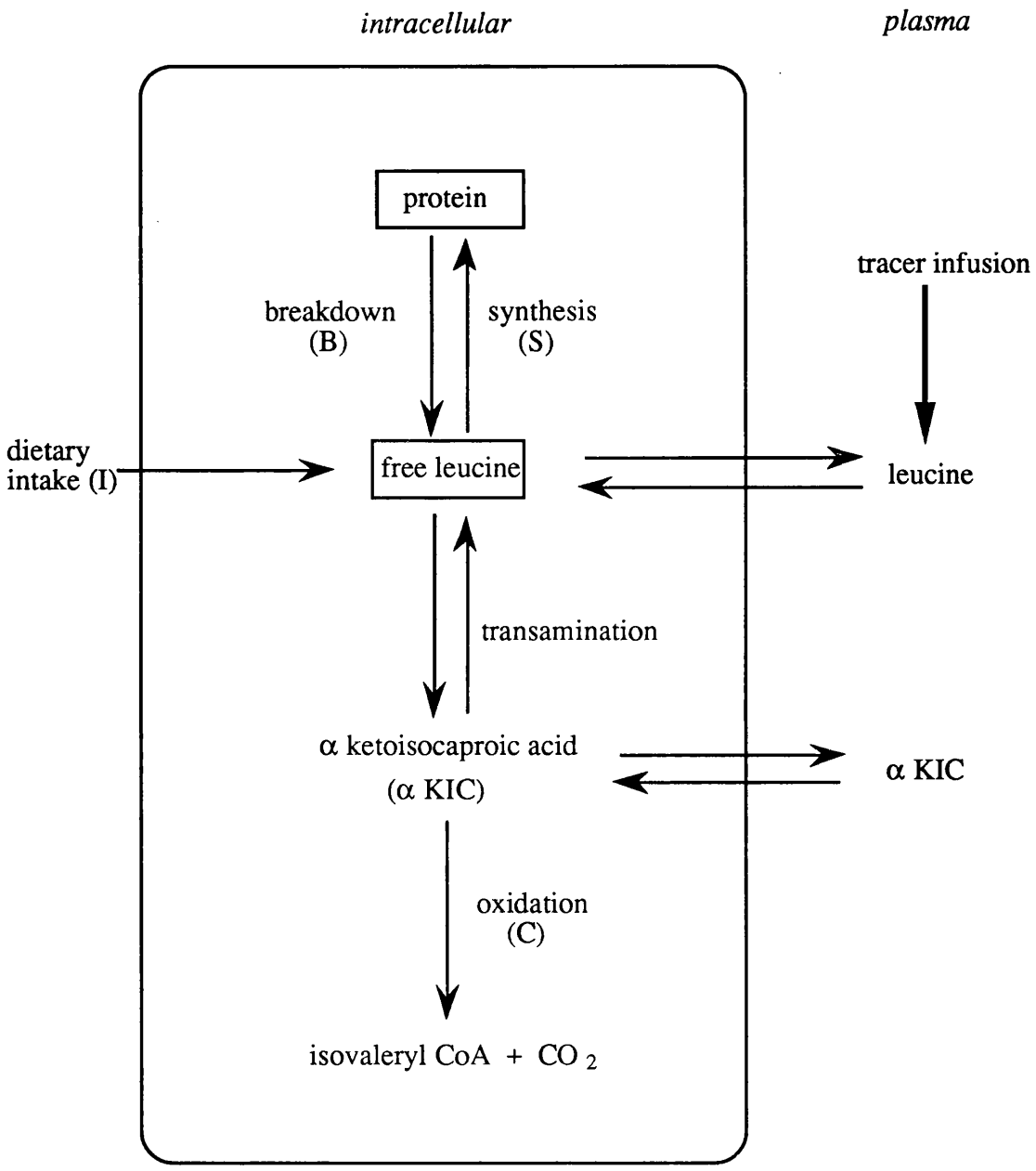
and Ep= isotopic enrichment of plasma

Hence, by sampling plasma at equilibrium, the enrichment and rate of appearance of substrate can be determined (Wolfe 1992).

c) Protein Turnover

To investigate protein turnover ^{13}C leucine was chosen as tracer because leucine is an essential amino acid and is metabolised primarily in muscle. Leucine turnover (Ra) was calculated from the primed continuous infusion technique using the theory of isotope dilution as above, assuming a two pool model. Leucine derived from the breakdown of muscle protein may undergo catabolism to CO_2 (C) after transamination to alpha ketoisocaproic acid, or may be reincorporated into protein (S). The irreversible loss of leucine to CO_2 may be quantitated by measurement of $^{13}CO_2$ in expired air and the CO_2 production rate. With a constant infusion of ^{13}C leucine, equilibrium enrichment in expired air may take 8-48 hours to be achieved. To shorten this time to reach equilibrium, a priming dose of sodium ^{13}C bicarbonate was given.

Fig 2.2. Two pool model of leucine kinetics



At equilibrium $Ra = S + C = B + I$

In the postabsorptive state, $I = 0$

therefore $S + C = B = Ra$

d) Leucine Oxidation

The rate of leucine oxidation was calculated from the proportion of plasma enrichment which had undergone oxidation to $^{13}CO_2$ and removing the contribution to oxidation due to tracer oxidation (Matthews et al. 1980).

$$F\,^{13}CO_2 = \frac{F_{CO_2}\ E_{CO_2}}{W}\left(\frac{60 \times 41.6}{100 \times 0.81}\right)$$

where $F\,^{13}CO_2$ is the rate of oxidation of $^{13}CO_2$ in expired air (μmol/kg/hr)

Fco_2 is the CO_2 production rate (ml/min)

Eco_2 is the $^{13}CO_2$ enrichment (APE) in expired air at isotopic steady state

W is the subject's weight (kg)

41.6 converts Fco_2 to μmol/min at Standard Temperature and Pressure

0.81 accounts for the fraction of $^{13}CO_2$ released by ^{13}C leucine oxidation but not released from the body bicarbonate pool into expired air (Allsop et al. 1978)

100 changes APE from a % to a fraction.

$$\text{the rate of leucine oxidation } C = F\,^{13}CO_2\left(\frac{1}{E_p} - \frac{1}{E_i}\right) \times 100$$

and the rate of leucine incorporation into protein $S = Ra - C$

Substrate oxidation takes place intracellularly, but during turnover studies plasma enrichment is measured. Some of the leucine released from protein breakdown is reincorporated into protein before it can equilibrate with the protein pool, and some is transaminated into αKIC. Measurement of plasma leucine turnover, therefore, underestimates the rate of leucine release from protein. In addition, some of the plasma leucine is derived from αKIC. In contrast, KIC is derived only from intracellular leucine, it has a small pool with rapid turnover and quickly achieves equilibrium in plasma and intracellularly. Once equilibrium is achieved in enrichment of plasma leucine, an equilibrium in enrichment of the KIC pool is achieved very shortly after.

We therefore measured plasma KIC enrichment as a more accurate reflection of intracellular leucine kinetics, and also to reduce errors due to the site of sampling (Thompson et al. 1988)

e) Clinical Studies

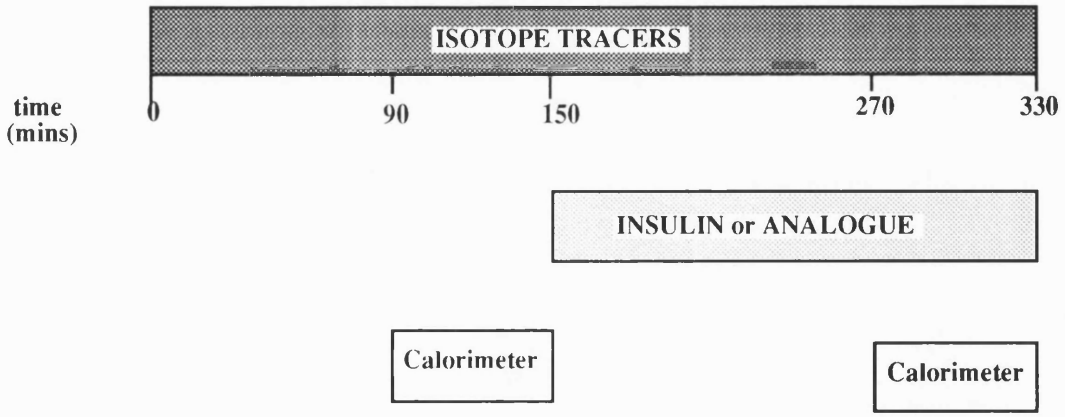
The development of this study protocol is described in chapter 4. After a 10-12 hour overnight fast, subjects attended the Metabolic Day Ward at 08.00 hours. An 18G intravenous cannula was inserted into a forearm vein under local anaesthesia for infusion of isotopes and insulin. A similar cannula was positioned in a dorsal hand vein of the opposite hand for sampling and was kept patent with a slow infusion of normal saline. The hand was placed in a warming chamber at 50°C to arterialise the venous blood. After a 30 minute rest, basal samples of blood were drawn at times -15 and 0 minutes. This was followed by administration of the priming doses of stable isotopes [6,6 ^2H] glucose (19.2 μmol/kg) and [1,2,3 $^2H_5$] glycerol (0.5 μmol/kg). Immediately after these priming doses, constant infusions of [6,6 ^2H] glucose (10.0 μmol/kg/hr) and [1,2,3 $^2H_5$] glycerol (3.0 μmol/kg/hr) prepared in normal saline were commenced and continued for 330 minutes. The stable isotope solutions were prepared in sterile pyrogen-free 0.9% saline and were passed through a Ministart (Sartorius GmbH, Göttingen, Germany) 0.2μm filter before infusion. Over the period 90-150 minutes plateau conditions were achieved (Matthews et al. 1980). At time 150 minutes an infusion of soluble human insulin (Actrapid HM) 100u/ml diluted in Haemaccel® was begun using a Braun infusion pump (Perfusor^R B.Braun, Melsungen AG., Germany) and continued until the end of the study.

Ten blood samples were taken at intervals between 90 and 150 minutes after the isotope infusions were started to ensure equilibrium enrichment had been reached. Sampling was then continued at 15 minute intervals during the remainder of the study. The blood was taken for measurement of immunoreactive insulin, glucose, glycerol and NEFA concentrations and glucose and glycerol tracer enrichment. Plasma glucose was measured at the bedside (Beckman Instruments, High Wycombe, UK).

Insulin Analogue Studies

In these studies (1-^{13}C) leucine tracer, in priming dose 3.8 μmol/kg and infusion 3.8 μmol/kg/hr, was also administered for measurement of protein turnover in addition to the glucose and glycerol tracers. A priming bolus of sodium ^{13}C bicarbonate (1.0 μmol/kg) was given at time 0 to ensure equilibrium enrichment in expired air was attained. Samples of blood and expired air were collected for measurement of leucine tracer and $^{13}CO_2$ enrichment at the same sampling timings as above. Infusions of insulin or insulin analogue LA (B9-Asp, B27-Glu insulin) and HA (B10-Asp-insulin) 600nmol/ml = 100 U/ml (Novo, Bagsvaerd, Denmark) in equimolar doses (0.005 units/kg/hour) diluted in Haemaccel® were administered from 150-330 minutes on separate occasions as above. Carbon dioxide production rate was measured for one hour prior to the insulin or analogue infusion (90-150 minutes) and for the final hour of the study (270-330 minutes) when attainment of a new steady state was assumed, using a Deltrac metabolic monitor (Deltrac Datex Instrumentarium Corp., Helsinki, Finland).

Figure 2.3. Diagrammatic representation of stable isotope study design.



Stable isotopic tracers 6,6^{2}H glucose, 1,2,3 $^{2}H_5$ glycerol and 1-^{13}C leucine were infused for 330 minutes. Insulin, analogue or Haemaccel® infusion was commenced at 150 minutes until end of study. CO_2 production was measured with the Deltrac metabolic calorimeter from 90-150 minutes and 270-330 minutes.

2.3. LABORATORY METHODS

2.3.1. Collection and Storage of Samples

Blood samples (5ml) for immunoreactive insulin or insulin analogues, specific insulin, intact and 32, 33 split proinsulin, were collected in lithium heparin tubes. For measurement of plasma glucose, NEFA and glycerol concentrations during the stable isotope studies blood (5ml) was collected in fluoride oxalate tubes. In the metabolic studies blood samples (1.5ml) for glycerol, 3-hydroxybutyrate, lactate, pyruvate and alanine were collected in preweighed plain glass tubes containing 5ml 5% weight/volume ice cold perchloric acid for deproteinisation. The tubes were then reweighed. During the stable isotope studies blood (4ml) was collected into fluoride oxalate tubes for measurement of glucose and glycerol tracers. Blood (3ml) for measurement of leucine tracer was collected into lithium heparin tubes. Samples for lipids (10ml) were collected in plain glass tubes and allowed to clot. All the above samples were collected on ice prior to centrifugation at 2500 revs/min for 15 minutes at 4°C as soon as possible, usually within 30 minutes. Plasma, supernatant or serum was separated immediately and stored at -20°C until analysis. During the leucine studies, expired air was collected in 20 ml venoject Vacutainers for estimation of $^{13}CO_2$ enrichment.

2.3.2. Assays

a) Glucose

Glucose was analysed during the clamp studies and the stable isotope infusions using a glucose oxidase method (Beckman instruments, High Wycombe, UK).

$$\text{glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

Glucose concentration was determined by measurement of the rate of O_2 consumption. Oxygen was consumed at the same rate as that at which glucose reacted to form gluconic acid. The peroxide was destroyed without producing oxygen by catalase and molybdate.

In the metabolic studies glucose was measured by a hexokinase method using a COBAS BIO automated analyser (Roche, Welwyn Garden City, Herts., UK).

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose 6-phosphate} + \text{ADP}$$

$$\text{glucose 6-phosphate} + \text{NAD} \xrightarrow{\text{G6PDH}} \text{gluconate 6-phosphate} + \text{NADH} + \text{H}^+$$

NADH absorption was measured at 340nm.

b) Non-esterified fatty acids (NEFA)

NEFA were measured using an enzymatic colorimetric method (NEFA C, WAKO Chemicals GmbH, Neuss, Germany) on the COBAS BIO analyser.

$$\underset{\text{(NEFA)}}{\text{R COOH} + \text{ATP} + \text{CoA}} \xrightarrow{\text{acyl CoA synthetase}} \text{acyl CoA} + \text{AMP} + \text{pyrophosphate}$$

$$\text{Acyl CoA} + \text{O}_2 \xrightarrow{\text{acyl CoA oxidase}} \text{H}_2\text{O}_2 + \text{2, 3 transenoyl CoA}$$

The hydrogen peroxide was oxidised by peroxidase in the presence of 3 methyl-N-ethyl N-aniline and 4 amino antipyrine. The coloured product formed was measured by absorption at 550nm.

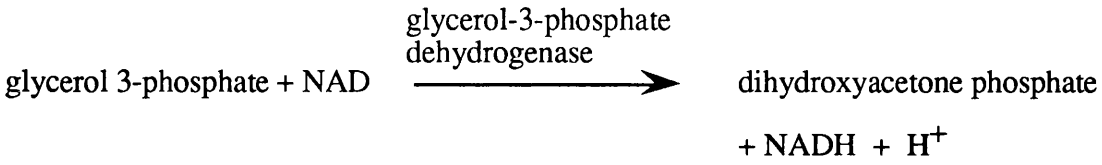
c) Intermediary metabolites

In the metabolic studies glycerol, 3-hydroxybutyrate, lactate, pyruvate and alanine were measured in deproteinised blood on the COBAS BIO centrifugal analyser using fluorimetric methods modified from Harrison et al. (1988).

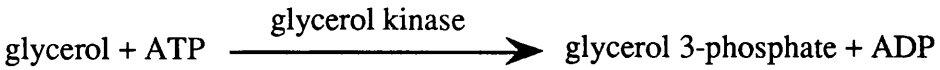
1) Glycerol

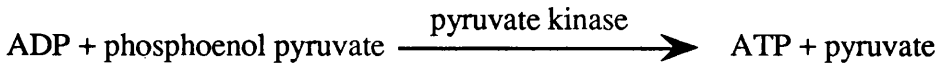
Glycerol was analysed by the following reaction, measuring the absorption of NADH at 340nm.

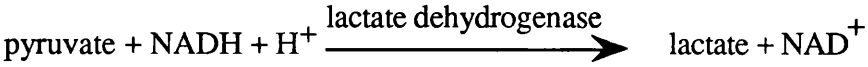
$$\text{glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol 3-phosphate} + \text{ADP}$$



In the stable isotope studies, plasma glycerol was measured by an alternative enzymatic colorimetric method (Boehringer Mannheim, Lewes, Sussex, UK).



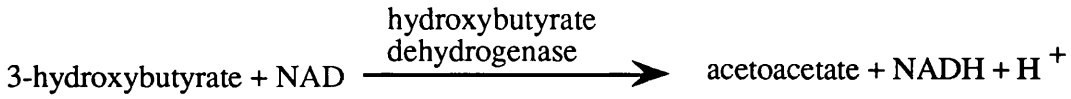




The decrease in absorbance of NADH at 340nm was determined.

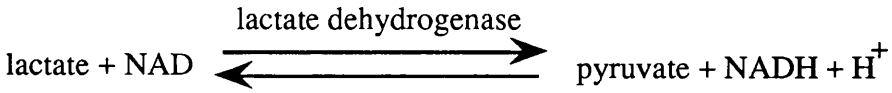
2) 3-hydroxybutyrate

3-hydroxybutyrate was measured by the following enzymatic reaction and by determining the increase in absorbance of NADH at 340nm in the presence of hydrazine.



3) Lactate and Pyruvate

Lactate was measured by the enzymatic method shown below, using hydrazine to trap pyruvate and an alkaline medium to trap protons. The absorption of NADH at 340nm was measured.



Pyruvate was analysed by the reverse reaction carried out at pH 7.4.

4) Alanine

Alanine was assayed by an enzymatic reaction with the addition of hydrazine to trap pyruvate at low pH. The increase in absorbance of NADH at 340nm was measured.

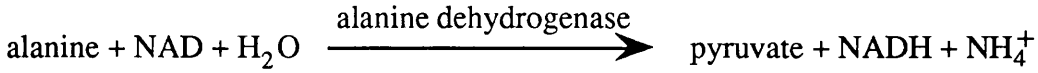


Table 2.1. Intra and inter assay precision of variation.

metabolite		mean (μmol/l)	intra assay c.v.(%)	inter assay cv (%)
glucose		4400	2.4	2.3
NEFA		74	7.4	4.3
		520	2.0	2.0
glycerol	blood	37	2.0	5.0
		57	1.8	5.1
	plasma	71	9.2	10.5
3-hydroxybutyrate		13	1.7	1.3
		46	1.5	10.6
lactate		358	1.8	4.0
		1050	2.8	2.9
pyruvate		23	3.0	5.0
		58	1.3	4.0
alanine		118	3.0	3.9
		152	0.7	3.6

d) Lipids

Serum total cholesterol and triglyceride concentrations were measured enzymatically using a Centrifichem centrifugal analyser (Baker instruments, Windsor, UK). High density lipoprotein (HDL) was isolated by dextran sulphate-$MgCl_2$ extraction and the HDL-cholesterol was measured as above. Low density lipoprotein (LDL) -cholesterol was calculated using the Friedewald equation (Friedewald et al. 1972). The interassay coefficients of variation for total cholesterol were 0.5% at 4.2 mmol/l and 1.3% at 10mmol/l, for triglyceride were 3.8% at 0.6mmol/l and 2.8% at 3.4mmol/l and for HDL-cholesterol 3.6% at 1.0mmol/l.

e) Insulin

Insulin was measured by radioimmunoassay using a double antibody technique. The first antibody was raised in the guinea pig and had 100% cross reactivity with human biosynthetic insulin and proinsulin (Hampton et al. 1988). A trace amount of

^{125}I-labelled insulin was added to a limited amount of antibody to form a labelled antigen-antibody complex. Antibody-bound labelled insulin was separated from the free labelled hormone by polyethylene glycol 6000 (BDH, Poole, UK) accelerated donkey antiguinea pig second antibody (Guildhay Antisera Ltd., Guildford, UK). Unlabelled insulin in the sample competed with the labelled hormone for the limited number of antibody binding sites. The change in percentage of labelled hormone bound, observed with unknown amounts of insulin in the sample, was determined by comparison with standard solutions. This measurement is termed 'immunoreactive' insulin (IRI) and is expressed in mU/l.

The conversion to pmol/l $= \frac{\text{mU/l}}{0.134}$

The intraassay coefficient of variation was 3.5%. Between assay coefficients of variation using Liphocheck immunoassay control serum (Biorad, Watford, UK) are shown below:

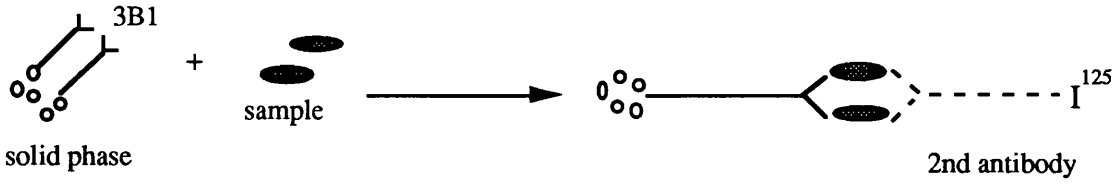
insulin concentration (mU/l)	coefficient of variation (%)
11.6	8.4
54.4	3.8
207	4.8

f) Proinsulin and specific insulin assays

The problems of lack of specificity and sensitivity with insulin radioimmunoassays (Temple et al. 1992) have led to the development of immunoradiometric assays. Intact proinsulin, 32, 33 split proinsulin and specific insulin were measured by specific immunoradiometric assays (Sobey et al. 1989).

In each case the first monoclonal antibody (3B1) was bound in excess to a solid phase. Fifty µl of sample were added and then a second monoclonal antibody labelled with ^{125}I was added. Although these assays have significant cross reactivity with 65, 66 split proinsulin (see below), this precursor is usually present only in such low concentrations that, in practice, it is negligible.

Figure 2.4. Diagrammatic representation of two-site immunoradiometric assay.



1. Intact proinsulin

The assay for proinsulin was modified from Sobey et al. (1989). The antibody 3B1 was bound to a Titertek immunoassay plate (Flow Labs., Thame, UK) as solid phase. After addition of the sample and overnight incubation, ^{125}I labelled A6 mouse antibody was added as the second antibody. The assay had a sensitivity of 0.5 pmol/l and does not cross react with 32, 33 split proinsulin or insulin. It has 66% cross reactivity with 65, 66 split proinsulin.

2. 32, 33 split proinsulin

32, 33 split proinsulin was measured by specific immunoradiometric assay with 3B1 as first antibody and PEP 001 as labelled second antibody (Novo, Bagsvaerd, Denmark). The sensitivity of the assay was 1.0 pmol/l and it cross reacted 40% with proinsulin and 60% with 65, 66 split proinsulin. The assay does not cross react with insulin. Assay values of 32, 33 split proinsulin were corrected by subtraction of the proinsulin cross reactivity.

3. Specific insulin

In chapters 7, 8, 9 and 11 insulin was measured by specific immunoradiometric assay using ^{125}I labelled 14B as second antibody (Novo, Bagsvard, Denmark). This assay had a sensitivity of 5.0 pmol/l and cross reacted 5.3% with intact proinsulin, 62% with 65, 66 split proinsulin and 5.0% with 32, 33 split proinsulin.

Table 2.2. Immunoradiometric assay inter and intra assay coefficients of variation

	over range (pmol/l)	inter assay cv (%)	intra assay cv (%)
intact proinsulin	6.6 - 51.0	12	<10
32, 33 split proinsulin	7.6 - 47.9	12	<10
insulin	30.5 - 250	15	<10

g) Insulin analogues

Plasma concentrations of the biosynthetic insulin analogues of low affinity (LA) and high affinity (HA) were measured by radioimmunoassay using each analogue for its own standard curve and guinea pig antiserum M8309 donated by Novo Industries (Bagsvaerd, Denmark). The values were corrected for cross reactivity with human insulin, 88% for LA and 91% for HA.

h) Glycosylated haemoglobin.

HbA_1 was measured by Corning gel electroendosmosis (normal range <8.0%).

2.3.3. Stable Isotope Tracer Analysis

Plasma for determination of stable isotope enrichment was treated by deproteinisation, deionisation and derivatisation.

a) Glucose

1) protein precipitation

Plasma (200µl) was pipetted into 1.5ml microcentrifuge tubes (Eppendorf, Netherler, Germany) with 100µl of water. Sulphosalicylic acid 0.275molar (BDH Ltd., Poole, UK) (300µl) was added and the tube mixed and centrifuged at 1000 revs/min for 1 hour at 4°C. The supernatant (400µl) was pipetted into 1.5ml microcentrifuge tubes.

2) cation absorption

Duolite MB5113 resin (BDH Ltd, Poole, UK) 30g/100 tubes was mixed with 1M acetic

acid (25% v/v) and stirred for 30 minutes. After being allowed to settle, the acetic acid was decanted off and the acetified resin was washed 20 times with 10 volumes of ion free water. The treated Duolite resin (250µl) was added to each sulphosalicylic acid supernatant and shaken for 20 minutes. The resin was allowed to settle and then the supernatant (375µl) was sampled into Wheaton screw top vials (Chromatography Services Ltd., Wirral, UK).

3) derivatisation

The supernatant was freeze dried in a rotary drier. Butane boronic acid (50µl) (Sigma, Poole, UK) in pyridine (5mg/ml) (Fisons, Loughborough, UK) was added, the solution mixed and placed in an oven at 90°C for 60 minutes. After addition of acetic anhydride (50µl) (Sigma, Poole, UK) and mixing, the solution was heated again for 60 minutes at 90°C. The vials were placed in a freeze drier and the derivatising reagents were removed under vacuum. Hexane (Fisons, Loughborough, UK) (500µl) was added and the vials sonicated for 10 minutes. The hexane solution (200µl) was then transferred to autosampler vials containing 200µl inserts for analysis.

b) Glycerol

1) acetone extraction

Plasma (200µl) was pipetted into 1.5µl microcentrifuge tubes and ice cold acetone (Fisons, Loughborough, UK) (800µl) added. The solution was mixed and centrifuged at 1000revs/min for 1 hour at 4°C. The supernatant (700µl) was sampled into 1.5 ml microcentrifuge tubes.

2) cation absorption

To each sample treated Duolite resin (500µl) 60g/100 tubes prepared as above, was added and shaken for 20 minutes. After being left to settle, the supernatant (500µl) was transferred to Wheaton screw top vials.

3) derivatisation

The vial contents were frozen and freeze dried overnight. Then trimethylsilyl imidazole (Fisons, Loughborough, UK) in pyridine (25µl) (21% v/v) was added and mixed. The vials were heated in an oven at 70°C for 30 minutes, after which hexane (250µl) was

added and mixed again. Following addition of ion free water (100µl) and further mixing, any precipitate was allowed to settle. The upper hexane layer (100µl) was then transferred to autosampler vials containing 0.1ml inserts.

c) α–Ketoisocaproic acid (αKIC)

Samples for αKIC enrichment were prepared following the method of Ford et al. (1985).

1) deproteinisation

Ethyl alcohol (1ml) and ketovaleric acid (50µl) were added to the plasma sample (100µl), the solutions mixed and centrifuged at 1000 revs/min at 4°C for 5 minutes. The supernatant was then evaporated to dryness under nitrogen at 50°C.

2) derivatisation

The residue was dissolved in water (200µl) and freshly prepared phenylenediamine solution (Flurochem Ltd., Glossop, UK) (2% in 4M HCl) (100µl). The mixture was heated at 90°C for 1 hour. The solution was then cooled and extracted twice with ethyl acetate (1ml) and the combined extract dried over anhydrous sodium sulphate. The solvent was evaporated to dryness under nitrogen at room temperature and the residue dissolved in pyridine (100µl). This solution was then reacted with BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) +1% TMCS (trimethylchlorosilane) 100µl (Pierce, Cheshire, UK) at 120°C for 30 minutes. Just before analysis the excess reagent was removed under nitrogen at room temperature and the residue was dissolved in dry n-decane containing 5% v/v BSTFA (200µl).

d) Mass spectrometer

Tracers were detected using electron impact ionization selected-ion-monitoring gas-chromatography mass spectrometry. This technique is based on the principle that the path of an ionised molecule may be controlled by an electric field in a mass-dependent manner.

The chemical substances in the sample are initially separated by gas chromatography. In order to be separated, organic compounds must first be converted to thermally-stable chemically inert, volatile derivatives. The sample is vapourised in the heated injector and carried through the column by helium, an inert carrier gas. As the outlet pressure of the gas chromatograph is much higher than that which the mass spectrometer can tolerate, the pressure is reduced by a vacuum pump. The sample then passes to the mass spectrometer and enters the ionisation chamber. A beam of electrons is emitted from a heated filament and attracted towards a positive plate. As the sample passes through the beam, the molecule may be hit by an electron, causing loss of an electron from the molecule and producing a positively charged ion. Any excess energy is dissipated by fragmentation of the ion, the pattern of fragmentation being characteristic for the particular sample. The ions pass through a mass analyser and their detection is improved using an electron multiplier.

For glucose tracer, ions 297 and 299 were monitored using a VG40-250 (VG Mass Lab Ltd., Altrincham, UK) mass spectrometer. The intra assay coefficient of variation (cv) was 1.3% at isotope ratio 0.0368 and the interassay cv was 4.2% at isotope ratio 0.0900 and 6.2% at isotope ratio 0.0400.

For glycerol tracer, ions 205 and 208 were monitored using a VG40-250 mass spectrometer as above. The assay had an intra assay cv of 2.2% at isotope ratio 0.0311 and an interassay cv of 7.1%. For αKIC enrichment, ions 232 and 233 were monitored using a Finnigan 4500 (Finnigan MAT Ltd., Hemel Hempstead, UK) mass spectrometer. The enrichment of $^{13}CO_2$ in expired air was measured by isotope ratio mass spectrometry using an established technique (Halliday and Read 1981).

2.4. STATISTICAL METHODS

Most statistical analyses were performed using STATVIEW SE statistical package (Abacus Concepts, Berkley, CA, USA) on an Apple Macintosh SE computer. Data are expressed as mean±se when normally distributed and as median (range) when non-normally distributed. For comparison of 2 group data Mann Whitney U tests were performed for non parametric data, and unpaired student's t tests for parametric data. Three-way analysis of variance for turnover results in the insulin analogue studies was performed using the statistical package SOLO (BMDP Statistical Software, CA., USA). Correlations were sought using Pearson's or Spearman's rank correlation coefficients for parametric and non parametric data respectively. The individual statistical tests used are described in each chapter as appropriate. A p value of less than 0.05 was considered statistically significant.

CHAPTER 3:

VALIDATION OF THE LOW DOSE SHORT INSULIN TOLERANCE TEST FOR EVALUATION OF INSULIN SENSITIVITY

3.1. INTRODUCTION

Insulin resistance is increasingly being recognised as a major contributory factor in the development of diabetes and other pathological states (DeFronzo 1988). The evaluation of insulin sensitivity is therefore an important aspect in their investigation, but the technique employed should ideally be simple, safe, reliable, accurate and reproducible. The insulin tolerance (suppression) test (Shen et al. 1970), one such technique, involves the measurement of the blood glucose response to insulin administration, but this response is complicated by the antagonism of counter-regulatory hormones induced by the fall in blood glucose and the risk of the subject developing dangerous hypoglycaemia.

To address these problems this test has been modified to measure the blood glucose decline over the initial 15 minutes, before the onset of the counter-regulatory response. The method, using an insulin bolus of 0.1units/kg body weight, has been validated against the euglycaemic hyperinsulinaemic clamp in normal, obese and diabetic individuals (Bonora et al. 1989; Akinmokun et al. 1992). In healthy subjects symptomatic hypoglycaemia remains a potential hazard. To reduce this risk we have further modified the test using an insulin bolus of 0.05 units/kg for use in the healthy relatives of patients with NIDDM, but in order for it to be of value as a simple screening method to rank individuals in terms of insulin sensitivity, it must be shown to be a reproducible and valid technique.

The aim of this study therefore, was to assess the reproducibility of the short insulin tolerance test in healthy subjects employing the insulin dose of 0.05 units/kg and validate the method against the euglycaemic clamp.

3.2. SUBJECTS

Twenty-one healthy subjects were recruited. None had a history of diabetes mellitus or was taking any medication. Eleven subjects, 4 male and 7 female, aged 21-34 years and of body mass index (BMI) 18.8-26.7 kg/m^2 underwent the short insulin tolerance test on two separate occasions, at least one week apart, to assess the reproducibility of the test (Table 3.1). The remaining 10 subjects, 2 male and 8 female, aged 23-50 years and with a BMI range 21.0-36.1 kg/m^2 were studied with the short insulin tolerance test on one occasion, followed by a euglycaemic hyperinsulinaemic clamp study on another day, at least one week later (Table 3.2). Subjects were asked to observe their usual diet for the 3 days preceding each test and to abstain from alcohol or strenuous exercise for the previous 24 hours. The women were studied within the first 10 days of their menstrual period on each occasion.

3.3. METHODS

The short insulin tolerance test employed a bolus of soluble insulin 0.05 units/kg body weight, as described in chapter 2. Insulin sensitivity was derived from the linear slope of the blood glucose concentration from 3-15 minutes and was expressed as μmol/l glucose fall per minute (IS). The euglycaemic clamp studies were performed as detailed in chapter 2. The coefficient of variation of blood glucose during the last 30 minutes of the clamp studies was (mean±se) 5.9±0.8%. The mean glucose infusion rate over the final 30 minutes of the study was calculated to represent the amount of glucose metabolised (M) and with the average plasma insulin level (I) over the same period, a measure of insulin sensitivity (M/I) was derived.

3.4. ANALYTICAL METHODS

Plasma glucose was measured by a glucose oxidase method at the bedside during the euglycaemic clamp and by a hexokinase method in the insulin tolerance test as described in chapter 2. Plasma insulin was measured by radioimmunoassay.

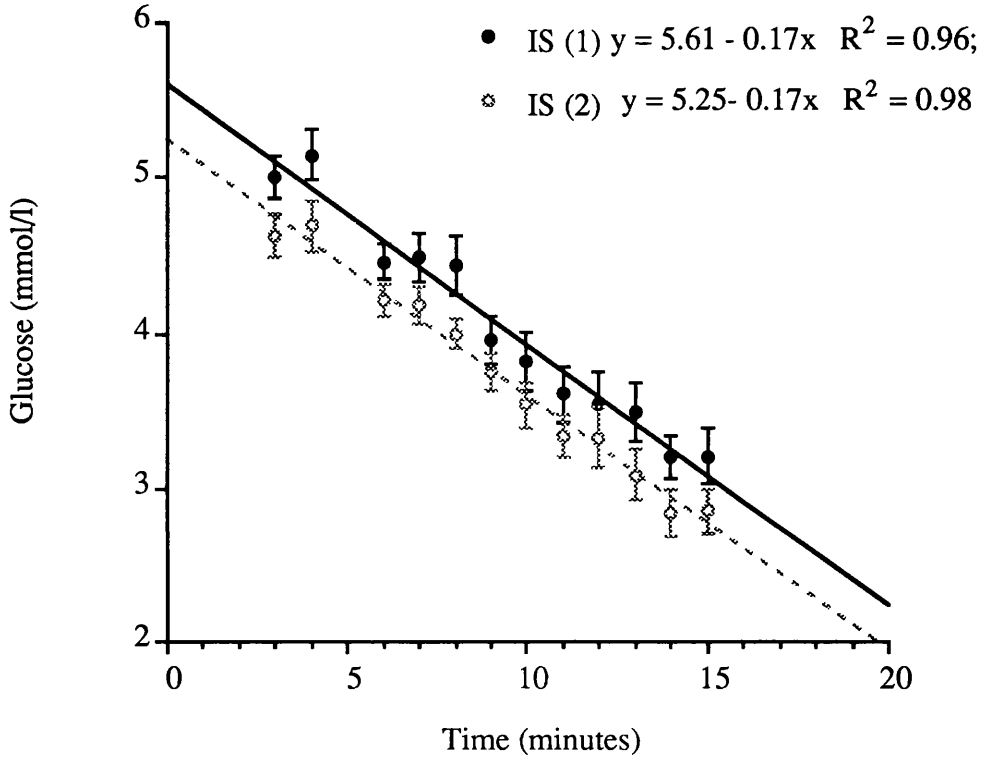
3.5 STATISTICAL ANALYSIS

Results are expressed as mean±se. In the insulin tolerance test, the slope of the glucose concentration against time was analysed using the statistical computer programme In Plot 4, with the curve fitted individually. The total sum of squares was calculated using both linear and exponential models, but the total sum of squares was less, indicating better fit, for the linear model, which was therefore adopted. The reproducibility of the insulin tolerance test was assessed by calculation of the coefficient of variation based on the difference between the two IS values obtained for each subject who underwent two insulin tolerance tests (Reed and Henry 1974). Correlations were sought using Pearson's correlation coefficient.

3.6. RESULTS

Figure 3.1 shows the mean±se blood glucose concentrations during the insulin tolerance tests for the 11 subjects studied twice and their individual plasma glucose disappearance rates (IS) are shown in Table 3.1.

Figure 3.1. The linear relation between plasma glucose concentration and time in 11 subjects (mean±se) from 3-15 minutes during the two insulin tolerance tests (IS(1) and IS(2)).



The mean±se insulin sensitivity on the two occasions were 174±10 and 179±11 μmol/l/min with a coefficient of variation of 6.9±2.6%. The insulin response peaked at 4 minutes (492±82mU/l). The mean blood glucose concentration achieved at 15 minutes was 3.0±0.2 mmol/l and no subject developed symptoms of hypoglycaemia nor a blood glucose below 2.2 mmol/l in any of the insulin tolerance tests.

Table 3.1. Clinical characteristics, fasting plasma glucose concentration (FPG) and insulin sensitivity derived from the short insulin tolerance tests on two separate occasions (IS (1) and IS (2)) in 11 subjects.

subject	sex	age (years)	BMI (kg/m^2)	FPG (mmol/l)	IS (1) (μmol/l/min)	IS (2) (μmol/l/min)
1	f	23	23.2	4.7	170	159
2	f	21	20.8	5.0	171	168
3	f	28	19.8	4.6	143	139
4	f	26	20.5	4.5	153	149
5	m	34	22.6	4.8	136	183
6	f	22	23.2	4.7	177	173
7	m	23	26.7	4.7	243	261
8	f	32	18.8	5.4	165	187
9	f	21	24.0	3.9	224	216
10	m	23	22.0	4.9	182	182
11	m	30	22.6	5.7	144	150

In the euglycaemic clamp experiments the mean±se fasting plasma insulin level was 6.9±1.0 mU/l. At steady state the plasma insulin level achieved was 68.3±5.8 mU/l and the amount of glucose required for maintaining euglycaemia was 153.9±23.7 $mg/kg/m^2$. In the 10 subjects who underwent both the euglycaemic clamp and a short insulin tolerance test the insulin sensitivity derived from the M/I ratios obtained from the euglycaemic clamp studies was 2.35±0.33$mg/kg/m^2$/mU/l and the IS for these subjects was 188±22 μmol/l/min (Table 3.2).

