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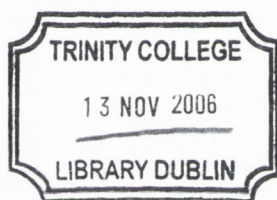
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**THE PATHOPHYSIOLOGY OF INSULIN
RESISTANCE AND MODULATION BY AEROBIC
EXERCISE IN OBESE AND TYPE 2 DIABETES
SUBJECTS**

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Submission to the Faculty of Health Science, University of Dublin for
the degree of PhD by thesis, September 2004



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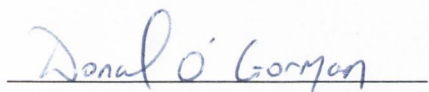
This thesis is dedicated to Lucy and our future

and

In recognition of my parents, Breda and John

AUTHOR'S DECLARATION

1. The work contained herein has not been submitted as an exercise for a degree at this or any other University.
2. The collection of data and content of this thesis is entirely my own work. The analysis of collected serum using an *in vitro* model (Chapter 3) was conducted by Dr. Philip Newsholme, University College Dublin.
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Donal O'Gorman

SUMMARY

1. Insulin resistance is a major contributing factor to the development of Type 2 diabetes and cardiovascular disease. It has been difficult to characterise the aetiological progression because of the complex metabolic milieu that is influenced by environmental and genetic factors.
2. Within the broad classification of diabetes, it is becoming clear that sub-phenotypes exist which are characterised by varying rates of development for insulin resistance and insulin secretory dysfunction. Determining the phenotypic characteristics could help to devise more specific treatment strategies for these patients.
3. Only recently have pharmacological agents been developed to specifically treat insulin resistance. The most underutilised, yet most effective, form of treatment for insulin resistance is exercise as it can improve insulin sensitivity in addition to improving a wide range of health parameters.
4. In order to further our knowledge of insulin resistance, two distinct sub-phenotypes of Type 2 diabetes were examined. In a separate experiment, modulation of insulin-mediated glucose disposal by exercise was used to investigate the cellular regulation of insulin resistance.
5. The presence of glutamic acid decarboxylase (GAD) antibodies is associated with an immune response in the β -cell and impaired insulin secretion. However, between 9-16% of patients initially diagnosed with Type 2 diabetes are GAD positive and insulin resistant. With a frequently sampled intra-venous glucose tolerance (FSIGT) test, we examined the relationship between insulin resistance and insulin secretion in subjects who were either GAD-antibody positive or negative. An *in vitro* model was also used to investigate the role of serum containing factors in the development of β -cell dysfunction. Both groups were severely insulin resistant though the GAD-negative group was more resistant and had a better insulin secretory response. The insulin secretory data was supported by the *in vitro* experiments and indicated that the presence of activated complement in serum was related to insulin secretory dysfunction. Therefore, GAD-positive subjects share characteristics of both Type 2 diabetes and Type 1 diabetes. We describe an insulin resistant sub-phenotype who fail to compensate for insulin resistance because of insulin secretory dysfunction, resulting in an earlier onset of Type 2 diabetes.

6. The worldwide prevalence of Type 2 diabetes has increased dramatically and has led to a decrease in the age of diagnosis. Early onset Type 2 diabetes, where the subjects are less than 25 years, is associated with at least two generations of a family history, obesity, low or high birth weight, and the onset of puberty. These characteristics have been reported most frequently in minority ethnic groups. In addition to diabetes control, these individuals are at risk of diabetes-related complications, especially cardiovascular disease, throughout their lives. We used the FSIGT test to determine insulin sensitivity and insulin secretion in a group of young subjects with Type 2 diabetes, an age-matched obese group and an older group of Type 2 diabetes subjects. Both Type 2 diabetes groups were severely insulin resistant and the older group had a lower insulin secretory response. The young obese subjects were insulin resistant but maintained glucose tolerance by hyperinsulinemia. Early onset Type 2 diabetes in Caucasian subjects is similar to 'classical' Type 2 diabetes but confers a greater long-term risk to patients, the health service, society and the economy.
7. Exercise has been shown to improve glucose disposal during and in response to exercise by two distinct mechanisms. During exercise glucose transport is facilitated by an insulin-independent mechanism while following exercise, whole body insulin-mediated glucose disposal is augmented. Type 2 diabetes and obesity are characterised by insulin resistance and decreased whole body glucose disposal. The present study was designed to determine the function of IRS-1- and IRS-2-associated PI3-kinase activity and the expression of GLUT-4 in obese and Type 2 diabetes subjects following acute and chronic exercise. Subjects underwent a $40\text{mU}\cdot\text{m}^{-2}\cdot\text{min}^{-1}$ euglycaemic-hyperinsulinemic at baseline, 16-hrs after a bout of exercise and 16-hrs after 7 consecutive days of exercise training. Glucose infusion rates increased significantly in the Type 2 diabetes following 7-days of exercise. The obese group also improved, though not significantly. However, their insulin sensitivity was close to the normal range after the 7 days of exercise. Changes in IRS-1- and IRS-2-associated PI3-kinase activity were similar but do not explain changes in glucose disposal. Phosphotyrosine-associated PI3-kinase was elevated in the Type 2 diabetes subjects but reduced in the obese group, indicating that factors other than IRS-1 and IRS-2 may be responsible for PI3-kinase activity in these groups. GLUT-4 protein expression also did not change, indicating the functional changes in the signalling cascade or alternative mechanisms are responsible.

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Chapter 1

Introduction and background

The changing trends in Type 2 diabetes and obesity

The incidence of chronic disease has increased dramatically since the latter half of the 20th century. During this time there have been tremendous technological development that has drastically changed our lifestyles. Dietary habits have changed with the advent of processed foods, pre-packaged foods and fast foods. Physical activity levels have been curtailed directly by automated systems such as motorised transport and indirectly by leisure time activities including television and computers. The combination of adverse dietary habits and diminished physical activity contributes to the increased incidence of chronic disease.

Obesity, according to the World Health Organisation, has increased to epidemic proportions (12). In the United States 61% of the population are considered either overweight (34%) or obese (27%) (246). Some 300,000 deaths each year are attributed to unhealthy dietary habits and inactive lifestyles (7). The increase in prevalence of obesity is also associated with increased economic cost. In 1995 the direct and indirect costs associated with obesity were \$99 billion. This figure rose to an estimated \$117 billion in 2000 and will continue to place economic stress on health services and resources (365). Type 2 diabetes, hypertension and cardiovascular disease account for most of the obesity-related costs. This trend is not exclusive to the United States. Obesity rates among the adult population in Ireland have increased from 11% in 1990 to 18% in 2000 (253). Other countries are experiencing increases in obesity and the International Task Force on Obesity estimates that in Europe 10-20% of males and 10-25% of females are obese (171,396)

Weight gain, obesity and fat distribution are all risk factors for Type 2 diabetes with body mass index (BMI) being the dominant predictor (55,67). In 1985 the World Health Organisation estimated that 30 million people around the world had diabetes. By 2000 it was estimated that 130 million had the disease, and this is expected to rise to 300 million by 2025 (12,201,412). In the U.S., it is estimated that the number of people diagnosed with diabetes will rise 165% to 29 million by 2050 (43). The current cost of

diabetes care for the 12.1 million people in the United States was \$132 billion in 2002 (9). Of the \$91.8 billion in direct medical costs, treatment for complications and the excess prevalence of general medical conditions accounted for \$68.7 billion. These costs do not include the 22.6% of overweight Americans who have pre-diabetes (26). In Ireland, 6% of the healthcare budget is spent on diabetes treatment with 59% of this amount spent on the treatment of diabetes-related complications.

With obesity and Type 2 diabetes now recognised as epidemics, their strong inter-relationship and the fact that they are the two biggest contributors to cardiovascular disease require drastic action. Our understanding of the pathophysiology of the disease as well as the development of effective treatment and preventative strategies are paramount to overcome this spiralling problem.

Insulin resistance in Type 2 diabetes and obesity

Type 2 diabetes and obesity are both characterised by insulin resistance or decreased insulin-mediated glucose disposal. Insulin resistance is one of the earliest detectable metabolic defects, also present in first-degree relative of patients with Type 2 diabetes (262), indicating a genetic predisposition. However, environmental factors including physical activity and diet strongly influence the development of insulin resistance. Insulin resistance alone is not enough to develop Type 2 diabetes, as insulin secretory dysfunction is also required, but it does increase the risk of disease. In addition to being a risk factor for Type 2 diabetes, insulin resistance is an independent risk factor for cardiovascular disease (93) and hypertension (116,286) and is associated with a cluster of risk factors known as the Metabolic Syndrome (4). Varying definitions of the Metabolic Syndrome have been devised by the WHO (4), the National Cholesterol Education Programme (111) and the European Group on Insulin Resistance (19).

In Scandanavia, 10% of females and 15% of males with normal glucose tolerance have the Metabolic Syndrome (176). This increases to 42% and 64%, respectively, in those with impaired fasting glucose or impaired glucose tolerance and to 78% and 84%, respectively, in patients with Type 2 diabetes. The Metabolic Syndrome confers elevated risk for coronary heart disease (176,214,93,139,45,399). In a large population-based study the risk of coronary heart disease and stroke was 3-fold greater in subjects with the Metabolic Syndrome and cardiovascular mortality was 12% versus 2% for

those without the syndrome (176). A recent meta-analysis of 11 prospective European studies showed that the metabolic syndrome was present in 15% of Europeans without diabetes. These individuals had an increased all-cause and cardiovascular mortality (167). The Bogalusa Heart Study found that obesity and hyperinsulinemia in parents may underlie familial clustering of the Metabolic Syndrome (57) but also that childhood obesity and fasting insulin were predictive of adult development of the syndrome (335).

Background to the experimental projects

Insulin resistance is a major contributing factor to the development of Type 2 diabetes and cardiovascular disease. In order to further our knowledge of insulin resistance, the following experiments were designed to investigate the contribution of insulin resistance to the aetiology of Type 2 diabetes and the physiological adaptations resulting from improved insulin sensitivity.

TABLE 1.1. Overview of research study design

Study I The physiological role of insulin resistance in the early development of diabetes. This study will focus on GAD antibody positive subjects initially diagnosed with Type 2 diabetes	Approximate age at onset of Type 2 diabetes 10 20 30 40 50 60 70 years Typical Type 2 diabetes GAD antibody T2DM
Study II The relationship between insulin resistance and secretion in young Type 2 diabetes subjects; a comparison with older Type 2 diabetes subjects and young obese subjects	Approximate age 10 20 30 40 50 60 70 years Young onset T2DM Obese age-matched Typical Type 2 diabetes
Study III The effects of 1- and 7-days of exercise as on insulin sensitivity in obese and Type 2 diabetes subjects	Typical Type 2 diabetes Obese age-matched Exercise sessions 1 2 3 4 5 6 7 8

Study I. Insulin resistance in GAD antibody positive subjects initially diagnosed with Type 2 diabetes

Type 2 diabetes accounts for approximately 90% of all diabetes patients. Despite decades of intensive investigation, the pathogenesis of Type 2 diabetes is not fully understood due to the complex interplay between genetic and environmental factors (144,370). It is now recognised that, in the broad characterisation of diabetes, sub-phenotypes exist with differing rates of progression of insulin resistance and insulin secretory dysfunction. Among these sub-phenotypes is a form of slowly progressive immune diabetes in adults who initially present with features typical of Type 2 diabetes (361), also known as Latent Autoimmune Diabetes in Adults (LADA). According to the American Diabetes Association the definition of Type 2 diabetes ranges 'from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with insulin resistance' (112), yet the World Health Organisation classify LADA as slow onset Type 1 diabetes (5). The relative contribution of insulin resistance and insulin secretory dysfunction to the pathophysiology of diabetes in this sub-phenotype is not fully understood.

LADA is characterised by circulating antibodies directed to islet cells (135,364), insulin (182) and more frequently to glutamic acid decarboxylase (GAD) (360,364,411) that appear to contribute to the destruction of β -cells in a similar manner to Type 1 diabetes. As GAD antibody status is most common and stable over time, the presence of these antibodies has become the focus of this particular study. Small (184,346) and large population (364,360) based studies have reported a prevalence of 9-16% for GAD antibody positive patients with a clinical presentation of Type 2 diabetes similar to the 12% of patients in our clinic. This sub-phenotype receives relatively little attention in clinical practice but understanding the relative contribution of insulin resistance and insulin secretory dysfunction is essential for the treatment of this common cohort of patients.

While LADA is associated with β -cell destruction, on clinical presentation GAD-positive patients tend to possess many of the phenotypic characteristics of Type 2 diabetes. Patients usually present with non-ketotic hyperglycaemia, overweight, insulin resistance, blunted insulin secretion and a positive response to diet and/or oral hypoglycaemic agents. However, when antibody status is also known, it is possible to

differentiate this sub-phenotype from the broad categorisation of Type 1 and Type 2 diabetes. GAD-positive patients are overweight, but usually less obese than GAD-negative Type 2 diabetes subjects (184,364,411) and not as lean as typical Type 1 diabetes subjects (360). GAD-positive patients tend to be as insulin resistant but have a lower fasting and glucose stimulated insulin and C-peptide response, even allowing for duration of diabetes (411,251,51,360). In 5- and 10-year follow-up studies, GAD-positive subjects had significantly greater rates of insulin deficiency (251).

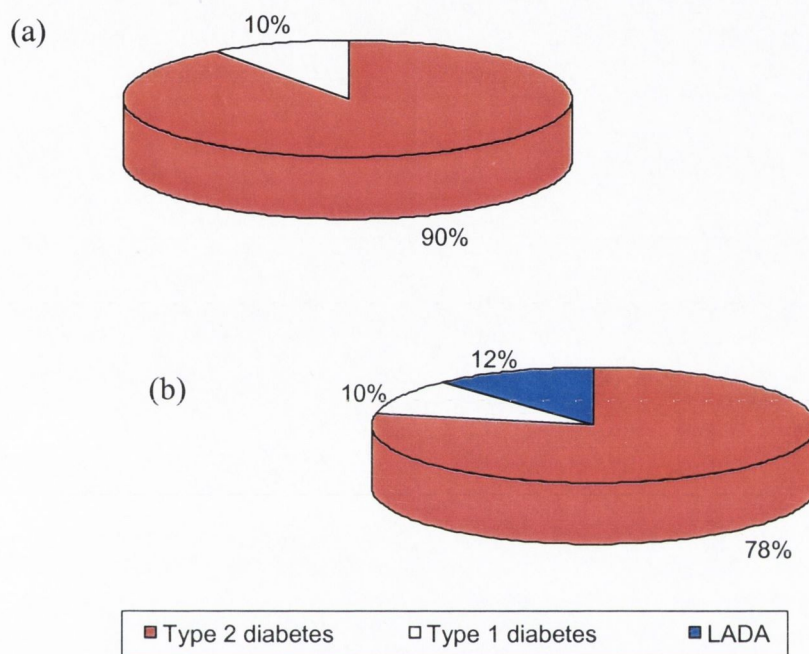


FIGURE 1.1. (a) The prevalence of Type 2 diabetes is far greater than Type 1 diabetes; (b) Antibody positive patients, initially diagnosed with Type 2 diabetes, account for 9-16% of this group; comparable to the total number of Type 1 diabetes patients. In our clinic, 12% of patients initially diagnosed with Type 2 diabetes are GAD antibody positive.

However, not all GAD-positive patients become insulin deficient and even after 10 years still have insulin and C-peptide responses significantly greater than Type 1 diabetes (360). GAD-positive patients also tend to have more features of the metabolic syndrome than Type 1 diabetes and have few differences in the prevalence of micro or macrovascular complications compared with GAD-negative Type 2 diabetes (360,177).

Study II. Insulin resistance and early onset Type 2 diabetes

Obesity is strongly associated with Type 2 diabetes and an increase in the prevalence of obesity will result in a parallel increase in Type 2 diabetes. These trends have been

dramatic in very young children. In the United States there was an increase in obesity in 4-5 year old girls (defined as BMI greater than the 95th percentile) from $5.8 \pm 1.2\%$ between 1971-74 to $10.8 \pm 1.4\%$ between 1988-94 (259). During this time the incidence amongst boys did not change significantly (4.4 ± 0.8 vs. $5.0 \pm 0.9\%$). However, even since 1994 there have been further increases with 2-5 year olds increasing from $7.2 \pm 0.7\%$ to $10.4 \pm 1.7\%$, 6-11 year olds increasing from $11.3 \pm 1.0\%$ to $15.3 \pm 1.7\%$ and 12-19 year olds increasing from $10.5 \pm 0.9\%$ to $15.5 \pm 1.2\%$ (258). This trend is not unique to the United States and increases have been reported in many other countries including Brazil, China, Denmark and Malaysia (119,379).

The prevalence of Type 2 diabetes is increasing in adults less than 40 years, including children and adolescents (113,297,326). In Pima Indians the prevalence of Type 2 diabetes amongst adolescents has increased 6-fold to 50.9 per 1000 from 1967-76 to 1988-94 (113). A further study (79) looking at age and gender breakdown between 1967-76 and 1987-96 found an increased prevalence of Type 2 diabetes in 10-14 year old males (0 vs. 1.4%) and females (2.73 vs. 5.31%) as well as 15-19 year old males (2.43 vs. 3.78%) and females (0.72 vs. 2.88%).