Table 3.2. Clinical characteristics, fasting concentrations of plasma glucose (FPG) and insulin (FPI), glucose disappearance constants derived from the short insulin tolerance test (IS) and M/I ratios (mg/kg/m^2/mU/l) derived from the euglycaemic clamp in 10 subjects.

subject	sex	age (years)	BMI (kg/m^2)	FPG (mmol/l)	FPI (mU/l)	M/I	IS (μmol/l/min)
1	f	36	25.7	5.5	8.7	2.56	160
2	f	28	27.8	4.6	3.0	4.14	292
3	m	50	36.1	4.9	13.5	1.07	49
4	m	36	33.5	5.4	9.7	1.09	185
5	f	26	20.7	5.1	6.0	3.14	218
6	f	33	21.0	4.9	5.0	3.18	284
7	f	26	23.7	4.3	4.9	2.25	180
8	f	40	26.1	4.6	7.9	2.98	196
9	f	42	22.3	4.8	4.6	1.51	158
10	f	23	18.6	4.9	5.3	1.55	162

There was a significant correlation between the M/I ratios derived form the euglycaemic clamp studies and the IS values in the same subjects ($r=0.81$; $p<0.005$) (Figure 3.2).There was also a close correlation between the IS and the clamp derived M values ($r= 0.68$; $p<0.05$) (Figure 3.3).

Figure 3.2. The relation between insulin sensitivity derived from the euglycaemic hyperinsulinaemic clamp (M/I) and that derived from the low dose short insulin tolerance test (IS) in 10 subjects.

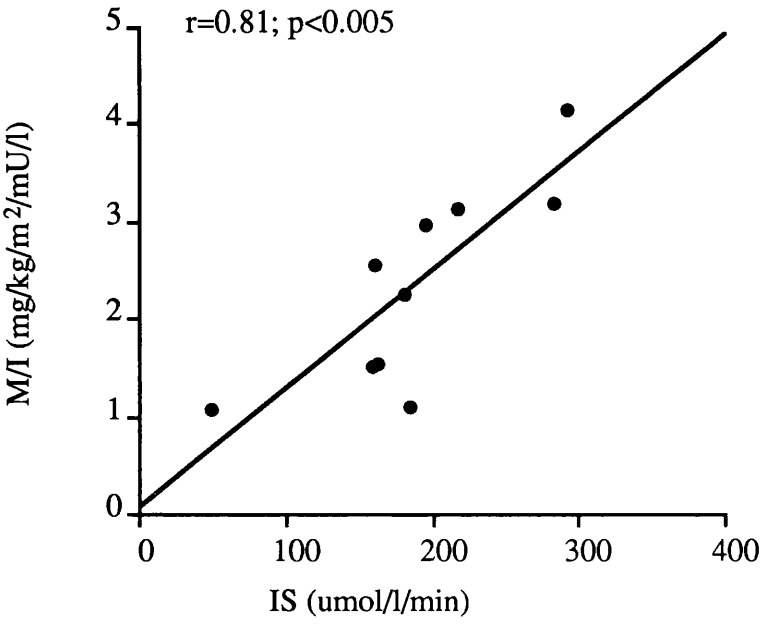
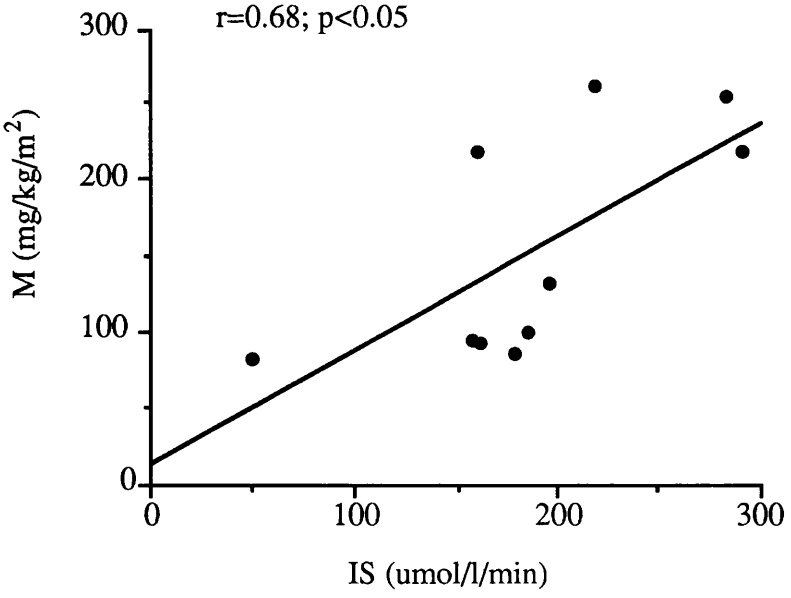


Figure 3.3. The relation between the amount of glucose metabolised during the euglycaemic hyperinsulinaemic clamp study (M) and insulin sensitivity derived from the low dose short insulin tolerance test (IS).



3.7. DISCUSSION

The pathogenesis of a variety of clinical disorders is characterised by resistance to the actions of insulin (DeFronzo 1988). The measurement of insulin sensitivity therefore, has become an important tool for investigating the contribution of diminished insulin sensitivity to the aetiology of such conditions and for evaluating subsequent therapeutic intervention. The simplest way of assessing insulin action *in vivo* is to measure the blood glucose response following administration of insulin. The pharmacological insulin doses usually employed in insulin sensitivity tests suppress hepatic glucose output, enabling the glucose uptake by peripheral tissues to be assessed. These original studies took several hours and also resulted in symptomatic hypoglycaemia. This promoted a counter-regulatory hormone response that would antagonise the fall in blood glucose (Shen et al. 1970).

The euglycaemic hyperinsulinaemic clamp (DeFronzo et al. 1979) has long been recognised as the gold standard for assessment of insulin sensitivity. However, it requires sophisticated equipment, expertise, is time consuming and is expensive. It relies on rapid measurement of blood glucose and is more suited to the research setting than to the clinical situation. More recently, several other techniques have been developed for measuring insulin sensitivity (Harano et al. 1977; Bergman et al. 1979), but these also require multiple sampling over several hours and complicated mathematical models. The short insulin tolerance test (Bonora et al. 1989) provides a simple alternative method for the assessment of whole body insulin sensitivity and is particularly suited to the rapid screening of large numbers of subjects on repeated occasions. Conventionally, an insulin bolus of 0.1units/kg body weight has been used which produces a fall in blood glucose concentration adequate for the assessment of subjects with varying degrees of insulin sensitivity. However in healthy individuals, who may well be included in large population screening surveys for which the test is designed, it may cause symptomatic hypoglycaemia. To overcome this problem we have used the lower insulin dose of 0.05units/kg. The test is terminated at 15 minutes ensuring that no subject becomes hypoglycaemic and thereby avoiding the counter-regulatory response (Bonora et al. 1989). Although we did not specifically measure levels of counter-regulatory hormones in our subjects during the insulin tolerance test,

Bonora et al. (1989) have demonstrated that levels of these antagonists do not rise until 30 minutes after administration of an insulin bolus of 0.1 units/kg; it is unlikely therefore, that the lower insulin dose which we employed would have triggered a response in the initial 15 minutes.The rate of fall of plasma glucose during the test is therefore purely a function of the action of insulin.

Any measure of insulin sensitivity must be sufficiently precise and accurate to allow comparison between individuals and ranking of insulin sensitivity. It must also compare favourably with the widely accepted standard, the euglycaemic hyperinsulinaemic clamp study. We have demonstrated the short insulin tolerance test employing 0.05 units/kg insulin to be reproducible. In addition, we have shown that a close correlation exists between the total body insulin sensitivity measurement derived from the low dose insulin tolerance test and the M/I ratio calculated from the clamp technique.

Unlike the euglycaemic clamp, the short insulin tolerance test does not provide a direct quantitation of glucose metabolism by peripheral tissues but gives an overall assessment of whole body sensitivity to insulin. This is adequate for the initial identification of insulin resistant subjects within high risk populations who may then be targeted for more detailed study and may benefit from early intervention. It may be used for repeated studies in the same individual, is minimally invasive and is safe. The relative simplicity and speed of this technique renders it suitable for the study of subjects, such as pregnant women,who may be unable to tolerate procedures of greater length, though its use in pathological states may require further validation.

In conclusion, this study has demonstrated the low dose short insulin tolerance test employing an insulin bolus of 0.05 units/kg body weight to be a safe, highly reproducible method of assessing insulin sensitivity which has been validated against the euglycaemic hyperinsulinaemic clamp.

CHAPTER 4:

DEVELOPMENT OF TRIPLE ISOTOPE TECHNIQUE AND MEASUREMENT OF INSULIN SENSITIVITY USING ISOTOPIC GLYCEROL

4.1. INTRODUCTION

Tracer techniques have long been used for measurement of substrate turnover (Wolfe 1992). In chapter 10, insulin sensitivity with respect to lipolysis was investigated in subjects predisposed to later NIDDM by examining the effects of a very low dose insulin infusion on glycerol turnover using stable isotopic tracers. This protocol involved a novel technique, in which two stable isotopes (6,6 ^2H glucose and 1,2,3 $^2H_5$ glycerol) were administered simultaneously as metabolic tracers for measurement of basal glucose and glycerol turnovers, and the change in the rate of appearance of each substrate in plasma during the insulin infusion provided an index of insulin sensitivity. A very low dose insulin infusion was chosen to specifically investigate insulin sensitivity with respect to lipolysis, a process which is inhibited at much lower insulin concentrations than is required for its action on glucose metabolism. Consequently, the degree of suppression of glycerol appearance in plasma is dependent on the sensitivity of adipose tissue to insulin.

To ensure that the technique could distinguish differences in insulin action, the method was employed initially to compare with human insulin the effects of biosynthetic insulin analogues with high (HA) or low affinity (LA) for the insulin receptor on glucose, glycerol and leucine turnovers (chapter 5). Some of the methods involved are established techniques (Matthews et al. 1980; Haigh et al. 1982), but the effects of the low dose insulin infusion on substrate turnover and laboratory analysis of the glycerol samples required considerable development. In particular, when a tracer is administered as a constant infusion, the calculation of substrate turnover relies on attaining isotopic equilibrium at steady state (Wolfe 1992). At isotopic steady state the enrichment of the pool of substrate is constant over time. It is important, therefore, that the tracer infusion is of sufficient duration to allow steady state to be reached, both before and after the addition of insulin. To optimise the study design, a series of preliminary studies were performed in individual subjects prior to undertaking the full protocol. These are discussed below.

4.2. CLINICAL STUDIES

4.2.1. Low dose insulin infusion

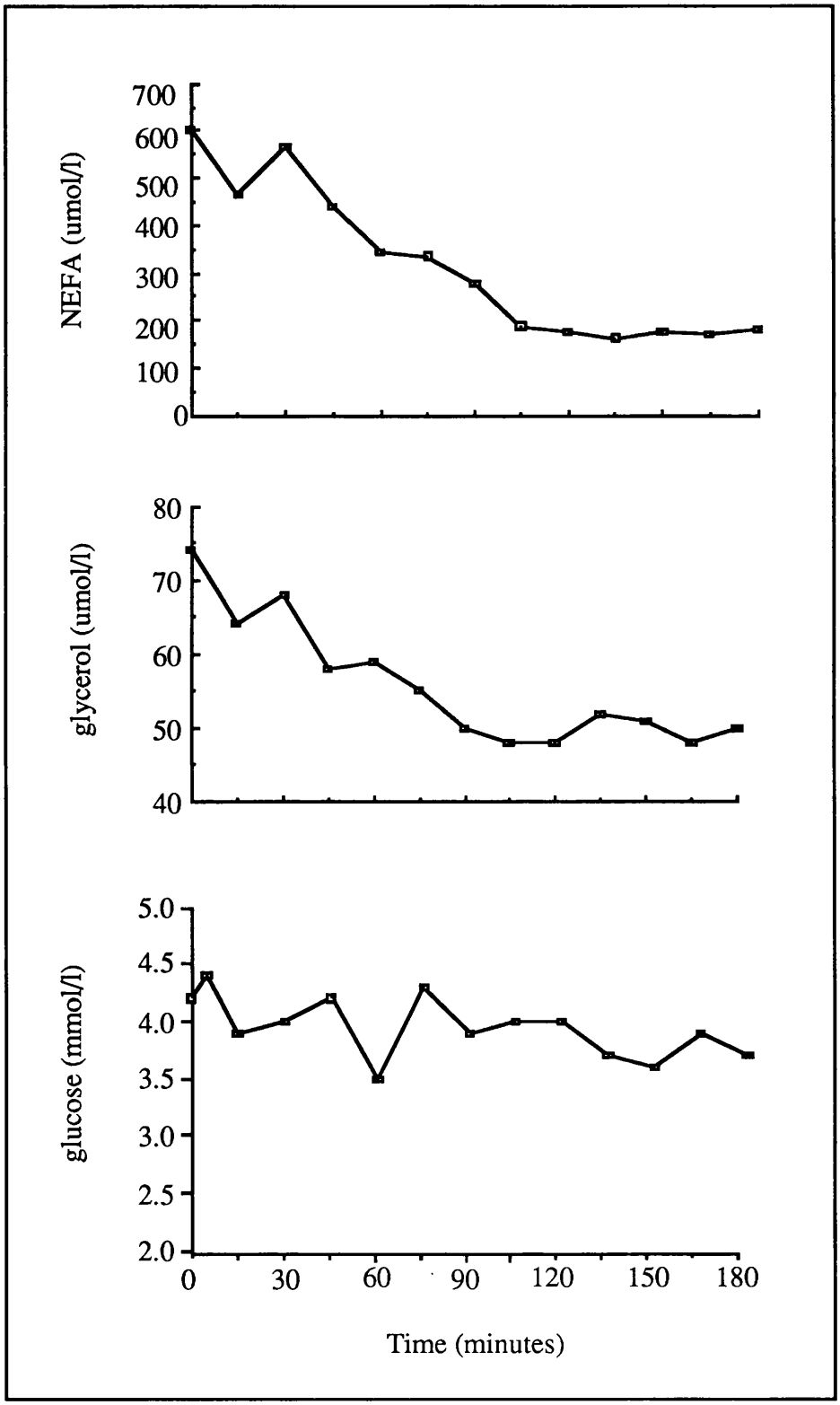
The effect of a very low dose insulin infusion on plasma glucose, glycerol and NEFA levels was investigated. One healthy male subject (age 24 years, body mass index 20.0 kg/m^2) was studied after an overnight fast. Two intravenous cannulae were inserted under subcutaneous lignocaine anaesthesia, one into a dorsal hand vein for sampling and the other into an antecubital vein of the opposite arm for the infusion. The sampling hand was placed in a warming chamber for arterialisation. After taking basal samples, the insulin infusion (Actrapid HM) 100U/ml diluted in Haemaccel® was commenced at 0.005 units/kg/hour and continued for 180 minutes. Blood was sampled at 15 minute intervals for measurement of glucose, glycerol, NEFA and insulin as detailed in chapter 2.

Results

Plasma NEFA, glycerol and glucose levels during the study are shown in Figure 4.1. The basal plasma insulin concentration was 27 pmol/l and during the infusion a steady state insulin level of 38 pmol/l was achieved. The plasma glucose concentration at steady state was 3.9 mmol/l, a mean fall of 0.5 mmol/l from the fasting value. Plasma NEFA and glycerol concentrations decreased during the infusion and reached a plateau by 105 minutes (Figure 4.1). The plasma NEFA concentration fell by 71% from a mean basal value of 602 to 176 μmol/l and glycerol fell by 34% from 74 to 49 μmol/l. The subject remained asymptomatic throughout.

This study confirmed that insulin infused at this low dose rate produces minimal falls in blood glucose concentration, which remains well above the threshold for symptomatic hypoglycaemia (Amiel 1991). Circulating levels of glycerol and NEFA, however, showed marked reductions during insulin administration. This dose of insulin, therefore, can be used safely in healthy individuals to examine lipolysis.

Figure 4.1. Plasma non-esterified fatty acid (NEFA), glycerol and glucose concentrations during 180 minute insulin infusion in one subject.



Insulin infusion was started at time 0.

4.2.2. Glucose turnover in response to insulin or analogue infusion.

1). Glucose turnover and insulin

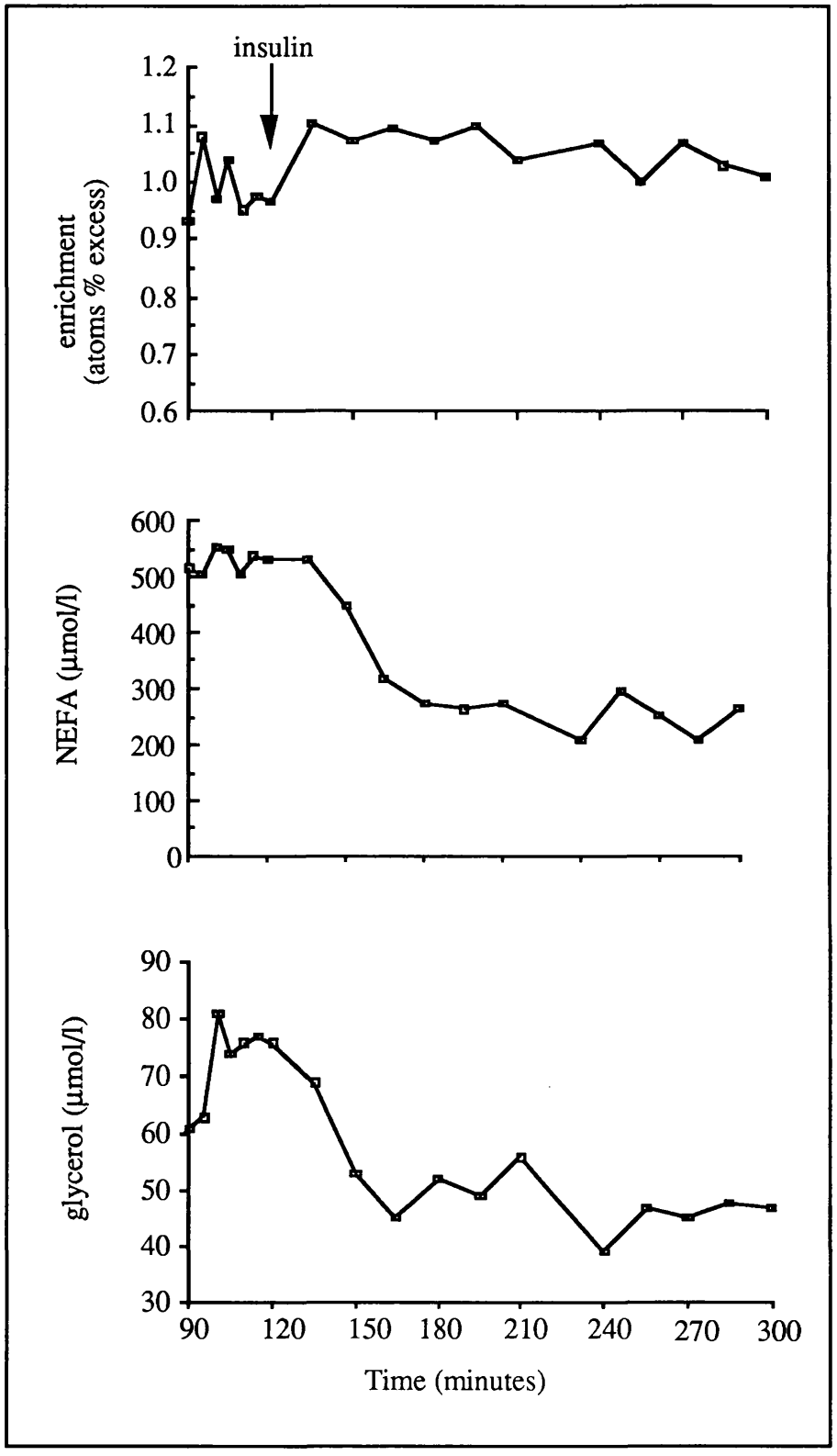
This study investigated the effect of the low dose insulin infusion on the rate of appearance of glucose measured isotopically. A healthy male volunteer (age 23 years, body mass index 23.4 kg/m^2) received a primed dose constant infusion of 6,6,^{2}H glucose for 5 hours as descibed in chapter 2. After 120 minutes, a low dose insulin (Actrapid HM) infusion (0.005 units/kg/hour) was commenced as above and continued until the end of the study. Blood samples were taken for measurement of glucose, glycerol, NEFA and insulin concentrations and for ^{2}H glucose enrichment at times -15, 0, 90 minutes and then at 5 minute intervals until the insulin was started; sampling continued thereafter every 15 minutes as described in chapter 2.

Results (Figure 4.2)

A plateau in isotopic enrichment was observed over the period 110-120 minutes. The mean basal enrichment in plasma (Ep) was 0.994 atoms percent excess (APE) and the enrichment in the infusate (Ei) was 88.8%. Hence by the theory of isotope dilution (chapter 2), the rate of appearance (Ra) of glucose was 2.65 mg/kg/min.

During the insulin infusion enrichment increased to reach a new steady state (Figure 4.2), when the mean Ep was 1.056 APE and calculated Ra glucose was 2.49 mg/kg/min. This fall in the rate of appearance of glucose was accompanied by decreases in plasma NEFA and glycerol concentrations of 51% and 37% respectively (Figure 4.2). Plasma glucose concentration declined from a fasting value of 5.0 mmol/l to 4.3 mmol/l during the insulin infusion and plasma insulin levels rose from the basal concentration of 28 pmol/l to 48 pmol/l.

Figure 4.2. Plasma ^{2}H glucose enrichment, non-esterified fatty acid (NEFA) and glycerol concentrations before and during insulin infusion in one subject.



The insulin was commenced at time 120 minutes.

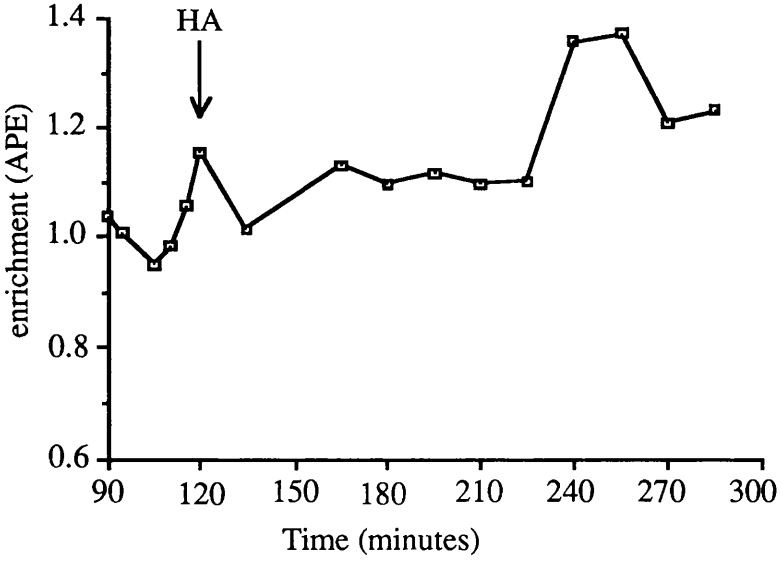
2). Glucose turnover and analogue HA

The above protocol was repeated in a healthy female subject, age 23 years and body mass index 21.2 kg/m^2, using the high affinity biosynthetic insulin analogue HA (B10-Asp-insulin; 600nmol =100U/ml) in place of human insulin. The insulin analogue was diluted in Haemaccel® and infused at a dose of 0.005 units/kg/hour as above. Blood was sampled at intervals for analysis of glucose, glycerol, NEFA, insulin and ^{2}H glucose enrichment as previously described.

Results

The basal insulin concentration was 27 pmol/l and during the analogue infusion the steady state immunoreactive insulin concentration was 28 pmol/l. The calculated rate of glucose appearance in the basal state was 2.55 mg/kg/min, though the enrichment did not appear to reach a steady plateau by 120 minutes (Figure 4.3).

Figure 4.3. Deuterated glucose enrichment in one subject before and during infusion with the insulin analogue HA.



Analogue infusion was started at 120 minutes.

Following the analogue infusion, there was a late rise in enrichment from 240 minutes and the corresponding calculated glucose Ra fell to 2.24 mg/kg/min. Plasma glucose

concentration fell from a basal value of 4.6 mmol/l to 4.4 mmol/l during the analogue infusion. The corresponding plasma levels of glycerol were 118 μmol/l before and 132 μmol/l during the analogue infusion; plasma NEFA concentation remained virtually unchanged from the basal value of 582 to 573 μmol/l at the second isotopic plateau. These minor changes in plasma concentrations of insulin, glycerol and NEFA from the basal values compared to the greater magnitude of change in those studies employing insulin, may be a consequence of the reduced receptor affinity of analogue HA. This is discussed in more detail in chapter 5.

3). Glucose turnover and analogue LA

In the first of the preceding two studies the deuterated glucose appeared to reach a steady state plateau by 100 minutes, but this did not occur in the second study. There were small increases in tracer enrichment in both studies after the insulin or analogue infusion and these were reflected in the change in the calculated values for the rates of glucose appearance. To verify that a steady state can indeed be attained in the initial basal period, a further study was performed with the pre-insulin/ analogue period extended to 180 minutes.

A healthy male volunteer (age 23 years, body mass index 21.6 kg/m^2) received a primed dose-constant infusion of 6,6 ^2H glucose for 5 hours as above. After 180 minutes, an infusion of the low affinity biosynthetic insulin analogue LA (B9-Asp, B27-Glu insulin) was commenced and continued for the remainder of the study. Blood samples were collected as previously.

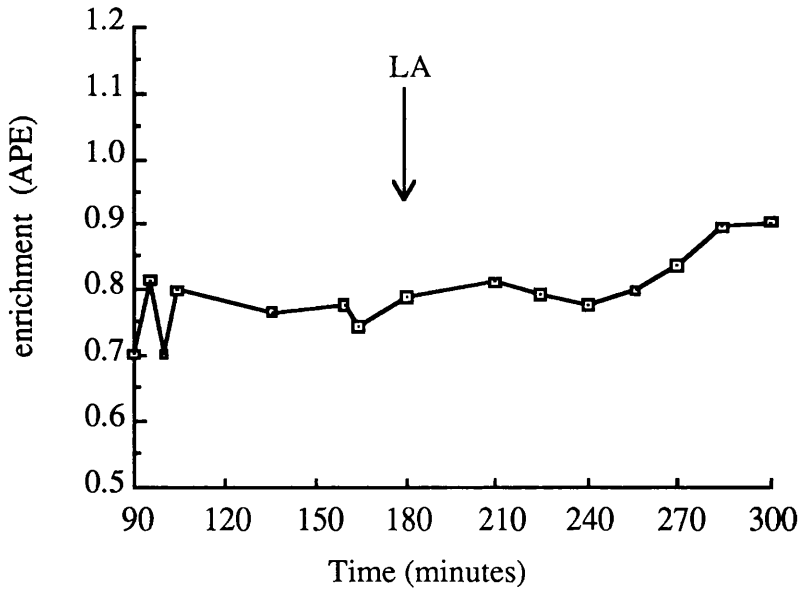
Results

A plateau in isotopic enrichment was reached by 110 minutes and was maintained until 180 minutes (Figure 4.4). The calculated basal Ra glucose was 3.33 mg/kg/min. After the analogue infusion was commenced a new steady state was not attained until 285 minutes. The calculated Ra glucose for this post-analogue infusion period was 2.93 mg/kg/min. Plasma glycerol concentrations fell by 42%, from a mean value of 96 μmol/l prior to the analogue infusion to 56 μmol/l and plasma NEFA levels decreased

by 50%, from an initial concentration of 313 μmol/l to 156 μmol/l. The basal plasma immunoreactive insulin concentration was 23 pmol/l and increased to 46 pmol/l over the steady state.

This study confirmed that a plateau in isotopic enrichment is achieved over the period 120-180 minutes. In later studies, therefore, the basal sampling period was extended to 120-150 minutes with insulin or analogue infusion initiated at 150 minutes. Since these early studies also suggested that the post-insulin plateau was only attained by 285 minutes, the total length of the study was later prolonged to 330 minutes.

Figure 4.4. Deuterated glucose enrichment before and during infusion of insulin analogue LA in one subject.



Analogue infusion was started at time 180 minutes.

4.2.3. Glucose and leucine kinetics in response to insulin or analogue infusion

The protocol was expanded to include infusion of two stable isotope tracers simultaneously.

1). Response to insulin

After an overnight fast, a healthy female subject (aged 28 years and of body mass index

20.0 kg/m^2) received primed dose constant infusions of 6,6 ^{2}H glucose and 1-^{13}C leucine for 300 minutes, as described in chapter 2. A priming dose of sodium ^{13}C bicarbonate was also administered at time 0 to accelerate attainment of equilibrium. At 120 minutes an infusion of human insulin (0.005 units/kg/hour) was begun and continued until the end of the study. Expired air was sampled at 15 minute intervals for analysis of $^{13}CO_2$ enrichment and CO_2 production rate was measured from 90-120 minutes and from 270-300 minutes using a Deltrac metabolic monitor. Blood samples were taken as previously described (chapter 2) for measurement of tracer enrichment and for glycerol, glucose and NEFA concentrations.

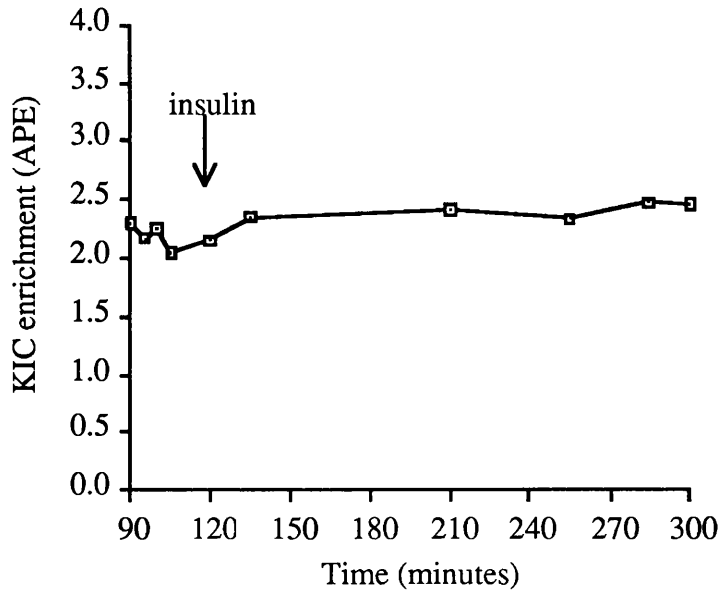
Results

Basal α-ketoisocaproic acid enrichment (a marker of leucine metabolism, as explained in chapter 2) over the plateau period 90-120 minutes was 2.184 APE and the calculated leucine flux was 168 μmol/kg/hour. The mean rate of oxidation of $^{13}CO_2$ in expired air over this period (F $^{13}CO_2$) was 1.078 μmol/kg/hour and the measured CO_2 production rate was 226 ml/min. Hence, the calculated rate of leucine oxidation (C) was 48 μmol/kg/hour and by subtraction, the rate of leucine incorporation into protein (S) was 120 μmol/kg/hour.

During the insulin infusion a small rise in ^{13}C α–KIC enrichment was observed to a mean value of 2.417 APE and a new steady state was attained (Figure 4.5). The corresponding leucine flux fell to 151 μmol/kg/hour. The CO_2 production rate was 191 ml/min and F$^{13}CO_2$ was 0.937 μmol/kg/hour. Thus, the calculated rate of leucine oxidation fell to 38 μmol/kg/hour and the resulting rate of leucine incorporation into protein (S) also decreased to 113 μmol/kg/hour. The plasma insulin concentration increased from a basal level of 24 pmol/l to 33 pmol/l at steady state and produced a small fall in plasma glucose concentration from 4.5 mmol/l to 4.1 mmol/l. Plasma glycerol levels decreased from basal by 22% from 107 to 77 μmol/l and plasma NEFA levels declined by a similar degree (20%) from 475 to 379 μmol/l.

Glucose turnover exhibited a small decrease from 2.38 mg/kg/min in the basal state to 2.25 mg/kg/min during the insulin infusion.

Figure 4.5. Leucine tracer enrichment before and during insulin infusion in one subject.



Insulin was commenced at 120 minutes.

2). Response to analogue LA.

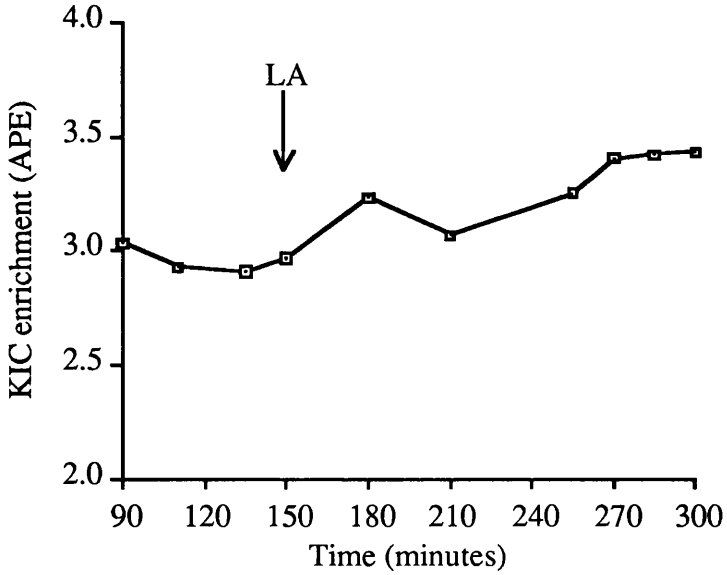
The study was then repeated in a second female subject (age 23 years, body mass index 21.2 kg/m^2) but using an infusion of the low affinity analogue LA in place of human insulin. In view of the findings in the earlier study (4.2.2.(2)), the initial period was extended from 120 to 150 minutes before the analogue infusion was commenced.

Results

A steady state in α-KIC enrichment was obtained over 120-150 minutes, during which the calculated leucine flux rate was 123 μmol/kg/hour, the leucine oxidation rate was 14 μmol/kg/hour and by subtraction, the rate of leucine incorporation into protein was 109 μmol/kg/hour. After the analogue infusion was started, there was a small rise in α-KIC enrichment and a new steady state was reached by 270 minutes. (Figure 4.6). The corresponding leucine flux rate decreased to 106 μmol/kg/hour, the rate of oxidation

increased to 15 μmol/kg/hour and the rate of leucine incorporation into protein fell to 91 μmol/kg/hour.

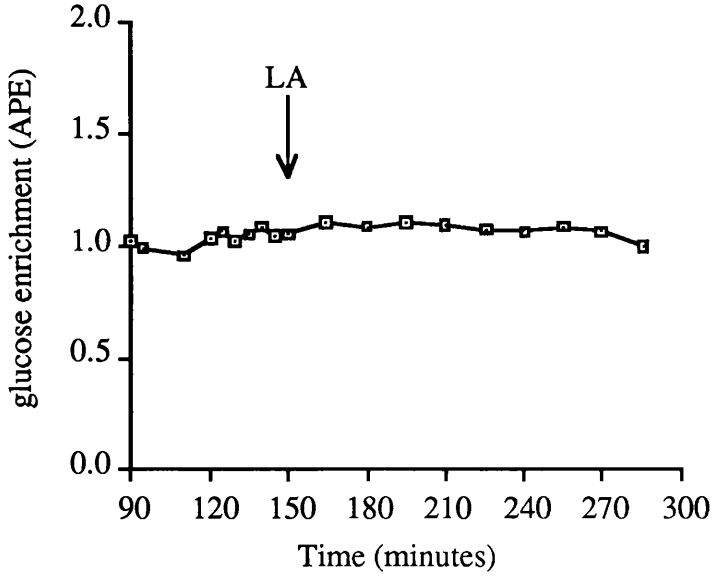
Figure 4.6. Leucine tracer enrichment before and during infusion with analogue LA in one subject.



The analogue infusion was commenced at 150 minutes
^{13}C enrichment was measured as α-ketoisocaproic acid (KIC).

Glucose enrichment was in steady state over the periods 120-150 minutes and 180-270 minutes (Figure 4.7). The basal glucose Ra was 2.56 mg/kg/min which declined slightly with the analogue infusion to 2.42 mg/kg/min.

Figure 4.7. Deuterated glucose enrichment before and during infusion with analogue LA in one subject.



The analogue infusion was started at 150 minutes.

Circulating immunreactive insulin levels increased from 23 pmol/l in the basal state to 63 pmol/l during the second steady state. This produced a fall in glucose concentration from 4.8 mmol/l before the analogue infusion to 4.3 mmol/l after this. Plasma levels of glycerol decreased by 41% from 84 to 50 μmol/l and plasma NEFA concentrations fell by 69% from 650 to 200 μmol/l over the same periods.

4.2.4. Glycerol turnover in response to insulin infusion.

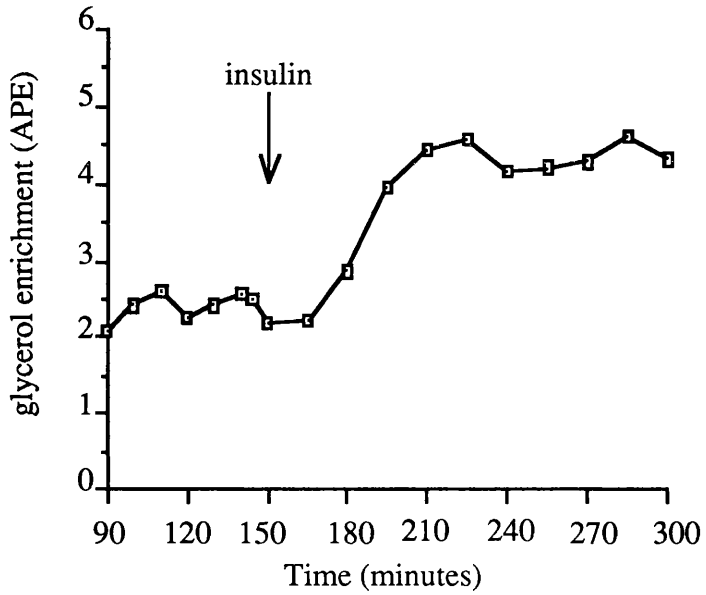
A healthy male volunteer (age 22 years, body mass index 26.4 kg/m^2) received a prime dose constant infusion of 1,2,3 $^2H_5$ glycerol after an overnight fast, as detailed in chapter 2. After 150 minutes, a constant infusion of human insulin (0.005 units/kg/hour) was commenced and continued until 300 minutes. Arterialised blood samples were taken at intervals for ^2H enrichment, as previously described.

Results

An isotopic plateau was reached over the period 90-150 minutes (Figure 4.8) and the calculated basal Ra glycerol was 2.09 μmol/kg/min. After starting the insulin infusion a new plateau in enrichment was reached and the calulated Ra glycerol fell to 1.10

μmol/kg/min. Circulating insulin levels rose from 30 pmol/l in the basal state to 48 pmol/l during the insulin infusion. Plasma concentrations of glycerol, NEFA and glucose exhibited similar changes to those observed in the earlier studies (glycerol fell by 34% from 67 to 44 μmol/l ; NEFA by 57% from 407 to 175 μmol/l ; glucose decreased from 4.6 mmol/l in the basal state to 4.3 mmol/l during the final hour of the study).

Figure 4.8. Deuterated glycerol enrichment before and during insulin infusion in one subject.



The insulin was started at 150 minutes.