As early onset Type 2 diabetes is relatively new, there are not many well-documented case or population based reports in the literature. A case-based study in Cincinnati studied 1027 consecutive patients diagnosed with diabetes between 0-19 years of age. They reported the incidence of Type 2 diabetes amongst adolescents increasing 10-fold, from 0.7/100000 per year to 7.2/100000 per year between 1982 and 1994 (273). As a proportion of new cases of diabetes, Type 2 diabetes accounted for 2-4% before 1992 and 16% by 1994. Amongst 10-19 year olds, Type 2 diabetes accounted for 33% of all new cases. In Canadian aboriginal children with obesity rates of 48% for females and 51% for males, the prevalence of Type 2 diabetes for 10-19 year old girls was 3.6%. Though no boys had Type 2 diabetes, the prevalence of impaired fasting glucose was 2.7% for boys and girls aged 8-18 years (83). Though not population based, other studies have reported young Type 2 diabetes in Hong Kong Chinese patients <40 years (219,250), Mexican Americans (249) and reports have also come from Japan, Libya, New Zealand, Australia and Bangladesh (297,326).

Sinha et al. (329) found that 25% of 55 obese children and 21% of 112 obese adolescents attending an obesity clinic had impaired glucose tolerance. Of the obese adolescents, 4% were found to have Type 2 diabetes. Therefore, the prevalence of Type 2 diabetes amongst obese adolescents also appears to be increasing. However, not all studies have found a high prevalence of impaired glucose tolerance as Invitti et al. (173) found a 4.5% incidence of impaired glucose tolerance in a large cohort of obese Italian children.

One of the difficulties associated with early onset Type 2 diabetes is the correct classification. Until recently, most young people diagnosed with diabetes were presumed to have Type 1 diabetes and commenced on insulin. As the rates of childhood obesity increase, it is more difficult to use physical characteristics to classify Type 1 or Type 2 diabetes. Even individuals < 40 years could have been mistaken for LADA or maturity onset diabetes of youth (MODY). The American Diabetes Association (10) identified this problem and developed a consensus statement to assist in the classification of young onset Type 2 diabetes. They recommend using a combination of antibody presence, fasting insulin/C-peptide and obesity to clinically classify a patient as having Type 1, Type 2, MODY or idiopathic diabetes.

Study III. Exercise as a treatment of insulin resistance and Type 2 diabetes

The clinical treatment of Type 2 diabetes usually involves education, dietary counselling, advice on physical activity but most often the prescription of pharmacological agents. Metformin and sulfonylureas are the most common form of oral medication, suppressing hepatic glucose production and increasing insulin secretion, respectively. However, neither of these medications effectively target the aetiology of the disease, insulin resistance and insulin secretory dysfunction. Thiazolidinediones are a relatively new class of drug that improve insulin resistance by binding to the peroxisome proliferator activated receptor gamma (PPAR γ) isoform, increasing glucose disposal (252). However, physical activity in the treatment of diabetes is not widely used by the medical professional and undervalued as a tool for understanding the cellular, molecular and genetic bases of disease (41). Muscle contraction is known to acutely increase glucose uptake and also enhance insulin-mediated glucose disposal (129). Therefore, the cellular and molecular mechanisms

regulating contraction-mediated glucose transport and enhanced insulin sensitivity could provide attractive alternative therapeutic targets.

Exercise training has many cardiovascular and metabolic benefits. Enhanced oxygen transport and uptake is facilitated by cardiac hypertrophy and increased cardiac contractility resulting in greater stroke volume and cardiac output (257). Increased blood volume, arterial distensibility, capillary recruitment and capillary-to-fibre ratio augment blood flow and distribution (238,145,64). Muscle fibre cross-sectional area increases and aerobic exercise training leads to an increase in the glycolytic/oxidative type IIa fibres (64,145,157). The number and size of mitochondria increase with increased Krebs cycle and β -oxidation enzyme activity (333,64,65,157). In conjunction with increased myoglobin and hexokinase activity, decreased phosphofructokinase and lactate dehydrogenase activity (64), cellular substrate utilisation shifts toward greater free fatty acid oxidation (FFA) at rest and during sub-maximal exercise with decreased lactate production (227). Muscle contraction is a key regulator of the gene expression of structural and functional proteins, including myosin heavy chain IIx, glucose transporter GLUT-4, hexokinase, glycogen synthase, lipoprotein lipase, insulin signalling intermediates, fatty acid transporter CD36, carnitine palmitoyltransferase I and angiogenic regulators (147,41). Therefore, the absence of regular physical activity will lead to decreased rates of protein synthesis and over time impair metabolic and cardiovascular function (41).

Physical inactivity is recognised as an independent risk factor for more than 25 chronic diseases, including Type 2 diabetes and cardiovascular disease, as well as all-cause mortality (41,380). Exercise training is known to improve insulin sensitivity (204) and glycaemic control (42), decrease blood pressure (179), improve plasma lipoprotein and triglycerides levels (92) and improve vascular structure and endothelial function (238). In conjunction with a calorie-reducing diet, exercise is also effective for weight loss and maintenance (365). Taken together, exercise has the potential not only to be an effective treatment for Type 2 diabetes but also for the prevention of diabetes-related complications.

Significantly, lifestyle modification incorporating exercise and dietary factors, has been shown to reduce the incidence of Type 2 diabetes in high risk individuals (97,362). The

Finnish Diabetes Prevention Trial (362) and on a larger scale the U.S. Diabetes Prevention Program (DPP) (97) both found intensive lifestyle modification reduced the incidence of Type 2 diabetes by 58% in subjects with impaired glucose tolerance. Physical activity was a key component of both programmes, in addition to dietary restriction, decreased energy intake from fat and increased dietary fibre. The DPP was stopped one year early because of clearly positive results. The lifestyle intervention group lost on average 7% of body weight in the first year of the study and maintained an average 5% weight loss for the 3 year duration of the study. While 29% of the control group developed Type 2 diabetes, only 14% of the lifestyle modification group progressed. Another group were treated with metformin but 22% of this group developed Type 2 diabetes, making lifestyle modification the more effective strategy.

The Finnish Diabetes Prevention Trial (362) also found that fasting and 2-hr glucose and insulin concentrations were significantly lower in the lifestyle modification group as were triglycerides and blood pressure. There was a trend toward increased HDL-cholesterol ($p=0.06$). The goals of the intervention strategy were (i) to reduce body weight $>5\%$; (ii) Decreased fat intake $<30\%$ of energy intake; (iii) Decrease saturated fat intake $<10\%$ of energy intake; (iv) fibre intake $>15\text{g}/1000\text{kcal}$; (v) exercise >4 hrs/week. A subject who reached four of these five goals, whether they were in the intervention or control group, did not develop Type 2 diabetes. This is the most important finding to date in the field of diabetes prevention.

The role of exercise in the prevention and clinical management of Type 2 diabetes is significantly underutilised. The Nurses Health Study followed 84,941 female nurses from 1980 to 1996 and found 3,300 new cases of Type 2 diabetes (166). They noted that overweight or obesity was the single best predictor of diabetes but those that had a low risk lifestyle had a relative risk of diabetes of 0.09. The per capita medical expenses for people with diabetes in the U.S. in 2002 were \$13,243 compared to \$2560 for people without diabetes (9). The DPP lifestyle programme cost the health care system \$750 per person per annum, including the cost of identifying the IGT subjects (98). Societal costs, which include the direct medical and indirect costs were approximately \$1180 per person per year. Considering that 12 million people in the U.S., or 22.6% of overweight people have prediabetes (26), and the decrease in the age of onset, it would be more beneficial to prevent the development of the disease.

Therefore, lifestyle modification is a cost effective way of preventing Type 2 diabetes, treating high risk individuals and those who have Type 2 diabetes.

Purpose of research projects

The purpose of this series of experiments is to investigate the role of insulin resistance in the pathophysiology of Type 2 diabetes. In particular, the identification of common sub-phenotypes and evaluation of the relative contribution of insulin resistance and insulin secretion to disease development will help to identify more specific prevention and treatment strategies. The effectiveness of exercise as a treatment strategy, and in particular its impact on insulin resistance and the insulin signalling cascade in skeletal muscle, will be determined.

Aims and Objectives

- I. Evaluation of clinical and phenotypic characteristics associated with glutamic acid decarboxylase (GAD) antibody positive subjects diagnosed with Type 2 diabetes.
 - i. To compare the degree of insulin resistance in antibody positive and antibody negative subjects with Type 2 diabetes.
 - ii. To examine the impact of GAD antibodies on *in vivo* and *in vitro* insulin secretion.
 - iii. To determine if complement activation downregulates insulin secretion.
- II. Characterisation of the metabolic and phenotypic traits of early onset Type 2 diabetes
 - i. To quantify insulin sensitivity and compare to an obese, age-matched control group and an older, typical group of Type 2 diabetes patients.
 - ii. To determine if the acute insulin response to glucose is altered in early onset Type 2 diabetes compared to an obese, age-matched control group and an older, typical group of Type 2 diabetes patients.
 - iii. To compare the clinical, metabolic and cardiovascular risk characteristics of subjects with early onset Type 2 diabetes to an

obese, age-matched control group and an older, typical group of Type 2 diabetes patients.