4.3. SUMMARY

These pilot experiments were undertaken to find the optimal protocol for investigation of insulin action on lipolysis, glucose and protein metabolism using stable isotopic tracers. Each study was performed in a single subject as the aim was to improve the methodology before initiating larger investigations. The experiments described involve different combinations of isotope tracers, insulin, analogues HA or LA and varying durations of infusion and this prevented the results from different studies from being combined or analysed statistically. They are, however, adequate to allow development of the full protocol which was then utilized in the larger, more detailed studies described in chapters 5 and 10.

The insulin dose of 0.005 units/kg/hour was sufficient to produce marked decreases in plasma glycerol and NEFA concentrations without invoking hypoglycaemia, as has been reported by Hale et al. (1985 and 1988). No subject experienced any adverse effect. Measurement of glucose turnover using ^{2}H glucose infusion as tracer is not a new technique (Haigh et al. 1982), but the duration of infusion required refinement to ensure that a steady state was reached both before and after insulin infusion. Although isotopic enrichment appeared to plateau by 110 minutes in one study, this was not the case in all the studies. Extending the pre-insulin tracer infusion period to 150 minutes ensured an isotopic plateau was attained prior to perturbing the system with insulin or analogue. Similarly, plasma enrichments of α-KIC and glycerol were also in steady state over the sampling period 120 to 150 minutes.

With the addition of insulin or biosynthetic analogue infusion, the equilibrium was disrupted and a new steady state had to be reached before turnover could be measured using the principle of isotope dilution (chapter 2). This occurred rapidly in the first study using ^{2}H glucose (by 210 minutes) and with the ^{2}H glycerol tracer, but in others, glucose and leucine tracers only reached steady state at 270 minutes. The full protocol was therefore extended to 330 minutes to ensure isotopic equilibrium was achieved both basally and again with the insulin or analogue infusion.

The results obtained for basal turnover of glucose, glycerol and leucine agree with previously published data (Matthews et al. 1980; Haigh et al. 1982; Fukagawa et al. 1985; Tessari et al. 1986; Beylot et al. 1987; Bennet et al. 1990; Matthews et al. 1991; Le Stunff and Bougnères 1992) validating this part of the protocol. Although studied in different subjects, the insulin/ analogue infusions resulted in small changes in glucose and leucine kinetics, but a marked decrease in glycerol turnover occurred with insulin. This demonstrates the value of this technique for the investigation of lipolysis and this, as well as the prevailing plasma concentrations of insulin or analogue during their infusion, are discussed further in chapters 5 and 10.

4.4. PROCESSING OF GLYCEROL TRACER

The method of processing the deuterated glycerol tracer for detection required considerable development. For tracer detection by gas chromatography-mass spectrometry (GCMS) the sample must undergo deproteinisation and deionisation followed by derivatisation to produce a volatile pure compound (chapter 2).

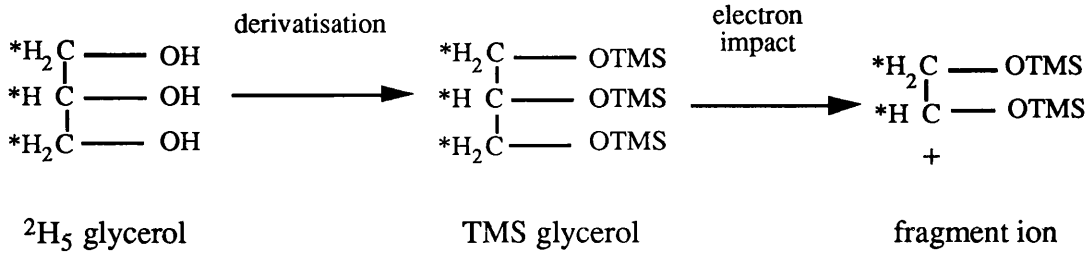
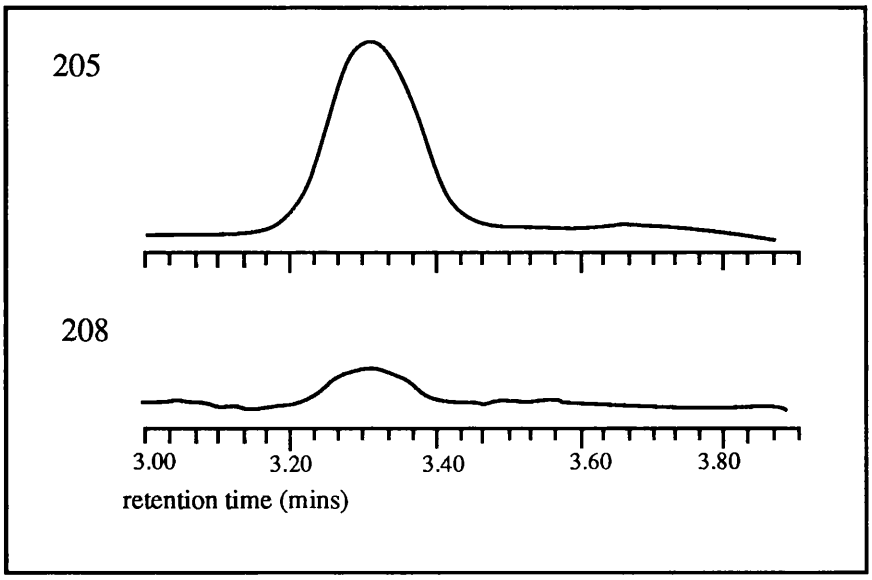


Figure 4.9. Derivatisation and fragmentation of deuterated glycerol.

Addition of trimethylsilyl imidazole (TMS) produces the volatile derivative. After electron impact in the mass spectrometer, the fragment ion is produced with only 3 remaining deuterium labels. Glycerol enrichment is measured by monitoring ions 205 (unlabelled fragment) and 208 (deuterated fragment).

Initially, plasma samples for glycerol tracer (200µl) were deproteinised with 0.275M sulphosalicylic acid (BDH Ltd., Poole, UK) (300µl) and the mixture centrifuged at 1000 revs/min for 1 hour at 4°C. The supernatant (400µl) was then deionised with 250µl acetified Duolite resin (BDH Ltd, Poole, UK) prepared as described in chapter 2 and shaken for 20 minutes. The supernatant (100µl) was separated, freeze-dried in a rotary drier and then derivatised with the addition of 100µl of trimethyl silane (TMS) (Fisons, Loughborough, UK) reagent to remove hydroxyl groups from the molecule. This helps to increase the volatility of the sample. After heating at 50°C for 15 minutes and freeze-drying again, the sample was dissolved in 50µl hexane (Fisons, Loughborough, UK) and injected into the gas chromatography injection port. Unfortunately, this method resulted in inadequate purification (Figure 4.10) with poor sensitivity and precipitation of the sample.

Figure 4.10. Diagrammatic representation of selected ion chromatogram of TMS derivative of glycerol at 205 and 208 with inadequate purification.

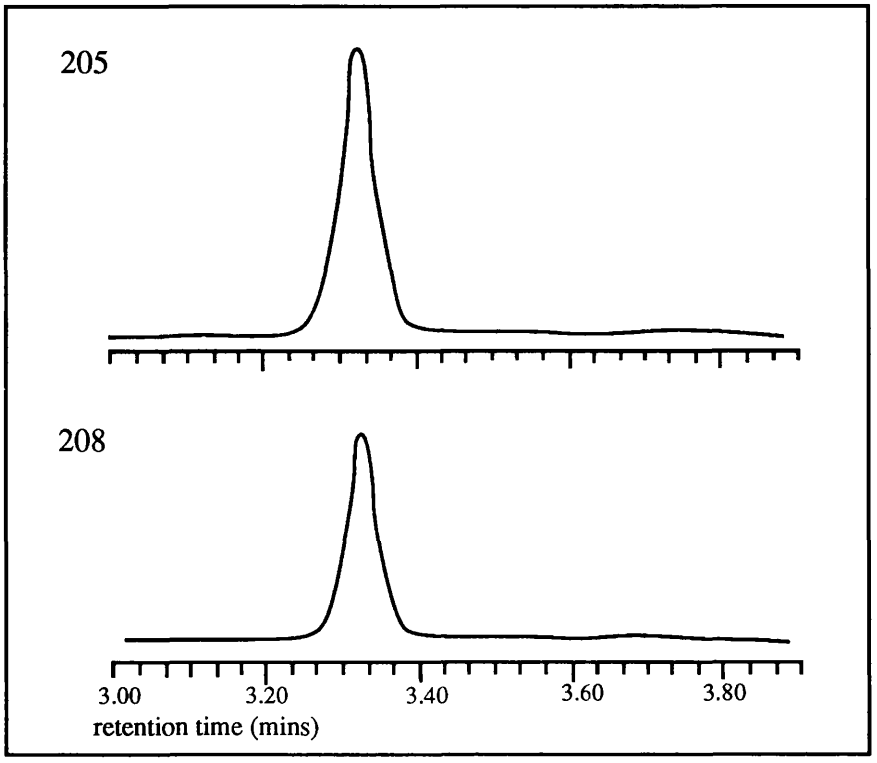


In order to improve sensitivity, the initial sample volume was increased to 500µl plasma and after being treated as above, it was dissolved in 100µl hexane, but isotope detection remained poor. A number of other adjustments were made to the method in an attempt to overcome this problem. For deproteinisation, methanol was substituted for the sulphosalicylic acid and oxygen-free nitrogen was used in the derivatisation step instead of freeze drying to reduce loss of the isotope. However, this still resulted in a broad isotope peak of low area on the chromatogram, indicating poor sensitivity and specificity.

Further modifications were therefore made to the method of processing the sample. Deproteinisation was performed with 800µl ice cold acetone added to 200µl plasma. After centrifugation, the sample was deionised with Duolite resin as previously. A larger volume of supernatant (500µl) than before was then freeze dried. Derivatisation was achieved with 25µl trimethylsilyl imidazole in pyridine, vortex mixed and heated at 70°C for 30 minutes. The volume of hexane added was then increased to 250µl and the solution was vortex mixed again. This was followed by the addition of ion free water (100µl) and further mixing. Any precipitate was allowed to settle prior to pipetting 100µl of sample into vials for the GC-MS. The processing adapted in this way

considerably improved sensitivity and ion detection (Figure 4.11). The full procedure used in the clinical studies is detailed in chapter 2.

Figure 4.11. Diagrammatic representation of selected ion chromatogram for glycerol derivative using modified method for sample processing.



CHAPTER 5:

VALIDATION OF MEASUREMENT OF INSULIN SENSITIVITY WITH RESPECT TO LIPOLYSIS USING STABLE ISOTOPIC TRACERS:

Differential Metabolic Actions of Insulin and Monomeric Insulin Analogues Assessed by Stable Isotopic Tracers.

5.1. INTRODUCTION

The investigation of insulin sensitivity has tended to concentrate predominantly on the action of insulin on glucose homeostasis, rather than its effects on fat or protein metabolism. As discussed in chapter 1, lipolysis is exquisitely sensitive to small increments in insulin concentration and this property may be utilized for the detection of subtle differences in insulin action. In this chapter, a novel technique was employed for the examination of insulin sensitivity with respect to lipolysis in which differences in action were sought between insulin and biosynthetic insulin analogues. The change in glycerol turnover measured isotopically in response to a very low dose insulin or analogue infusion was assessed as a measure of insulin sensitivity.

Biosynthetic insulin analogues have been developed in order to overcome the limitations of conventional insulin therapy. Soluble insulin in pharmaceutical concentrations exists in hexamers which must dissociate into dimers and monomers in order to be absorbed. This limits the rate of absorption from the subcutaneous tissue, resulting in unphysiological plasma insulin profiles. Monomeric insulin analogues have been produced by amino acid substitution of uncharged amino acids at the monomer-monomer interface with negatively charged residues and the resulting charge repulsion reduces self association (Brange et al. 1988). These analogues are absorbed more rapidly than insulin via the subcutaneous route (Vora et al. 1988) and have altered *in vitro* potencies compared to insulin.

One such analogue has amino acid substitutions at position B9 of serine to aspartic acid and B27 of threonine to glutamic acid, and another has a substitution at position B10 of histidine to aspartic acid. *In vitro* bioassays and receptor binding studies have demonstrated the first to have low affinity (LA: 20%) and the second to have high affinity (HA: 300-500%) relative to insulin (Schwartz et al. 1987). *In vivo* studies measuring the effects of these analogues on glucose metabolism suggest they have similar potency to insulin in normal (Brange et al. 1988; Ribel et al. 1990) and in diabetic subjects (Kang et al. 1990 and 1991). These studies have used upper physiological or supraphysiological concentrations for measurement of the effects on glucose metabolism and have not explored the effects of these analogues on amino

acids or other aspects of carbohydrate metabolism.

In order to investigate the actions of the analogues LA and HA on lipolysis, we administered a very low dose infusion of each analogue to healthy subjects and compared with insulin their effects on glucose, glycerol and protein turnover using stable non-radioactive isotope tracers.

5.2. SUBJECTS

Seven healthy volunteers were studied, six male and one female, with an age range of 22-32 years and body mass index range 18.5 - 26.2 kg/m^2. Each subject was studied on three separate occasions with human insulin, analogue LA or analogue HA in random order, at least one week apart. In addition, five of these subjects (four men and one woman) were studied on a fourth occasion with Haemaccel® infusion, as a control study. The woman was studied within the first 10 days of her menstrual cycle in each case. Individuals were asked to follow their usual diet for the five days preceding each test and to abstain from alcohol and strenuous exercise for three days prior to each study.

5.3. METHODS

The development of this protocol is described in chapter 4. Subjects received primed constant infusions of the stable isotopes [6,6 ^{2}H] glucose, [1,2,3 ^{2}H] glycerol and [1-^{13}C] leucine for 330 minutes as described in chapter 2. A priming dose of sodium ^{13}C bicarbonate was also administered to ensure equilibrium enrichment in expired air was attained (Allsop et al. 1978). Over the period 90-150 minutes plateau conditions were achieved (Matthews et al. 1980) and human insulin or analogue in equimolar doses (0.005 units/kg/hr) diluted in Haemaccel® was then infused until the end of the study. In the five control studies the same volume of Haemaccel®, without insulin or analogue added, was infused over this period. Samples of blood and expired air were taken at intervals and carbon dioxide production rate was measured with a metabolic monitor as detailed in chapter 2.

5.4. ANALYSES

Plasma glucose was measured at the bedside using a Beckman glucose oxidase analyser and plasma glycerol and NEFA concentrations were measured by enzymatic assays as described in chapter 2. Blood samples for tracer enrichment were treated as noted in chapter 2, prior to tracer detection by gas chromatography-mass spectrometry. Plasma insulin was measured by radioimmunoassay. Plasma concentrations of analogues LA

and HA were measured with each analogue used for its own standard curve and corrected for cross-reactivity with human insulin as described in chapter 2. The rates of appearance (Ra) of glucose, glycerol and leucine were calculated by the theory of isotopic dilution and leucine flux, oxidation and incorporation into protein were derived using a two-pool model. The rate of leucine oxidation was calculated from the rate of appearance of $^{13}CO_2$ in expired air (Matthews et al. 1980).

5.5. STATISTICAL ANALYSIS

The changes in glycerol, glucose, NEFA and insulin concentrations during the insulin or analogue infusions were analysed by calculating the areas under the curve from the preinfusion baseline values by the trapezoidal rule. Comparisons were made using analysis of variance and where significant differences were found, the effects of each insulin or control infusion were compared by the Kruskal-Wallis test for non-parametric data.

Turnover rates for glycerol, glucose and leucine and rates of leucine oxidation and incorporation into protein were calculated over the steady states at baseline (90-150 minutes) and during the final hour of the infusions (270-330 minutes). Due to variation in the baseline values, three-way analysis of variance was performed on the difference between baseline and post-infusion values and the Kruskal-Wallis test applied to determine differences between the insulin, analogue and control studies, using the statistical package SOLO (BMDP Statistical Software, CA., USA). All results are expressed as median (range).

5.6. RESULTS

Basal serum insulin levels during the control and insulin infusions were 25 (17 to 34) and 28 (16 to 40) pmol/l respectively. The changes in circulating "immunoreactive insulin" produced with each infusion were significantly different (area under curve, median (range): insulin 3009 (1749 to 3683) pmol/l.hr vs analogue LA 4256 (1603 to 8083) pmol/l.hr, $p<0.05$; analogue HA 246 (-8348 to 7686) pmol/l.hr, $p<0.05$ vs LA) and these levels of insulin and analogues were statistically different from the change produced with the control infusion (1331 (-1044 to 3240) pmol/l.hr, $p<0.0001$). Equimolar infusions of insulin, analogue LA and analogue HA all produced similar falls in blood glucose concentration. Each of these differed from the control infusion, which produced no significant change in plasma glucose level (area under curve, median (range): control -3 (-16 to 13); insulin -33 (-101 to -24), $p<0.01$ vs control; LA: -53 (-101 to -18), $p<0.01$ vs control; HA: -41 (-164 to 5) mmol/l.hr, $p<0.01$ vs control) (Table 5.1).

Plasma glycerol concentrations fell with insulin, LA and HA infusions but not in the control study, though the differences in response to the infusions did not reach statistical significance (area under curve: insulin -1507 (-3150 to 750), LA -2497(-5632 to 5213), HA -1597 (-3855 to 615), control +120 (-908 to 2370) μmol/l.hr) (Table 5.1).

Plasma NEFA levels showed a significant fall with insulin and LA, but not with HA, the effect of which did not differ from that in the control study. The change in NEFA concentration was significantly different between insulin and the control study (control +15 (11 to 53), insulin -23 (-41to 10) mmol/l.hr, $p<0.05$), LA and the control study (LA -28 (-42 to19) mmol/l.hr, $p<0.05$) and between LA and HA (LA -28 (-42 to 19) vs HA -8 (-28 to 35) mmol/l.hr, $p<0.05$) (Table 5.1).

Table 5.1. Incremental areas under the curves for immunoreactive insulin, glucose, glycerol and non-esterified fatty acid (NEFA) levels following insulin, analogue or control infusions.

	Insulin	LA	HA	control
Immunoreactive insulin (pmol/l.hr)	3009[a,b] (1749 to 3683)	4256[b] (1603 to 8083)	246[a,b] (-8348 to 7686)	1331 (-1044 to 3240)
Glucose (mmol/l.hr)	-33[c] (-101 to -24)	-53[c] (-101 to -18)	-41[c] (-164 to 5)	-3 (-16 to 13)
Glycerol (μmol/l.hr)	-1507 (-3150 to 750)	-2497 (-5632 to 5213)	-1597 (-3855 to 615)	120 (-908 to 2370)
NEFA (mmol/l.hr)	-23[d] (-41 to 10)	-28[d] (-42 to 19)	-8[e] (-28 to 35)	15 (11 to 53)

[a]$p<0.05$ vs low affinity analogue (LA); [b]$p<0.0001$ vs control; [c]$p<0.01$ vs control; [d]$p<0.05$ vs control; [e]$p<0.05$ vs LA.

Glucose and glycerol isotopic turnover rates are shown in Table 5.2. Glucose turnover exhibited a decrease from baseline with LA infusion and non significant falls with insulin and HA, but none of these changes in glucose flux from baseline were statistically different from that in the control study. Glycerol turnover showed a marked fall with insulin and LA infusions and these were significantly different from the effects of the control infusion and HA (change from baseline: control +0.60 (0.10 to 1.96) μmol/kg/min; insulin - 0.54 (-1.50 to 0.63) μmol/kg/min, ($p<0.01$ vs control); LA -0.74 (-1.76 to 0.72) μmol/kg/min, ($p<0.01$ vs control); HA -0.19 (-0.74 to 1.13) μmol/kg/min, ($p<0.05$ vs LA)).

There was a significant fall in α-ketoisocaproic acid flux with insulin, LA, HA and control studies, but these changes were not significantly different in the four infusions. Similarly, changes in α-ketoisocaproic acid oxidation and incorporation into protein (synthesis) were similar with insulin, both analogues and the control (Table 5.3).

Table 5.2. Glucose and glycerol turnover rates (Ra) measured isotopically before infusion and during the final hour of each infusion.

	insulin	LA	HA	control
Ra glucose (mg/kg/min)				
baseline	1.97 (1.52 to 2.40)	2.09 (1.73 to 2.22)	2.04 (1.43 to 2.34)	1.70 (1.55 to 2.02)
after infusion	1.84 (1.73 to 2.18)	1.85 [a] (1.54 to 2.06)	1.86 (1.36 to 2.38)	1.63 (1.43 to 1.98)
Ra glycerol (μmol/kg/min)				
baseline	2.16 (1.84 to 3.13)	2.10 (1.66 to 3.81)	2.77 (1.07 to 3.56)	1.81 (1.72 to 2.62)
after infusion	1.75 [b] (1.10 to 2.59)	1.67 [b] (1.33 to 2.38)	2.73 [c] (0.87 to 4.17)	2.62 (1.91 to 4.26)

Glucose turnover fell significantly only with the low affinity analogue (LA) infusion ([a]$p<0.01$). The changes in glycerol turnover with LA and with insulin were significantly different from the control study ([b]$p<0.01$), and the change with LA differed from that with the high affinity analogue (HA) ([c]$p<0.05$).

Table 5.3. Flux, oxidation and synthesis of α-ketoisocaproic acid (μmol/kg/hour).

	insulin	LA	HA	control
Baseline				
flux	138.9 (129.6 to 171.1)	136.6 (117.3 to 162.4)	144.5 (112.0 to 171.5)	131.3 (109.0 to 141.8)
oxidation	21.9 (16.9 to 34.7)	20.3 (18.0 to 32.9)	20.3 (14.0 to 24.2)	24.0 (19.8 to 28.8)
synthesis	113.7 (104.2 to 145.5)	112.4 (96.9 to 138.4)	127.9 (95.2 to 153.7)	109.4 (88.0 to 113.0)
After infusion				
flux	121.2[b] (117.9 to 172.6)	130.1[b] (99.2 to 149.7)	129.7[b] (99.0 to 153.8)	121.3[a] (103.3 to 130.6)
oxidation	23.7 (15.8 to 32.0)	22.1 (20.7 to 29.9)	20.5 (16.1 to 31.7)	21.7 (19.0 to 23.1)
synthesis	103.0 (89.4 to 140.6)	104.4 (78.1 to 119.8)	109.7 (82.9 to 133.2)	98.6 (84.3 to 108.9)

Kinetics for α-ketoisocaproic acid are shown for the baseline period before insulin, analogue or control infusions and for the final hour of the infusions. Flux fell significantly with all four infusions. [a]$p<0.01$ vs baseline, [b]$p<0.005$ vs baseline.

5.7. DISCUSSION

Lipolysis was significantly suppressed by insulin and analogue LA, but not by analogue HA, as demonstrated by a reduction in glycerol production measured isotopically and a decrease in NEFA concentrations. No change in glucose or leucine turnover was observed with any preparation at the low concentrations employed. This demonstrates for the first time different effects of equimolar doses of biosynthetic insulin analogues in normal man.

In vitro studies have characterised these analogues by their degree of binding to human hepatoma $HEPG_2$ receptors. Compared to insulin, LA has low affinity (18%) and HA high affinity (333%) (Brange et al. 1988; Ribel et al. 1990). This estimate of potency corresponds well with that derived from the incorporation of tritium labelled glucose into lipids in mouse free adipocytes, (31% and 207% respectively). Despite these differences between analogues *in vitro*, *in vivo* studies have found the effects of both LA and HA to be similar to that of insulin. *In vivo* work has mainly concentrated on their action on glucose metabolism. During euglycaemic clamp studies in pigs, the total amount of glucose infused during and after insulin infusion was similar whether insulin, LA or HA was infused (Vølund et al. 1988; Ribel et al. 1990). Heineman et al. (1990) performed corresponding clamp studies in healthy men with subcutaneous insulin and came to the same conclusion. The present study is in agreement with these; there were small falls in glucose concentration with all three preparations but none significantly altered glucose turnover. The dose administered was chosen specifically to have minimal effect on glucose concentration, permitting lipolysis to be investigated at low concentrations and without a counter-regulatory response (Hale et al. 1985).

Infusion of LA achieved the greatest increase in circulating immunoreactive insulin concentrations, with insulin causing a lesser rise (63% of that observed with LA). Although individual variation was greater, no change in circulating immunoreactive insulin was observed with HA, despite administration of equimolar quantities of insulin and both analogues. A similar phenomenon has been observed in previous animal and human studies (Ribel et al. 1990; Vølund et al. 1991; Robertson et al. 1992) with the plasma level being inversely related to the potency *in vitro*. It may be explained by

different metabolic clearance rates which have been demonstrated (insulin 20ml/kg/min; LA 13ml/kg/min; HA 27ml/kg/min) (Robertson et al. 1992). Endogenous C-peptide release is suppressed to an equal extent by insulin and both analogues (Robertson et al. 1992). The low affinity analogue has decreased plasma clearance possibly due to reduced receptor-mediated degradation, resulting in a higher steady state plasma concentration (Brange et al. 1988). It also may reflect the relative distribution of analogue between the plasma and the receptor compartments (Ribel et al. 1990). The absence of an increase in immunoreactive insulin with the HA infusion in the present low dose study presumably reflects its rapid clearance. As clearance and biological action are linked, the lack of action on lipolysis suggests that the clearance occurred predominantly in the liver, as discussed later.

Leucine kinetics were chosen as an index of the actions of the analogues on muscle, as leucine is an essential amino acid metabolised primarily in muscle. The reduction in leucine turnover with all three preparations as well as with the control infusion suggest that the changes observed may reflect the metabolic response to fasting over the duration of the study (Fryburg et al. 1990). The small degree of the changes with insulin and analogues is consistent with the documented dose-dependent effect of insulin on leucine turnover and proteolysis (Fukagawa et al. 1985; Tessari et al. 1986). Any changes in flux due to amino acid label recycling (Schwenk et al. 1985) would be similar with all four infusions. A small increase in leucine oxidation was observed with the insulin and analogue infusions as has also been reported by other workers at low insulin infusion rates (Fukagawa et al. 1985). The lack of any significant change in leucine incorporation into protein with either analogue or insulin is consistent with other studies, in which basal insulin levels were only sufficient to stimulate protein synthesis when adequate amino acid substrate was supplemented (Gelfand and Barrett 1987; Tessari et al. 1987; Fukagawa et al. 1989; Bennet et al. 1990).

This study has demonstrated a significant difference in the degree of inhibition of lipolysis achieved by LA, HA and insulin, which has not previously been reported. Selective metabolic changes have been reported in normal pigs given subcutaneous insulin or analogues LA and HA (Falholt et al. 1987 and 1988) when the differential

actions were attributed to the faster absorption of the analogues resulting in preferential glycogen synthesis and decreased lipid deposition (Falholt et al. 1989). This could not explain the findings in the present study in which all three preparations were given by intravenous infusion, not via the subcutaneous route.

The discrepancy between our findings and those of other workers (Robertson et al. 1992) may reflect the sensitivity of the methods used. The effects on lipolysis are observed at much lower concentrations of insulin than those conventionally employed for the study of insulin's effects on glucose metabolism. Using a very low dose infusion this study, like that of Robertson et al. (1992), did not show a significant difference in effect of the analogues on glycerol concentration compared to that of insulin, but measurement of glycerol production using a stable isotopic tracer method provides a more accurate determination of turnover at low concentration (Wolfe 1992) and since glycerol cannot be re-esterified in adipose tissue (Lin 1977), its appearance in plasma is a direct reflection of the rate of lipolysis. Using a constant infusion technique, the baseline values for glycerol turnover were similar to those previously reported in normal subjects (Beylot et al. 1987; Matthews et al. 1991; Le Stunff and Bougnères 1992) and we detected a greater inhibition of lipolysis by analogue LA than by insulin, and HA had no effect. A similar pattern was observed with the NEFA responses.

There are several possible factors which may contribute to these differences observed. The analogues may vary in their hepatic and peripheral distribution. There is evidence from radiolabelled studies that HA is preferentially taken up by the liver, whereas LA is distributed mainly in peripheral tissues (Kruse et al. 1989; Sodoyez-Goffaux et al. 1991). Such differential distribution has also been demonstrated with chemically modified insulin analogues *in vitro* (Zeuzem et al. 1984) and *in vivo* (Tompkins et al. 1981). Their different activities were explained on the basis of a predominant hepatic or peripheral site of action (Chap et al. 1987). Tissue-specific changes in insulin receptor structure may also be of relevance (Burant et al. 1986). Analogues LA and HA also differ in their effects on cell growth. Unlike LA, HA has been shown to have a higher binding affinity for the insulin-like growth factor 1 (IGF-1) receptor with greater tyrosine kinase activation than insulin (Drejer et al. 1991) and to have enhanced growth

promoting activity *in vitro* (Wolpert et al. 1990; Bornfeldt et al. 1991).These reports, as well as the increased immunogenicity of LA, have limited further study of these analogues.

In conclusion, equimolar doses of biosynthetic insulin analogues with altered receptor affinity *in vitro* possess different antilipolytic activities compared to insulin *in vivo*. At the very low concentrations used, no differences of effect on glucose or leucine turnover were detected between either analogue or insulin. The study demonstrates the value of this technique employing low dose insulin and glycerol tracer infusions for the investigation of subtle differences in insulin sensitivity with respect to lipolysis.

CHAPTER 6:

THE GLYCEROL CLAMP

6.1. INTRODUCTION

Resistance to the hypoglycaemic actions of insulin in NIDDM is well recognised and can be measured using the euglycaemic hyperinsulinaemic clamp (DeFronzo et al. 1979) and the short insulin tolerance test, as described in chapter 3. These techniques, however, are of limited value in the assessment of lipolysis, since the high doses of insulin employed are far in excess of those required to produce maximal antilipolytic effect. In chapter 5 lipolysis was assessed using glycerol isotopic turnover measurements in response to a low dose insulin infusion. In this chapter an alternative method of quantifying lipolysis, **the glycerol clamp**, is described. The glycerol clamp is analogous to the euglycaemic hyperinsulinaemic glucose clamp, but with modifications to allow for the more marked sensitivity of lipolysis to insulin. During a very low dose insulin infusion plasma glycerol levels are clamped at fasting concentration by the administration of exogenous glycerol infused at a variable rate. The clamp technique, however, requires rapid estimation of plasma glycerol concentrations. This has been achieved by the development of a glycerol biosensor by Dr Lindy Murphy at St Mary's (Albery et al. 1993).

6.2. THE GLYCEROL BIOSENSOR

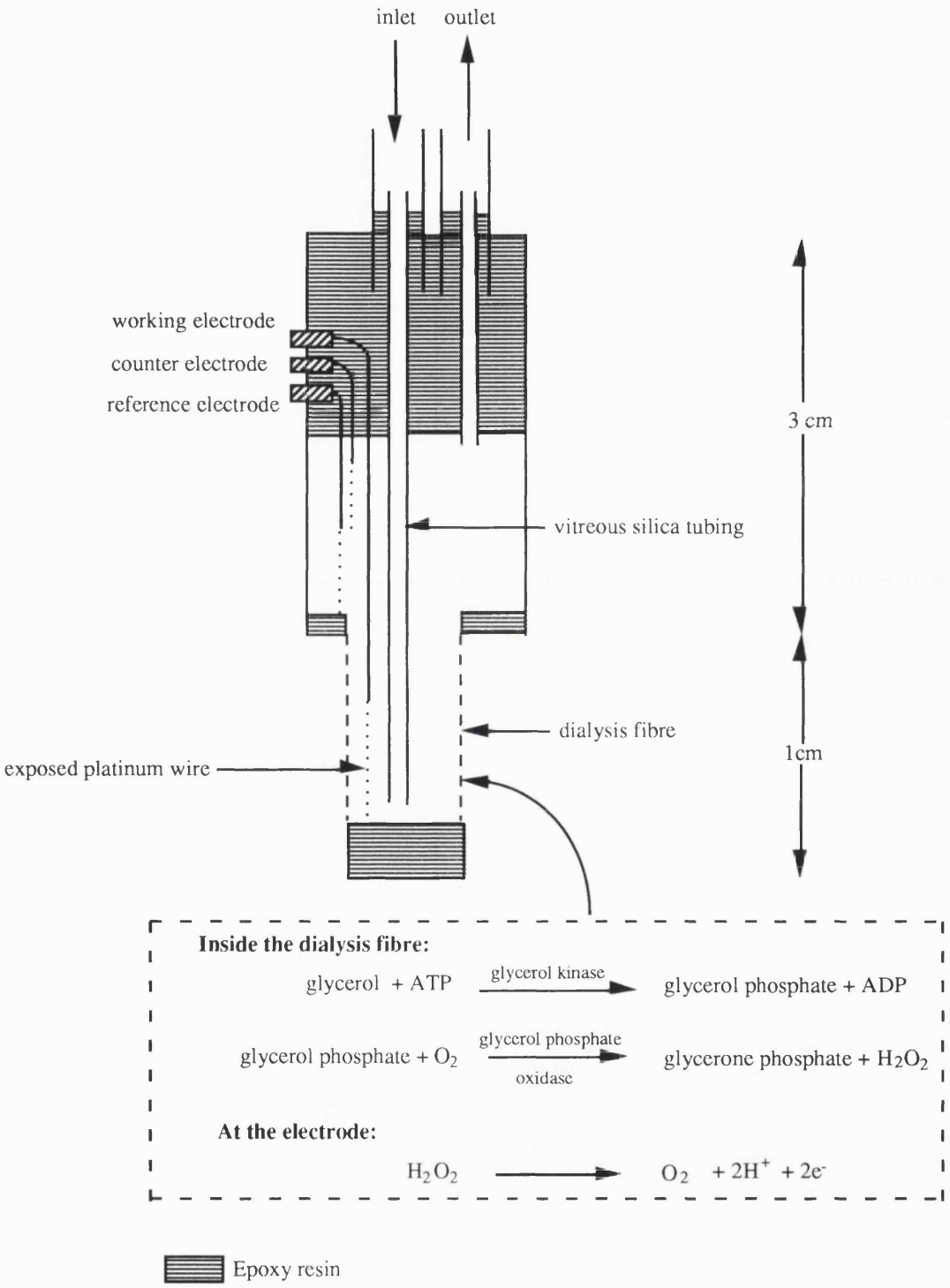
The glycerol biosensor is a microdialysis electrode which comprises a platinum electrode system encased within a semipermeable dialysis membrane. Within the membrane, glycerol is converted to glycerol phosphate using glycerol kinase and the glycerol phosphate is oxidised by glycerol phosphate oxidase to glycerone phosphate, with the generation of hydrogen peroxide. At the electrode, hydrogen peroxide is oxidised and the electrons released are detected as electrical current. The magnitude of this current is directly proportional to the concentration of glycerol in the sample.

The microdialysis electrode requires three electrodes for its operation: a silver/silver chloride reference electrode which gives a reference potential against which the potential of the working electrode is held; a silver counter electrode which forms a circuit with, and allows current to flow through the working electrode and the platinum working electrode at which hydrogen peroxide is oxidised (Figure 6.1). The electrode is filled with enzyme solution via the silica tubing. To measure the concentration of glycerol in a plasma sample the dialysis fibre is first dipped into buffer solution and the sample to be measured is added, giving a background current reading. ATP, the cofactor for glycerol kinase, is then added. Glycerol in the sample diffuses through the dialysis membrane into the internal solution, giving rise to the glycerol derived current.

Adsorption of albumin on to the outside of the dialysis fibre stabilises the response of the electrode by preventing adsorption of plasma proteins which may otherwise block the pores of the dialysis fibre. Certain interfering substances, both naturally occurring such as ascorbate, urate and L-cysteine, or adminstered such as paracetamol, give rise to a non-specific oxidation current at the electrode. Some of these interferents may also act as oxidising agents and reduce the amount of hydrogen peroxide detected at the electrode, causing a decrease in the measured glycerol concentration. Ascorbate, urate and paracetamol are removed by pretreating the plasma with peroxidase and catalase. The remainder of the interferents can still give rise to a small current at the electrode and this is greatly reduced by electrochemically coating the electrode with poly (1,2-diaminobenzene).

The electrode can measure glycerol linearly up to 500μmol/l and has a fast response time (30 seconds). It is capable of measuring the glycerol concentration of a pretreated sample every 5 minutes and the values for the glycerol concentration obtained give excellent agreement with those obtained by the spectrophotometric analyser.

Figure 6.1. The Microdialysis Electrode



6.3. CLINICAL STUDIES

The microdialysis glycerol electrode has been used in pilot studies of the measurement of insulin sensitivity with respect to lipolysis using an *in vivo* glycerol clamp.

6.3.1. Subjects

Four healthy volunteers, 2 male and 2 female, were studied, with an age range of 20-27 years and body mass index range 19.9-27.2 kg/m^2. None had a history of diabetes mellitus or was taking any medication.

6.3.2. Methods

After an overnight fast, the subject attended the Metabolic Day Ward. Body composition was measured using a Holtain monitor and bioelectrical impedance (Lukaski et al. 1986). An intravenous cannula was positioned under subcutaneous lignocaine anaesthesia into a deep forearm vein for infusion of glycerol and insulin. A second cannula was positioned in a dorsal hand vein of the contralateral hand and kept patent with a slow infusion of normal saline. The hand was placed in a warming chamber at 50°C to arterialise the sampled blood.

After resting for 30 minutes, three basal blood samples were taken at -20, -10 and 0 minutes. At time 0 an infusion of Human Actrapid insulin (0.005 unit/kg/hour) diluted in Haemaccel® was begun and continued until the end of the study. After 10 minutes an infusion of 1% glycerol (0.11 mol/l; 100ml of 10% glycerol solution diluted in 900ml normal saline) was commenced at 1ml/hour using a volumetric infusion pump (IMED, Abingdon, Oxon UK). Plasma glycerol concentration was measured using the glycerol electrode every 10 minutes and the glycerol infusion rate was adjusted at 10 minute intervals to maintain the arterialised plasma glycerol concentration at fasting level. The required rate of glycerol infusion was estimated.

Blood (2ml) for glycerol measurement was collected in EDTA tubes and an aliquot was centrifuged for 30 seconds at 13 000 revs/min to obtain plasma for the electrode. Blood glucose concentration was measured at the bedside using a Beckman glucose analyser.

Blood samples were also taken for measurement of immunoreactive insulin every 20 minutes. Plasma C peptide concentrations were measured on the same samples by radioimmunoassay using a polyethylene-glycol accelerated method as previously described (Hampton et al. 1986), with an interassay coefficient of variation of 11.0% at 300 pmol/l and 9.8% at 1767 pmol/l. The studies were continued until a steady state was attained and the glycerol infusion rate was constant (140-180 minutes). At the end of the study the subject was given a meal.

6.3.3. Calculations

The insulin infused caused a suppression of lipolysis and consequent decrease in plasma glycerol concentration. As discussed above (chapters 1 and 5), glycerol cannot be re-esterified in adipose tissue due to absence of glycerol kinase. Thus, insulin can only influence glycerol production and not its disappearance; hence, the amount of glycerol infused is a direct index of the rate of lipolysis.

In the steady state, plasma glycerol concentration is constant and the amount of glycerol infused is equal to the suppression of glycerol production.

$$\text{Insulin sensitivity of lipolysis (µmol/kg)} \quad M = \left(\frac{I}{2} \times 0.11\right)\frac{1}{W} \times 1000$$

where

I is the mean glycerol infusion rate over the 30 minute steady state (ml/hr)

2 converts ml/hr to ml

0.11 converts ml to mmol as the glycerol solution concentration is 110 mmol/l

W is body weight in kg

1000 converts mmol to µmol.