III. Evaluation of the role and effectiveness of exercise as a treatment strategy for Type 2 diabetes

- i. To measure changes in insulin sensitivity following an acute bout of exercise and 7 consecutive days of exercise in obese and Type 2 diabetes subjects
- ii. To measure the impact of acute and chronic exercise on the insulin signalling pathway in obese and Type 2 diabetes subjects.
- iii. To determine if exercise changes glucose transporter expression or activation following acute and chronic exercise in obese and Type 2 diabetes subjects.

Chapter 2

Review of Literature

Insulin and the Regulation of Metabolic Homeostasis

Metabolic homeostasis is regulated by a complex interaction between substrate appearance, the neuroendocrine response, and tissue utilisation. This process is dependent on the pleiotrophic actions of insulin, a potent anabolic hormone secreted from pancreatic β -cells. Though insulin is most commonly associated with the facilitation of cellular glucose uptake (84,141,317), it has a diverse range of functions that regulate many physiological processes. However, maintaining glucose homeostasis by regulating the flux between carbohydrate and lipid metabolism is central to insulin action. This is particularly evident in diabetes (262,86,323), obesity (185), dyslipidemia (128), hypertension (116), cardiovascular disease (45,128) and the metabolic syndrome (139), all associated with insulin resistance.

Glucose and free fatty acids (FFA) are the main substrates for energy production. Both can be derived from exogenous food digestion and endogenous stores. Absorption of digested glucose and triglycerides across the intestinal lumen results in an increase in plasma concentrations, providing substrate for energy production and storage. Insulin is responsible for the regulation of many of these processes, as an increase in plasma glucose stimulates insulin secretion. Insulin stimulates glucose uptake in muscle (141,317) and adipose tissue (260) with glucose sensing, in addition to insulin, necessary for hepatic glucose uptake (265). Cytoplasmic glucose is either stored as glycogen or oxidised in preference to FFA in skeletal muscle. Insulin action in hepatic tissue also inhibits glycogenolysis and gluconeogenesis, leading to net hepatic glucose uptake (265,270). In adipose tissue insulin-stimulated glucose uptake is used to re-esterify FFA as triglycerides for storage and simultaneously suppresses lipolysis, preventing the release of FFA (285). At other times, when plasma glucose is not elevated above baseline, insulin is secreted in a low pulsatile manner, allowing lipolysis to liberate FFA with glycogenolysis and gluconeogenesis maintaining plasma glucose, satisfying tissue energy requirements. Therefore, insulin is the key metabolic regulator because it is a promoter of exogenous substrate disposal and a regulator of endogenous energy availability.

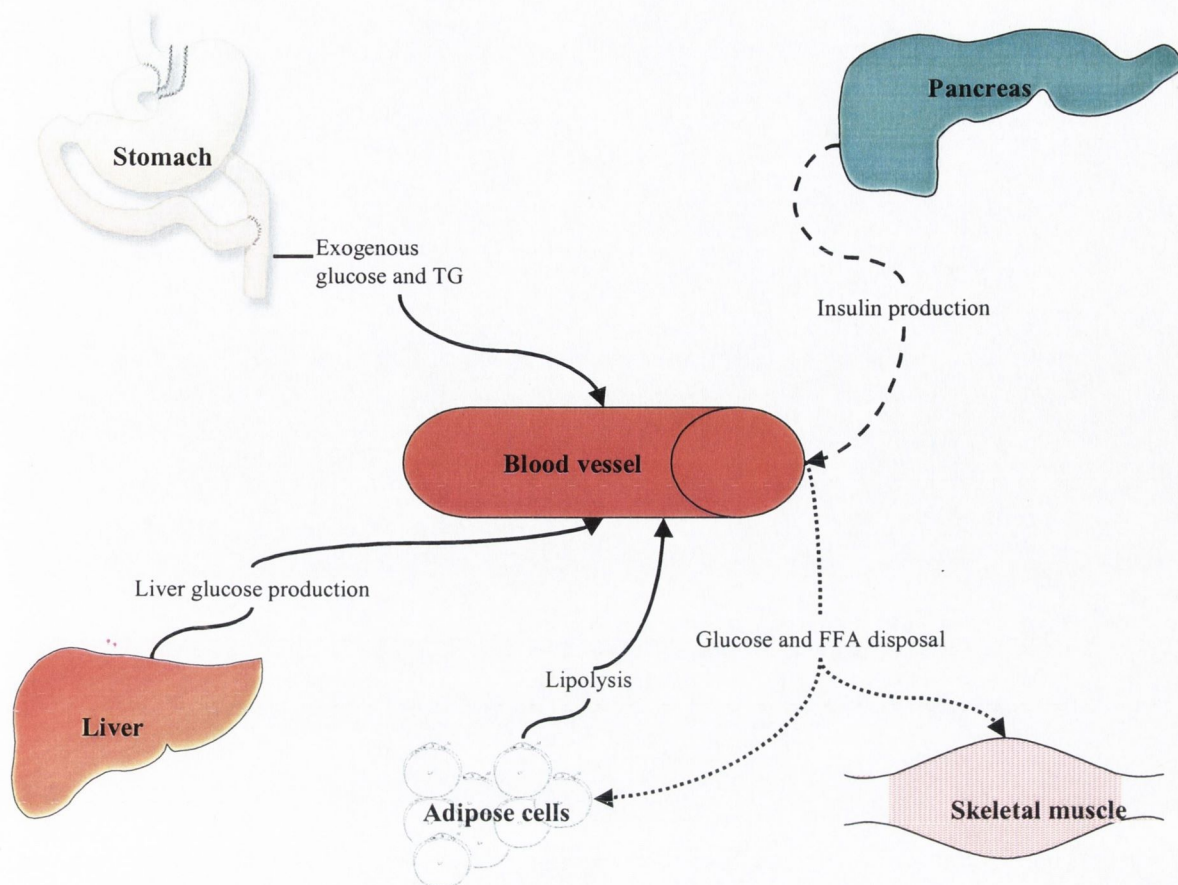


FIGURE 2.1 Regulation of glucose and free fatty acid appearance and disappearance. The solid lines represent glucose and lipid appearance in the blood while the dashed line reflects insulin secretion in response to elevated blood glucose. The dotted line indicates the main disposal sites for glucose and FFA.

Aetiology of Type 2 diabetes

Genetic and environmental factors determine insulin sensitivity

The progression from normal glucose tolerance (NGT) to impaired glucose tolerance (IGT) and Type 2 diabetes results from a progressive deterioration in the physiological regulation of glucose homeostasis. In classical Type 2 diabetes this process is precipitated by a combination of genetic and environmental factors. The genetic loci associated with Type 2 diabetes are difficult to identify but while genetic predisposition or a positive family history confer risk, disease manifestation is most strongly associated with an increase in body weight leading to the development of obesity.

Insulin resistance is characterised by a decrease in whole-body insulin-mediated glucose disposal, mainly accounted for by decreased uptake in skeletal muscle (410,322). Obesity, especially abdominal obesity, is an insulin resistant condition accompanied by increased circulating triglycerides and free fatty acids (94). Decreased insulin action *per se* does not result in Type 2 diabetes as normal glucose tolerance can be maintained by increased β -cell insulin secretion. Hyperinsulinemia compensates for insulin resistance to maintain NGT but those who progress to develop IGT (121,187) or Type 2 diabetes (262,86,126,115) exhibit a loss in first-phase insulin secretion and an inadequate insulin response when matched for plasma glucose. It was thought that insulin resistance was the primary defect but it now appears that insulin secretory dysfunction is also manifested early in the pathogenesis of Type 2 diabetes.

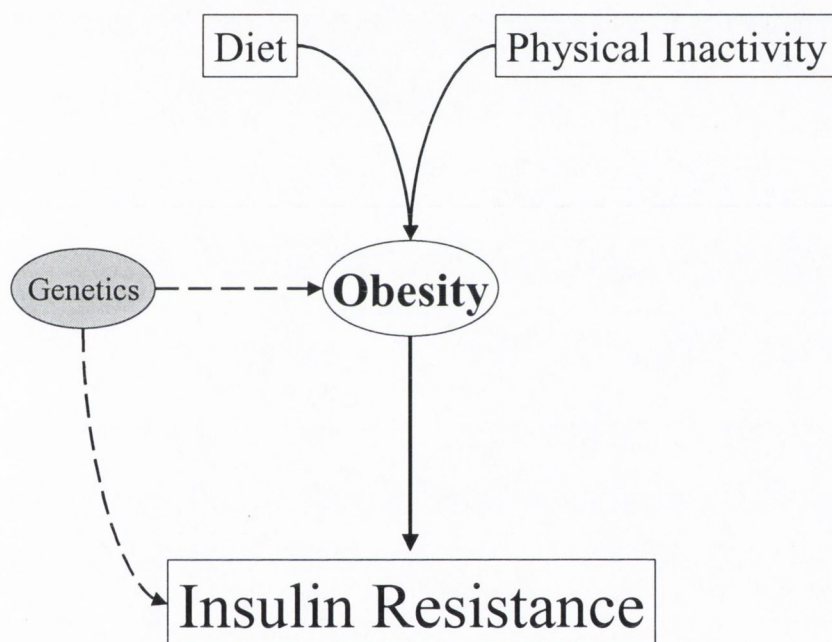


FIGURE 2.2. Genetic predisposition and environmental factors such as physical inactivity and dietary nutrient intake contribute to the development of insulin resistance.

The accumulation of body weight over time in the presence of a genetic predisposition for insulin resistance or insulin secretory dysfunction will most likely result in the development of IGT and Type 2 diabetes. A positive family history for Type 2 diabetes is associated with decreased insulin mediated glucose disposal and reduced β -cell compensation (367,105,220,267,374,275,125,34,372). In monozygotic and dizygotic twins 60% of the variance in glucose stimulated insulin secretion and 40-60% of the

variance in insulin mediated glucose disposal can be accounted for by genetic variance (220,275). Despite these facts, weight loss improves insulin sensitivity and insulin secretory dysfunction in IGT (384) and obese Type 2 diabetes patients (240) indicating that environmental factors associated with weight gain are important for disease development.

Insulin resistance, impaired fasting glucose and impaired glucose tolerance

The progression from NGT to Type 2 diabetes involves a gradual and simultaneous deterioration in insulin action and insulin secretion (372,278,54,386). At all stages in the development of the disease both insulin resistance and the acute insulin response to glucose are independent predictors of decreasing glucose tolerance and therefore are both primary targets for the prevention and treatment of the disease (386). The decrease in insulin sensitivity is inversely related to weight gain, up to a point that corresponds with the onset of obesity, with other factors contributing to the development of IGT and Type 2 diabetes (38,126,117,252). In contrast, the insulin secretory response is markedly different during each of these stages. Pima Indians with IGT but not IFG have well preserved β -cell function (278,383,277), indicating that IFG may be related to insulin secretory dysfunction with IGT a better predictor of insulin resistance (310). There is a progressive deterioration when IFG and IGT are present but subjects with Type 2 diabetes display the lowest insulin sensitivity and insulin secretory responses (277).

The regulation of hepatic glucose production

The severity of Type 2 diabetes and the rate of progression are dependent on factors other than glucose disposal and insulin secretion alone. Splanchnic glucose uptake, endogenous glucose production and FFA availability all have an impact on Type 2 diabetes. Splanchnic glucose uptake is an important contributing factor to circulating blood glucose. Ludvik et al. (225) examined splanchnic glucose uptake by hepatic vein catheterisation and an oral glucose clamp in Type 2 diabetes subjects. They found that insulin stimulated splanchnic glucose disposal in Type 2 diabetes subjects was decreased after oral glucose administration. This may partly be explained by increased net hepatic glucose output in Type 2 diabetes (262,86) resulting from a combination of impaired insulin-mediated suppression of glycogenolysis (86,240,226) and increased endogenous glucose production (328,72). In obese NGT and IGT, endogenous glucose

production is not elevated and only appears with the hyperglycaemia associated with Type 2 diabetes (86). Hepatic autoregulation appears sufficient to prevent increased endogenous glucose production during IGT but under the increasing metabolic stress accompanying hyperglycaemia, suppression of endogenous glucose production fails.

The development and progression of hyperglycaemia acts as a further complicating and deteriorating factor in Type 2 diabetes. In NGT subjects glucose-mediated glucose disposal decreases endogenous glucose production (375) but in Type 2 diabetes subjects hyperglycaemia increases metabolic stress, hepatic glucose production is incompletely suppressed and contributes to elevated fasting glucose (181), insulin resistance and insulin secretory dysfunction (86). Hyperglycaemia begets further hyperglycaemia, leading to the term ‘glucotoxicity’, and is related to hypertriglyceridemia (151,266) and the development of most diabetes-related complications and increases the severity of the disease.

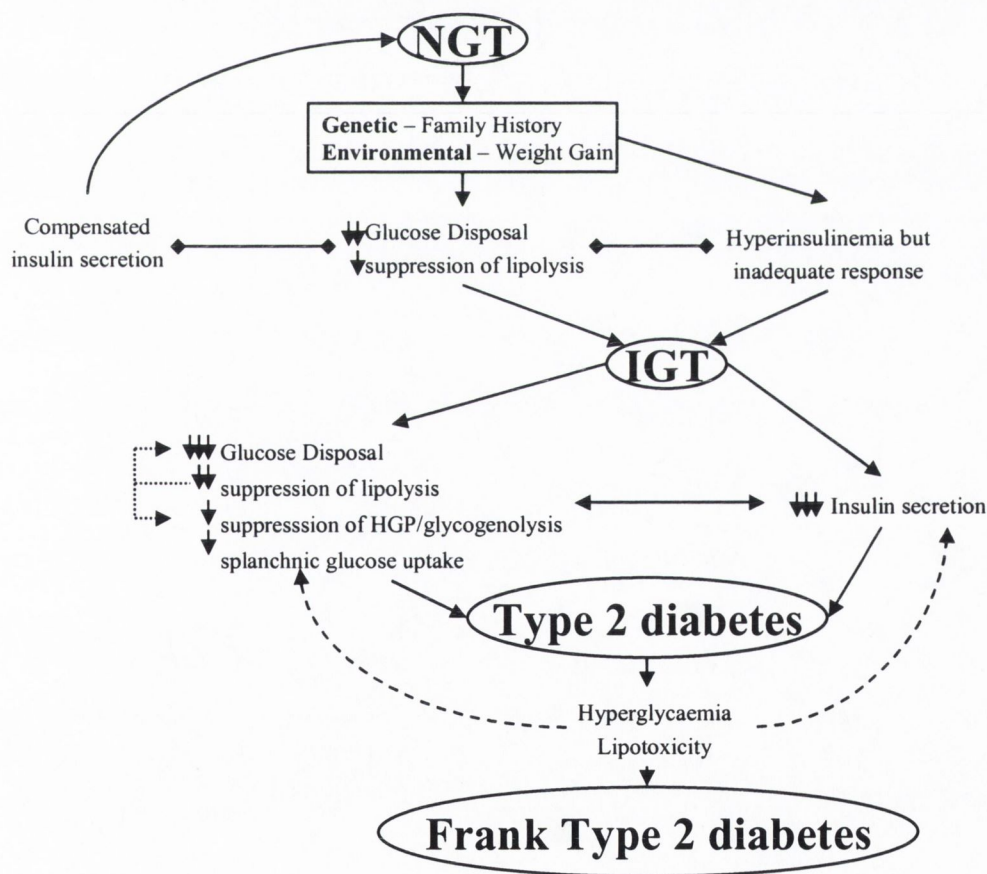


FIGURE 2.3. A proposed model for the aetiological development of impaired glucose tolerance (IGT) and Type 2 diabetes.

The role of adipose tissue, free fatty acids and intramuscular triglycerides

While adipose tissue lipolysis is normally suppressed by relatively small physiological doses of insulin (180), the anti-lipolytic effect is attenuated with visceral obesity (160) and Type 2 diabetes (254,21). The failure of insulin to suppress lipolysis increases the triglyceride-FFA flux (285). Along with diet-induced visceral adiposity (94), and an increase in hepatic lipogenesis (151,128) the failure to suppress lipolysis results in elevated plasma triglyceride and FFA concentrations. Increased triglyceride concentrations are one the earliest detectable signs of insulin resistance (86).

Recent studies have shown that FFA's are not just a metabolic substrate but play an important role in the regulation of metabolic processes. Increased FFA's are associated with a decrease in whole body glucose disposal (37,291,35,52,18,290), an increase in gluconeogenesis (254,292,215) and decreased capacity to suppress glycogenolysis leading to increased hepatic glucose production (36,18,289). In addition to decreased insulin action, elevated FFA results in decreased insulin secretion in IGT and Type 2 diabetes but not NGT (338,289). Evidence suggests that insulin sensitivity and secretion may be improved while endogenous glucose production suppressed by decreasing circulating FFA with acipimox (369,283). Chronic FFA elevation termed 'lipotoxicity' may also be responsible for progression of Type 2 diabetes, contributing further to the severity of insulin resistance and insulin secretory dysfunction.

A proposed "unifying hypothesis" (289) suggests that FFA's may play a central role in the aetiology of Type 2 diabetes because of their impact on insulin resistance, hepatic glucose production and insulin secretion. The proposed aetiology outlined in Figure 2.3 suggests that, initially increased FFA availability may result from inactivity and a high fat diet. Insulin resistance develops because of defects in the regulation of carbohydrate and FFA metabolism contributed to by the accumulation of intramuscular triglycerides and leading to the development of IGT and Type 2 diabetes (193,107,222,72). Impaired insulin action and FFA's progressively impair glucose disposal and insulin secretion while contributing to endogenous glucose production. This process continues in a cyclical manner with the progressive deterioration of metabolic function. The relationship between insulin action and insulin secretion becomes even further strained as insulin secretory dysfunction fails to control blood glucose. Finally, it is proposed that chronic exposure to this lipotoxic and glucotoxic environment may contribute to

insulin deficiency, resulting in a further failure to facilitate glucose metabolism and the development of frank Type 2 diabetes.