M provides a measure of insulin sensitivity per kg total body weight. Lipolysis, however, predominantly occurs in adipose tissue and it may therefore be more appropriate to express this value per kg fat mass (M_f).

Lean body mass was measured by bioelectrical impedance. The principle of this method is that lean tissues comprise largely of electrolyte-containing water and so readily

conduct applied electrical current, whereas fat acts as an insulator and conducts poorly. An 800 microAmp alternating current at 50kHz is passed from the right hand to the ipsilateral foot. The impedance obtained enables calculation of total body water (TBW).

$$\underset{(kg)}{TBW} = \left(\frac{h^2}{\rho} \times 0.585\right) + 1.825$$

(after Kushner 1986) where h = height in cm

ρ = impedance in Ω

0.585 = specific resistivity of lean tissue (Ω/kg)

and $$\text{Fat free mass} = \frac{TBW}{0.73}$$

where 0.73 = average hydration fraction of lean body mass.

$$\underset{(FM)}{\text{Body fat mass}} = \underset{(W)}{\text{Total weight}} - \underset{(FFM)}{\text{Fat free mass}}$$

Thus $$\underset{(\mu mol/kg)}{M_f} = \left(\frac{I}{2} \times 0.11\right)\frac{1}{FM} \times 1000$$

6.3.4. Statistical analysis

Since this pilot study involved a large amount of development work, only four experiments were completed in the time available. This prevented formal statistical analysis of the data. Results are therefore presented for individual subjects.

6.3.5. Results

The individual M values for the four normal subjects studied are shown in Table 6.1.

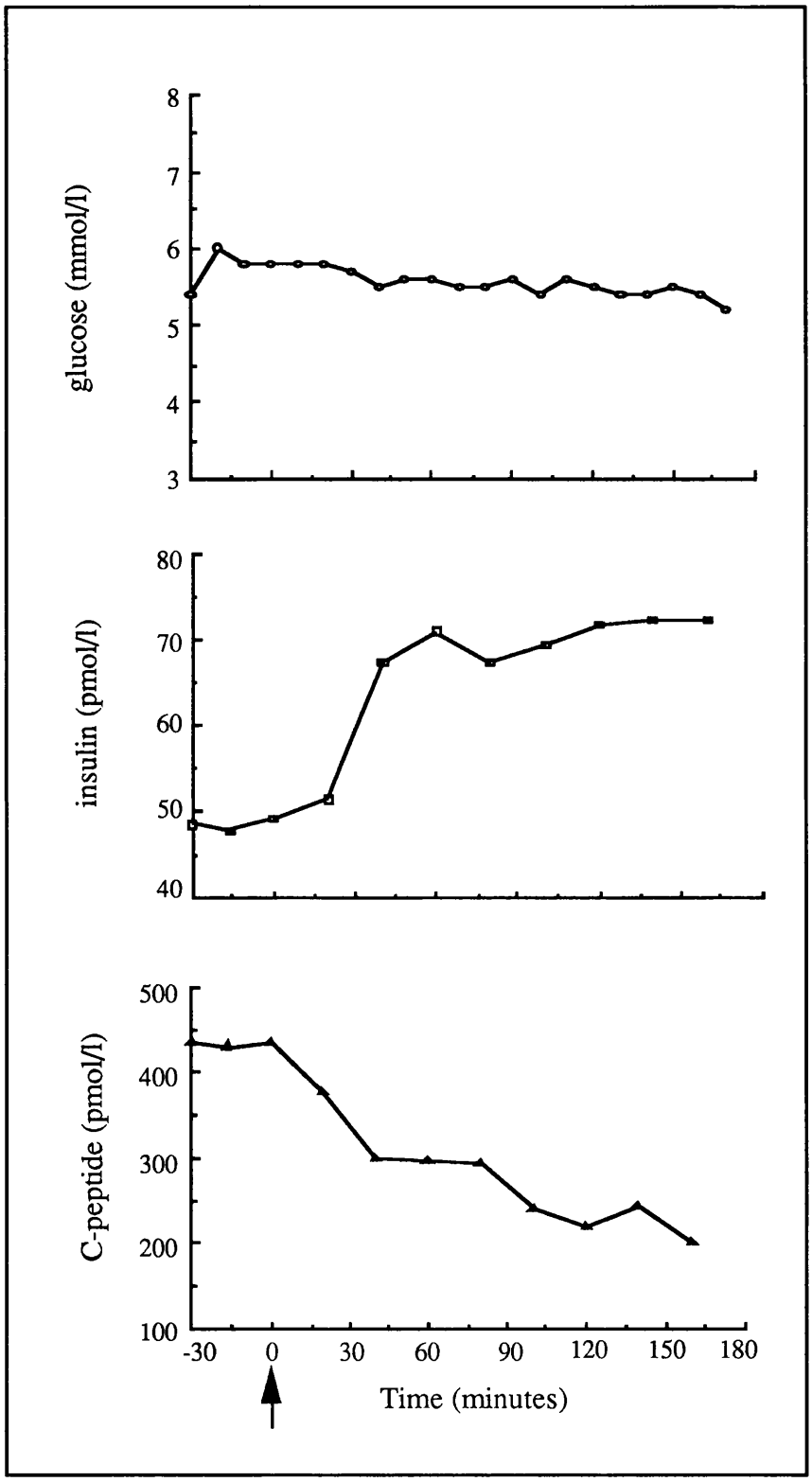
Table 6.1. Subject characteristics and insulin sensitivity measured by the glycerol clamp.

subject	sex	age (years)	BMI (kg/m^2)	fasting glycerol (μmol/l)	fat mass (kg)	M (μmol/kg)	M_f (μmol/kg)
1	M	26	27.2	41	18.1	33.0	15.7
2	M	21	25.9	40	20.9	8.8	36.9
3	F	27	20.7	44	13.6	22.0	97.3
4	F	20	19.9	41	11.5	5.7	24.0

M = insulin sensitivity per kg body weight; BMI = body mass index;
M_f = insulin sensitivity per kg fat mass

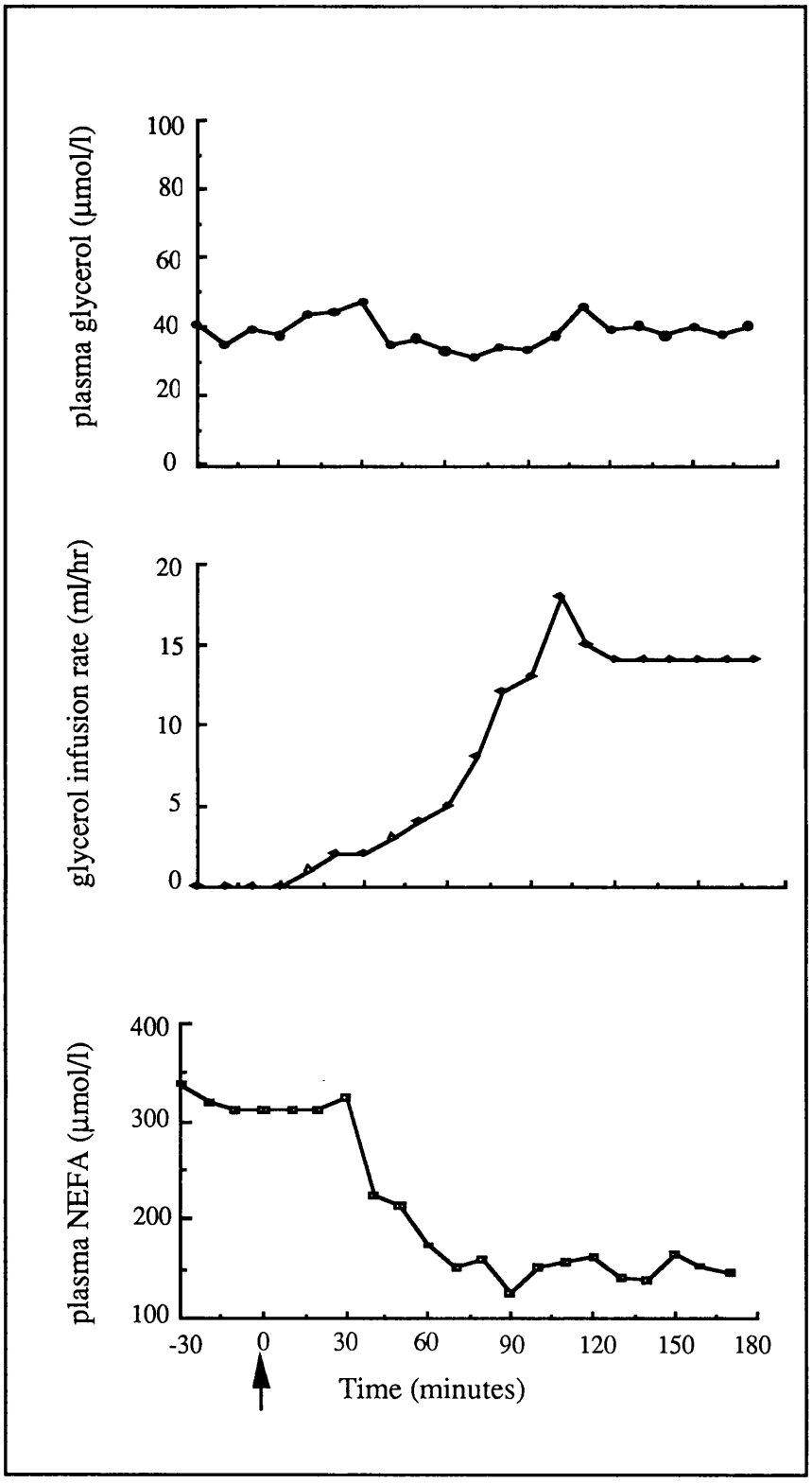
Plasma insulin levels achieved at steady state were 45, 57, 64 and 72 pmol/l in the four subjects and plasma C peptide concentrations decreased by 50% (Figure 6.2). A steady state in plasma glycerol was not attained until 100-120 minutes after commencing the insulin infusion. Plasma glucose concentrations exhibited minor decreases with insulin of 0.2 to 0.5 mmol/l from fasting levels. No subject experienced symptomatic hypoglycaemia. Plasma NEFA concentrations decreased by 50-70% during the insulin infusion in the four subjects (Figure 6.3).

Figure 6.2. Plasma glucose, insulin and C-peptide concentrations during the glycerol clamp in one subject.



Arrow denotes start of insulin infusion.

Figure 6.3. Plasma glycerol and non-esterified fatty acid (NEFA) concentrations and glycerol infusion rate during the glycerol clamp study in one subject.



Arrow denotes start of insulin infusion.

6.3.6. Discussion

These pilot studies have explored the feasability of the measurement of insulin sensitivity with respect to lipolysis by the novel glycerol clamp technique. The results suggest that this method with suitable development can provide a measure of insulin-induced suppression of lipolysis *in vivo*.

The glycerol clamp is based on the well established hyperinsulinaemic euglycaemic clamp (DeFronzo et al. 1979), but with appropriate adaptations. To allow for the more marked sensitivity of lipolysis to insulin (Newsholme and Leech 1983) compared to glucose metabolism, a low dose insulin infusion was utilized as in chapter 5. This produced minimal changes in glucose concentration as previously observed by ourselves and others (Hale et al. 1985; Robertson et al. 1992), but was however, sufficient to suppress lipolysis as demonstrated by the decline in plasma glycerol and NEFA concentrations. Rapid estimations of plasma glycerol concentration were achieved with the microdialysis enzyme electrode, allowing the glycerol infusion rate to be suitably adjusted to halt the insulin-induced decline in plasma glycerol level. Thus, plasma glycerol concentration was successfully clamped at fasting level.

For these pilot studies the required glycerol infusion rate was estimated, assuming that the half life of glycerol is 4 minutes, its volume of distribution in the body is 0.22 - 0.41 l/kg and the endogenous glycerol production rate in normal subjects is approximately 2 μmol/kg/min (Beylot et al. 1987; Puhakainen et al. 1992). If lipolysis were totally suppressed, an infusion rate of 2 μmol/kg body weight/min would be required. In the four studies performed the glycerol infusion rates at steady state were 1.1, 0.3, 0.8 and 0.2 μmol/kg body weight/min for the four individuals. Although these values may appear lower than expected, they are within published normal ranges of glycerol production rates (Nurjhan et al. 1992). Furthermore, the infusion rates required may have been low if lipolysis was not totally suppressed with the dose of insulin employed. It is important to ensure that a steady state was indeed attained and this was initially achieved by prolonging the study period from 140 to 180 minutes. In these four subjects the steady state insulin levels achieved were quite similar and endogenous insulin secretion, as measured by the C peptide concentration, was

suppressed. Expression of insulin sensitivity per unit insulin (M/I) would enable comparison of M lipolysis values at different steady state insulin concentrations.

These preliminary studies provoke a number of experiments for optimisation of the technique. It is planned to perform the clamp in a larger number of healthy individuals to obtain a normal range for M lipolysis. An algorithm for the glycerol infusion rate may then be devised for future studies and this could accelerate attainment of a steady state. The reproducibility of the technique should be confirmed by repeated studies in the same subjects. The glycerol clamp provides a quantitative measure of lipolysis and using graded doses of insulin a dose-response curve for insulin action on lipolysis could be obtained. The clamp may then be applied to other situations, such as patients with NIDDM, obesity and polycystic ovary syndrome in whom insulin insensitivity to glucose is well documented (Caro 1991; DeFronzo 1992; Robinson et al. 1993), but quantitation of insulin sensitivity with respect to lipolysis has not been as well investigated.

Further validation of this technique could be achieved by combining the clamp with a prior infusion of isotopic glycerol to quantitate glycerol rate of appearance, which may then be included in the algorithm to more accurately predict the required glycerol infusion rate. Values obtained from the clamp for insulin sensitivity to lipolysis could also be compared with those obtained on a separate occasion using the method of isotopically measured glycerol turnover in response to low dose insulin infusion as described in chapter 5.

The glycerol clamp provides a more subtle assessment of insulin sensitivity than the glucose clamp and the greater sensitivity of lipolysis to insulin suggests that it will allow very early defects in insulin action to be detected, which may not be apparent using other methods of assessment. Serial studies in the same subject could also be used to monitor the effect of therapeutic interventions.

A particular feature of the glycerol clamp is its safety. It does not involve radioisotopes, making it useful in children and pregnant women. The plasma insulin concentrations

achieved produce only minor changes in blood glucose concentrations, without risk of hypoglycaemia. Insulin sensitivity measurements at these insulin levels are more representative of the physiological situation rather than the pharmacological doses employed in glucose clamps. Peripheral infusion of glycerol has previously been demonstrated to be quite safe (Johnston et al. 1982; Carpentier et al. 1984) and does not appreciably affect endogenous glycerol release (Carpentier et al. 1984; Beylot et al. 1987).

The glycerol clamp has been made possible by the development of the microdialysis electrode which provides rapid and accurate measurement of plasma glycerol levels. Conventional enzymatic measurement of low plasma glycerol concentrations tend to be unreliable, whereas the lower limit of detection of the glycerol biosensor is 1μmo/l allowing accurate determination of plasma glycerol levels during insulin infusion. At present the clamp requires one operator for the electrode and one clinician to perform the study. Work is in progress to produce electrodes which can be stored and reused and this should enable wider applicability of the technique. It is thus comparatively inexpensive in terms of both manpower and resources.

In conclusion, the glycerol clamp has been shown to be a safe and practical method for quantitation of insulin sensitivity of lipolysis in healthy subjects. It has enormous potential in the assessment of insulin sensitivity and should help provide new insight into the pathogenesis and evaluation of insulin resistant states. Validation studies of the method are currently in preparation.

CHAPTER 7:

INSULIN SENSITIVITY IN NON-DIABETIC RELATIVES OF PATIENTS WITH NON-INSULIN DEPENDENT DIABETES OF ASIAN AND EUROPEAN ORIGIN.

7.1. INTRODUCTION

Non-insulin dependent diabetes (NIDDM) is characterised by insulin insensitivity and insulin deficiency (DeFronzo 1988), but which is the primary defect remains unknown. In order to establish which is the earliest abnormality, several groups have studied subjects with an increased risk of later developing the disease (first-degree relatives (Barnett et al. 1981a; Beaty et al. 1982; Newman et al. 1987), populations such as the Pima Indians). Unaffected individuals in populations with a high prevalence of NIDDM are hyperinsulinaemic before diabetes develops (Haffner et al. 1988; Saad et al. 1989; Zimmett et al. 1992), suggesting that insulin insensitivity is a primary feature. However both hyper- and hypoinsulinaemia have been reported in other genetically predisposed subjects (Barnett et al. 1981b; Dornhorst et al. 1992). Some of these discrepancies may be related to the contribution of biologically inactive insulin precursor peptides to the measured insulin concentrations (Temple et al. 1989). Ethnic origin may be another confounding factor, as ethnic differences in insulin secretion in the normal population have been reported in Britain (McKeigue et al. 1991 and 1992; Dornhorst et al. 1992).

First-degree relatives have about a 40% risk of developing NIDDM (Köbberling et al. 1985) and so study of such at risk individuals, at a time when glucose tolerance is normal, should provide information concerning the earliest defect in the progression to NIDDM.

In this study therefore, insulin sensitivity and insulin and proinsulin responses to oral glucose were examined in glucose-tolerant first-degree relatives from two different ethnic groups

7.2. SUBJECTS

Twenty-four first-degree relatives of patients with NIDDM were studied (12 of European and 12 of Asian (Indian subcontinent) origin). The relatives were matched with control subjects for ethnic origin, age, sex and body mass index. No subject had known cardiac, renal or hepatic disease or was taking any regular medication. Each subject had a normal physical examination. Premenopausal women were studied within the first 10 days of their menstrual period.

7.3. STUDY DESIGN

Subjects were studied on two separate occasions at least one week apart. On the first occasion they received a 75g oral glucose tolerance test as described in chapter 2. Venous blood samples were taken at times 0, 30, 60, 90 and 120 minutes for glucose, insulin, and intermediary metabolites (non-esterified fatty acids (NEFA), glycerol, lactate, pyruvate, 3-hydroxybutyrate and alanine), and at 0, 30 and 120 minutes for proinsulin. All relatives and controls had normal glucose tolerance by WHO criteria (WHO 1985).

Insulin sensitivity was assessed on a second occasion in 10 European relatives and 8 Asian relatives and their controls by means of the short insulin tolerance test, employing a bolus of Human Actrapid insulin 0.05 units/kg body weight. The full protocol is described in chapter 2. Insulin sensitivity was derived from the slope of blood glucose concentration from 3-15 minutes.

7.4. ANALYSES

Sample collection, preparation and assays are described in detail in chapter 2. Plasma concentrations of glucose and blood levels of intermediary metabolites were measured enzymatically. Plasma insulin was measured by a double antibody radioimmunoassay and proinsulin was assayed using a two-site immunoradiometric assay. To allow for its cross reactivity in the insulin assay, the proinsulin measured was subtracted from the total insulin concentration to give the "true" insulin level.

7.5. STATISTICAL ANALYSES

Results are expressed as mean ± standard error for glucose and metabolites (NEFA, glycerol, pyruvate, lactate and alanine) and as median (range) for insulin, proinsulin, true insulin and 3-hydroxybutyrate. Data from relatives and controls were compared using unpaired Student's t-tests or a Mann Whitney U test when data were not normally distributed. Plasma 3-hydroxybutyrate levels were log transformed before analysis. Glucose and insulin areas were calculated by the trapezoidal rule (Matthews et al. 1990).

7.6. RESULTS

Subject characteristics are shown in Table 7.1.

Table 7.1. Characteristics of subjects who underwent the oral glucose tolerance test (OGTT) and the insulin tolerance test (ITT).

	Asian		European	
	relatives	controls	relatives	controls
OGTT				
n	12	12	12	12
m:f	7:5	7:5	5:7	5:7
age (years)	31±2	33±3	36±3	42±3
BMI (kg/m^2)	24.1± 0.9	22.7±0.6	24.9±1.1	24.5±0.8
ITT				
n	8	8	10	10
m:f	4:4	4:4	5:5	5:5
age (years)	29±2	30±3	35±4	34±3
BMI (kg/m^2)	23.8±1.2	22.9±0.9	25.2±1.2	24.0±0.8

Values are expressed as mean±se where appropriate. BMI: body mass index

7.6.1. Fasting values (Table 7.2)

All subjects and controls had normal glucose tolerance by WHO criteria. Fasting glucose concentrations were similar in subjects and controls, but Asian relatives had significantly higher fasting insulin levels than their controls (66 (8-203) vs 33 (16-84) pmol/l $p<0.05$). Fasting proinsulin levels were not significantly different in Asian relatives compared with their controls, and when subtracted from the total insulin concentration, the fasting "true" insulin in Asian relatives remained elevated compared to that of the Asian controls (62 (6-188) vs 30 (12-82) pmol/l $p<0.05$).

Fasting total insulin, proinsulin and "true" insulin levels, although higher in relatives of European origin than in their controls, were not significantly different (total insulin 55 (8-178) vs 33 (8-84) pmol/l; true insulin 54 (5-173) vs 34 (11-85) pmol/l).

Table 7.2. Fasting concentrations of metabolites, insulin and proinsulin for subjects who underwent an oral glucose tolerance test.

	Asian		European	
	relatives	controls	relatives	controls
glucose (mmol/l)	5.0±0.2	4.9±0.1	5.1±0.2	4.9±0.1
total insulin (pmol/l)	66 (8-203)	33 * (16-84)	55 (8-178)	33 (8-84)
proinsulin (pmol/l)	3 (1-15)	3 (1-5)	2 (0-5)	2 (1-6)
true insulin (pmol/l)	62 (6-188)	30 * (12-82)	54 (5-173)	34 (11-85)
glycerol (µmol/l)	83±9	51±4 **	65±4	67±9
NEFA (µmol/l)	504±66	403±49	511±47	422±53
3-hydroxybutyrate (µmol/l)	59 (12-133)	43 (11-469)	54 (9-324)	39 (6-247)
lactate (µmol/l)	991±135	1061±212	804±107	856±154
pyruvate (µmol/l)	88±15	68±9	62±6	68±8
lactate:pyruvate	12.2±1.1	15.1±1.7	12.6±0.9	12.0±1.1
alanine (µmol/l)	343±37	313±31	256±19	305±35

Results are expressed as median (range) for total insulin, proinsulin, true insulin and 3-hydroxybutyrate; all other values are expressed as mean±se. Asian relatives had higher fasting total insulin, true insulin and glycerol than their controls. *p<0.05; **p<0.005.

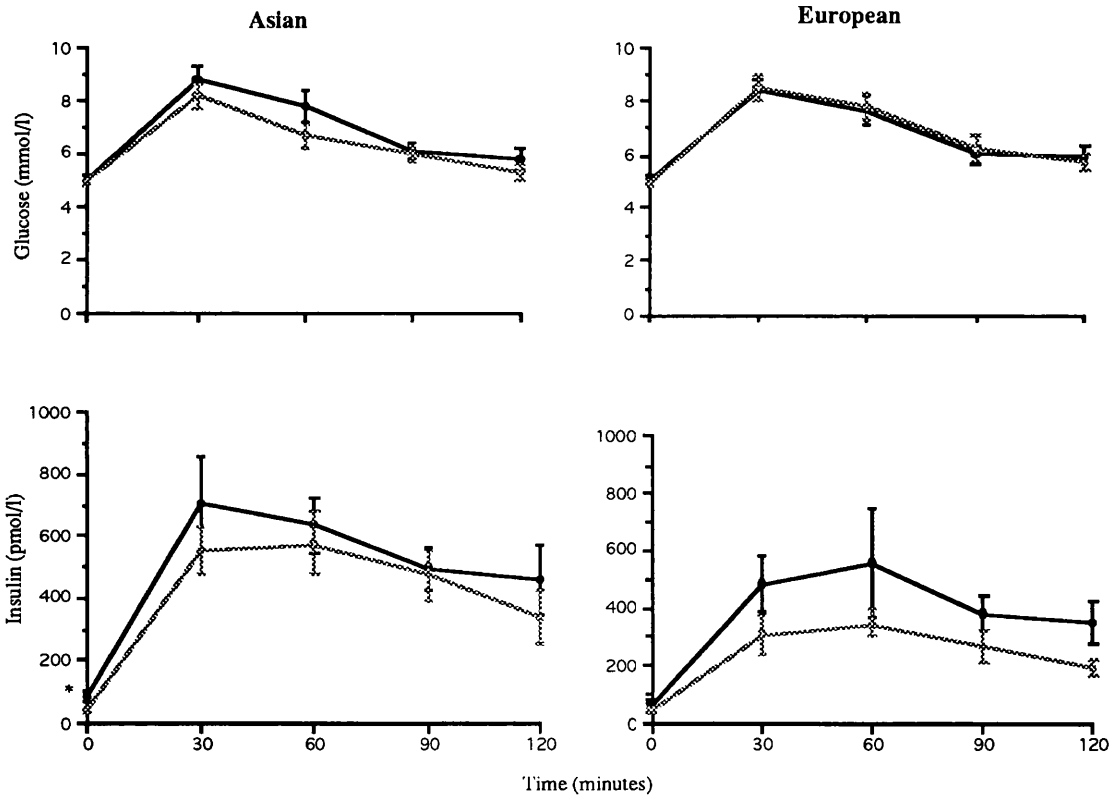
Fasting glycerol concentrations were significantly elevated in Asian relatives compared to their controls (83±9 vs 51±4 µmol/l $p<0.005$).This difference was not observed in the European group (65±4 vs 67±9 µmol/l NS). Fasting concentrations of non-

esterified fatty acids, lactate, pyruvate, alanine and the fasting lactate:pyruvate ratio were similar in relatives and controls within each ethnic group.

7.6.2. Response to oral glucose

The blood glucose reponses to oral glucose were similar in patients and controls (Figure 7.1).

Figure 7.1. Plasma glucose and insulin concentrations during the oral glucose tolerance test in relatives —●— and control subjects ·····△····· of Asian and European origin.



Results are expressed as mean±se. Fasting insulin concentrations in Asian relatives at time 0 were significantly higher than in Asian controls (*p<0.05).

Within each ethnic group, relatives had higher total insulin, "true" insulin levels and in the Asians, proinsulin concentrations after glucose but these differences did not reach statistical significance (Figure 7.1 and Table 7.3. Total insulin, areas under curve (mean±se): Asian *relatives* 57 375 (18 716 -145 735) vs *controls* 43 075 (14 519 - 103 052); European *relatives* 42 073 (8988 - 130 133) vs *controls* 33 655 (6965 - 55 767) pmol/l.min).

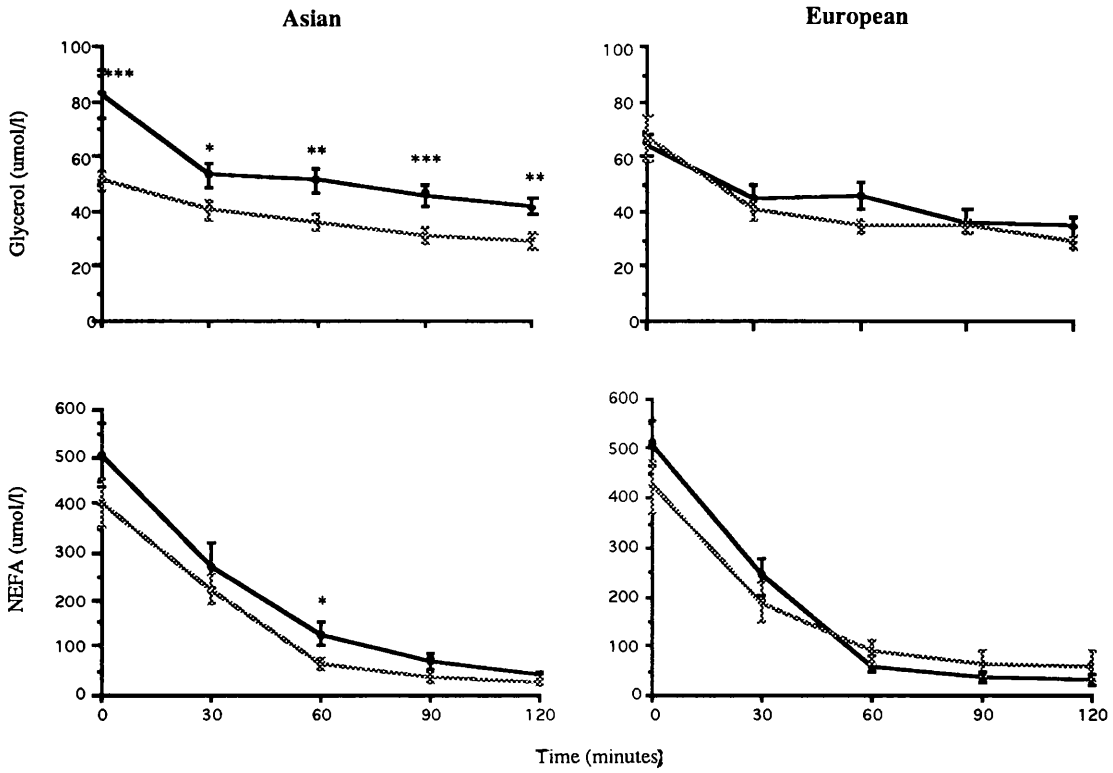
Table 7.3. Proinsulin and insulin concentrations during the oral glucose tolerance test.

	time (minutes)	Asian		European	
		relatives	controls	relatives	controls
proinsulin (pmol/l)	0	3 (1-15)	3 (1-5)	2 (0-5)	2 (1-6)
	30	11 (2-87)	11 (5-23)	6 (1-21)	6 (2-28)
	120	20 (5-71)	14 (8-55)	8 (2-41)	12 (2-45)
true insulin (pmol/l)	0	62 (6-188)	30 * (12-82)	54 (5-173)	34 (11-85)
	30	535 (287-2189)	615 (147-956)	364 (70-1077)	300 (11-917)
	120	247 (108-845)	137 (36-892)	341 (23-729)	198 (36-312)

Results are expressed as median (range). *p<0.05 Asian relatives vs Asian controls.

Asian relatives had significantly higher blood glycerol levels at all time points than their controls (Figure 7.2). These differences were not seen in the European groups. Plasma NEFA concentrations were also higher in Asian relatives than their controls, although this was significant only at 60 minutes (127±27 vs 65±12 μmol/l $p<0.05$). Plasma NEFA levels in relatives of European origin were not different from those of their controls. Blood 3-hydroxybutyrate, alanine, pyruvate and lactate responses to oral glucose were similar in relatives and controls of each group (Figure 7.3).

Figure 7.2. Blood glycerol and plasma non-esterified fatty acid (NEFA) concentrations in relatives ——●—— and control subjects ▒▒▒▒▒▒▒▒ of Asian (left panel) and European (right panel) origin during the oral glucose tolerance test.

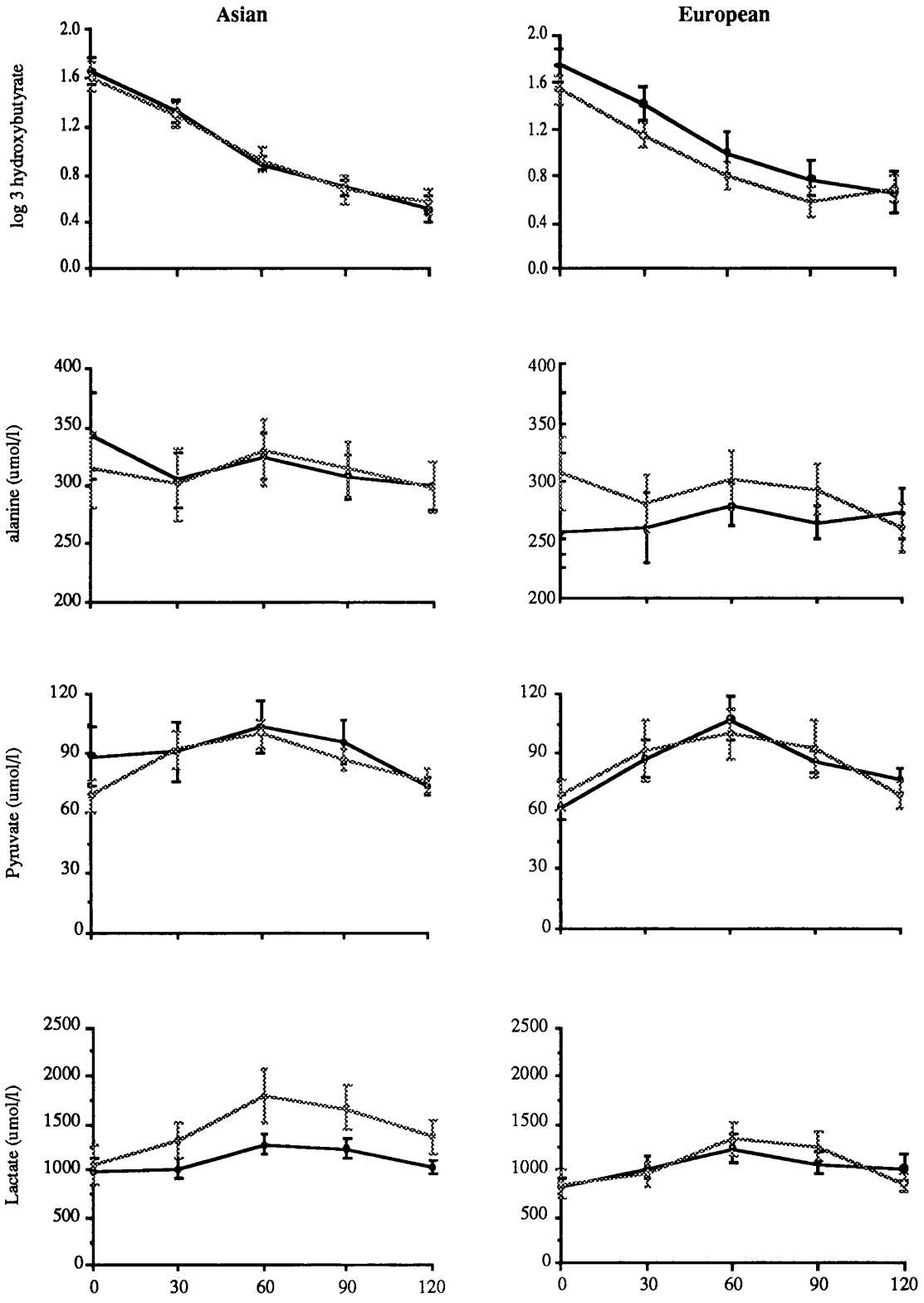


Results are expressed as mean±se. Blood glycerol concentrations were higher in Asian relatives at all time points compared to Asian control subjects. ***p<0.005; **p<0.01; *p<0.05. Plasma NEFA levels were significantly higher at 60 minutes in Asian relatives than in Asian controls.

7.6.3. Insulin Tolerance Test

Peak insulin concentrations after the insulin bolus were similar in relatives and controls in each group. Fasting insulin concentrations in these Asian relatives were also significantly higher than in their controls (62 (11-279) vs 28 (8-31) pmol/l; $p<0.05$); the relatives and controls of European origin had similar fasting insulin levels. Insulin sensitivity was significantly reduced in the Asian relatives compared to their controls (183±7 vs 139±12 μmol/l/min; $p<0.01$). In contrast, insulin sensitivity in European relatives was similar to that of their controls (167±11 vs 160±11 μmol/l/min).

Figure 7.3. Blood 3-hydroxybutyrate, alanine, pyruvate and lactate concentrations in relatives —●— and control subjects ···○··· of Asian (left panel) and European (right panel) origin during the oral glucose tolerance test.



Results are expressed as mean±se and as log concentration for 3-hydroxybutyrate.

7.6.4. OGTT specific insulin and 32, 33 split proinsulin responses

During the progress of this study methods became available for measurement of plasma insulin and 32, 33 split proinsulin by specific immunoradiometric assays as described in chapter 2. Plasma samples from the subjects in this study (at timings 0, 30 and 120 minutes) were also analysed using these assays (Table 7.4). In both the Asian and European groups the plasma levels of insulin and 32, 33 split proinsulin were similar in relatives and controls, both fasting and after oral glucose.

Table 7.4. Plasma concentrations of insulin and 32, 33 split proinsulin measured by immunoradiometric assay during the oral glucose tolerance test in relatives and controls of Asian and European origin.

time (minutes)	Asian		European	
	relatives	controls	relatives	controls
insulin (pmol/l)				
0	48 (34-174)	56 (12-110)	50 (17-103)	46 (22-89)
30	343 (219-949)	445 (165-1020)	326 (101-511)	331 (202-542)
120	204 (136--622)	152 (66-588)	270 (32-386)	182 (48-358)
32, 33 split proinsulin (pmol/l)				
0	2 (0-20)	2 (0-4)	2 (1-24)	1 (0-6)
30	10 (4-66)	16 (5-25)	7 (1-41)	5 (2-24)
120	12 (5-80)	10 (7-48)	13 (3-42)	8 (2-39)

Results are expressed as median (range).

7.7. DISCUSSION

This study has demonstrated disturbances in insulin secretion and insulin action in subjects with normal glucose tolerance but predisposed to the later development of NIDDM. The abnormalities were detected in predisposed individuals of Asian origin. First-degree relatives of NIDDM patients of Asian origin were hyperinsulinaemic, both fasting and in response to oral glucose. Lipolysis, assessed by fasting glycerol concentrations, was increased. The suppression of lipolysis after oral glucose was impaired, as reflected in the elevated glycerol and NEFA concentrations. Asian relatives were also less sensitive to exogenous insulin, as measured by the initial rate of decline of plasma glucose following an intravenous insulin bolus. These findings indicate that insulin insensitivity is an early feature of NIDDM in this ethnic group. First-degree relatives of NIDDM patients of European origin did not exhibit insulin insensitivity, suggesting that the primary defect in the pathogenesis of NIDDM may not be the same in the two ethnic populations.

A similar approach has been adopted by workers who studied glucose-tolerant individuals in populations with an exceptionally high prevalence of NIDDM. In the Pima Indians of Arizona and in the inhabitants of the island of Nauru, the progression from normal to impaired glucose tolerance was associated with the development of hyperinsulinaemia and reduced glucose disposal (Lillioja et al. 1988; Saad et al. 1989; Zimmett et al. 1992). Progressive hyperinsulinaemia with increasing strength of family history has been demonstrated in glucose-tolerant offspring of diabetic Mexican Americans (Haffner et al. 1988) Our findings in Asian subjects are in agreement with these earlier studies. In the subjects of European origin, the observations are less clear cut. Basal and stimulated insulin levels were elevated in the relatives, although the differences were not significant statistically. Lipolysis was not increased and the glucose response to exogenous insulin was similar to that in controls. Although there have been reports of impaired ß cell function in relatives (Barnett et al. 1981b; O'Rahilly et al. 1986 and 1988a) they were also glucose intolerant or mildly hyperglycaemic; we observed no evidence for ß cell dysfunction in our subjects of European origin, all of whom had normal glucose tolerance.

In view of the recent demonstration of overestimation of insulin levels in NIDDM due to cross reactivity with intact and split proinsulins (Temple et al. 1989), it was possible that the hyperinsulinaemia in the Asians was not indicative of biologically active insulin. We therefore measured proinsulin and calculated biologically active "true" insulin concentrations. The proinsulin contribution to immunoreactive insulin was small and "true" insulin levels remained elevated in Asian relatives after correction. As the glucose responses to oral glucose were similar, this provides strong evidence for insulin insensitivity in this at-risk group. The hyperinsulinaemia was not confirmed using an immunoradiometric assay measuring specific insulin. Furthermore, since the plasma levels of 32, 33 split proinsulin were not significantly elevated in the Asian relatives compared to their controls, increased secretion of these precursor peptides could not explain the greater immunoreactive insulin concentrations. These findings are difficult to reconcile and possibly reflect hitherto unrecognised discrepancies in the assay methods. Nevertheless, as discussed below, insulin insensitivity in the Asian relatives was also detected using measurement of circulating intermediary metabolites and the glucose response to exogenous insulin.

The observation in relatives of European origin of a tendency to higher insulin and proinsulin concentrations is similar to that of other workers, in whose studies the differences did (Røder et al. 1990) or did not (Clark et al. 1992) reach statistical significance. These small differences in insulin in European subjects in this and other studies may be insignificant. Alternatively, they may reflect real but small differences not discernible with the number of subjects studied. First-degree relatives have only a 40% risk of later NIDDM and inevitably family studies such as this will include relatives who are not destined to develop the disorder. Proinsulin:total insulin ratios were not different between relatives and controls in either ethnic group. This observation is similar to that reported in the Pima Indian population (Saad et al. 1990).

In addition to the differences observed in insulin levels, Asian relatives had persistently elevated blood glycerol concentrations compared to their controls, both fasting and after oral glucose. Plasma NEFA levels were also higher. Enhanced lipolysis and a failure of its suppression after glucose, despite higher circulating insulin levels, provide further

evidence for insulin insensitivity in these family members.

Abnormalities of intermediary metabolism have been reported in the offspring (Leslie et al. 1986) and unaffected co-twins (Barnett et al. 1981b) of NIDDM patients, though the findings were not consistent between studies. Barnett et al. (1981b) observed elevated concentrations of glycerol in co-twins, fasting and after oral glucose. The insulin response was reduced in the co-twins. Leslie et al. (1986) demonstrated raised circulating levels of lactate and pyruvate, which as in this study, were associated with hyperinsulinaemia. Lactate and pyruvate levels were not increased in this study, despite the increase in insulin. Higher concentrations of blood glycerol and 3-hydroxybutyrate following oral glucose have been reported in another at-risk group for NIDDM, glucose-tolerant women with previous gestational diabetes (Chan et al. 1992). In that study the disturbances of intermediary metabolism were associated with a reduced insulin response. This may reflect the varied ethnic mix of the group, as when analysed according to ethnic origin, Asian women with previous gestational diabetes are not insulin deficient (Dornhorst et al. 1992).

In the present study insulin resistance in glucose-tolerant relatives of Asian origin was confirmed by measurement of insulin sensitivity with the short insulin tolerance test. Asian relatives were significantly less sensitive than matched controls, but relatives and controls of European origin were of similar insulin sensitivity. Relatives of NIDDM patients of Asian origin therefore exhibit resistance to both exogenous and endogenous insulin.

The molecular mechanism underlying this insulin insensitivity remains to be clarified. Other workers have demonstrated reduced insulin sensitivity in glucose-tolerant first-degree relatives of NIDDM patients using an insulin suppression test (Laws et al. 1989; Ho et al. 1990), an insulin tolerance test (Ramachandran et al. 1990) and the intravenous glucose tolerance test (Warram et al. 1990; Osei et al. 1991a). These tests, like the short insulin tolerance test employed in this study, do not distinguish between hepatic and peripheral insulin insensitivity. Osei et al. (1990) have reported increased hepatic glucose output in first-degree relatives compared to controls, though the values

were within the normal range. This was not the case in all ethnic groups (Osei et al. 1992), nor has it been a consistent finding between studies. Eriksson et al. (1989) documented impaired insulin-mediated glucose metabolism but normal hepatic glucose output in first-degree relatives of NIDDM patients. The defect was accounted for by reduced glucose storage of similar magnitude to that observed in NIDDM patients themselves. The recent demonstration that this defect in NIDDM is due to impaired glycogen synthesis (Shulman et al. 1990) has led to further investigation of this metabolic pathway in first-degree relatives. Defective activation of muscle glycogen synthase by insulin has been reported in glucose-tolerant relatives (Schalin-Jäntti et al. 1992; Vaag et al. 1992) as well as a reduced number of insulin receptors (Migdalis et al. 1992) and decreased insulin-stimulated receptor tyrosine kinase activity (Handberg et al. 1993).

In this study resistance to the action of insulin was not limited to glucose metabolism. Asian relatives also exhibited resistance to the inhibition of lipolysis following glucose-stimulated insulin release. Non-esterified fatty acids may contribute to the pathogenesis of NIDDM through the glucose-fatty acid cycle (Randle et al. 1963). Although other workers have failed to show a difference in insulin-mediated suppression of NEFA and lipid oxidation in relatives compared to controls (Eriksson et al. 1991), they used high doses of insulin which may have failed to detect differences in lipolysis, a process which is exquisitely sensitive to insulin. In addition, the plasma concentration of NEFA reflects re-esterification as well as lipolysis. Circulating glycerol concentrations are a better guide to lipolysis as glycerol kinase, which is necessary for further metabolism of glycerol, is absent in adipose tissue.The glycerol is released into the circulation and is avidly taken up by the liver. The finding of persistently elevated blood glycerol levels in Asian relatives in the presence of normal or elevated insulin concentrations is firm evidence for insulin insensitivity at the adipocyte.

A most striking finding of the present study is the demonstration of resistance of glucose metabolism to exogenous insulin in Asian relatives but not in relatives of European origin. This resistance to both the glucose-lowering and the anti-lipolytic actions of insulin was most prominent in the Asian at-risk group. Insulin insensitivity is

a feature of the Asian compared to European populations in the UK (McKeigue et al. 1991 and 1992), as well as the higher prevalence and earlier age of onset of NIDDM (Mather and Keen 1985; Viswanathan et al. 1985; Simmons et al. 1991) and in the present study, the Asians (relatives and controls) were hyperinsulinaemic using insulin radioimmunoassay in comparison with the groups of European origin. This study was, however, designed to investigate differences within ethnic groups rather than between them, and the Asian and European groups were not matched to permit cross-racial analyses. The insulin resistance observed in the Asians was in comparison with their Asian matched controls and represents a difference in an at-risk group before the development of diabetes.

In conclusion, this study has demonstrated that glucose-tolerant first-degree relatives of NIDDM patients of Asian background are insulin resistant and have increased circulating levels of immunoreactive insulin. Relatives of patients with NIDDM of European origin did not exhibit these features. These differences in insulin sensitivity, in a group at increased risk of future diabetes, suggest that insulin resistance is an early feature of NIDDM in the Asian population.

CHAPTER 8:

INSULIN SECRETION AND INSULIN SENSITIVITY IN NON DIABETIC RELATIVES OF PATIENTS WITH NON-INSULIN DEPENDENT DIABETES OF AFRO-CARIBBEAN ORIGIN

8.1. INTRODUCTION

In non-insulin dependent diabetes (NIDDM) defective insulin secretion and insulin insensitivity have been associated with a relative increase in the secretion of proinsulin (Ward et al. 1987; Reaven et al. 1993). It is not clear whether these abnormalities precede the onset of clinical diabetes or are secondary to the hyperglycaemia.

As discussed in chapter 1, the recent introduction of two-site immunoradiometric assays has enabled insulin and its precursors to be measured specifically (Sobey et al. 1989; Temple et al. 1990) This has revealed reduced secretion of insulin in NIDDM in some studies (Temple et al. 1989; Davies et al. 1993a) but not in all patients (Reaven et al. 1993).

In the previous chapter, insulin insensitivity and elevated immunoreactive insulin levels were demonstrated in first-degree relatives of patients with NIDDM of Asian origin, at a time when glucose tolerance was normal. It is not known if such metabolic defects are typical of other ethnic groups.The Afro-Caribbean population in Britain is another group with a high prevalence of NIDDM (Cruikshank et al. 1991). In this chapter therefore, insulin sensitivity and the secretion of insulin and its conversion intermediates has been investigated in glucose-tolerant first-degree relatives of NIDDM patients of Afro-Caribbean origin.

8.2. SUBJECTS

Nine first-degree relatives of patients of Afro-Caribbean origin with NIDDM were recruited as described in chapter 2. The relatives were matched individually with healthy control subjects for age, sex, ethnic origin and body mass index or matched as a group when individual matching was not possible. Premenopausal women were studied within the first 10 days of their menstrual period.

8.3. STUDY DESIGN

The subjects were studied on three separate occasions and for the three days prior to each study they were asked to observe their usual diet. On the day of the study, individuals attended the Metabolic Day Ward at 08.00 hours after a 10-12 hour overnight fast. On the first visit, subjects received a 75g oral glucose tolerance test. Venous blood samples were taken at times 0, 30, 60, 90 and 120 minutes for glucose, insulin, and intermediary metabolites (non-esterified fatty acids (NEFA), glycerol, lactate, pyruvate, 3-hydroxybutyrate and alanine), and at 0, 30 and 120 minutes for intact and 32, 33 split proinsulin, and specific (IRMA) insulin measurements as described in chapter 2.

On a second occasion insulin sensitivity was measured in 9 relatives and 8 control subjects using the short insulin tolerance test, employing a bolus of insulin 0.05 units/kg body weight, as described in chapters 2 and 3. Two of the male subjects (1 relative and 1 control) had normal glucose tolerance on an oral glucose tolerance test but were not also included in the OGTT results analysis as metabolite and insulin data were incomplete. Insulin sensitivity was derived from the linear slope of the blood glucose concentration from 3-15 minutes.

On the third occasion secretion of insulin and that of its precursor peptides was assessed in 6 relatives and their controls with an intravenous glucose tolerance test using a glucose bolus of 0.3g/kg body weight. As described in chapter 2, blood was sampled for measurement of glucose, immunoreactive insulin, specific (IRMA) insulin, intact and 32, 33 split proinsulin and for intermediary metabolites basally and at frequent intervals during the test. The glucose disappearance constant (K_G) was

calculated from the slope of log glucose concentration over 10-60 minutes plotted against time.

8.4. ANALYSES

Blood samples were collected and stored as described previously. Samples for glucose and metabolite estimation were assayed enzymatically as described in chapter 2. Plasma insulin was measured by radioimmunoassay. Intact proinsulin, 32, 33 split proinsulin and specific insulin were measured using two-site immunoradiometric (IRMA) assays . These assays are described in detail in chapter 2.

8.5. STATISTICAL ANALYSIS

Results are expressed as mean±se with comparison of data from relatives and controls made by Student's unpaired t-tests. For those parameters not normally distributed (total immunoreactive insulin, intact and 32, 33 split proinsulins, IRMA insulin and 3-hydroxybutyrate) results are expressed as median (range) and comparisons made using a Mann Whitney U test. Areas for the glucose, insulin and precursor peptide responses to oral and intravenous glucose were calculated using the trapezoidal rule (Matthews et al. 1990). During the intravenous glucose tolerance test, the first phase response was calculated from the incremental area under the curve from 0-10 minutes, and the second phase response was calculated similarly from 10-60 minutes. Since the starting point for calculation of the second phase is dependent on the magnitude of the first phase, the overall insulin response was also calculated as the cumulative area from 0-40 minutes; this analysis did not alter the results and so for clarity only the first and second phase responses are presented.

8.6. RESULTS

Table 8.1. shows the characteristics of the subjects and controls who underwent each test.

Table 8.1. Characteristics of relatives and controls who underwent the oral glucose tolerance test (OGTT), the insulin tolerance test (ITT) and the intravenous glucose tolerance test (IVGTT).

		relatives	controls
OGTT	n	9	9
	m:f	2:7	2:7
	age (years)	30 (18-53)	29 (18-47)
	BMI (kg/m^2)	24.4 (20.1-32.3)	24.6 (23.3-33.7)
ITT	n	9	8
	m:f	3:6	3:5
	age (years)	30 (18-49)	28 (20-47)
	BMI (kg/m^2)	24.3 (20.0-32.8)	24.0 (20.5-31.9)
IVGTT	n	6	6
	m:f	1:5	2:4
	age (years)	27 (18-49)	28 (25-47)
	BMI (kg/m^2)	25.4 (23.3-32.3)	23.8 (22.7-31.9)

Results are expresed as median (range). BMI = body mass index.

8.6.1. Fasting values

All relatives and controls had normal glucose tolerance by WHO criteria. Fasting concentrations of glucose, immunoreactive insulin, IRMA insulin, intact and 32, 33 split proinsulin were not significantly different between relatives and controls. There was no difference in fasting levels of intermediary metabolites between relatives and controls (Table 8.2).

Table 8.2. Fasting concentrations of immunoreactive insulin (IRI), IRMA insulin, intact and 32, 33 split proinsulin, glucose and intermediary metabolites in 9 relatives and 9 control subjects.

	relatives	controls
IRI (pmol/l)	50 (13-135)	45 (5-149)
IRMA insulin (pmol/l)	53 (43-109)	53 (25-94)
intact proinsulin (pmol/l)	3 (0-5)	2 (1-4)
32, 33 split proinsulin (pmol/l)	2 (0-4)	2 (1-6)
glucose (mmol/l)	4.8±0.2	4.6±0.2
glycerol (μmol/l)	65±9	76±12
NEFA (μmol/l)	365±70	564±90
3-hydroxybutyrate (μmol/l)	34 (17-122)	32 (11-108)
lactate (μmol/l)	702±74	792±148
pyruvate (μmol/l)	58±5	53±8
lactate:pyruvate	12.7±1.3	14.9±1.8
alanine (μmol/l)	246±26	246±22

Results are expressed as median (range) for insulin, proinsulins and 3-hydroxybutyrate and as mean±se for glucose and the remaining metabolites.

8.6.2. Response to oral glucose

Following oral glucose the plasma immunoreactive insulin and blood glucose responses were not significantly different in relatives and controls (120 minute areas: IRI relatives 49 907 (19 665-69 045) vs controls 45 696 (22 938 - 107 172) pmol/l.min; glucose 769±26 vs 755±47 mmol/l.min.). Plasma concentrations of intact and 32, 33 split proinsulin and IRMA insulin, the ratio of IRMA insulin:glucose and the contribution of proinsulin-like molecules (intact + 32, 33 split proinsulin) to the total immunoreactive insulin were similar in relatives and controls (Table 8.3).

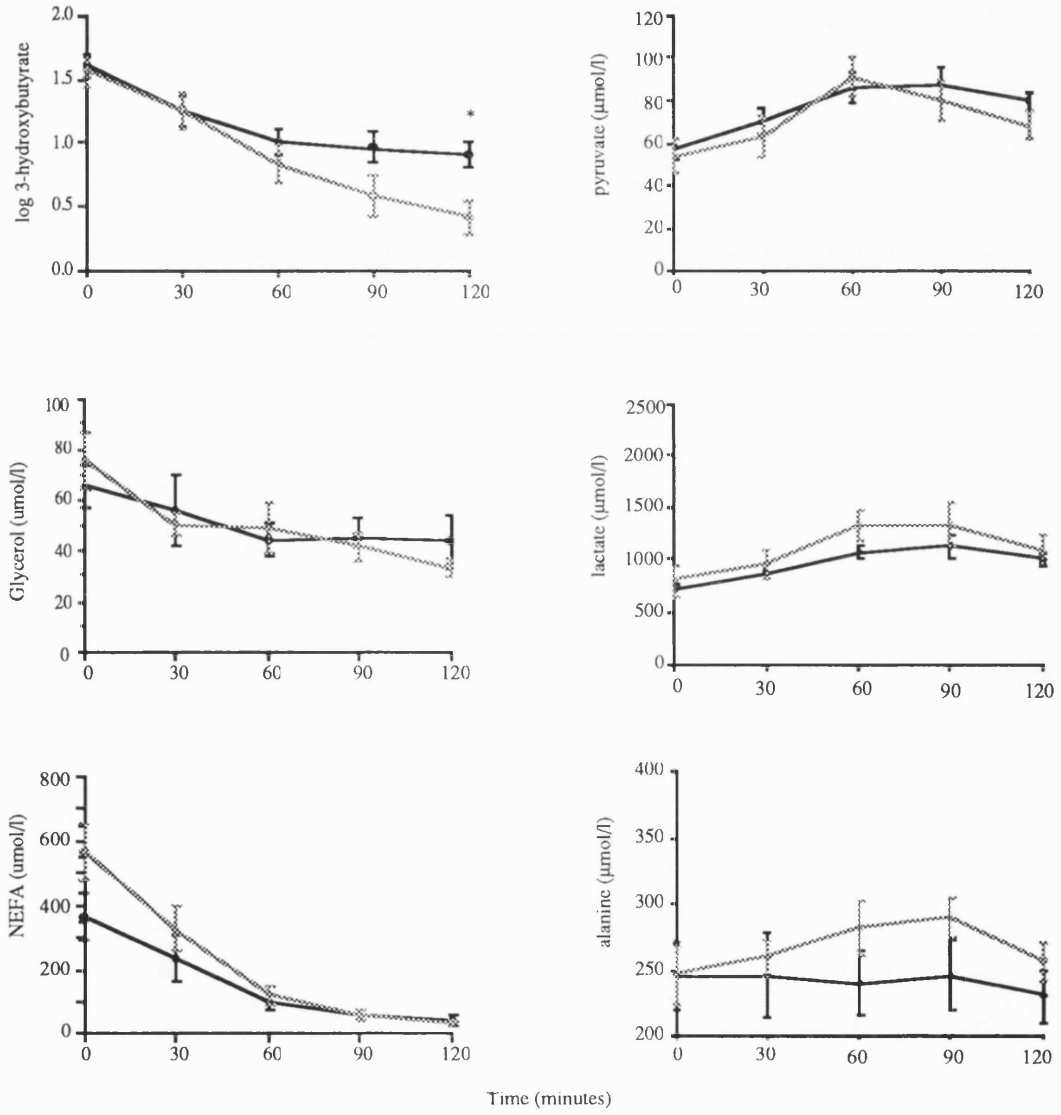
Table 8.3. Plasma concentrations of IRMA insulin, intact and 32, 33 split proinsulin before and following oral glucose.

		relatives	controls
IRMA insulin (pmol/l)	0	53 (43-109)	53 (25-94)
	30	322 (259-592)	308 (168-926)
	120	192 (32-336)	213 (74-613)
intact proinsulin (pmol/l)	0	3 (0-5)	2 (1-4)
	30	8 (2-15)	7 (2-27)
	120	13 (8-28)	13 (3-45)
32, 33 split proinsulin (pmol/l)	0	2 (0-4)	2 (1-6)
	30	12 (4-18)	8 (1-37)
	120	13 (0-18)	14 (0-29)
total proinsulin:IRI (%)	0	9 (2-42)	11 (3-33)
	30	4 (2-8)	3 (2-9)
	120	9 (6-22)	8 (5-14)
IRMA insulin:glucose	0	11.9 (8.5-24.2)	12.0 (4.9-19.9)
	30	43.9 (29.4-89.7)	47.4 (26.2-131.6)
	120	34.6 (5.2-53.6)	33.8 (16.9-107.5)

Results are expressed as median (range).

Blood glycerol, lactate, pyruvate and alanine concentrations and plasma NEFA responses to oral glucose were not significantly different in relatives and controls. Following glucose, the relatives exhibited slightly higher blood 3-hydroxybutyrate concentrations, but the difference was only significant at 120 minutes (relatives 7 (3-20) vs controls 3 (0-9) μmol/l. $p<0.05$) (Figure 8.1).

Figure 8.1. Blood 3-hydroxybutyrate, glycerol, pyruvate, lactate and alanine concentrations and plasma non-esterified fatty acid (NEFA) levels during the oral glucose tolerance test in relatives —●— and controls ···◇···.



Results are expressed as mean±se and 3-hydroxybutyrate as log concentration. *$p<0.05$.

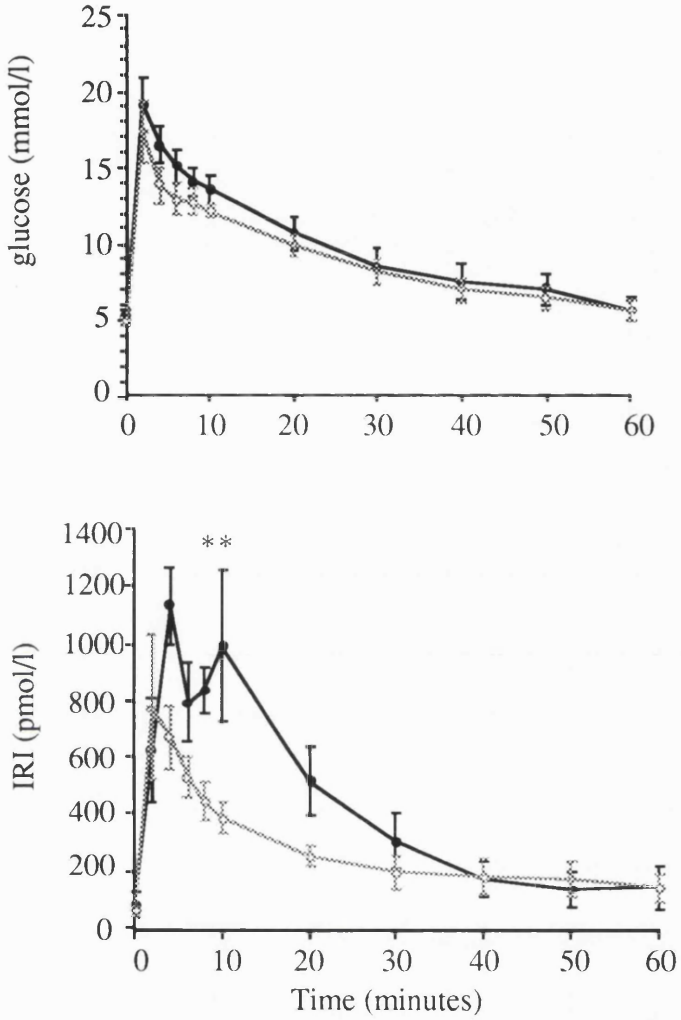
8.6.3. Insulin tolerance test

Insulin sensitivity measured by the low dose short insulin tolerance test was similar in relatives and control subjects (169±16 vs 156±9 μmol/l/min).

8.6.4. Intravenous glucose tolerance test

Following intravenous glucose, both groups had similar glucose values, but the relatives exhibited a greater immunoreactive insulin response, which peaked later, at 4 minutes, than that of the control group, at 2 minutes (Figure 8.2). However, analysis of the insulin areas for the first and second phases failed to reach statistical significance, and the glucose values were also similar in relatives and controls (1st phase: glucose, *relatives* 147±11 vs *controls* 127±10 mmol/l.min; IRI, *relatives* 7694 (5032 - 10 815) vs *controls* 5085 (2514-7698) pmol/l.min, p=0.06; 2nd phase: glucose, *relatives* 423±53 vs *controls* 399±35 mmol/l.min; IRI *relatives* 17 341 (7243 - 35 037) vs *controls* 9679 (5015 - 21 377) pmol/l.min; p=0.21).

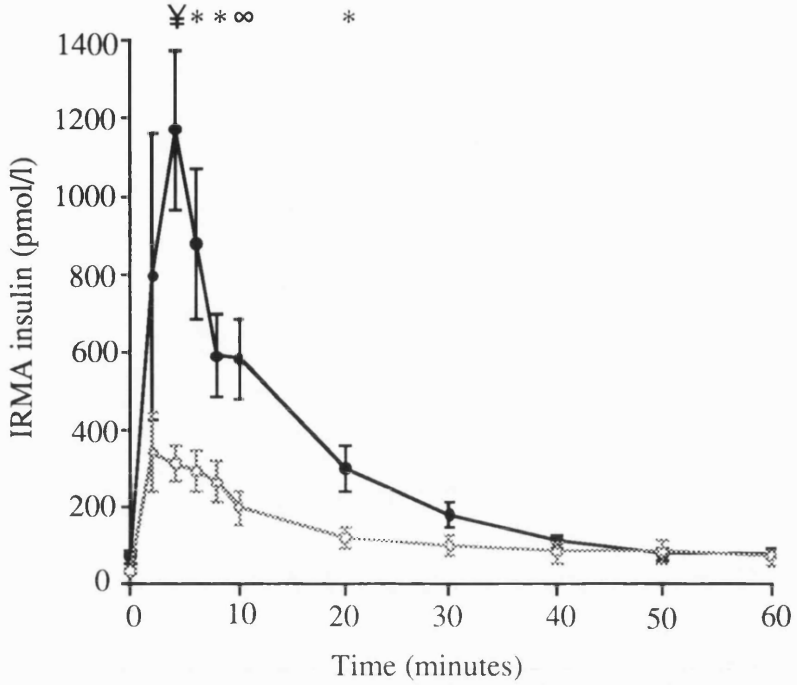
Figure 8.2. Plasma glucose and immunoreactive insulin (IRI) concentrations during the intravenous glucose tolerance test in relatives —●— and controls ⁘



Results are expressed as mean±se. *p<0.05

The difference in insulin secretion was more marked when the IRMA insulin and proinsulin levels were analysed. Relatives had significantly higher 1st phase IRMA insulin secretion (relatives 7228 (2905-13 062) vs controls 2680 (1473-3771) pmol/l.min p=0.01; Figure 8.3). The difference in 2nd phase IRMA insulin failed to reach statistical significance (relatives 11 033 (4912-14 091) vs controls 5943 (820-7730) pmol/l.min p<0.08).

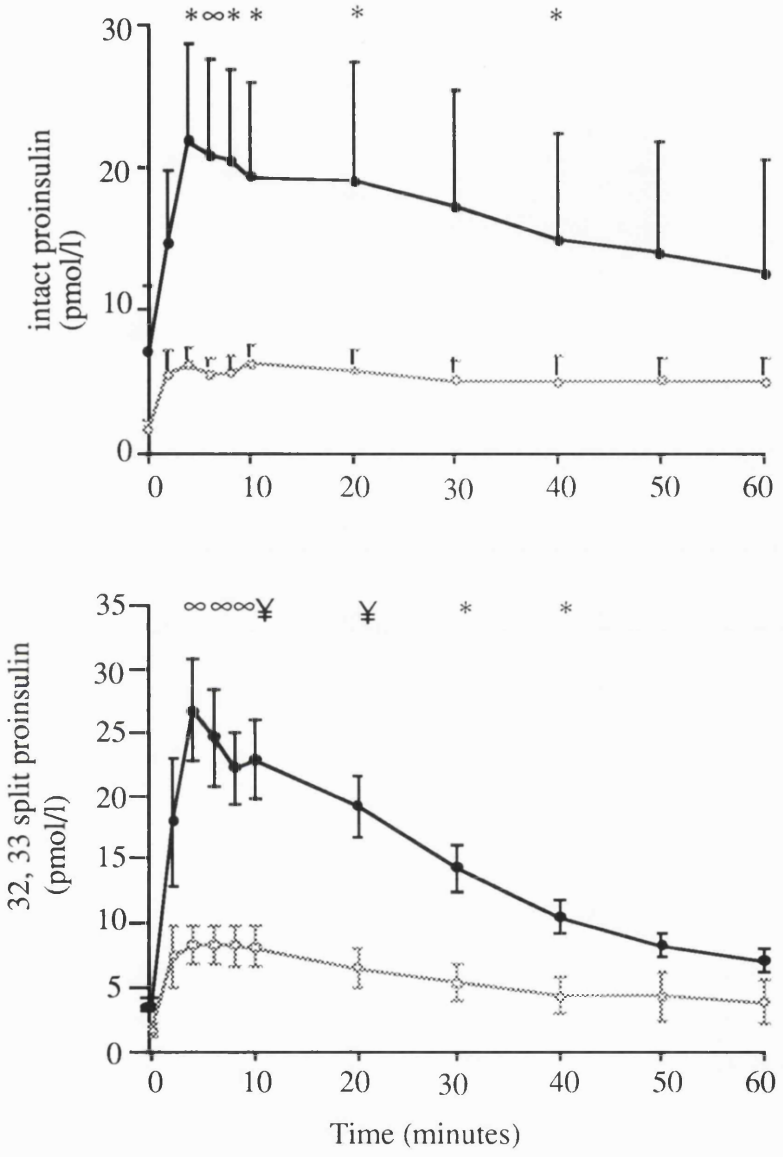
Figure 8.3. Plasma IRMA insulin concentrations during the intravenous glucose tolerance test in relatives —●— and controls ·····○·····



Results are expressed as mean±se. ¥ p<0.005; ∞ p<0.01; * p<0.05.

Intact proinsulin secretion was also greater in the relatives (1st phase: relatives 156 (57-473) vs controls 52 (17-97) pmol/l.min. p<0.05; 2nd phase: relatives 459 (232-2732) vs controls 243 (104-603) pmol/l.min. p<0.08) (Figure 8.4). The 32, 33 split proinsulin response was significantly higher in relatives throughout the test (1st phase: relatives 211 (118-324) vs controls 72 (20-130) pmol/l.min, p<0.01; 2nd phase: 670 (402-882) vs 261 (40-500) pmol/l.min. p<0.01; Figure 8.4). The sum total secretory response (IRMA insulin+intact+ 32, 33 split proinsulin) was increased in the relatives compared to the controls, though the second phase just failed to reach statistical significance (1st phase: relatives 7741 (3392-13 265) vs controls 2881 (1522-3877) pmol/l.min p=0.01; 2nd phase: relatives 12 089 (5948-16 836) vs controls 6441 (963-8355) pmol/l.min p=0.06).

Figure 8.4. Plasma intact (upper panel) and 32, 33 split proinsulin (lower panel) concentrations during the intravenous glucose tolerance test in relatives —●— and controls ·····◇·····



Results are expressed as mean+se for intact proinsulin for clarity and mean±se for 32, 33 split proinsulin. ¥p<0.005; ∞p<0.01; *p<0.05.

The larger insulin secretory response in the relatives compared to the controls was accompanied by a higher percentage of proinsulin-like molecules (intact + 32,33 split proinsulin) to total IRI at 2 minutes (5.7 (2.1-18.4) vs 2.3 (0.8-4.2)% $p<0.05$), at 6 minutes (5.7 (2.9-20.4) vs 2.8 (0.8-4.0)% $p<0.05$), at 40 minutes (13.8 (7.5-26.7) vs 6.1 (3.5-19.3)% $p<0.05$) and at 60 minutes (14.5 (10.6-30.1) vs 7.4 (4.5-25.5) % $p<0.05$).

In addition, the IRMA insulin:glucose ratio was higher in relatives than controls at 4, 6, 10, 20 and 30 minutes after glucose. With analysis of the ratio of IRMA insulin area: glucose area for each secretory phase, the 1st phase responses just failed to reach statistical significance (IRMA insulin area:glucose area, 1st phase: *relatives* 42.1 (20.1-127.4) vs *controls* 18.5 (14.3-32.8) $p=0.06$; 2nd phase: *relatives* 23.8 (14.6-33.1) vs *controls* 14.7 (2.8-16.6) $p<0.05$). Furthermore, the relatives had higher ratios of intact proinsulin:glucose (1st phase, intact proinsulin area:glucose area 1.1 (0.4-2.6) vs 0.5 (0.1-0.6) $p<0.05$; 2nd phase, 1.3 (0.6-4.5) vs 0.7 (0.2-1.2) $p<0.06$) and of 32, 33 split proinsulin:glucose (1st phase, 32, 33 split proinsulin area:glucose area 1.3 (0.8-2.2) vs 0.7 (0.2-0.7) $p<0.005$; 2nd phase, 1.6 (1.1-2.4) vs 0.7 (0.1-1.0) $p<0.005$). The glucose disappearance constants K_G were similar in relatives and controls (relatives 905±180 vs controls 737±92 min^{-1} NS).

Relatives exhibited impaired suppression of blood glycerol and 3-hydroxybutyrate concentrations after intravenous glucose compared to controls (% glycerol suppression to minimum value: relatives 36±5 vs controls 52±4 % $p<0.05$; % 3-hydroxybutyrate suppression: relatives 76 (63-80) vs controls 98 (76-100) % $p<0.05$). Blood NEFA levels fell similarly in both subject groups (% suppression: relatives 83±5 vs controls 84±5 % NS). Relatives had significantly lower blood pyruvate concentrations at 60 minutes (relatives 60±7 vs controls 88±9 μmol/l $p<0.05$) after intravenous glucose. The lactate and alanine responses and the ratio lactate:pyruvate were similar in relatives and controls throughout the test (Table 8.4).

Table 8.4. Intermediary metabolite concentrations (μmol/l) and lactate:pyruvate ratio in relatives and controls during the intravenous glucose tolerance test.

Time (mins)	glycerol		NEFA		3-hydroxybutyrate		pyruvate		lactate		lactate:pyruvate		alanine	
	relatives	controls	relatives	controls	relatives	controls	relatives	controls	relatives	controls	relatives	controls	relatives	controls
0	53±5	53±6	361±32	465±78	47 (24-154)	53 (6-157)	46±6	53±9	579±71	673±105	12.8±1.3	13.8±2.5	228±30	254±27
4	52±6	53±7	323±32	456±103	47 (22-123)	46 (7-153)	44±8	61±7	582±95	739±113	13.5±1.0	12.6±1.9	233±47	243±17
10	47±5	51±8	296±30	389±68	40 (18-120)	33 (2-149)	48±8	61±5	680±104	726±120	14.3±0.8	12.3±2.3	231±47	232±19
30	36±5	32±5	131±49	106±26	16 (4-45)	14 (1-48)	74±8	75±5	907±114	873±86	12.4±0.8	11.8±1.2	240±38	244±20
60	39±6	28±5	82±24	78±17	14 (6-31)	5 (0-17)	60±7 *	88±9	811±122	922±152	13.2±0.6	10.6±1.5	252±54	256±31

Results are expressed as median (range) for 3-hydroxybutyrate and as mean±se for all other values.
*$p<0.05$ relatives vs controls. NEFA = non-esterified fatty acids.

8.7. DISCUSSION

This study has assessed the secretion of insulin and that of its precursor peptides and insulin sensitivity in Afro-Caribbean subjects genetically predisposed to NIDDM, whilst glucose tolerance was still normal. The first-degree relatives were demonstrated to have exaggerated secretion of insulin and of intact and 32, 33 split proinsulin following intravenous glucose compared to control subjects. No difference in insulin sensitivity of glucose metabolism was found between the two groups using the short insulin tolerance test, and the insulin and proinsulin responses to oral glucose in relatives were also normal. These findings suggest that subtle disturbances in insulin secretion are present in the Afro-Caribbean population prior to the onset of glucose intolerance.

Previous investigations of insulin secretion in individuals predisposed to diabetes have produced conflicting results. Fasting insulin concentrations adopt familial aggregation (Schumacher et al. 1992a). As discussed in chapter 7, in those populations with an exceptionally high prevalence of NIDDM increased plasma insulin levels in non-diabetic members are associated with later diabetes (Haffner et al. 1988). Hyperinsulinaemia when glucose tolerance is normal predicts the later development of diabetes in Pima Indians (Lillioja et al. 1988; Saad et al. 1989) and Nauruans (Zimmett et al. 1992). In other populations, defective insulin secretion has been documented in relatives of patients with NIDDM with impaired glucose tolerance (O'Rahilly et al. 1986 and 1988a; Eriksson et al. 1989). However fasting and glucose-stimulated insulin levels in glucose-tolerant relatives have been variously reported as elevated (Leslie et al. 1986; Ramachandran et al. 1990; Osei et al. 1991b), normal (O'Rahilly et al. 1986; Eriksson et al. 1989; Johnston et al. 1990) or reduced (Barnett et al. 1981; Cook et al. 1993b).

These previous studies relied on measurement of plasma insulin concentrations by radioimmunoassay, which cross reacts with intact and split proinsulins and thus may have overestimated insulin levels (Temple et al. 1990). In established NIDDM there is an increase in the proportion of proinsulin-like molecules to insulin (Ward et al. 1987; Saad et al. 1990; Reaven et al. 1993) and increased fasting proinsulin levels have been

reported in their normal relatives (Røder et al. 1990). The use of specific immunoradiometric assays has revealed increased intact and 32, 33 split proinsulin concentrations, both fasting and after oral glucose in patients with NIDDM, but reduced insulin levels 30 minutes after oral glucose (Davies et al. 1993a). Similar results have been reported for subjects with impaired glucose tolerance (IGT) (Williams et al. 1991; Krentz et al. 1993; Davies et al. 1993b). In this study, we have demonstrated for the first time increased glucose-stimulated secretion of intact and 32, 33 split proinsulin in individuals with normal glucose tolerance, but who are at risk of future NIDDM. Unlike the reports in IGT and NIDDM, these glucose-tolerant relatives also exhibited raised insulin levels and increased insulin:glucose ratios, as plasma glucose concentrations were similar in relatives and control subjects.

These raised plasma levels may represent either increased secretion or decreased clearance. Proinsulin clearance is mediated by the kidney and insulin is cleared by the liver. Although clearance was not specifically measured, all relatives had normal renal and hepatic function. It is possible that selective removal of insulin by the liver may have exaggerated the ratio of proinsulin:insulin peripherally, but this could not explain the increased insulin concentrations observed. Reduced hepatic extraction of insulin has been reported in offspring of white diabetic patients, but it is normal in black relatives and controls (Osei et al. 1992). Thus exaggerated secretion of insulin, intact and 32, 33 split proinsulin in response to intravenous glucose is likely to account for our findings.

In the present study the raised levels of IRMA insulin in relatives at matched glucose concentrations could represent insulin resistance, yet insulin sensitivity measured by the short insulin tolerance test was normal. It is possible that a subtle defect in insulin sensitivity exists which was not apparent from the glucose response to exogenous insulin. Alternatively, the increased insulin and proinsulin responses may represent abnormal ß cell processing. The finding of increased fasting proinsulin levels in the nondiabetic twins of patients with insulin dependent diabetes has been suggested to indicate increased ß cell responsiveness to glucose (Heaton et al. 1988). The disproportionate increase in proinsulin-like molecules in the current study may result from increased insulin demand (Ward et al. 1987). There is no evidence for abnormal

amylin secretion (which would compete with proinsulin for processing) in such genetically predisposed individuals (Eriksson et al. 1992).

Similar abnormalities in insulin and proinsulin were not demonstrated in response to oral glucose. This may be a reflection of the limitations of the method used, as the OGTT suffers from wide variablility and poor reproducibility (McDonald et al. 1965) unlike the IVGTT (Rayman et al. 1990) It may also be attributed to the nature of the stimulus employed; oral glucose is a less powerful stimulus than intravenous glucose for examining the first phase insulin response, when differences between relatives and controls were most marked. This may explain why other workers have been unable to demonstrate similar changes in intact and split proinsulins in family members (Clark et al. 1992) We did not measure plasma levels of 65, 66 split proinsulin, but as these are present only in very small quantities (Sobey et al. 1989; Temple et al. 1989), their contribution is likely to be negligible.

There was no difference in the glucose disappearance constant (K_G) between relatives and controls. This is consistent with most other studies (Johnston et al. 1990; Osei et al. 1991a). The lower K_G reported in the offspring of patients with NIDDM in one study (Warram et al. 1990) may have been due to increased obesity in those relatives studied.