Cellular Mechanisms of Insulin Resistance

The primary defect: glucose transport or regulation of glycogen synthesis?

The decrease in insulin-mediated glucose disposal in Type 2 diabetes can be accounted for by decreased rates of glucose transport (61,211,409) and glycogen synthesis (39,87,83). Intra-cellular glucose is phosphorylated by hexokinase to glucose-6-phosphate (G-6-P) and subsequently partitioned between glycolysis and glycogen synthesis. The ratio of storage to utilisation is dependent on the initial glycogen concentration and the energy requirements of the cell, though under insulin stimulated conditions the greater proportion is synthesised as glycogen (323). Glycogen synthase, the rate-limiting enzyme in this process, is covalently regulated by G-6-P and allosterically by at least nine protein kinases (217). Insulin stimulation dephosphorylates glycogen synthase, increasing the rate of synthesis. Glycogen synthase activity is decreased in Type 2 diabetes (39,350), impaired glucose tolerance (309) and in first degree relatives of patients with Type 2 diabetes (368) making it an attractive locus for insulin resistance by mediating a negative feedback signal to glucose transport.

If this were the primary mechanistic defect in Type 2 diabetes there would be an accumulation of intracellular G-6-P to act as a negative feedback. However, Rothman et al. (310), using NMR, found that during hyperglycaemia and hyperinsulinemia G-6-P was unchanged in patients with Type 2 diabetes and increased 0.1 mM in controls, indicating that the defect in Type 2 diabetes was in glucose transport/phosphorylation. The same group found decreased glucose transport/phosphorylation in first-degree relatives of patients with Type 2 diabetes (299). Earlier studies showed that when glucose disposal rates were normalised by hyperinsulinemia (349) or hyperglycaemia (195), glycogen synthase activity also increased proportionally, indicating that decreased glycogen synthesis was a secondary effect. In another series of experiments, Cline et al. (61) using nuclear magnetic resonance spectroscopy (NMR) found that the rate of intracellular glucose accumulation, when hyperinsulinemia was increased from

40mU/m²/min to 400mU/m²/min, was only 4% of the expected value if hexokinase were the rate-limiting step. Taken together these data suggest that glucose transport is the primary defect in the insulin resistance of Type 2 diabetes. However, impaired glucose phosphorylation may play a more significant role in obesity as Williams et al. (390) found by positron emission tomography (PET), that glucose disposal was decreased in patients with Type 2 diabetes, when compared to obese and lean controls, but glucose phosphorylation was lower in the obese and Type 2 diabetes groups. Though methodological differences may exist between PET and NMR it is generally accepted that glucose transport is rate limiting for glucose disposal in Type 2 diabetes.

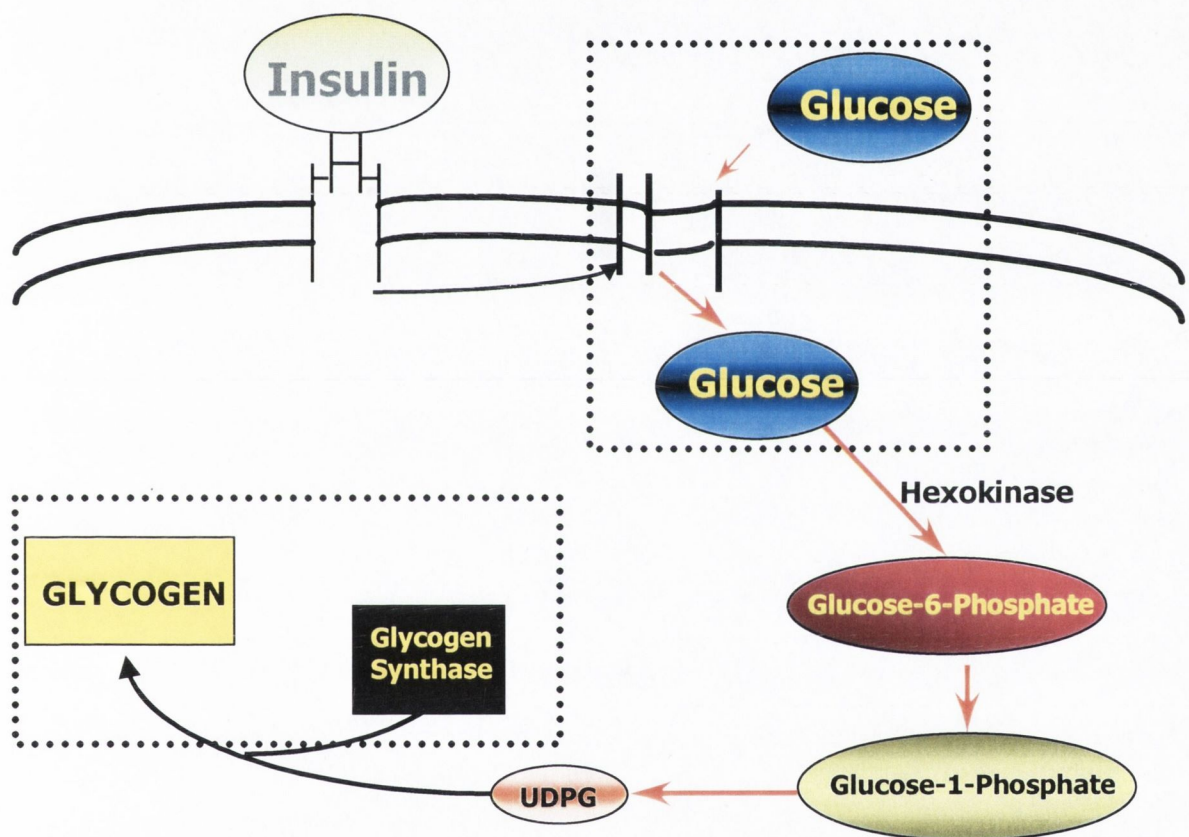


FIGURE 2.4. The regulation of glucose transport and glycogen synthesis were identified as the primary targets for cellular defects in glucose disposal.

The Glucose/Free Fatty Acid Cycle

Glucose not stored as glycogen undergoes anaerobic glycolysis resulting in an increase in cytosolic pyruvate. Under aerobic conditions the majority of pyruvate is oxidised to acetyl CoA by pyruvate dehydrogenase, transported into the mitochondria to produce adenosine triphosphate (ATP) by oxidative phosphorylation. The remainder is shuttled

to lactate, or remains as pyruvate, and is transported out of the cell providing substrate for gluconeogenesis. In Type 2 diabetes glycolytic flux is also altered. Glucose oxidation rates are lower and glycolytic production of lactate and pyruvate is increased in patients with Type 2 diabetes under euglycaemic-hyperinsulinemic conditions (349). Though it appears that glucose is being shunted to anaerobic glycolysis because of down regulated glucose oxidation, no mechanistic defect has been identified to support this. The decrement in glucose oxidation is less than that of glycogen synthesis, proportional to the decrease in glucose disposal and not evident in insulin resistant relatives of patients with Type 2 diabetes (368) making it a defect subsequent to insulin resistance. However, though Kelley and Mandarino (195) found that when hyperglycaemia was used to normalise glucose disposal rates, the decrease in glucose oxidation was reversed, other studies do not support these findings during hyperglycaemia or hyperinsulinemia (87,349). Therefore, a defect in glucose oxidation may exist in Type 2 diabetes. The regulation of this process is not fully understood.