No difference was detected in sensitivity to exogenous insulin between the individuals with and without a family history of NIDDM. This is in agreement with some studies (O'Rahilly et al. 1986; Johnston et al. 1990) involving European relatives but, in contrast, several other workers have reported reduced insulin sensitivity in normoglycaemic relatives (Eriksson et al. 1989; Laws et al. 1989; Ho et al. 1990; Ramachandran et al. 1990; Osei et al. 1991a; Gulli et al. 1992; Martin et al. 1992). The inability to detect impaired insulin sensitivity in this study could be due to the relatively small number of subjects. As discussed previously, only 40-60% of first-degree relatives would be expected to develop NIDDM in later life (Köbberling et al. 1985) and so any abnormalites present may be obscured by those normal relatives not

destined to become diabetic. However, these numbers were sufficient to detect differences in insulin secretion compared to the controls. The conflicting observations from other studies could be due to the method used. The studies cited above employed various different techniques, but the short insulin tolerance test used in this chapter has been well validated for the assessment of insulin sensitivity in chapter 3 and by others (Bonora et al. 1989; Akinmokun et al. 1992).

A major reason for the discrepancy between this and other studies is the ethnic origin of the subjects. In chapter 7 insulin insensitivity was reported in first-degree relatives of Asian origin but not in relatives of European origin, consistent with other studies in white Europeans (O'Rahilly et al. 1986; Johnston et al. 1990). Those reports of reduced insulin sensitivity have been in relatives from Finland, India, Taiwan, American Whites and Mexican Americans.The present study involved relatives of Afro-Caribbean origin. There is evidence to suggest that insulin secretion and action may be different in black and white subjects, although the data from different studies are conflicting. In the USA, Osei et al. (1992) have reported higher glucose-stimulated insulin levels and hepatic glucose output with lower hepatic insulin extraction, in the normal black compared to the normal white population. Comparison of relatives of NIDDM patients and controls revealed basal and glucose-stimulated hyperinsulinaemia, increased hepatic glucose output and reduced hepatic insulin extraction in white relatives, but no differences between relatives and controls were observed in the black population. Banerji and Lebovitz (1989) subdivided American blacks with NIDDM and near normal glycaemia into an insulin-sensitive variant with severely reduced insulin secretion and an insulin-resistant variant with only mildly impaired insulin secretion. These variants may have different genetic associations (Banerji et al. 1993) and do not exhibit the same clustering of cardiovascular risk factors reported in the white population (Osei et al. 1991b; Saad et al. 1991; Chaiken et al. 1993; Dowling and Pi-Sunyer 1993). In contrast with results from the American black studies, blacks from the normoglycaemic population of southern Africa are reported to have reduced insulin secretory capacity compared to the white population (Joffe et al. 1992).

Although the plasma glucose response to exogenous insulin was normal in the relatives, the suppression of blood glycerol and 3-hydroxybutyrate by endogenous insulin in the IVGTT was significantly impaired. This suggests that a defect in insulin action on lipolysis exists in these predisposed individuals. Similar findings were observed in relatives of Asian origin in chapter 7 and in normoglycaemic women with a previous history of gestational diabetes, who are at risk of NIDDM in the future (Chan et al. 1992). Barnett et al. (1981b) have also reported raised blood levels of glycerol, both fasting and after oral glucose, and a relative reduction in blood pyruvate concentrations in nondiabetic co-twins.

In conclusion, glucose-tolerant relatives of Afro-Caribbean patients with NIDDM demonstrate increased and disproportionate secretion of insulin, intact proinsulin and 32, 33 split proinsulin in response to intravenous glucose. This suggests that disturbance of ß cell processing is an early feature of NIDDM in the Afro-Caribbean population. The increased IRMA insulin levels and the impaired glycerol and 3-hydroxybutyrate responses to intravenous glucose suggest a subtle defect in insulin sensitivity also exists prior to the development of glucose intolerance.

CHAPTER 9:

INSULIN SECRETION AND INTERMEDIARY METABOLITE RESPONSES TO INTRAVENOUS GLUCOSE IN EUROPEAN AND ASIAN RELATIVES WITH NORMAL GLUCOSE TOLERANCE

9.1. INTRODUCTION

Non-insulin dependent diabetes (NIDDM) is a heterogeneous disease (Porte Jr 1991; DeFronzo 1992; Yki-Järvinen 1994). In order to discover whether the primary disturbance in NIDDM is insulin resistance or impaired insulin secretion, other workers have investigated subjects with impaired glucose tolerance or mildly elevated fasting glucose concentrations, but these results are complicated by the deleterious effects of hyperglycaemia on insulin action and ß islet cell secretion (Leahy 1990; Davies et al. 1993b; Yki-Järvinen 1994).

Studies of insulin secretion by other groups in genetically predisposed individuals prior to the development of glucose intolerance have been conflicting, with insulin secretion in unaffected relatives variously reported as increased (Leslie et al. 1986; Haffner et al. 1988; Saad et al. 1989; Ramachandran et al. 1990; Warram et al. 1990; Osei et al. 1991b), normal (O'Rahilly et al. 1986; Eriksson et al. 1989; Johnston et al. 1990) or reduced (Barnett et al. 1981b; Cook et al. 1993b). Use of the more specific immunoradiometric assays for measurement of insulin and its precursor peptides (Temple et al. 1990) has demonstrated increased levels of intact and split proinsulins in NIDDM in some studies (Temple et al. 1989; Nagi et al. 1990; Davies et al. 1993a; Reaven et al. 1993), but normal proinsulin levels in others (Clark et al. 1992; Levy et al. 1993).

In the previous chapter, increased and disproportionate secretion of insulin, intact and 32, 33 split proinsulin was reported following intravenous glucose in a group of glucose-tolerant relatives of NIDDM patients of Afro-Caribbean origin. There is evidence to suggest that the pathogenesis of NIDDM may not be the same in all ethnic groups (Banerji and Lebovitz 1989; Lillioja et al. 1991; Saad et al. 1991; McKeigue et al. 1991 and 1992; Joffe et al. 1992; Osei et al. 1992; Simmons and Powell 1993; Osei et al. 1993). In this chapter therefore, insulin secretion and that of its conversion intermediates has been investigated in response to intravenous glucose in first-degree relatives from two other ethnic groups, Europeans and subjects of Asian origin.

9.2. SUBJECTS

Seventeen first-degree relatives of patients with NIDDM were recruited for study, 10 of White European and 7 of Asian (Indian-subcontinent) origin. They were matched for age, ethnic background and body mass index with 17 control subjects. All relatives and controls had normal glucose tolerance by WHO criteria (WHO 1985).

9.3. METHODS

Each subject underwent an intravenous glucose tolerance test employing a bolus of glucose (0.3g/kg) as described in chapter 2. Blood samples were collected and stored as detailed in chapter 2 for later measurement of glucose, immunoreactive insulin by radioimmunoassay, insulin, intact and 32, 33 split proinsulin by immunoradiometric assays and intermediary metabolites.

9.4. STATISTICAL ANALYSIS

Results are expressed as mean±se for those data adopting a normal distribution and the values for relatives and controls were compared using unpaired Student's t tests. For those parameters not normally distributed (immunoreactive insulin, intact and 32, 33 split proinsulin, specific insulin and 3-hydroxybutyrate) data are expressed as median (range) and comparisons made using a Mann Whitney U test. The glucose disappearance constant K_G was calculated from the slope of $\log_{10}$ glucose concentration over 10-60 minutes plotted against time.

Areas for the glucose, insulin, intact and 32, 33 split proinsulin responses to intravenous glucose were calculated using the trapezoidal rule (Matthews et al. 1990). The first phase response was calculated from the incremental area under the curve from 0-10 minutes, and the second phase response was calculated from the area from 10-60 minutes. Where significant differences were found, correlations were sought using Spearman's correlation coefficient. As in chapter 8, results were also analysed using the cumulative area 0-40 minutes to allow for the influence of the first phase response on the calculated second phase value; this analysis did not alter the results and so for clarity, only the first and second phase responses are presented.

9.5. RESULTS

Subject characteristics are shown in Table 9.1.

9.5.1. Fasting values

Fasting concentrations of glucose, immunoreactive insulin, specific insulin, intact and 32, 33 split proinsulin were similar in European relatives and controls (Table 9.1). Amongst the Asian group, fasting glucose concentrations were lower in the relatives than in the controls, despite subjects being well matched for age and body mass index. There were no significant differences in fasting levels of plasma immunoreactive insulin, specific insulin (by IRMA), intact and 32, 33 split proinsulin between Asian relatives and controls (Table 9.1).

Table 9.1. Subject characteristics and fasting concentrations of plasma glucose, immunoreactive insulin (IRI), specific insulin, intact and 32, 33 split proinsulin.

	European		Asian	
	relatives	controls	relatives	controls
n	10	10	7	7
m:f	5:5	5:5	3:4	6:1
age (years)	34±3	37±3	31±2	28±4
BMI (kg/m^2)	24.4±1.3	25.8±1.5	24.5±1.5	24.2±2.1
glucose (mmol/l)	5.3±0.2	5.1±0.2	4.9±0.2	5.5±0.2 *
IRI (pmol/l)	43 (27-228)	40 (21-71)	68 (8-157)	49 (8-146)
specific insulin (pmol/l)	52 (8-118)	49 (14-72)	75 (21-106)	76 (30-117)
intact proinsulin (pmol/l)	2.4 (0.6-5.0)	1.3 (0.6-3.1)	3.4 (1.1-8.7)	2.9 (1.7-15.3)
32, 33 split proinsulin (pmol/l)	2.9 (0.2-12.3)	2.2 (0.4-4.5)	2.9 (0.5-9.3)	5.1 (0.3-8.0)

Results are expressed as mean±se for age, body mass index (BMI) and glucose, and as median (range) for all other values. Plasma glucose concentration was significantly lower in Asian relatives compared to Asian controls *$p<0.05$.

Asian relatives had significantly higher fasting circulating concentrations of glycerol (65±7 vs 44±4 μmol/l $p<0.05$), NEFA (569±59 vs 375±64 μmol/l $p<0.05$) and 3-hydroxybutyrate (147 (44-187) vs 35 (21-57) μmol/l $p<0.01$) than their controls.These differences were not observed in the European group (Table 9.2).

Table 9.2. Blood glycerol and 3-hydroxybutyrate concentrations and plasma non-esterified fatty acid (NEFA) levels during the intravenous glucose tolerance test.

	Time (minutes)	European		Asian	
		relatives	controls	relatives	controls
Glycerol (μmol/l)	0	56±3	61±7	65±7	44±4 *
	4	52±3	62±7	68±6	46±5 *
	10	47±4	52±5	66±8	44±3 *
	30	42±5	40±6	43±5	33±2
	60	39±5	38±6	37±6	30±2
NEFA (μmol/l)	0	360±44	442±56	569±59	375±64 *
	4	340±45	404±47	548±52	362±63 *
	10	302±42	345±49	509±59	316±49 *
	30	134±39	135±30	292±88	139±26
	60	76±17	70±17	149±31	84±10
3-hydroxybutyrate (μmol/l)	0	30 (12-114)	53 (4-161)	147 (44-187)	35** (21-57)
	4	26 (11-114)	51 (0-115)	120 (42-171)	39 ** (18-58)
	10	26 (9-103)	46 (0-158)	156 (43-165)	27** (17-49)
	30	11 (5-32)	17 (0-63)	53 (25-77)	17*** (5-27)
	60	8 (3-15)	10 (0-26)	17 (8-28)	12 (2-28)

Results are expressed as mean±se for glycerol and non-esterified fatty acids (NEFA), and as median (range) for 3-hydroxybutyrate. Asian relatives vs Asian controls * $p<0.05$; ** $p<0.01$; *** $p<0.005$

Fasting concentrations of pyruvate, lactate and alanine (Table 9.3) and the fasting lactate:pyruvate ratio were similar between relatives and controls within each ethnic group.

Table 9.3. Blood pyruvate, lactate and alanine concentrations during the intravenous glucose tolerance test (mean±se).

	Time (minutes)	European		Asian	
		relatives	controls	relatives	controls
Pyruvate (μmol/l)	0	73±11	50±5	62±7	74±9
	4	73±9	46±5 *	67±8	71±8
	10	83±12	57±5	71±8	79±9
	30	109±17	65±12 *	93±9	95±10
	60	82±9	65±8	91±11	81±9
Lactate (μmol/l)	0	864±142	725±117	661±78	808±191
	4	930±181	739±128	705±75	841±173
	10	928±170	805±145	792±88	810±162
	30	1209±206	982±194	926±109	1049±211
	60	1016±156	879±169	855±104	882±148
Alanine (μmol/l)	0	290±56	253±19	299±16	289±21
	4	294±48	247±17	294±22	303±24
	10	287±48	248±18	306±19	293±21
	30	297±55	247±22	294±16	298±21
	60	290±46	258±19	301±23	287±17

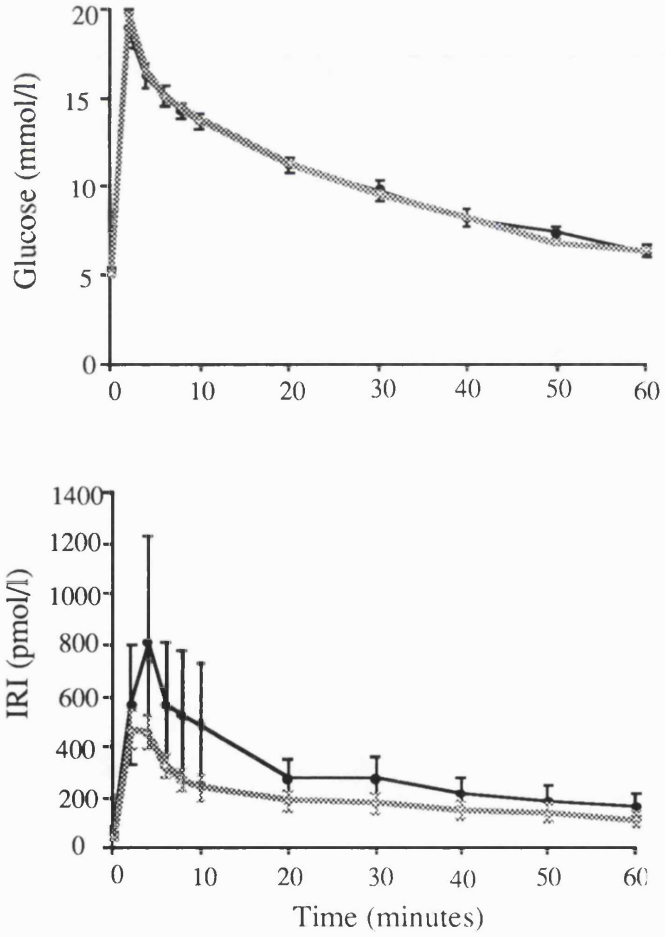
European relatives vs European controls * $p<0.05$

9.5.2. Response to intravenous glucose

1. Europeans

European relatives and controls exhibited very similar glucose responses to the intravenous glucose bolus (Figure 9.1) (1st phase: relatives 148±6 vs controls 158±7 mmol/l.min; 2nd phase: relatives 467±19 vs controls 464±21 mmol/l.min) and the glucose disappearance constants K_G were similar in relatives and controls (relatives 725±101 vs controls 668±47 min^{-1}). There were no significant differences in the immunoreactive insulin concentrations between relatives and controls (1st phase: IRI, relatives 3349 (806-28 113) vs controls 3617 (1114-5106) pmol/l.min; 2nd phase: IRI, relatives 8252 (3922-45 463) vs controls 6680 (2810-22 313) pmol/l.min) (Figure 9.1).

Figure 9.1. Plasma glucose and immunoreactive insulin (IRI) concentrations during the intravenous glucose tolerance test in European relatives —●— and controls ⋯○⋯ .

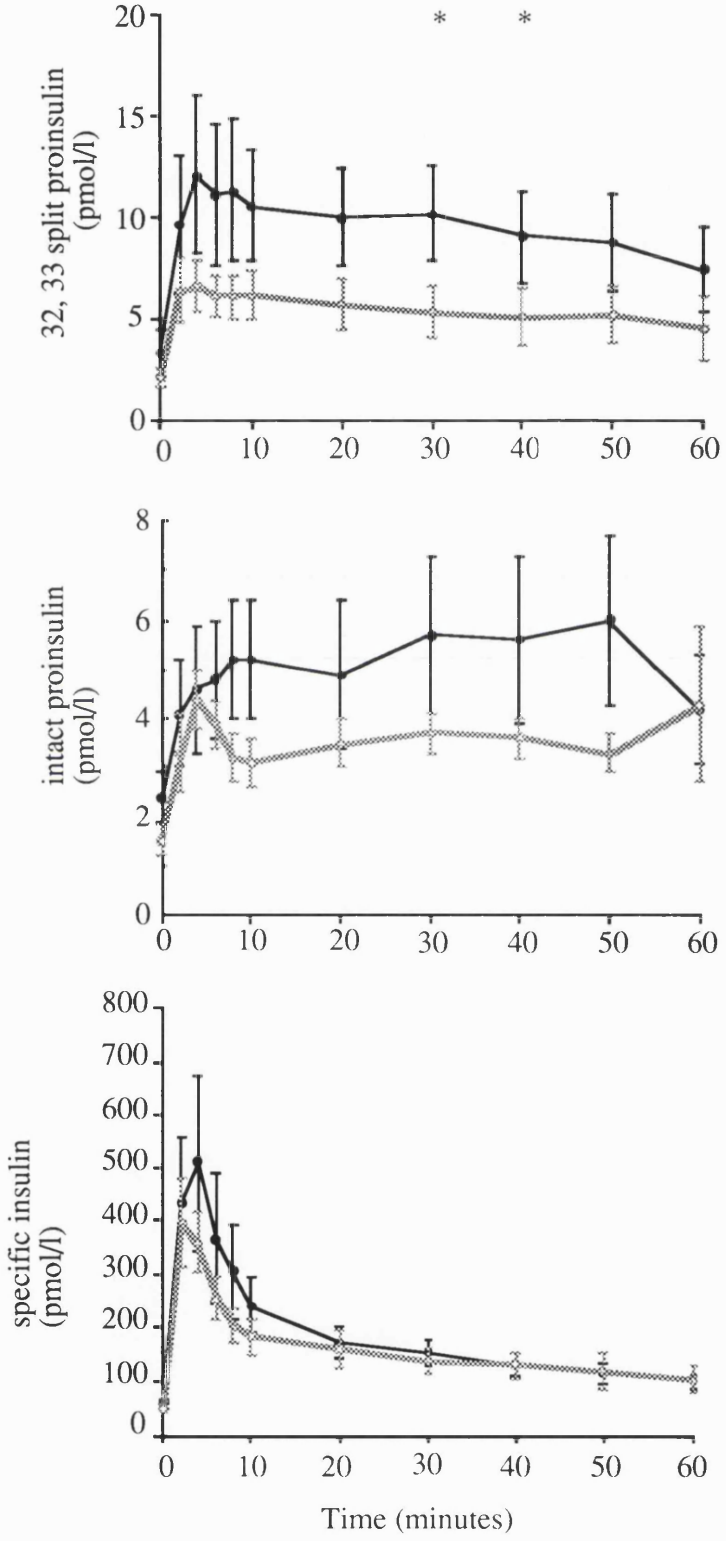


Results are expressed as mean±se.

The specific insulin response (by IRMA) was also similar in relatives and controls (1st phase: relatives 2757 (700-10 969) vs controls 2830 (632-4682) pmol/l.min; 2nd phase: relatives 6387 (3006-15 865) vs controls 5284 (2060-18 605) pmol/l.min) (Figure 9.2).

The European relatives exhibited significantly greater late secretion of 32, 33 split proinsulin in response to intravenous glucose than their controls, but 1st phase 32, 33 split proinsulin, though higher in the relatives than in the controls, was not statistically different (1st phase: relatives 71 (7-352) vs controls 55 (17-118) pmol/l.min, $p=0.17$; 2nd phase: relatives 433 (115-1459) vs controls 234 (55-745) pmol/l.min, $p<0.05$) (Figure 9.2). Secretion of intact proinsulin in Europeans however, did not differ significantly in relatives and controls (1st phase: relatives 31 (13-113) vs controls 32 (16-72) pmol/l.min; 2nd phase: relatives 174 (87-737) vs controls 159 (97-298) pmol/l.min) (Figure 9.2).

Figure 9.2. Plasma concentrations of 32, 33 split proinsulin, intact proinsulin and specific insulin during the intravenous glucose tolerance test in European relatives —●— and controls ⟿



Results are expressed as mean±se. *p<0.05.

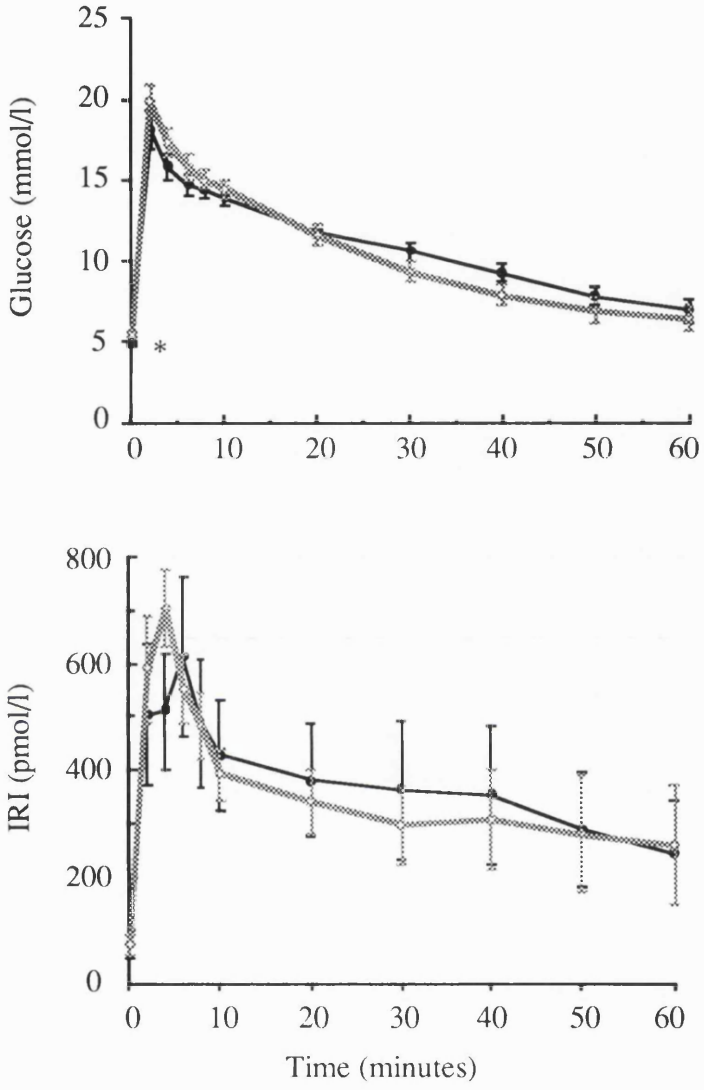
European relatives also had a significantly greater percentage of proinsulin-like molecules (intact + 32, 33 split proinsulin) to total insulin-like molecules (specific insulin plus intact + 32, 33 split proinsulin) during the second phase of secretion (1st phase: relatives 4.0 (2.5-10.2) vs controls 3.2 (2.7-6.6) % NS; 2nd phase: relatives 9.1 (5.0-11.8) vs controls 5.9 (4.3-12.6) % $p<0.05$). The ratio of 32, 33 split:intact proinsulin was significantly higher in the relatives at 4 minutes and 30 minutes after intravenous glucose (4 minutes: relatives 3.2 (0.9-4.5) vs controls 1.3 (0.8-4.5), $p<0.05$; 30 minutes: relatives 2.2 (0.8-7.4) vs controls 1.2 (0.4-5.5), $p<0.05$). Amongst the relatives, there were significant correlations between 1st and 2nd phase 32, 33 split proinsulin secretion and the 1st phase glucose response, but no such correlations held in the controls (1st phase secretion: relatives rho=0.77, $p<0.05$; controls rho=0.31, p=0.35; 2nd phase secretion: relatives rho=0.71, $p<0.05$; controls rho=0.36, p=0.28). There were no significant differences between relatives and controls in the ratios of 32, 33 split proinsulin:glucose, intact proinsulin:glucose, nor in the specific insulin:glucose ratio.

Following intravenous glucose, blood glycerol and 3-hydroxybutyrate and plasma NEFA concentrations fell to a similar extent in relatives and control subjects (Table 9.2). European relatives had higher circulating levels of pyruvate compared to controls at 4 minutes and 30 minutes after glucose (Table 9.3), but blood concentrations of lactate, alanine and the lactate:pyruvate ratio were not significantly different.

2. Asians

As in the European group, relatives and controls had similar glucose responses to the intravenous glucose bolus (1st phase: relatives 145±7 vs controls 156±7 mmol/l.min; 2nd phase: relatives 499±23 vs controls 464±34 mmol/l.min) and similar K_G values (relatives 610±97 vs controls 783±93 min^{-1}). The immunoreactive insulin concentrations were not significantly different between Asian relatives and controls (1st phase: relatives 5031 (1467-9464) vs controls 4876 (2621-7210) pmol/l.min; 2nd phase: relatives 12 335 (2072-46 500) vs controls 13 168 (3650-34 766) pmol/l.min) (Figure 9.3).

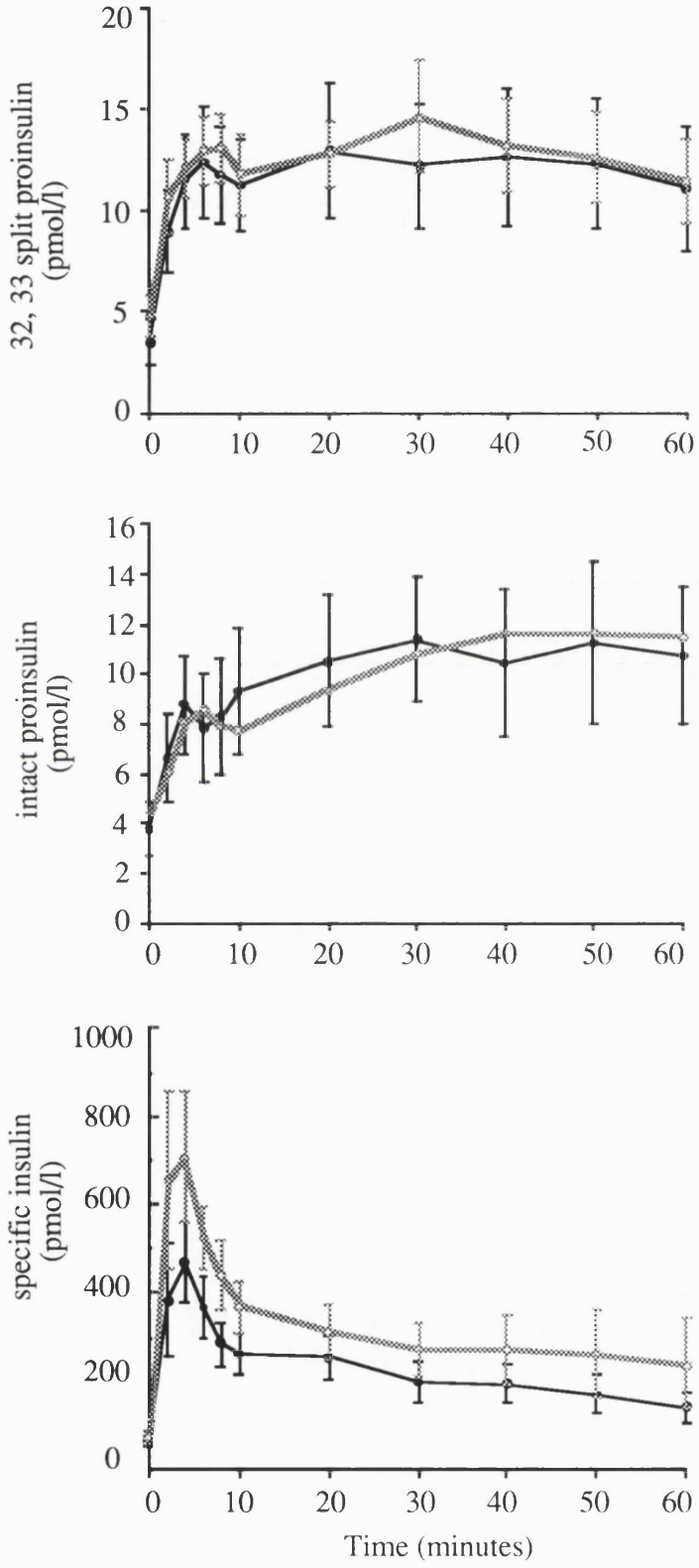
Figure 9.3. Plasma glucose and immunoreactive insulin (IRI) concentrations during the intravenous glucose tolerance test in Asian relatives —●— and controls ⟿◇⟿



Results are expressed as mean±se *p<0.05

Furthermore, secretion of specific insulin and intact proinsulin did not differ significantly between Asian relatives and controls (1st phase: specific insulin, relatives 2708 (1331-6389) vs controls 4404 (2408-9772) pmol/l.min; intact proinsulin, relatives 78 (15-154) vs controls 51 (32-204) pmol/l.min; 2nd phase: specific insulin, relatives 10 698 (2589-20 445) vs controls 10 505 (5726-28 950) pmol/l.min; intact proinsulin, relatives 706 (95-1007) vs controls 291 (192-1927) pmol/l.min) (Figure 9.4).

Figure 9.4. Plasma concentrations of 32, 33 split proinsulin, intact proinsulin and specific insulin during the intravenous glucose tolerance test in Asian relatives —●— and controls ⋯◇⋯



Results are expressed as mean±sc.

Unlike the Europeans however, Asian relatives and controls also had similar 32, 33 split proinsulin secretory responses (1st phase: relatives 97 (35-194) vs controls 122 (29-154) pmol/l.min, p=0.57; 2nd phase: relatives 412 (137-1334) vs controls 698 (150-1020) pmol/l.min, p=0.85) (Figure 9.4). In addition, the percentage of proinsulin-like molecules to total insulin-like molecules did not differ between Asian relatives and controls (1st phase: relatives 6.6 (1.0-7.1) vs controls 3.9 (1.6-8.8) %; 2nd phase: relatives 11.3 (2.1-16.0) vs controls 9.0 (4.0-12.7) %); nor were any differences observed in the ratios of 32, 33 split proinsulin:glucose, intact proinsulin:glucose, nor specific insulin:glucose.

In the Asian relatives, blood glycerol concentrations remained significantly higher than those of the control group at 4 minutes and 10 minutes after the glucose bolus (4 minutes: relatives 68±6 vs 46±5 μmol/l, $p<0.05$; 10 minutes: relatives 66±8 vs controls 44±3 μmol/l, $p<0.05$). Blood 3-hydroxybutyrate levels were also elevated in the Asian relatives compared to their controls (4 minutes: relatives 120 (42-171) vs controls 39 (18-58) μmol/l, $p<0.01$; 10 minutes: relatives 156 (43-165) vs controls 27 (17-49) μmol/l, $p<0.01$; 30 minutes: relatives 53 (25-77) vs controls 17 (5-27) μmol/l, $p<0.005$). In addition, Asian relatives had significantly greater plasma NEFA concentrations following intravenous glucose (4 minutes: relatives 548±52 vs controls 362±63 μmol/l, $p<0.05$; 10 minutes: relatives 509±59 vs controls 316±49 μmol/l, $p<0.05$) (Table 9.2). Circulating levels of pyruvate, lactate and alanine were not different in Asian relatives and controls following intravenous glucose (Table 9.3), nor were the lactate:pyruvate ratios.

3. Asians versus Europeans

Asian and European controls were selected to match their ethnic relatives rather than each other (notably age: 28±4 vs 37±3 years respectively). Nonetheless, some comparisons are warranted. They had similar glucose concentrations, fasting and after intravenous glucose, but basal and glucose-stimulated levels of intact and 32, 33 split proinsulin and specific insulin were significantly higher in the Asian controls than in their European counterparts, despite the Asian controls being younger and despite reasonable matching for body weight (Table 9.4).

Table 9.4. Plasma glucose, immunoreactive insulin (IRI), specific insulin, intact and 32, 33 split proinsulin concentrations fasting and during the intravenous glucose tolerance test in European and Asian controls.

		European	Asian
glucose (mmol/l.min)	fasting (mmol/l)	5.1±0.2	5.5±0.2
	1st phase	158±7	156±7
	2nd phase	464±21	464±34
IRI (pmol/l.min)	fasting (pmol/l)	40 (21-71)	49 (8-146)
	1st phase	3617 (1114-5106)	4876 (2621-7210) *
	2nd phase	6680 (2810-22 313)	13 168 (3650-34 766)
specific insulin (pmol/l.min)	fasting (pmol/l)	49 (14-72)	76 (30-117) *
	1st phase	2830 (632-4682)	4404 (2408-9772)
	2nd phase	5284 (2060-18 605)	10 505 (5726-28 950) *
intact proinsulin (pmol/l.min)	fasting (pmol/l)	1.3 (0.6-3.1)	2.9 (1.7-15.3) *
	1st phase	32 (16-72)	51 (32-204) *
	2nd phase	159 (97-298)	291 (192-1927) **
32, 33 split proinsulin (pmol/l.min)	fasting (pmol/l)	2.2 (0.4-4.5)	5.1 (0.3-8.0) *
	1st phase	55 (17-118)	122 (29-154) *
	2nd phase	234 (55-745)	698 (150-1020) *

Results are expressed as mean±se for glucose and median (range) for all other values.
1st phase = 0-10 minutes; 2nd phase = 10-60 minutes after glucose bolus. *p<0.05 **p<0.01

9.6. DISCUSSION

This study has demonstrated increased and disproportionate secretion of 32, 33 split proinsulin in response to an intravenous bolus of glucose in glucose-tolerant first-degree relatives of NIDDM patients of European origin. No significant defects in secretion of insulin or intact proinsulin were observed. This suggests that European subjects at risk of future NIDDM possess disturbances of ß cell processing, at a time when glucose tolerance is still normal and implies that ß cell dysfunction is an early feature of NIDDM in this ethnic group.

Disproportionate secretion of insulin precursors has previously been demonstrated in established NIDDM, with increased fasting intact proinsulin: immunoreactive insulin ratios (Ward et al. 1987; Saad et al. 1990). Using more specific immunoradiometric assays, higher proportions of fasting intact and 32, 33 split proinsulin to total insulin have been observed in NIDDM patients compared to control subjects (Temple et al. 1989; Clark et al. 1992; Davies et al. 1993a) and this was associated with either increased (Temple et al. 1989; Levy et al. 1993; Davies et al. 1993a) or normal (Clark et al. 1992) plasma concentrations of these precursor peptides. Furthermore, exaggerated secretion of intact and 32, 33 split proinsulin is also a feature of impaired glucose tolerance (Williams et al. 1991; Krentz et al. 1993; Davies et al. 1993b). In these situations however, ß cell dysfunction could be secondary to the hyperglycaemia (Leahy 1990). In this study increased glucose-stimulated secretion of 32, 33 split proinsulin was observed in individuals with normal glucose tolerance and who are genetically predisposed to NIDDM. These relatives had elevated plasma concentrations of 32, 33 split proinsulin, despite circulating glucose levels very similar to those of control subjects. This was accompanied by an increased proportion of proinsulin-like molecules to total insulin. The study, therefore, has demonstrated that defects in insulin secretory function similar to those found in patients with NIDDM are present prior to the development of clinical diabetes or impaired glucose tolerance.

Several other studies have investigated insulin secretion in first-degree relatives of NIDDM patients with normal glucose tolerance, but most have relied solely on insulin measurement by radioimmunoassay. Immunoreactive insulin responses to intravenous

glucose in this study were normal, consistent with many other reports (O'Rahilly et al. 1986; Eriksson et al. 1989; Widén et al. 1992; Osei et al. 1993), but some have observed increased insulin levels (Haffner et al. 1988; Warram et al. 1990; Osei et al. 1991; Gulli et al. 1992). However, there are few reports of insulin precursor secretion in such relatives and altered concentrations of these conversion intermediates could account for some of the above discrepancies. Unlike in this study, Røder et al. (1990) found fasting proinsulin concentrations were elevated in relatives of NIDDM patients, but Beer et al. (1990) reported normal fasting and glucose-stimulated proinsulin levels in glucose-tolerant relatives and only family members with impaired glucose tolerance exhibited a relative increase in proinsulin secretion after glucose infusion. These studies, however, did not examine the contribution of 32, 33 split proinsulin to the measured insulin concentrations, and the 32, 33 split form may be a more sensitive guide to ß cell dysfunction than intact proinsulin (Davies et al. 1994). In the fasting state, plasma levels of intact and 32, 33 split proinsulin were normal in the relatives in the present study and this is consistent with other reports (Clark et al. 1992). In the previous chapter more marked abnormalities were observed, with increased 32, 33 split proinsulin, increased insulin and increased intact proinsulin responses to intravenous glucose in glucose-tolerant relatives of Afro-Caribbean patients, suggesting that processing abnormalities are an early defect in more than one ethnic group. The more marked disturbance in Afro-Caribbean relatives may reflect the fact that a higher proportion will go on to get NIDDM in later life. Evidence for this is lacking however, although NIDDM in the Afro-Caribbean population is more prevalent than in the European (Cruikshank et al. 1991).

In the present study, the exaggerated 32, 33 split proinsulin response to intravenous glucose suggests a defect in insulin processing. This may be a consequence of increased insulin demand (Ward et al. 1987) or glucose toxicity. In the relatives studied, but not in the controls, the elevated 32, 33 split proinsulin secretion correlated significantly with the plasma glucose response (although overall glucose responses were very similar to controls'). This may reflect increased ß cell responsiveness to glucose and may be reversible, in view of the recently reported decline in 32, 33 split proinsulin concentrations in frank NIDDM with improved glycaemic control (Davies et

al. 1994). The lower biological activity of 32, 33 split proinsulin compared to mature insulin may promote deterioration of glucose tolerance in genetically predisposed individuals, although direct evidence for this is lacking. An elevated concentration of 32, 33 split proinsulin has also been implicated as a cardiovascular risk factor associated with NIDDM (Nagi et al. 1990; Hales et al. 1991; Davies et al. 1993a); the elevated levels several years before the onset of clinical diabetes may explain why the risk of macrovascular disease is not dependent on the duration of diabetes (Reaven 1988). The higher circulating levels of 32, 33 split proinsulin could represent impaired renal clearance rather than increased secretion; this is unlikely, as all subjects had normal renal function and levels of intact proinsulin, which is also cleared by the kidney, were similar in relatives and controls.

There were no significant differences in secretion of insulin, intact or 32, 33 split proinsulin in glucose-tolerant relatives of Asian patients compared to Asian controls. The Asian relatives had similar fasting insulin concentrations to their controls, despite significantly lower fasting glucose concentrations and this could be interpreted as insulin resistance. The different basal glucose levels complicate interpretation of the insulin response. However, following intravenous glucose intact and 32, 33 split proinsulin levels and insulin concentrations were similar in Asian relatives and controls at matched glucose concentrations. Thus, no evidence of ß cell dysfunction was found in this Asian group.

There are several possible explanations for the discrepancy with the findings in the European group. It is possible that the Asian relatives studied were too few in number to achieve statistically significant results compared to controls, but numbers were sufficient to detect differences in circulating levels of intermediary metabolites. NIDDM in the Asian community may have a different metabolic basis to that in the European population (Mather and Keen 1985; Simmons et al. 1991). There is considerable evidence for higher immunoreactive insulin levels in the Asian (diabetic and non diabetic) population (McKeigue et al. 1991; Dornhorst et al. 1992), as was also observed in chapter 7. In this, the Asian population resembles those ethnic groups such as the Pima Indians in whom insulin resistance and hyperinsulinaemia are the

predominant findings (Lillioja et al. 1991 and 1993) and the proportion of proinsulin:immunoreactive insulin is normal in normoglycaemic relatives of NIDDM patients (Saad et al. 1990).

In both the European and Asian relatives the glucose disappearance constants were not significantly different from those of their respective controls. This is consistent with other studies (Johnston et al. 1990; Osei et al. 1991a). One study has reported lower K_G in relatives (Warram et al. 1990), but they were more obese than the control group. Asian relatives in this study were noted to have persistently elevated circulating levels of glycerol, NEFA and 3-hydroxybutyrate compared to their controls. This implies increased lipolysis and hepatic ketogenesis in the basal state. It confirms the earlier findings of chapter 7 in Asian relatives of a failure to suppress lipolysis after oral glucose and in the presence of normal basal insulin levels, is evidence of insulin resistance. Similar metabolic defects have been observed in women with previous gestational diabetes (Chan et al. 1992), in offspring of Mexican American NIDDM patients (Gulli et al. 1992) and in the Afro-Caribbean relatives studied in chapter 8. Lipolysis is normally very sensitive to small increments in circulating insulin and defects in insulin action at the adipocyte may represent an early abnormality in the pathogenesis of NIDDM. The resultant increase in circulating NEFA concentrations may impair muscle glucose utilization by the glucose-fatty acid cycle (Randle et al. 1963) and enhanced gluconeogenesis can contribute to fasting hyperglycaemia (Nurjhan et al. 1992; Puhakainen et al. 1992). In this study levels of these intermediary metabolites were not different in European relatives and controls, in agreement with other reports (Leslie et al. 1986; Eriksson et al. 1991) and with the findings of chapter 7 in response to oral glucose. The relative contribution of disturbances in insulin sensitivity and ß cell function in the evolution of NIDDM may thus be different in the two ethnic groups, with insulin secretory defects predominating in the Europeans and insulin resistance in the Asians.

Although the study was designed to compare relatives and controls within ethnic groups rather than between them, comparison of data from Asian and European control groups revealed significantly increased secretion of specific insulin, intact and 32, 33

split proinsulin in the Asians, despite similar glucose levels. This provides further evidence for the heterogeneity of ß cell function and underlines the importance of close matching for ethnic origin in metabolic studies.

In conclusion, this study has demonstrated that first-degree relatives of European patients with NIDDM exhibit exaggerated and disproportionate secretion of 32, 33 split proinsulin in response to intravenous glucose, whilst glucose tolerance is still normal. No such defect was observed in glucose-tolerant relatives of Asian patients. The findings suggest that ß cell dysfunction is an early characteristic of NIDDM in the European population, but these conclusions cannot necessarily be extrapolated to NIDDM in all ethnic groups.

CHAPTER 10:

INSULIN RESISTANCE WITH RESPECT TO LIPOLYSIS IN NON-DIABETIC RELATIVES OF EUROPEAN PATIENTS WITH NON-INSULIN DEPENDENT DIABETES.

10.1. INTRODUCTION

Impaired insulin action in established NIDDM extends both to glucose metabolism at the level of the liver and peripheral tissues (DeFronzo 1992) as well as to lipid metabolism (Groop et al. 1989; Nurjhan et al. 1992). Resistance to the antilipolytic action of insulin results in an enhanced supply of glycerol and non-esterified fatty acids (NEFA) which could contribute to the increased hepatic gluconeogenesis. The prevalence and presentation of NIDDM vary in different ethnic groups (Mather and Keen 1985; Simmons et al. 1991), suggesting that the pathogenesis may not be the same in all populations. In chapter 7, insulin resistance was demonstrated in first-degree relatives of NIDDM patients originating from the Indian-subcontinent. When glucose tolerance was still normal, these relatives exhibited impaired suppression of plasma glycerol concentrations following oral glucose and a reduced initial rate of fall of plasma glucose after a bolus of intravenous insulin. Glucose-tolerant first-degree relatives of patients of European origin, however, did not demonstrate these features, but are also predisposed to later diabetes.

This discrepancy may reflect the extreme sensitivity of lipolysis to small increments in circulating insulin levels. Discrete differences in insulin sensitivity may be masked if supraphysiological doses of insulin are administered. In addition, slight differences in plasma glycerol concentration may have been difficult to detect using conventional enzymatic assays. Plasma NEFA levels are a less reliable indicator of lipolysis as, unlike glycerol, NEFA can be re-esterified in adipose tissue (Newsholme and Leech 1983); plasma NEFA appearance in the circulation thus reflects the balance between release from lipolysis on the one hand and re-esterification on the other. Subtle defects in insulin action on lipolysis may therefore have been obscured in the European group in the earlier studies. To overcome these limitations, glycerol turnover can be measured as an index of lipolysis, as demonstrated in chapter 5.

In this chapter, lipolysis has been investigated in glucose-tolerant relatives of NIDDM patients of European origin by examining the effect of a very low dose insulin infusion on glycerol turnover measured with stable non-radioactive isotopic tracers using a similar protocol to that employed in chapter 5.

10.2. SUBJECTS

Eight healthy first-degree relatives of NIDDM patients of European origin and nine healthy control European subjects with no family history of diabetes were recruited for study. The relatives and controls were matched for age and body mass index (Table 10.1) and all had normal glucose tolerance by WHO criteria (WHO 1985). Each subject had a normal physical examination, blood tests of renal and hepatic function and a normal electrocardiogram. None was taking any regular medication.

10.3. STUDY DESIGN

The development of this protocol is detailed in chapter 4 and the full protocol is described in chapter 2. Subjects were asked to follow their usual diet and to abstain from alcohol and strenuous exercise for the 3 days preceding the study. They attended the Metabolic Day Ward at 08.00 hours after a 10-12 hour overnight fast and received primed constant infusions of the stable isotopic tracers [1,2,3 $^{2}H_5$] glycerol and [6,6 ^{2}H] glucose for 330 minutes (priming doses 0.5 μmol/kg and 19.2 μmol/kg, infusion doses 3.0 μmol/kg/hr and 10.0 μmol/kg/hr respectively) for measurement of glycerol and glucose turnovers. At 150 minutes an infusion of soluble insulin (0.005units/kg/hour) diluted in Haemaccel® was started and continued until the end of the study. Blood samples were taken at intervals for measurement of glycerol and glucose tracer enrichments, and glycerol, glucose, insulin and NEFA concentrations as described in chapter 2.

10.4. ANALYSES

Plasma glucose concentration was measured at the bedside with a Beckman glucose oxidase analyser and plasma glycerol and NEFA concentrations were measured enzymatically as described in chapter 2. Plasma insulin levels were measured by radioimmunoassay. Plasma for glucose and glycerol tracer enrichment was treated and prepared for detection by gas chromatography-mass spectrometry as discussed in chapter 2. The rates of appearance of glycerol and glucose measured isotopically (Ra) were obtained by the principle of isotope dilution.

10.5. STATISTICAL ANALYSIS

Steady state plasma concentrations of glycerol, glucose, NEFA and insulin were calculated over the baseline period (90-150 minutes) and over the final hour of the infusion (270-330 minutes). Changes in plasma concentration induced during the insulin infusion were analysed by calculating the incremental areas under the curve from the preinfusion baseline values using the trapezoidal rule. The changes in concentration of glycerol, glucose, NEFA and insulin were also analysed from the steady state plasma concentrations before and after insulin by subtraction, but this did not alter the findings; for consistency with results from chapter 5 therefore, the data are presented here as areas under the curve. The rates of appearance of glycerol and glucose were calculated over the same periods and the rate of appearance over the final hour (270-330 minutes) was subtracted from the basal Ra in each case to obtain the change in glycerol and glucose Ra induced by the insulin infusion.

Data from relatives and controls were compared using Student's unpaired t tests for all parameters except insulin levels, for which the Mann Whitney U test was used as these data were not normally distributed. Isotopic measurements of glycerol and glucose rates of appearance were compared before and after insulin infusion in each group using unpaired Student's t tests. Correlations were sought using Spearman's rank correlation coefficient. Results are expressed as median (range) for insulin and as mean±se for all other parameters.

10.6. RESULTS

Fasting insulin concentrations were significantly higher in the relatives than in the control subjects (49 (30 to 113) vs 28 (18 to 66) pmol/l $p<0.05$), but the fasting plasma concentrations of glycerol, NEFA and glucose were not different between the two groups (Table 10.1). The basal plasma rates of appearance of glycerol and glucose measured isotopically were similar in relatives and controls (Table 10.1).

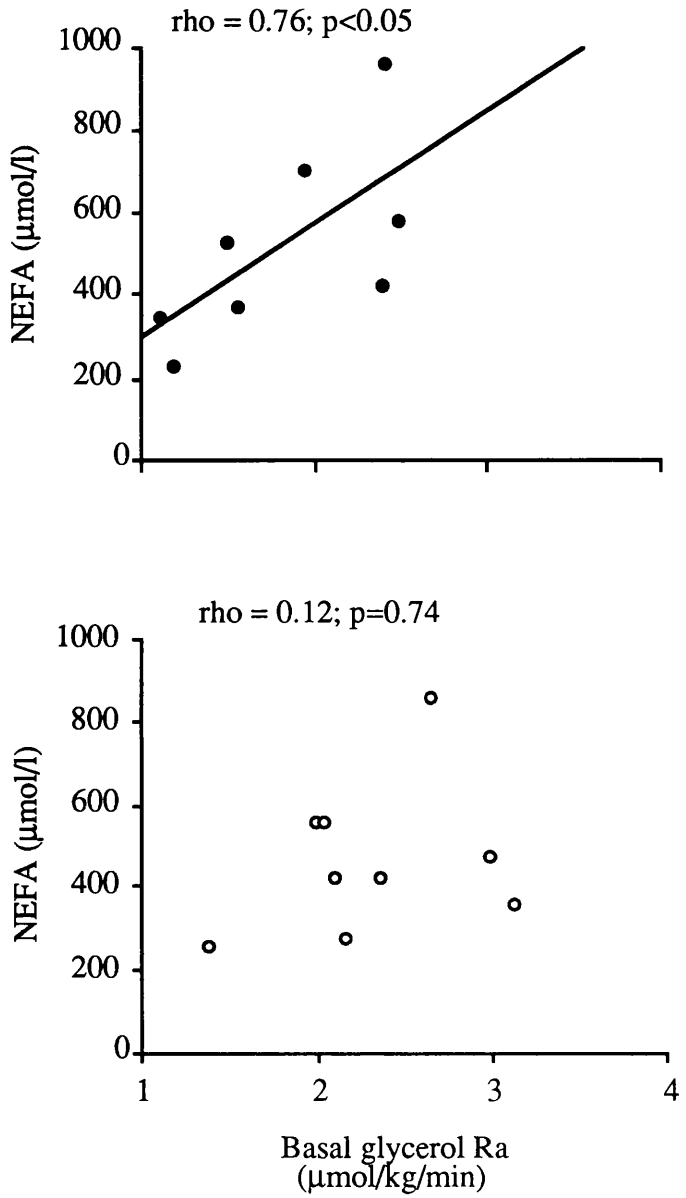
Table 10.1. Subject characteristics, fasting plasma concentrations of glycerol, non-esterified fatty acids (NEFA), glucose and insulin and basal rates of appearance (Ra) of glycerol and glucose measured isotopically in relatives and controls

	relatives	controls
n	8	9
m:f	4:4	5:4
age (years)	30 (19 to 49)	27 (22 to 37)
BMI (kg/m^2)	26.2 (20.3 to 33.1)	25.5 (18.5 to 39.9)
fasting glycerol (μmol/l)	46±4	55±6
fasting NEFA (μmol/l)	520±82	467±61
fasting glucose (mmol/l)	4.8 ±0.1	4.8 ±0.1
fasting insulin (pmol/l)	49 (30 to 113)	28 (18 to 66) *
basal glycerol Ra (μmol/kg/min)	1.81±0.20	2.31±0.18
basal glucose Ra (mg/kg/min)	1.61±0.11	1.83±0.15

BMI = body mass index. Age and BMI are expressed as mean (range). Insulin is expressed as median (range) and all other values as mean±se. Fasting insulin levels were significantly higher in the relatives than in the controls *$p<0.05$.

In the relatives only, there was a significant correlation between the basal rate of glycerol appearance and the fasting NEFA concentration (rho=0.76, $p<0.05$) but this was not observed in the control group (rho=0.12, p=0.74) (Figure 10.1).

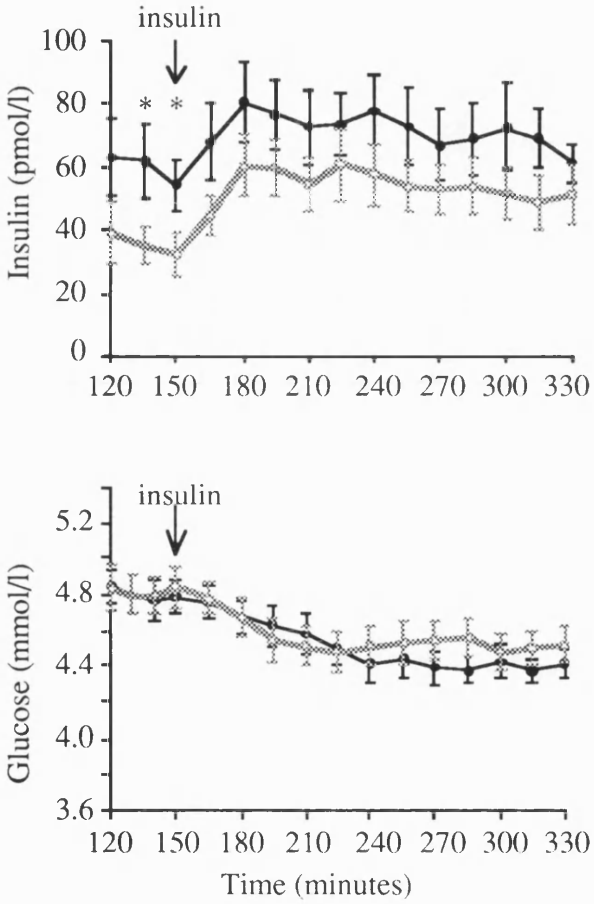
Figure 10.1. The relationship between basal plasma rate of appearance (Ra) of glycerol and basal non-esterified fatty acid (NEFA) concentrations in relatives • (upper panel) and controls o (lower panel).



During the insulin infusion, steady state plasma insulin levels of 61 (51 to 142) pmol/l in the relatives and 43 (30 to 112) pmol/l in the controls were achieved. These values were not statistically different between the groups and nor were the proportional increases in insulin concentration above fasting level. The steady state concentrations of glucose, glycerol and NEFA attained during the insulin infusion were not significantly

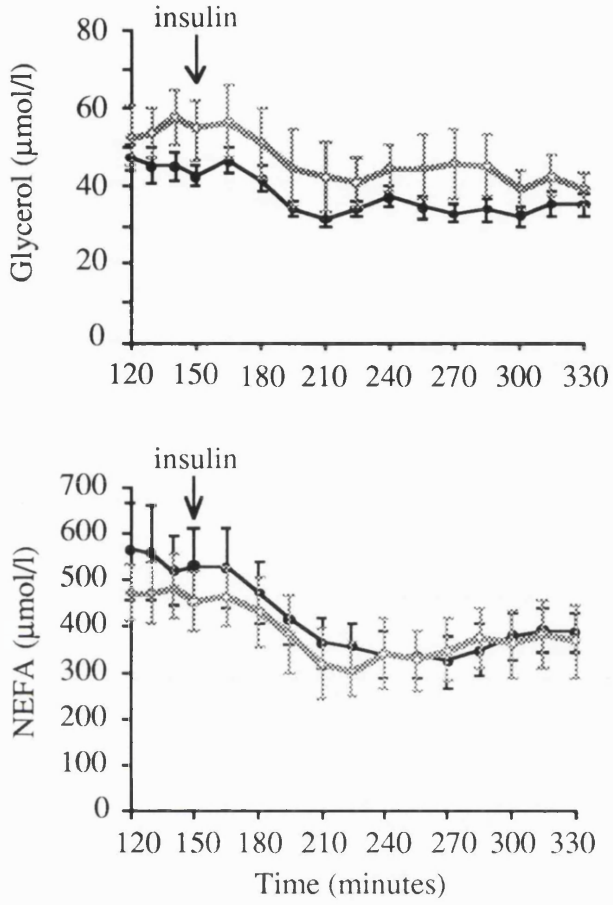
different in relatives and controls (steady state levels (mean±se): glucose, *relatives* 4.4±0.1, *controls*, 4.5±0.1 mmol/l; glycerol, *relatives* 35±2 µmol/l, *controls* 47±8 µmol/l; NEFA, *relatives:* 368±48 µmol/l, *controls* 337±66 µmol/l) (Figures 10.2 and 10.3).

Figure 10.2. Plasma concentrations of insulin (upper panel) and glucose (lower panel) before and during the insulin infusion in relatives —●— and controls ⟿○⟿



The insulin infusion was commenced at 150 minutes. Results are expressed as mean±se Relatives had significantly higher basal insulin concentrations compared to controls *p<0.05.

Figure 10.3. Plasma concentrations of glycerol (upper panel) and non-esterified fatty acids (NEFA) (lower panel) before and during the insulin infusion in relatives —●— and controls ⋯◇⋯



Results are expressed as mean±sc.
The insulin infusion was commenced at 150 minutes.

As expected, this physiological hyperinsulinaemia produced only small decreases in plasma glucose concentration, which were similar in relatives and controls (Table 10.2). Plasma glycerol concentrations decreased with the insulin infusion in both relatives and controls to a similar extent (area under curve: relatives -1.77±0.46, controls -1.53±0.34 mmol/l.hr NS). Plasma NEFA levels also declined similarly in relatives and controls (relatives -23±7, controls -20±4 mmol/l.hr NS) (Table 10.2).

Table 10.2. Changes in the rates of appearance (Ra) of glycerol and glucose and in the plasma concentrations of glycerol, non-esterified fatty acids (NEFA), glucose and insulin following the insulin infusion in relatives and controls.

	relatives	controls
Ra glycerol (μmol/kg/min)	+0.06±0.21	-0.51±0.16*
Ra glucose (mg/kg/min)	-0.10±0.03	-0.07±0.06
glycerol (mmol/l.hr)	-1.77±0.46	-1.53±0.34
NEFA (mmol/l.hr)	-23±7	-20±4
glucose (mmol/l.hr)	-47±7	-46±16
insulin (pmol/l.hr)	2110 (-1008 to 6371)	3370 (1982 to 5993)

Results are expressed as median (range) for insulin and as mean±se for all other values. Changes in plasma concentration are calculated as incremental area under the curve. The change in glycerol Ra was significantly less in the relatives than in the controls *$p<0.05$.

In the control group the rate of appearance of glycerol measured isotopically decreased significantly following the insulin infusion (Glycerol Ra: basal 2.31±0.18, after insulin 1.82±0.18 μmol/kg/min $p<0.05$). In contrast, in the relatives there was no significant change in glycerol Ra after insulin (Glycerol Ra: basal 1.81±0.20, after insulin 1.87 ±0.34 μmol/kg/min NS). Furthermore, the suppression in glycerol appearance in response to insulin was significantly less in the relatives than in the control subjects (change in glycerol Ra: relatives +0.06±0.21, controls -0.51±0.16 μmol/kg/min $p<0.05$) (Table 10.2). Although comparison of values for glucose rates of appearance before and after insulin showed significant differences in the relatives (Glucose Ra: basal 1.61±0.11, after insulin 1.51±0.12 mg/kg/min $p<0.05$) and not in the control

group (Glucose Ra: basal 1.83±0.15, after insulin 1.76±0.11 mg/kg/min NS), analysis of the change in glucose Ra for each subject revealed similar falls in both the relatives and controls (change in glucose Ra: relatives -0.10±0.03, controls -0.07±0.06 mg/kg/min NS) (Table 10.2). For one relative the incremental insulin area under the curve during the insulin infusion was a negative value (-1008 pmol/l.hr). This subject may have cleared insulin particularly rapidly from the circulation. However, analysis of the glycerol turnover data excluding results for this subject did not alter the findings.

10.7. DISCUSSION

This study was undertaken to examine insulin sensitivity with respect to lipolysis in healthy subjects who are predisposed to later NIDDM. Using a novel technique which was validated in chapter 5, first-degree relatives of NIDDM patients were demonstrated to have impaired suppression of glycerol appearance in plasma in response to a very low dose insulin infusion, at a time when glucose tolerance is normal. This suggests that the regulation of lipolysis is abnormal early in the development of NIDDM and implies that insulin resistance is an early feature in its pathogenesis.

Plasma glycerol is derived during lipolysis from the breakdown of triglyceride in adipose tissue. Non-esterified fatty acids are also released but unlike glycerol, they may undergo re-esterification. Glycerol cannot be re-esterified as it requires prior phosphorylation and adipose tissue lacks the necessary enzyme, glycerol kinase (Lin 1977). Hence, the appearance of glycerol in plasma is a direct reflection of the rate of lipolysis (Nurjhan et al. 1988). This principle was utilized in this study, with the rate of appearance of glycerol determined isotopically taken as an index of lipolysis. Under normal circumstances glycerol is a minor contributor to overall gluconeogenesis (Bortz et al. 1972), but in frank NIDDM lipolysis is augmented and gluconeogenesis from glycerol is disproportionately increased (Yki-Järvinen et al. 1987; Nurjhan et al. 1992; Puhakainen et al. 1992) contributing to the rise in hepatic glucose production and fasting hyperglycaemia. Furthermore, inhibition of lipolysis by insulin is impaired in overt NIDDM (Yki-Järvinen et al. 1987; Skowronski et al. 1991).

In this study the basal rate of glycerol appearance in the relatives was not significantly different from that of the control group, and was well within reported normal ranges (Beylot et al. 1987; Matthews et al. 1991). Hence basal lipolysis was normal in the relatives. This is not unexpected as these subjects were not diabetic and all had normal fasting glucose concentrations. Fasting plasma glycerol and NEFA concentrations were also similar to those of the control group. The notable finding was the difference between the two groups in the suppression of glycerol turnover by physiological hyperinsulinaemia. The relatives exhibited significantly less reduction in glycerol

appearance compared to the control group despite tending to have higher circulating insulin levels. This demonstrates that in those individuals who are genetically predisposed to NIDDM, the antilipolytic action of insulin is impaired prior to the development of glucose intolerance. This reduced response to physiological hyperinsulinaemia indicates the presence of insulin insensitivity.

The abnormality in insulin sensitivity with respect to lipolysis was not apparent from the effect of insulin on plasma glycerol concentration alone; both the relatives and controls exhibited similar falls in glycerol concentration during the insulin infusion.The impaired response was only manifest by analysis of glycerol turnover and this highlights the sensitivity of the stable isotope infusion technique. Measurement of glycerol appearance in plasma using the isotope tracer provides a more accurate determination of turnover at low concentration (Wolfe 1992) when enzymatic assays are imprecise. The findings in chapter 5 demonstrated that this technique can distinguish differences in insulin action which were not detected by determination of plasma glycerol concentration. A very low dose insulin infusion was specifically chosen to enable lipolysis to be investigated without a counter-regulatory response (Hale et al. 1985). This permitted small differences in glycerol metabolism to be revealed which were not apparent using cruder stimuli, such as the oral glucose tolerance test as in chapter 7. In this study no difference was observed between the relatives and control subjects in plasma NEFA concentrations, neither basally nor in response to insulin. This is in agreement with other workers (Eriksson et al. 1991) and remains consistent with the glycerol turnover results. As discussed above, NEFA concentrations reflect the balance between lipolysis and re-esterification.

Further evidence for the presence of insulin resistance in the relatives is provided by the significantly higher basal insulin levels compared to those of the control group, despite no difference in fasting glucose concentrations. Other workers have reported similar findings which have been taken to indicate insulin resistance (Haffner et al. 1988; Ramachandran et al. 1990; Warram et al. 1990). As discussed in earlier chapters, the possible contribution of proinsulin and its intermediates to the fasting insulin levels (Temple et al. 1989) however, make interpretation of raised fasting concentrations of

immunoreactive insulin alone a potentially unreliable indicator of insulin insensitivity. Proinsulin levels were not increased in these unaffected relatives in chapter 7 and in the present study, insulin resistance was confirmed by the decreased antilipolytic response to physiological hyperinsulinaemia. In addition, there is evidence that when lipolysis is increased, the excess NEFA released decrease hepatic clearance of insulin, contributing to systemic hyperinsulinaemia (Björntorp 1991). The hyperinsulinaemia in this study, therefore, may be related to impaired suppression of lipolysis at certain times of the day and signifies insulin resistance.

Basal glucose turnover was not significantly different in the relatives from that in the control subjects. This is in agreement with previous work (Osei and Holland 1987; Eriksson et al. 1989) where, as in this study, relatives and control groups had similar fasting glucose concentrations. Osei et al. (1992) have reported increased hepatic glucose output in unaffected family members, but those relatives also had significantly elevated fasting glucose levels compared to controls. The values for basal glucose appearance in this study were associated with significantly increased basal circulating insulin levels, which suggests that a mild impairment of insulin inhibition of hepatic glucose output may be present in the relatives. The insulin infusion produced small decreases in glucose turnover of similar magnitude in both the relatives and controls. This is not unexpected as the very low dose insulin protocol was designed primarily for investigation of insulin action on lipolysis rather than on glucose metabolism.

Basal glycerol turnover in the relatives correlated significantly with the fasting NEFA concentration. Similar findings were reported by Puhakainen et al. (1992) in NIDDM patients, but as in this study, no such correlation held in control subjects. This suggests that the relatives studied here may already possess a defect in the regulation of lipolysis which is similar to that found in frank diabetes. In NIDDM, gluconeogenesis from glycerol is disproportionately increased (Nurjhan et al. 1992) and the intrahepatic pathways responsible may be accelerated by the increased NEFA availability (Blumenthal 1983). The elevated NEFA, resulting from accelerated lipolysis, may also reduce glucose utilization by the glucose-fatty acid cycle (Randle et al. 1963). One potential explanation for the findings in this study is that the glucose-tolerant relatives

have early signs of abnormal control of lipolysis, but as basal NEFA levels are still normal they have not yet developed increased gluconeogenesis nor raised fasting glucose concentrations.

The defect in lipolysis in the relatives may not be present in adipose tissue at all sites. Evidence exists that compared to subcutaneous fat, visceral adipose tissue is less sensitive to the antilipolytic action of insulin and has a lower density of insulin receptors (Björntorp 1991). Body fat distribution was not assessed in this study, but it is unclear whether a relatively higher proportion of visceral to subcutaneous fat in the relatives compared to the controls could explain the difference in insulin sensitivity observed. The insulin sensitivity assessed reflects whole body lipolysis, but in view of the recent report of opposing actions of insulin on lipoprotein lipase activity in adipose tissue and skeletal muscle (Coppack et al. 1993) it is conceivable that the degree of suppression of lipolysis may vary in different tissues. Quantitatively, however, glycerol turnover should predominantly reflect lipolysis in adipose tissue (Newsholme and Leech 1983).

Few other studies have selectively studied lipolysis in unaffected relatives, but there is evidence for hypertriglyceridaemia (Laws et al. 1989; Schumacher et al. 1992b). Impaired suppression of plasma glycerol levels following oral glucose has also been reported in glucose-tolerant women with previous gestational diabetes (Chan et al. 1992), another group at risk of developing NIDDM. This further suggests that altered regulation of lipolysis may be an early feature in the pathogenesis of NIDDM. Not all the relatives in the present study would be expected to develop diabetes and this makes the finding of reduced insulin sensitivity in the relatives as a group more notable. The fact that impaired lipolysis was demonstrable in Asian relatives after oral glucose, but in these European relatives the defect was only detected with the low dose insulin infusion and glycerol turnover measurements, suggests that the metabolic abnormality is less obvious in this predisposed European population. This may be related to the earlier age of onset and greater prevalence of NIDDM in the Asian community (Mather and Keen 1985; Simmons et al. 1991).

In conclusion, first-degree relatives of European patients with NIDDM exhibited reduced suppression of glycerol turnover in response to a very low dose insulin infusion when glucose tolerance is normal. No abnormality was detected in basal glycerol turnover, nor in basal nor post-insulin plasma glycerol concentration. This subtle defect in insulin sensitivity was only manifest during physiological hyperinsulinaemia using isotopic tracer infusions, which provide a more reliable index of glycerol turnover than plasma glycerol concentrations. This study confirms that insulin resistance with respect to lipolysis is an early characteristic of NIDDM.

CHAPTER 11:

SERUM LIPOPROTEINS AND BASAL INSULIN SECRETION IN NORMOGLYCAEMIC RELATIVES OF PATIENTS WITH NON-INSULIN DEPENDENT DIABETES.

11.1. INTRODUCTION

Non-insulin dependent diabetes is associated with lipoprotein abnormalities, most commonly elevated levels of total or very low density lipoprotein (VLDL) triglyceride and reduced levels of high density lipoprotein (HDL) -cholesterol, and these may predispose to premature vascular disease (Merrin et al. 1992). It has been suggested that these abnormalities, together with the resistance to insulin-stimulated glucose uptake characteristic of NIDDM (DeFronzo 1992) and associated compensatory hyperinsulinaemia are part of a metabolic syndrome, linked by insulin resistance. The syndrome includes increased plasma triglyceride concentrations, decreased HDL-cholesterol concentrations, hypertension, glucose intolerance and hyperinsulinaemia, each of which increase the risk of coronary artery disease (Reaven 1988).

As discussed earlier, the presence of compensatory hyperinsulinaemia in NIDDM has recently been challenged (Temple et al. 1990). Using specific immunoradiometric assays (IRMA), several studies have demonstrated reduced insulin levels in NIDDM (Temple et al. 1989) and either increased (Nagi et al. 1990; Davies et al. 1993b) or normal (Levy et al. 1993) proinsulin concentrations.

In chapters 7 and 10 insulin resistance was demonstrated in first-degree relatives of NIDDM patients whilst glucose tolerance was still normal; similar observations have been reported by other workers (Eriksson et al. 1989; Laws et al. 1989; Martin et al. 1992). It is not known whether the dyslipidaemia typical of frank NIDDM is also present prior to the development of clinical diabetes. The present study, therefore proposed firstly, to examine lipid profiles in glucose-tolerant relatives of NIDDM patients in order to investigate whether the diabetic dyslipidaemia is also an early feature of the disease, and secondly to characterise their insulin and proinsulin levels by specific IRMA assays.

11.2. SUBJECTS AND METHODS

Thirty-six first-degree relatives of patients with NIDDM (13 European, 15 of Asian (Indian-subcontinent) and 8 of Afro-Caribbean origin) and 36 control subjects were recruited for study as described in chapter 2. Relatives and controls were matched individually for sex and ethnic origin and the two groups were matched for age and body mass index when individual matching was not possible. All individuals had a normal physical examination and none was taking any regular medication.

Subjects attended the Metabolic Day Ward at 08.00 hours after a 10-12 hour overnight fast. They were allowed to sit quietly for 20 minutes, after which venous blood was sampled for measurement of total and HDL-cholesterol, triglycerides, HbA_1, glucose, insulin, intact and 32, 33 split proinsulin and total immunoreactive insulin. All except 2 relatives and 8 controls had previously undergone a 75g oral glucose tolerance test and had normal glucose tolerance by WHO criteria. The remaining subjects all had normal fasting glucose and HbA_1 values.

11.3. ANALYSES

Blood samples were collected and analysed as described in chapter 2. Serum total cholesterol, high density lipoprotein cholesterol and triglyceride concentrations were measured enzymatically. Insulin, intact and 32, 33 split proinsulin were measured using specific two-site immunoradiometric assays and total immunoreactive insulin was measured by radioimmunoassay Plasma glucose was measured using a hexokinase method and HbA_1 was measured by electroendosmosis (normal range <8.0%).

11.4. STATISTICAL ANALYSIS

Results are expressed as mean±se for data normally distributed. For skewed data (insulin, intact and 32, 33 split proinsulin, immunoreactive insulin and triglyceride) results are expressed as median (range). Low density lipoprotein (LDL) -cholesterol was calculated using the Friedewald equation (Friedewald et al. 1972). Data from relatives and controls were compared using Student's unpaired t test, or the Mann Whitney U test for non-normally distributed data. Results were also analysed after subdividing the relatives and their controls according to ethnic origin, in order to remove any potential masking of differences by combining subjects of different ethnic background.

11.5. RESULTS

Subject characteristics are shown in Table 11.1.

Table 11.1. Subject characteristics in 36 relatives and controls

	relatives	controls
m:f	17:19	17:19
age (years)	33±2	34±2
body mass index (kg/m^2)	23.7±0.5	23.7±0.6
HbA_1 (%)	6.0±0.1	5.9±0.1
glucose (mmol/l)	5.0±0.1	4.9±0.1

Results are expressed as mean±se

Relatives and control subjects had similar fasting glucose concentrations and HbA_1 values (6.0±0.1 vs 5.9±0.1%). Total cholesterol, HDL-cholesterol and triglyceride concentrations were not significantly different between the two groups (Table 11.2). There were no differences in calculated LDL-cholesterol levels between relatives and controls. Total immunoreactive insulin concentrations, though higher in the relatives than the controls, were not significantly different (53 (8-203) vs 42 (5-134) pmol/l, p=0.07). Plasma levels of insulin measured by immunoradiometric assay were similar in relatives and controls. Intact and 32, 33 split proinsulin concentrations did not differ significantly between the groups (Table 11.2). The proportion of proinsulin-like molecules (intact + 32, 33 split proinsulin) to total insulin-like molecules (insulin + intact + 32, 33 split proinsulin) was also similar in relatives and controls (8.0 (2.7-24.1) vs 8.5 (2.5-33.6)%).

Table 11.2. Fasting concentrations of serum lipids and plasma immunoreactive and IRMA insulin, intact and 32, 33 split proinsulin in 36 relatives and controls.

	relatives	controls
total cholesterol (mmol/l)	4.51±0.13	4.54±0.17
HDL-cholesterol (mmol/l)	1.21±0.06	1.10±0.05
LDL-cholesterol (mmol/l)	2.84±0.14	2.96±0.14
triglyceride (mmol/l)	0.78 (0.44-2.45)	0.83 (0.41-4.03)
immunoreactive insulin (pmol/l)	53 (8-203)	42 (5-134)
insulin (pmol/l)	50 (19-174)	52 (10-118)
intact proinsulin (pmol/l)	3 (0-15)	2 (1-6)
32, 33 split proinsulin (pmol/l)	2 (0-24)	2 (0-6)

Results are expressed as mean±se for total, HDL and LDL cholesterol and as median (range) for all other values.

When the data for relatives and controls were analysed for each ethnic group separately, the findings did not differ from those of the multiethnic cohort (Tables 11.3, 11.4 and 11.5). Within the Asian group (Table 11.3), despite similar fasting glucose concentrations, immunoreactive insulin levels tended to be higher in the relatives, although again the differences were not significant statistically (64 (8-203) vs 38 (17-109) pmol/l; p=0.07). Intact proinsulin concentrations were not significantly different in the Asian relatives than in their controls (3 (1-15) vs 2 (1-6) pmol/l; p=0.34), nor were fasting insulin and 32, 33 split proinsulin concentrations. Total cholesterol, HDL- and LDL-cholesterol concentrations did not differ between the two Asian groups. The median triglyceride level was higher for the Asian relatives than for their controls, but the scatter was wide and there was no statistical difference (1.24 (0.49-2.45) vs 0.82

(0.41-4.03) mmol/l; p=0.58). The proportion of proinsulin-like molecules to total insulin-like molecules was no different between Asian relatives (9.3 (2.7-24.1)%) and controls (9.5 (4.7-22.5)%).

Table 11.3. Subject characteristics and fasting concentrations of serum lipids and plasma immunoreactive and IRMA insulin, intact and 32, 33 split proinsulin in Asian relatives and controls.

	relatives	controls
m:f	9:6	9:6
age (years)	33±2	34±3
body mass index (kg/m^2)	23.1±0.8	23.0±1.2
HbA_1 (%)	6.2±0.2	5.9±0.2
glucose (mmol/l)	4.9±0.2	5.0±0.1
total cholesterol (mmol/l)	4.66±0.19	4.41±0.30
HDL-cholesterol (mmol/l)	1.05±0.07	1.01±0.06
LDL-cholesterol (mmol/l)	3.05±0.20	2.88±0.24
triglyceride (mmol/l)	1.24 (0.49-2.45)	0.82 (0.41-4.03)
immunoreactive insulin (pmol/l)	64 (8-203)	38 (17-109)
insulin (pmol/l)	49 (19-174)	48 (10-118)
intact proinsulin (pmol/l)	3 (1-15)	2 (1-6)
32, 33 split proinsulin (pmol/l)	2 (0-20)	2 (0-6)

Results are expressed as mean±se or median (range).

Amongst those of European origin, relatives and controls did not differ in any of the parameters (Table 11.4) and the proportion of proinsulin-like molecules to total insulin-like molecules was also similar (8.0 (3.5-22.0) vs 5.6 (2.5-33.6))%).

Table 11.4. Subject characteristics and fasting concentrations of serum lipids and plasma immunoreactive and IRMA insulin, intact and 32, 33 split proinsulin in European relatives and controls.

	relatives	controls
m:f	5:8	5:8
age (years)	37±3	37±3
body mass index (kg/m^2)	24.5±1.1	24.3±0.9
HbA_1 (%)	6.0±0.2	5.8±0.2
glucose (mmol/l)	5.2±0.2	5.0±0.1
total cholesterol (mmol/l)	4.65±0.21	4.87±0.26
HDL-cholesterol (mmol/l)	1.26±0.10	1.14±0.08
LDL-cholesterol (mmol/l)	2.96±0.23	3.27±0.23
triglyceride (mmol/l)	0.75 (0.60-1.63)	0.79 (0.60-1.95)
immunoreactive insulin (pmol/l)	40 (8-178)	40 (13-91)
insulin (pmol/l)	47 (20-103)	54 (14-78)
intact proinsulin (pmol/l)	2 (1-5)	2 (1-6)
32, 33 split proinsulin (pmol/l)	2 (0-24)	1 (0-5)

Results are expressed as mean±se or median (range).

Relatives of Afro-Caribbean patients tended to have lower triglyceride concentrations than their controls, but these were not statistically different (Table 11.5). Total, HDL and LDL-cholesterol concentrations did not differ significantly between Afro-Caribbean relatives and their controls. Although the median value of immunoreactive insulin was lower in relatives and the median value of IRMA insulin was higher in the relatives than in their controls, these differences did not reach statistical significance. There were no differences between Afro-Caribbean relatives and controls in levels of intact or 32, 33 split proinsulin, nor in the proportion of proinsulin-like molecules to total insulin-like molecules (6.3 (3.4-11.7) vs 9.2 (3.4-16.5)%).