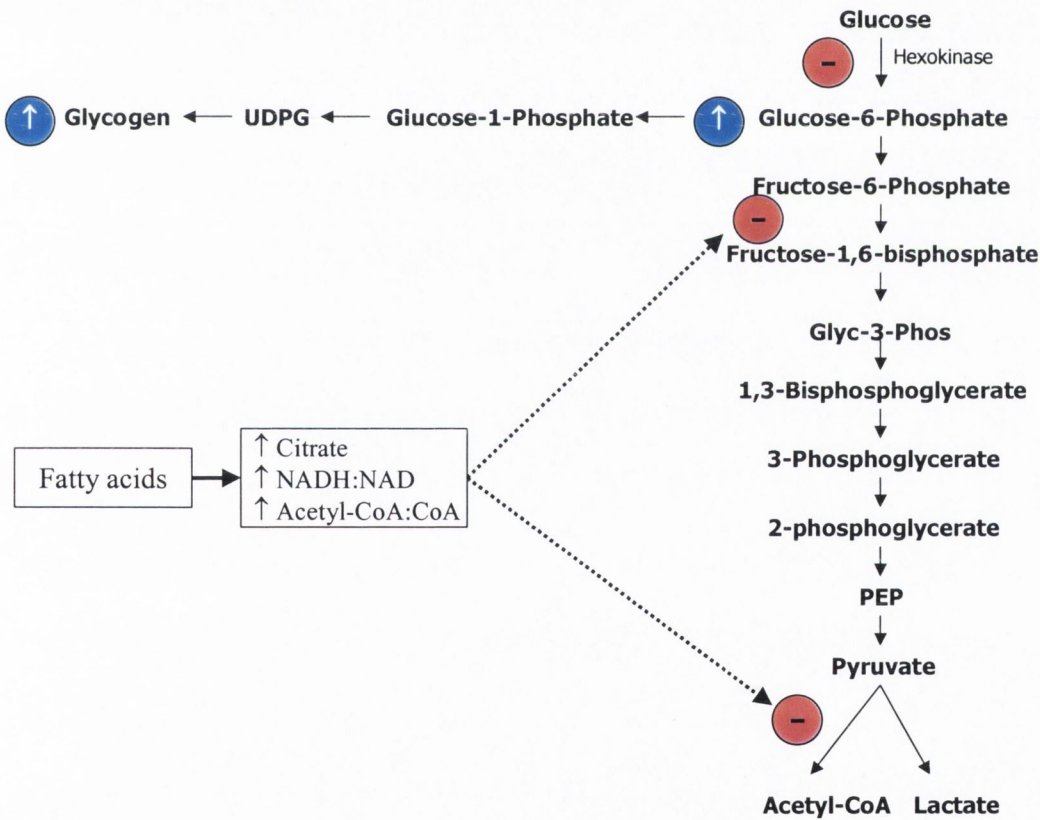


FIGURE 2.5. The glucose and free fatty acid cycle was proposed by Randle, as an explanation for competitive inhibition of glucose utilisation by increased free fatty acid availability.

The Glucose Free Fatty Acid Cycle (GFFA cycle), proposed by Randle et al. (284), provided an explanation for the interaction between glucose and FFA and how altered substrate utilisation could subsequently decrease glucose disposal. To be more specific, Randle et al. (284) proposed that an increase in FFA availability would lead to an increase in β -oxidation with a subsequent increase in mitochondrial citrate from the Krebs cycle. The accumulation of citrate would inhibit phosphofructokinase activity, thereby inhibiting glycolysis. The increase in FFA would also increase acetyl CoA and the cytoplasmic NADH:NAD ratio, increasing pyruvate dehydrogenase kinase and decreasing pyruvate dehydrogenase. The inhibition of glycolysis would decrease glucose oxidation, increase glucose 6-phosphatase and subsequently down-regulate hexokinase II resulting in negative feedback for glucose uptake.

Increasing FFA availability by intra-venous infusion of lipid and heparin, in people who do not have Type 2 diabetes, decreases glucose oxidation, increases lipid oxidation and decreases glucose disposal (35,37). Conversely, suppression of FFA, by inhibiting lipolysis, increases glucose disposal in patients with Type 2 diabetes (369) confirming the FFA mediated effects on insulin resistance and consistent with the Randle cycle. Kelley et al. (196) found that physiological hyperinsulinemia alone failed to suppress plasma FFA in Type 2 diabetes during a $120 \text{ mU/m}^2/\text{min}$ glucose clamp, and that plasma FFA were the best predictor of insulin resistance. However, results pertaining to the interaction between glucose and lipid oxidation, as measured by indirect calorimetry are equivocal. Del Prato et al. (87) found that when glucose disposal was matched by hyperglycaemia, lipid oxidation was not significantly suppressed, whereas hyperinsulinemia did suppress lipid oxidation similar to control subjects during a $20 \text{ mU/m}^2/\text{min}$ glucose clamp. However, Thorburn et al. (349) found that lipid oxidation was similarly suppressed during hyperglycaemia and hyperinsulinemia, though plasma glucose was higher during the hyperglycaemic clamp than the study by del Prato et al. (87) (20.7 ± 1.6 vs $14.9 \pm 1.2 \text{ mM}$, respectively). Henry et al. (152) also found that lipid oxidation was similarly suppressed across a range of glucose and insulin values in patients with Type 2 diabetes. The interaction between glucose and lipid metabolism is complex and not fully understood.

The reverse Glucose/FFA cycle

For the Randle cycle to explain substrate selection and insulin resistance there should be an increase in cytosolic citrate and G-6-P. Roden et al. (291) found that FFA decreased glucose disposal, G-6-P, glycogen synthesis and glucose oxidation, but not pyruvate dehydrogenase. Indeed, when FFA were suppressed during a euglycaemic-hyperinsulinemic clamp, intra-cellular G-6-P was significantly greater than during basal or elevated FFA (290). Boden et al. (37) did not find an increase in citrate or G-6-P during elevated plasma FFA therefore FFA may mediate the inhibition of glucose transport/phosphorylation. Dresner et al. (100) addressed this question and found a 50% decrease in glucose oxidation, glycogen synthesis and glucose disposal during a 1 mU/kg/min hyperinsulinemic clamp following 5-hrs of lipid infusion. There was a 90% reduction in G-6-P and no accumulation of intracellular glucose implying that elevated FFA impairs glucose transport in healthy volunteers.

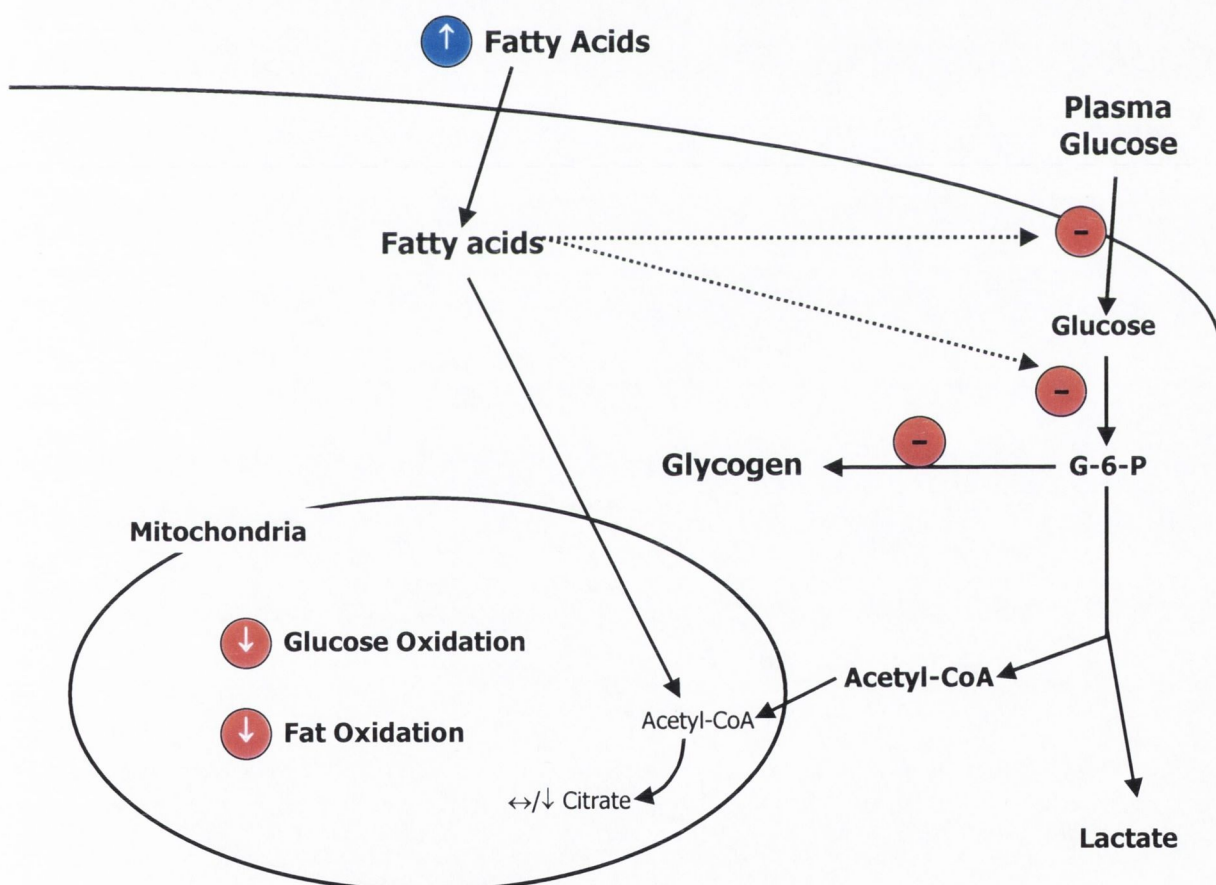


FIGURE 2.6. The reverse glucose/free fatty acid (GFFA) cycle proposed that increased free fatty acid (FFA) availability directly inhibits insulin-mediated glucose transport and phosphorylation and results in decreased glucose and FFA oxidation.