Table 11.5. Subject characteristics and fasting concentrations of serum lipids and plasma immunoreactive and IRMA insulin, intact and 32, 33 split proinsulin in Afro-Caribbean relatives and controls.

	relatives	controls
m:f	3:5	3:5
age (years)	26±3	28±1
body mass index (kg/m^2)	23.7±0.9	24.1±0.4
HbA_1 (%)	6.0±0.2	5.9±0.3
glucose (mmol/l)	4.8±0.2	4.8±0.2
total cholesterol (mmol/l)	4.00±0.32	4.23±0.30
HDL-cholesterol (mmol/l)	1.43±0.12	1.21±0.12
LDL-cholesterol (mmol/l)	2.24±0.21	2.60±0.25
triglyceride (mmol/l)	0.69 (0.44-1.53)	0.94 (0.49-1.71)
immunoreactive insulin (pmol/l)	36 (13-135)	55 (5-134)
insulin (pmol/l)	55 (43-109)	43 (25-94)
intact proinsulin (pmol/l)	2 (0-5)	3 (1-4)
32, 33 split proinsulin (pmol/l)	2 (0-4)	2 (0-6)

Results are expressed as mean±se or median (range).

11.6. DISCUSSION

Lipid metabolism is deranged in NIDDM, but the underlying mechanism is not fully understood. The strong evidence for the genetic basis for NIDDM suggests that similar lipid abnormalities may be present in the healthy relatives of NIDDM patients. This study found no evidence to support this view. Concentrations of total, HDL- and LDL-cholesterol and triglyceride did not differ between first-degree relatives and controls. The family members had normal glucose tolerance, and fasting glucose concentrations and HbA_1 values were similar to those of their controls. Hence, lipid and insulin levels were not influenced by the presence of hyperglycaemia. The results imply that the disturbances in lipids and lipoproteins characteristic of NIDDM are not apparent prior to the development of hyperglycaemia and are therefore likely to be a consequence of the metabolic derangement, rather than a primary feature.

These findings are in agreement with previous studies involving smaller numbers of subjects (Barnett et al. 1981b; Leslie et al. 1986; Zavaroni et al. 1990; Osei et al. 1991b; Velho et al. 1993). In contrast, Schumacher et al. (1992) reported an increased prevalence of dyslipidaemia in normoglycaemic relatives as defined by published population data; yet these results are difficult to interpret since the control group also had a high prevalence of dyslipidaemia and their family history was not specified; many of the relatives were from the same pedigrees and differences between relatives and controls disappeared after adjusting for higher insulin levels or when using alternative published normal criteria. In another study, Sarlund et al. (1991) observed abnormal lipid profiles only in those relatives with impaired glucose tolerance and normal lipid levels in normoglycaemic family members. Cook et al. (1992a) reported no differences in lipid levels between relatives with and without impaired glucose tolerance. Furthermore, normal lipid levels have been observed in Mexican American relatives with normal glucose tolerance, even in the face of reduced insulin-stimulated glucose disposal (Gulli et al. 1992). These results are thus consistent with most other publications. Although Laws et al. (1989) have reported increased plasma triglyceride and reduced HDL-cholesterol levels in glucose-tolerant relatives, these subjects were selected for their obesity and sedentary lifestyle which may have influenced the findings. Alternatively, the discrepancy with the present study may reflect the

heterogeneous nature of NIDDM.

As fasting plasma lipid levels are known to vary between different ethnic groups (McKeigue et al. 1991; Chaiken et al. 1993), the relatives and controls in this study were carefully matched for ethnic origin. After separate analysis of the data by ethnic group, there continued to be no differences in lipid levels between the relatives and controls. The European, Afro-Caribbean and Asian groups were not sufficiently matched to allow cross-racial analysis, as the study was specifically designed to investigate differences between relatives and controls. The clinical significance and validity of such a comparison between ethnic groups may be questioned, however, as there is evidence to suggest that the relationships between insulin sensitivity and lipid levels (Chaiken et al. 1993), body fat distribution (Dowling and Pi-Sunyer 1993) and blood pressure (Saad et al. 1991) are not the same in all ethnic groups. This may imply different genetic associations.

In established NIDDM the elevated triglyceride levels arise from overproduction and impaired catabolism of VLDL, associated with reduced lipoprotein lipase activity. The origin of the decreased HDL-cholesterol concentrations is not clear, but is thought to involve enhanced hepatic lipase activity resulting from resistance at the liver to the inhibitory action of insulin. In this study, the absence of significant abnormalities in lipid levels in unaffected relatives suggests that there is not a major derangement of hepatic and lipoprotein lipase in such predisposed individuals, though they have not been formally tested.

In the group as a whole, fasting plasma concentrations of immunoreactive insulin displayed a tendency to be higher in the relatives than in the controls (53 (8-203) vs 42 (5-134) pmol/l p=0.07). This was also true for the Asian relatives compared to their controls (64 (8-203) vs 38 (17-109) pmol/l p=0.07). Previous findings of elevated plasma insulin levels measured by radioimmunoassay at similar glucose concentrations have been interpreted as indicating insulin resistance (McKeigue et al. 1991) and some have postulated that it reflects altered hepatic handling of insulin (Cruikshank et al. 1991). In view of the possibility that these data were not representative of biologically

active insulin levels due to cross reaction with insulin precursor peptides in the assay (Temple et al. 1989 and 1990), specific immunoradiometric assays were also used in this study. These confirmed that levels of insulin, intact and 32, 33 split proinsulin were not different in relatives and controls, neither in the whole cohort nor in the individual ethnic groups. Furthermore, the proportion of proinsulin-like molecules to total insulin-like molecules was not different between subjects with and without a family history of NIDDM. These results are consistent with smaller, earlier studies on relatives (Clark et al. 1992) and on unaffected members of those populations with an exceptionally high prevalence of NIDDM (Saad et al. 1990). Thus, these normoglycaemic relatives did not possess the disturbances in ß cell function characteristic of patients with raised blood glucose levels, whether due to impaired glucose tolerance or NIDDM (Williams et al. 1991; Davies et al. 1993a and 1993b; Krentz et al. 1993). These results suggest that the disproportionate increase in concentrations of fasting intact and 32, 33 split proinsulin observed in those patients is a consequence of the hyperglycaemia, rather then a primary defect (Nagi et al. 1990; Davies et al. 1993a).

This study reflects insulin status only in the basal unstimulated state and these findings cannot necessarily be extrapolated to other situations; in chapter 8 increased secretion of insulin, intact and 32, 33 split proinsulin was reported in glucose-tolerant relatives of Afro-Caribbean origin after intravenous glucose, and in chapter 9 European relatives exhibited exaggerated 32, 33 split proinsulin levels, though other workers have recorded enhanced proinsulin responses only in relatives with impaired glucose tolerance (Beer et al. 1990). It is therefore uncertain if the abnormal proinsulin response is also dependent on the degree of hyperglycaemia, rather than being implicit to the diabetic state (Levy et al. 1993). In established NIDDM, the elevated levels of 32, 33 split proinsulin correlate with the diabetic dyslipidaemia (Nagi et al. 1990; Davies et al. 1993a). Correlations were not sought in this study as no significant differences were found either in lipid or insulin levels between relatives and controls. Indeed, where metabolic abnormalities have been found, the role of insulin in their development has been questioned (Jarrett 1992). In unaffected Mexican American (Haffner et al. 1990) and white American (Schumacher et al. 1992b) relatives lipid abnormalities decreased or

were abolished after adjusting for the higher immunoreactive insulin levels.

It should be remembered that only approximately 40-60% of the relatives studied would be expected to develop diabetes. The negative findings in this study could reflect the fact that many of these relatives were never destined to become diabetic. It is also possible that those relatives who will develop diabetes are being studied before any of the metabolic defects are manifest; this is unlikely in the light of other reports in normoglycaemic relatives (Eriksson et al. 1989) and in view of the findings in chapters 7-10.

In conclusion, relatives of NIDDM patients with normal glucose tolerance do not exhibit the lipid abnormalities characteristic of NIDDM. Fasting concentrations of total, HDL- and LDL-cholesterol and triglyceride are similar to those of control subjects with no family history of diabetes. In addition, the relatives do not manifest altered fasting levels of insulin, intact or 32, 33 split proinsulin. The absence of significant abnormalities in fasting lipids, insulin and proinsulin in this genetically susceptible population suggests that these metabolic disturbances are secondary to the diabetic state and are not inherited together with the predisposition to hyperglycaemia.

CHAPTER 12:

FINAL DISCUSSION

This thesis has sought to identify metabolic abnormalities in individuals at risk of later non-insulin dependent diabetes in an attempt to elucidate the primary derangement in the pathological sequence of events which ultimately culminates in clinical diabetes. In order to remove the confounding influence of hyperglycaemia, first-degree relatives of NIDDM patients with normal glucose tolerance were selected for study as a model for prediabetes and insulin action and insulin secretion were investigated in these subjects. In this chapter the main implications and limitations of these findings will be discussed.

12.1. INSULIN SENSITIVITY

A large part of the initial section of this work is devoted to the development and validation of the methods used for assessing insulin action. The study population comprised relatively young healthy subjects in whom use of more conventional methods of measuring insulin sensitivity developed for study in diabetic patients (Shen et al. 1970) is not appropriate.

In chapter 7, whole body insulin sensitivity measured with the low dose short insulin tolerance test was found to be markedly reduced in Asian relatives compared to matched controls. This insulin insensitivity was confirmed by the higher plasma insulin levels at equivalent glucose concentrations, both fasting and during the oral glucose tolerance test. In addition, the antilipolytic action of insulin was also impaired in these Asian relatives, in whom fasting circulating glycerol concentrations were elevated and both glycerol and NEFA concentrations failed to suppress after oral glucose. These findings provide unequivocal evidence for insulin insensitivity in relatives of Asian NIDDM patients, before glucose intolerance has developed.

The initial findings in the other two main ethnic groups studied (the Europeans and the Afro-Caribbeans) were less conclusive. Insulin sensitivity assessed by the insulin tolerance test was normal in relatives of white European patients. No disturbance of intermediary metabolite levels was observed in the fasting state, nor following oral glucose. Plasma glucose concentrations were similar to those of controls and although the immunoreactive insulin response to oral glucose was exaggerated, it was not statistically different to that of the control group. This may be explained by one of two

possibilities. Firstly, insulin sensitivity may be normal in these European relatives, as has been reported by other workers (Johnston et al. 1990). If we assume that many of these relatives will eventually develop diabetes, this would imply that a defect in insulin sensitivity is not an early feature of the disease. Although these individuals were being studied many years before we would expect hyperglycaemia to be manifest, if the genetic tendency to NIDDM is present (Barnett et al. 1981a; Köbberling et al. 1985; Newman et al. 1987); one might expect such a defect to be detectable even at this early stage.

The second possibility is that insulin action is defective but that this defect was not apparent with the methods employed. As discussed in chapter 1, inhibition of lipolysis by insulin occurs at ten-fold lower concentrations than that required to stimulate peripheral glucose uptake (Johnston 1989). A minor impairment in insulin action, as may occcur early in the development of NIDDM, could initially present as impaired suppression of lipolysis. However, the magnitude of the insulin response to oral glucose could have prevented the detection of small differences in insulin action on lipolysis between relatives and controls. Although plasma glycerol levels are an accurate guide to lipolysis, enzymatic assays are imprecise at low concentrations. Insulin sensitivity was therefore investigated in the European relatives in more detail by measurement of glycerol turnover assessed isotopically in response to a very low dose insulin infusion. This sensitive method, which was validated in chapter 5, revealed reduced suppression of glycerol appearance following insulin in the European relatives compared to controls. This occurred despite mildly elevated basal insulin levels in these relatives. Thus, insulin insensitivity was present in European relatives with normal glucose tolerance, though only with respect to lipolysis. Hepatic glucose production was normal, but in association with higher fasting insulin concentrations, implies a mild defect exists in the inhibitory action of insulin at the level of the liver as well.

Relatives of Afro-Caribbean patients also had normal insulin sensitivity when examined with the insulin tolerance test and plasma glucose, insulin and glycerol concentrations were similar to those of controls, both fasting and after oral glucose. However, in response to an intravenous glucose bolus these relatives of Afro-Caribbean origin

exhibited hyperinsulinaemia and impaired suppression of plasma glycerol and 3-hydroxybutyrate concentrations, despite having similar glucose levels to controls. This provides evidence of insulin resistance. Hence, insulin insensitivity has been demonstrated in glucose-tolerant relatives of all three ethnic groups studied, though the expression of this defect varied according to ethnic origin.

The consistent abnormality detected in the relatives, regardless of ethnic background, was reduced insulin sensitivity with respect to lipolysis; since this process is normally very sensitive to insulin, if insulin action was defective it is likely that this process would be one of the earliest disturbances to be identified. It suggests that insulin resistance is an early feature of NIDDM and although previous groups have demonstrated insulin resistance in unaffected family members (Lillioja et al. 1987; Eriksson et al. 1989; Warram et al. 1990; Gulli et al. 1992) they have measured reduced insulin action on glucose metabolism. The finding of insulin insensitivity with respect to lipolysis alone in European and Afro-Caribbean relatives implies that this defect is present at a more preliminary stage. The potential pathogenic mechanisms involved are discussed later in this chapter.

These results also imply that the short insulin tolerance test provides only a rough estimate of whole body insulin sensitivity. Values recorded for Afro-Caribbean relatives were normal with this test, but exaggerated insulin responses in the intravenous glucose tolerance test confirmed insulin resistance. Furthermore, European relatives had similar insulin sensitivity measurements to controls with the insulin tolerance test, but were insulin resistant when studied by stable isotopic glycerol turnover measurements. This highlights the importance of selecting the appropriate technique of investigation and justifies the sections of this thesis devoted to method development and validation.

12.2. INSULIN SECRETION

The insulin secretory response to oral and intravenous glucose has been investigated in chapters 7, 8 and 9. Although Asian relatives had fasting hyperinsulinaemia and exaggerated insulin responses to oral glucose using insulin radioimmunoassay, this was not confirmed with measurement of insulin concentrations by specific immunoradiometric assay; insulin levels were similar in relatives and controls, and intact and 32, 33 split proinsulin concentrations were not significantly different. This could suggest that the documented immunoreactive hyperinsulinaemia was not indicative of biologically active insulin; however, if intact and 32, 33 split proinsulin levels were not elevated and 65, 66 split proinsulin levels have previously been reported as negligible in both normal controls and patients with diabetes (Sobey et al. 1989; Temple et al. 1989; Nagi et al. 1990), the cause of the higher insulin concentrations measured by radioimmunoassay requires further investigation. Nevertheless, since insulin resistance is defined as "a metabolic state in which physiological concentrations of insulin produce a less than normal biological response" (Caro 1991), the Asian relatives are indeed insulin resistant, despite the discrepancy in insulin assay results.

Plasma levels of IRMA insulin, intact proinsulin and 32, 33 split proinsulin were also similar in European and Afro-Caribbean relatives and their own controls, fasting and during the oral glucose tolerance test. However, in response to intravenous glucose, insulin secretory defects became apparent. European relatives exhibited exaggerated secretion of 32, 33 split proinsulin, which was disproportionate to the total secretion of insulin and proinsulin-like molecules. Immunoreactive insulin and insulin levels measured by IRMA assay were normal, as were the glucose and metabolite concentrations. Intact proinsulin secretion tended to be higher in relatives, but was not statistically different with the number of subjects studied.

In the Afro-Caribbean relatives the disturbances in insulin secretion following intravenous glucose were even more marked. These relatives possessed increased secretion of 32, 33 split proinsulin, intact proinsulin and insulin which was disproportionate for the plasma glucose levels. The proportion of proinsulin-like molecules to total insulin was also abnormal. This implies that a defect in ß cell insulin

processing exists in both the European and Afro-Caribbean relatives, though it appears more severe in the latter.

No significant differences in insulin secretory response to intravenous glucose were apparent in the Asian relatives with no evidence of a ß cell defect. Indeed, in this group despite the relatives having lower fasting glucose concentrations, immunoreactive insulin levels tended to be higher than in their controls, though this difference did not reach statistical significance, and IRMA insulin concentrations were similar. Blood glycerol, NEFA and 3-hydroxybutyrate concentrations were elevated in the relatives, both fasting and after intravenous glucose. Hence, this is further evidence for insulin resistance in these Asian relatives.

In chapter 11, in order to increase the number of subjects studied, fasting plasma levels of insulin, intact and 32, 33 split proinsulin were examined in 15 Asian relatives and controls. The previously observed tendency to higher immunoreactive insulin concentrations in the relatives persisted, but the levels of insulin, intact and 32, 33 split proinsulin measured by immunoradiometric assays were similar to those of controls.

Although no secretory defect in insulin secretion could be demonstrated in relatives of Asian origin this does not deny the existence of such an abnormality. A larger number of relatives had been studied but considerable difficulty was encountered with recruitment of controls, resulting in matching for the intravenous glucose tolerance test being possible for only seven subjects. The problems with ascertainment of control subjects in such studies are well recognised (O'Rahilly et al. 1988b) and are particularly evident in communities such as the Asians and Afro-Caribbeans (Mather and Keen 1985; Cruikshank et al. 1991) in whom the disease has a high prevalence and few subjects have no family history of diabetes. The control subjects were formally tested to ensure normal glucose tolerance, but absence of a positive family history was accepted from memory recall from controls, as it was not practical to test all their family members and thus subclinical diabetes in their families cannot be excluded. In addition, the presence of ethnic differences in disease pathogenesis emphasises the need for close matching of relatives and controls for ethnic origin. The ethnic groups chosen in this

thesis were broad to allow recruitment of sufficient numbers of subjects, but this may have been at the expense of masking small differences in ß cell function. Evidence exists for variation in the insulin secretory response between African and American blacks (Osei et al. 1993) and different prevalence rates and metabolic characteristics of NIDDM in different Asian regional and religious communities have been reported within the same geographical region (Ramaiya et al. 1991; McKeigue et al. 1991); Simmons et al. 1992; Simmons and Powell 1993). Thus, we cannot exclude the presence of insulin secretory abnormalities in Asian relatives at risk of future NIDDM. Although circulating plasma levels of insulin and proinsulins were taken as an index of secretion, as discussed in chapters 8 and 9, this assumes that peptide clearance is normal. Measurement of C-peptide levels may have confirmed the state of secretory function.

In chapters 7, 8 and 9 insulin secretory function as defined by glucose-induced levels of IRMA insulin, intact and 32, 33 split proinsulin, was abnormal in the Afro-Caribbean and European relatives in response to intravenous glucose but not to oral glucose. This most probably reflects the method of study, since oral glucose-stimulated insulin secretion integrates a variety of reponses including changing glucose absorption, the influence of gut hormones and cephalic-induced insulin release (Leahy 1990). The secretory responses induced by oral and intravenous glucose therefore are not equivalent (Gibby and Hales 1983) and the intravenous glucose tolerance test is a more reliable indicator of glucose-induced insulin secretion (McDonald et al. 1965; Rayman et al. 1990). Hence, the abnormalities of ß cell function recorded after the intravenous glucose bolus are likely to be genuine.

12.3. RELEVANCE TO THE PATHOGENESIS OF NIDDM

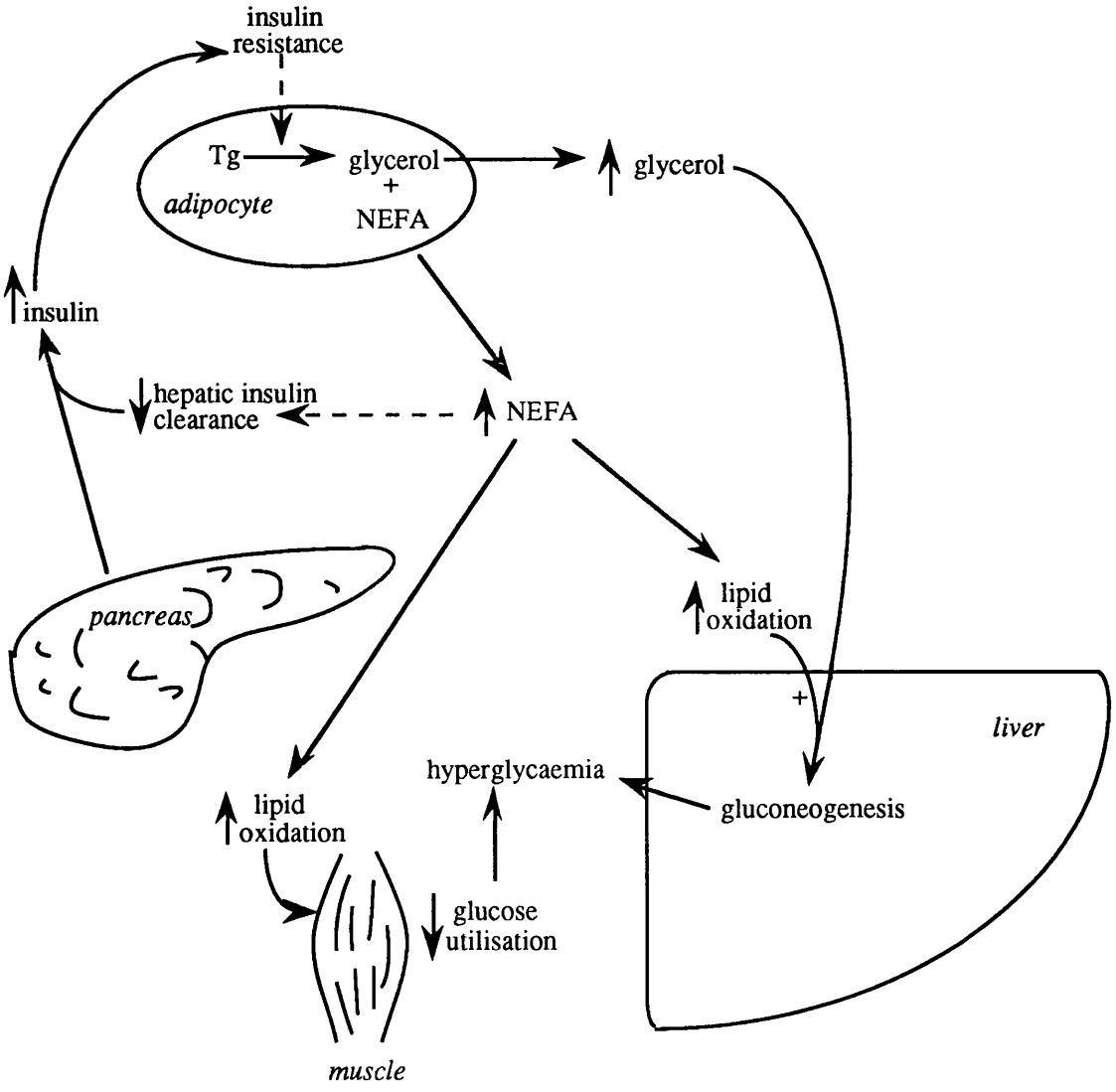
Throughout this thesis two prevailing themes have become apparent: the heterogeneous nature of NIDDM with a different combination of metabolic defects identified in the individual ethnic groups and the common finding of insulin resistance with respect to lipolysis in relatives irrespective of ethnic origin. At this juncture it is tempting to speculate how such a disturbance in insulin action could lead to the abnormalities typical of frank NIDDM.

Resistance to insulin's inhibition of hormone-sensitive lipase in adipose tissue would result in increased production of glycerol and NEFA. Compensatory hyperinsulinaemia would occur as insulin secretion is augmented to overcome the defect in insulin action. The elevated NEFA levels would also decrease hepatic clearance of portal insulin, contributing to the hyperinsulinaemia. In the early stages of the disease this disturbance may be mild, and as in the European relatives studied in chapter 10, only a small increase in glycerol turnover may be detected; the basal hyperinsulinaemia is sufficient to maintain hepatic glucose ouptut at a normal rate.

As resistance to insulin action becomes more marked, the increased glycerol produced undergoes conversion to glucose in the liver and the rise in NEFA availability causes elevated rates of lipid oxidation, which accelerate the glycerol gluconeogenesis. Indeed, in chapter 10, glycerol turnover was positively associated with the basal NEFA concentration in the European relatives but not in their controls, indicating that just such a defect in hepatic glycerol handling already existed (Nurjhan et al. 1992; Puhakainen et al. 1992). Hence in NIDDM, hepatic glucose production would rise due to both increased glycerol availablility and altered intrahepatic handling of glycerol to accelerate gluconeogenesis. Initially this may be counterbalanced by a reduction in hepatic glycogenolysis (DeFronzo 1992) so that net hepatic glucose production is normal, but eventually the restraining effect of hyperinsulinaemia would be lost and increased hepatic glucose production and fasting hyperglycaemia would result. The excessive rate of NEFA oxidation would also impair glucose oxidation via the glucose-fatty acid cycle described in chapter 1 and inhibit glycogen synthase activity. This would reduce insulin-stimulated glucose uptake in muscle, resulting in hyperglycaemia. Thus, the defect in

insulin action on lipolysis identified in relatives and common to all the ethnic groups studied could ultimately lead to overt NIDDM (Figure 12.1).

Figure 12.1. Diagrammatic representation of the metabolic derangement originating from insulin resistance with respect to lipolysis.



Tg = triglyceride

In the Asian relatives studied a number of these metabolic abnormalities were already present. Glycerol and NEFA suppression to oral and intravenous glucose-induced insulin secretion were impaired despite hyperinsulinaemia (chapters 7 and 9). Unlike in the European relatives, total body insulin sensitivity during the short insulin tolerance test which predominantly reflects insulin-stimulated glucose uptake, was also reduced

(chapter 7). Although the subjects were not characterised in terms of body fat distribution, the greater severity of the metabolic defect in the Asian population may relate to the increased central adiposity identified in this community (McKeigue et al. 1991); compared to subcutaneous fat, abdominal adipocytes are less sensitive to insulin and are up to four times more sensitive to lipolytic stimuli (Björntorp 1991). It may also relate to the higher prevalence of NIDDM and younger age of onset of the disease in this ethnic group so that these relatives are closer in time to developing overt NIDDM

A further consequence of the insulin resistance and hyperinsulinaemia is hypertriglyceridaemia, with increased VLDL secretion. Despite the presence of insulin insensitivity with respect to lipolysis in all the relatives studied, the absence of any lipid abnormality in these subjects reported in chapter 11 indicates that the hyperlipidaemia characteristic of NIDDM occurs as a later consequence of the metabolic disturbance and that possibly environmental factors are of significance.

The demonstration of a common initial metabolic abnormality in individuals genetically predisposed to NIDDM which could potentially lead to overt NIDDM begs the question *'where is the site of the genetic defect?'* As discussed in chapter 1, the identification of mutations in the glucokinase gene in certain MODY pedigrees was followed by the functional demonstration of a defect in insulin secretion (Velho et al. 1992). The challenge here is to examine candidate genes, mutations in which could lead to the pathological sequence outlined above. Possible candidate genes include those for the adipocyte insulin receptor, the SH_2 containing proteins interacting with the insulin receptor substrate, second messengers or hormone-sensitive lipase itself. The progress of genetic studies in NIDDM to date (O'Rahilly 1993; Leahy and Boyd 1993) however, suggests that this is unlikely to reveal a single mutation in all families, but that rather a polygenic aetiology with associated environmental influences will compound to produce the clinical phenotype expressed.

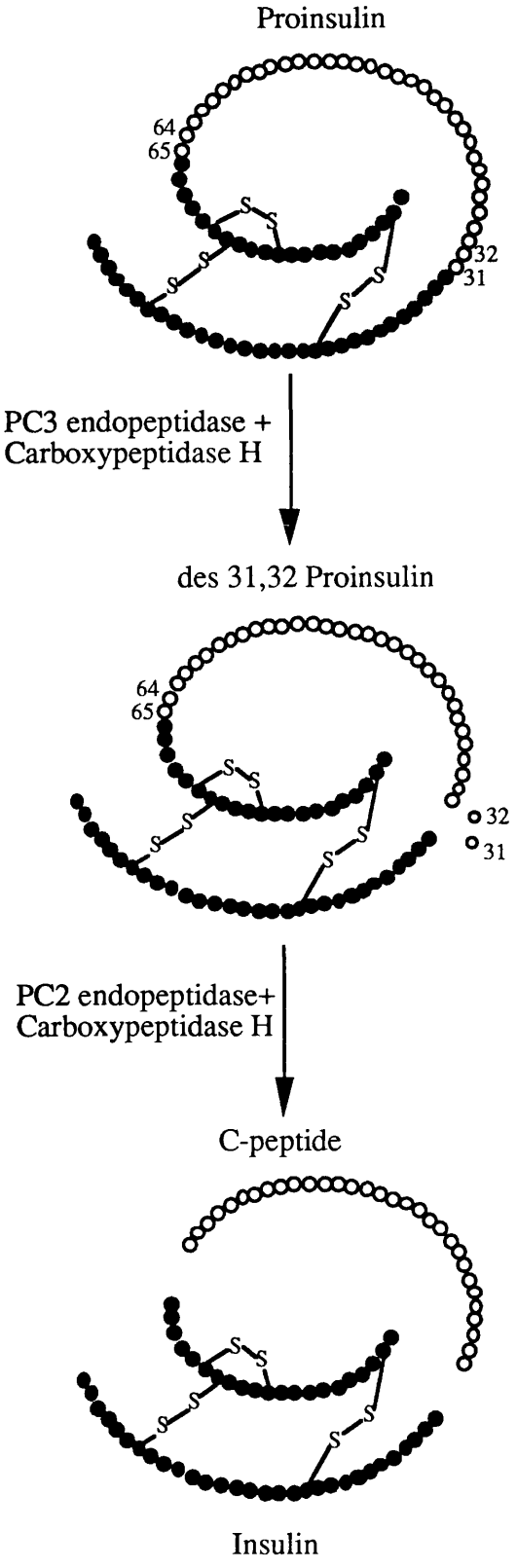
A complimentary approach is to quantify the severity of the defect in lipolysis and this could be accomplished fairly simply by the glycerol clamp technique described in chapter 6, or by the glycerol turnover method of chapter 10. Serial studies could be

undertaken in first-degree relatives of NIDDM patients to monitor the deterioration or otherwise of their insulin sensitivity with respect to lipolysis, which may identify those individuals progressing to diabetes and the underlying sequence of pathological events as they occur. A similar approach has been adopted for monitoring deterioration in glucose metabolism in other high risk populations (Lillioja et al. 1988; Saad et al. 1989), but the glycerol clamp allows examination of these processes at an earlier stage.

It should not be forgotten that this thesis has also identified abnormalities in insulin secretion as well as in insulin action. In chapter 9 the increased 32, 33 split proinsulin levels observed in response to intravenous glucose in European relatives, despite normal glucose levels, could represent a primary defect in ß cell insulin processing. The higher intact and 32, 33 split proinsulin responses to intravenous glucose exhibited by Afro-Caribbean relatives in chapter 8 compared to their controls support this conclusion. In recent years a number of advances have provided a clearer understanding of the proinsulin conversion mechanism and may help to provide clues to the abnormalities responsible for these results.

Although proinsulin may be cleaved at either the Arg^{31}, Arg^{32} site by PC3 or at the Lys^{64}, Arg^{65} site by PC2, the higher quantities of the 32, 33 split intermediate compared to the 65, 66 form present in the circulation (Sobey et al. 1989) and as transient intermediates (Sizonenko et al. 1993) suggest that PC3 is the preferential route of conversion (Figure 12.2). Furthermore, PC2 cleaves the des 31, 32 proinsulin intermediate more readily than it cleaves intact proinsulin; PC2 fails to perform cleavage efficiently if the Arg^{31}, Arg^{32} has undergone point mutation to Arg^{31}, Gly^{32} (Docherty et al. 1989) and the intact proinsulin cleavage at Lys^{64}, Arg^{65} by PC2 is relatively less efficient than PC3 cleavage at Arg^{31}, Arg^{32} (Rhodes et al. 1992). These findings suggest that the pathway via 32, 33 is the preferential route of proinsulin processing (over 95%).

Figure 12.2. Preferential route of proinsulin processing.



This is also reflected in the characteristics of the processing enzymes themselves (Sizonenko et al. 1993). They are both Ca^{2+} and pH dependent, but whereas PC2 is half maximally activated at between 50-100μM Ca^{2+}, PC3 is only active above 0.5mM Ca^{2+}. In addition, both enzymes have pH 5.5 optimum but PC2 is more active than PC3 at neutral pH. Thus PC3 is more strictly regulated. PC2 is active over a broader range of conditions and may also be active in the transgolgi complex where it can produce small quantities of des 64,65 proinsulin. The ß granule in the ß cell however, is the main site of proinsulin processing and has an acid pH 5.5 and free calcium concentration of 1-10mM. The narrow biochemical requirements for PC3 activity ensure that this enzyme is only active in the ß granule and PC3 is likely to initiate and regulate the proinsulin processing mechanism.

Carboxypeptidase H is normally present in high quantities in the ß granule so that most of the conversion intermediates present in the circulation are in the des peptide form. Since the immunoradiometric assays employed in this thesis do not distinguish between split-proinsulin molecules and their des peptide derivatives, the levels of 32, 33 split proinsulin includes both 32, 33 split proinsulin and des 31, 32 proinsulin.

Hyperproinsulinaemia could thus result from a number of potential defects in this mechanism. Structural mutations of the processing enzymes would produce hyperproinsulinaemia from accumulation of precursor peptides. Analysis of the PC2 gene in NIDDM families is currently underway. Such genetic variants provide an attractive explanation for the proinsulin abnormalities detected in family members, but are likely to be rare. Defective glucose sensing mechanisms by the ß cell may also cause secondary defects on insulin processing.

Other potential sites of defect relate to the regulation of the conversion mechanism. Proinsulin synthesis is stimulated by glucose both at the level of transcription and translation and at least in the short term, glucose stimulation produces a proportional increase in PC3 synthesis at the level of translation, confirming the importance of PC3 as the controlling enzyme in proinsulin processing (Alarcón et al. 1993). Synthesis of

PC2 and carboxypeptidase H, however, is not glucose-regulated, but during glucose stimulation proinsulin processing will proceed efficiently provided adequate PC2 and carboxypeptidase H are already present in the ß cell. It is not known whether transcription of the PC3 and PC2 genes is co-regulated by glucose in parallel with that of the preproinsulin gene. Defective transcriptional regulation of PC3 by glucose could account for the increased glucose-induced intact proinsulin concentrations observed. Glucose would increase proinsulin synthesis which might then outstrip the processing capability, resulting in a disproportionate increase in intact proinsulin. Deficient regulation of PC3 translation by glucose could also result in less efficient proinsulin processing due to deficiency of the initiating enzyme.

Glucose stimulation may produce a discrepancy in the ratio of PC3 and PC2 enzymes as the former is regulated at the translational level by glucose but the latter is not. PC3 synthesis would increase coordinate with that of proinsulin, but PC2 synthesis would lag behind until the amount of PC2 would become rate limiting for proinsulin processing (Rhodes and Alarcón 1994). This could explain the disproportionate elevation in concentrations of 32, 33 split proinsulin observed, although it is not clear whether this could occur in the situation of short term glucose stimulation of the intravenous glucose tolerance test. The endopeptidases PC2 and PC3 are glycoproteins which must undergo post-translational cleavage to produce their mature forms (Alarcón et al. 1993). Although it is not known whether this modification affects their proteolytic activities, a defect in the post-translational processing of PC2 and/or PC3 could theoretically reduce their activities and may lead to disproportionate elevation of intact or 32, 33 split proinsulins.

Several other possible explanations exist for the insulin secretory defects reported in the Afro-Caribbean and European relatives. Alteration in the Ca^{2+} and pH conditions in the ß granule would adversely affect PC2 and PC3 activity, for example due to defects in activation of the proton pump ATPase or Ca^{2+} transporting proteins. Inefficient targeting of PC2, PC3 and proinsulin to the ß granules would cause increased secretion by the constitutive pathway with deficient proinsulin processing (Halban 1991).

Hyperproinsulinaemia could also signify the ß cell response to increased demand for insulin induced by insulin resistance (Ward et al. 1987). When the ß cell is stressed newly synthesised proinsulin is preferentially released, but the biochemical environment outside the ß cell is not conducive for proinsulin processing and a disproportionate amount of proinsulin would accumulate in the circulation. It is possible that a combination of these speculated defects is responsible for the proinsulin abnormalitites found in the first-degree relatives studied.

The observed defects were not similar in all three ethnic groups studied suggesting that the causative abnormality is not necessarily fundamental to the initial metabolic insult. Alternatively, the elevated 32, 33 split proinsulin levels could be indicative of an early response to insulin resistance with respect to lipolysis, prior to the expression of increased secretion of mature insulin. This explanation is less likely as the ethnic population found to be most overtly insulin resistant, the Asians, were the only group of relatives in whom no demonstrable defect in proinsulin or insulin secretion was evident.

It could be argued that absolute insulin secretion or insulin sensitivity are of less significance in the pathogenesis of NIDDM, but rather it is the relationship between these two factors that is of paramount importance. This has been proposed in the aetiology of NIDDM in both the Pima Indians and in the American and European white populations (Johnston et al. 1990; Martin et al. 1992; Lillioja et al. 1993). Certainly the plasma levels of intact and 32, 33 split proinsulin comprised only a small proportion of the total insulin-like molecules, suggesting that they are unlikely to have a primary pathogenic role. The exact combination of genetic defects may depend on ethnic origin; in each ethnic group additional metabolic abnormalities were identifed, apart from insulin insensitivity with respect to lipolysis. This may reflect the heterogeneous nature of NIDDM and multiple inherited metabolic defects may produce the same clinical phenotype. The nature of the secretory defect may depend on the dynamics of insulin secretion which are known to be perturbed in NIDDM (Polonsky et al. 1988a) and in impaired glucose tolerance (O'Rahilly et al. 1988a). In chapter 8, the first-phase immunoreactive insulin response to intravenous glucose peaked later in the relatives

than in the controls, despite similar glucose values in the two groups. In the intravenous glucose tolerance studies, the tests were terminated before insulin or proinsulin values returned to baseline levels; theoretically this may have prevented recognition of differences in the secretory response in Asian relatives compared to their controls, but in practice values for insulin, intact and 32, 33 split proinsulin displayed such similar trends in both groups that prolonging the test was unlikely to change the interpretation of the results.

A further consideration is whether the defects in insulin secretion and action identified in the relatives could represent the consequences of poor fetal and early post natal nutrition (Hales and Barker 1992). Impaired development of the fetal pancreas has been purported to explain the signs of ß cell failure characteristic of NIDDM (Hales et al. 1991) and abnormal muscle structure to result in reduced insulin-stimulated glucose uptake in later life (Phillips et al. 1994). Insulin sensitivity with respect to lipolysis, the defect shared by all three populations studied, has not been explained on this basis. Such metabolic programming may have a role in the aetiology of the disturbance, but in view of the large body of evidence for the genetic basis of NIDDM discussed in chapter 1, if present it is likely to be minor.

12.4. CONCLUSIONS

1. First-degree relatives of patients with NIDDM possess abnormalities in insulin action and insulin secretion whilst glucose tolerance is still normal.

2. Insulin insensitivity to lipolysis can be detected using novel techniques employing low dose insulin, when not apparent by measurement of the response of glucose metabolism alone.

3. Insulin resistance with respect to lipolysis is a universal finding in first-degree relatives of all ethnic groups studied. It may thus represent the earliest defect in NIDDM and could explain the metabolic derangement characteristic of NIDDM. Relatives of Asian origin also demonstrate resistance to insulin-stimulated glucose disposal.

4. Disturbances in insulin processing with increased and disproportionate glucose-stimulated concentrations of 32, 33 split proinsulin exist in glucose-tolerant first-degree relatives of NIDDM patients of Afro-Caribbean or European origin.

5. The absence of any significant disturbance in serum lipoproteins in the glucose-tolerant relatives suggests that the diabetic dyslipidaemia is not a primary feature of NIDDM, but is a consequence of hyperglycaemia.

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