Randle et al. (284) also proposed that increased lipid oxidation occurs in the presence of elevated FFA leading to impaired glucose oxidation. However, when patients with Type 2 diabetes and control volunteers undergo a hyperinsulinemic clamp at fasting plasma glucose, fat oxidation was lower in Type 2 diabetes (195). Also when plasma FFA concentrations are maintained in normal controls during a hyperglycaemic-hyperinsulinemic clamp, total and plasma FFA oxidation was decreased (324). Those with impaired glucose tolerance also have decreased plasma FFA uptake and oxidation rates (239) while in obesity, palmitate oxidation is approximately 50% lower during intra-venous infusion (198) and FFA uptake and oxidation are not increased during hyperinsulinemia (66). Even in healthy individuals, those with lower insulin sensitivity indices had higher FFA at baseline, after isoproterenol stimulated lipolysis and following suppression by hyperinsulinemia (1). Therefore, FFA-mediated insulin resistance appears to be independent of diabetes, and therefore may be a primary mechanistic defect.

FFA-mediated inhibition of glucose disposal

The two key factors regulating FFA utilisation are cellular uptake and oxidation. FFA uptake is decreased in type 2 diabetes (195,239,33) while lipolysis is increased, contributing to elevated plasma FFA. There is also a decrease in enzymes involved in β -oxidation and the electron transport chain from patients with Type 2 diabetes (239,33,327). In normal volunteers oxidation of the long chain fatty acid (LCFA) oleate but not octanate, a medium chain fatty acid, was decreased during hyperglycaemia and hyperinsulinemia suggesting that glucose and/or insulin may regulate FFA oxidation through mitochondrial transport of LCFA (325). Therefore, decreased muscle FFA uptake and oxidation or the subsequent accumulation of intra-muscular triglycerides may be responsible for decreased glucose transport (150). Recently it has been possible to accurately quantify intra-muscular triglyceride content, independent of adipose tissue. These data show that patients with Type 2 diabetes have increased intra-muscular triglyceride stores, the quantity of which inversely correlates with the degree of insulin resistance (196,222). The accumulation of intra-muscular triglyceride may subsequently impair insulin-mediated glucose transport.

It now appears that the GFFA cycle does not fully explain substrate utilisation and insulin resistance. In addition to FFA mediated decreases in glucose disposal, recent evidence presented above suggests that the reverse of the GFFA cycle may also be true, that is, excess glucose may inhibit lipid oxidation (195,289). It has been demonstrated that basal glucose uptake (86) and oxidation (195) are increased in Type 2 diabetes. The subsequent increase in cytosolic citrate dephosphorylates and activates acetyl CoA carboxylase α (ACCA) in hepatic cells and ACC β in muscle cells *in vivo* (302). ACC β , with acetyl CoA as substrate, synthesizes malonyl CoA, a negative regulator of carnitine palmitoyltransferase I (CPT I), the rate limiting enzyme of LCFA entry to the mitochondria (302). Though many of the studies describing a glucose and insulin-mediated increase in malonyl CoA by citrate (304,305), and the effect of CPT I inhibition on LCFA accumulation (3), have been conducted in animal models there is also human data to support these findings. Båvenholm et al. (25) studied healthy volunteers during euglycaemia at 0.25 and 1 mU/kg/min insulin. Whole body fat oxidation was decreased at the low dose insulin, but there was no change in malonyl-CoA concentration. However, during the higher insulin dose, malonyl-CoA increased 20% with a further 41% decrease in whole body fat oxidation and a 35% increase in cytosolic citrate. Therefore, in the presence of high dose insulin and glucose, malonyl-CoA appears to play a role in decreased fatty acid oxidation in human skeletal muscle.

Taken together these data suggest that FFA metabolism, and the interaction with glucose utilisation, is regulated by a number of processes. As neither the GFFA cycle, nor the inverse GFFA cycle fully explain the dysmetabolism of Type 2 diabetes it is reasonable to assume at this time that defects in both carbohydrate and lipid metabolism contribute. Though it is likely that defects in substrate oxidation, glycogen synthesis and intra-muscular triglycerides are contributing factors, it is generally agreed that the primary defect in Type 2 diabetes is impaired glucose transport. Therefore, a more careful and detailed examination of the molecular processes regulating insulin-mediated glucose transport is necessary to fully explain the cellular defects.

Insulin stimulated intracellular signalling and Glut-4 translocation

Cell membranes, except the blood-brain barrier, are impermeable to glucose (180.2 kDa) which requires facilitated diffusion to cross the hydrophobic lipid bilayer of cells.

In the lumen of the intestine and the kidney Na^+ /glucose co-transporters are present, but in most tissues the family of specific glucose transporters (GLUT's) are primarily responsible for facilitated diffusion (156). Though 13 Glut isoforms have been characterised, the class 1 subgroup (Glut 1-4) is primarily responsible for glucose transport (183). Insulin stimulation results in the translocation, docking and binding of Glut-4 containing vesicles to the plasma membrane from intra-cellular stores in adipose, cardiac and skeletal muscle.

Insulin receptor activation

Insulin binds to the extra-cellular domain of the insulin receptor, initiating a pleiotrophic cascade of intracellular signals (56). The heterotetrameric receptor comprises of two extra-cellular α -polypeptides (135 kDa), responsible for ligand binding, and two β -polypeptides (95 kDa) that traverse the plasma membrane. The four polypeptides are linked by di-sulfide bonds. Decreased intra-cellular β -subunit serine/threonine activity and increased tyrosine activity are associated with insulin binding and intracellular insulin (233,387). There are four key regulatory areas on the β -subunit phosphorylated by ligand binding. The first area consists of three binding sites, Tyr¹¹⁵⁸, Tyr¹¹⁶² and Tyr¹¹⁶³ on the kinase regulatory domain, that are necessary for downstream insulin action (56,387). The second area consists of Lys¹⁰³⁰, the ATP-binding domain, whose phosphorylation is necessary for all subsequent actions of insulin,. *In vitro* mutagenesis of this amino acid inactivates the receptor though not preventing insulin binding (59). The third area, Tyr¹³²⁸ and Tyr¹³³⁴, may play a role in the activation of glycogen synthesis though this is controversial (56). Finally, the juxta-membrane domain is responsible for receptor internalization with single point mutations at Tyr⁹⁶⁵ and Tyr⁹⁷² completely inhibiting this process (114). Tyr⁹⁷² is also a site of autophosphorylation but more importantly phosphorylates insulin receptor-1 (IRS-1).

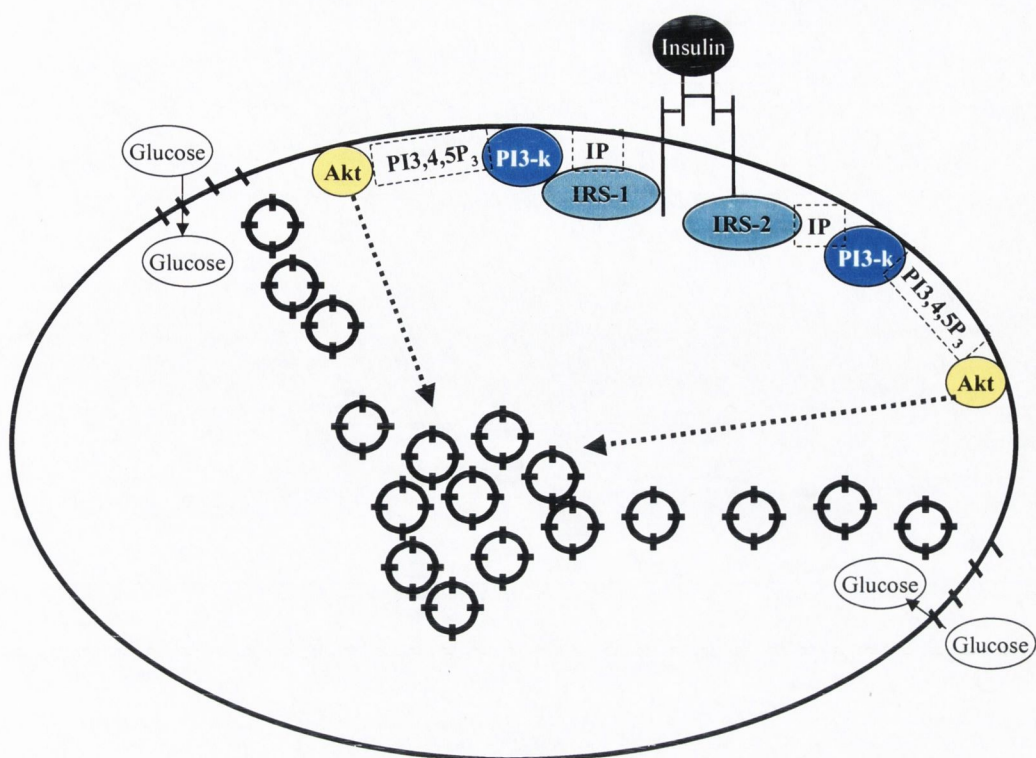


FIGURE 2.7. Schematic representation of insulin-mediated GLUT-4 translocation via insulin receptor substrate (IRS)-1 and -2 associated phosphatidylinositol 3-kinase (PI3-k) activity.

The insulin receptor substrate and phosphatidylinositol 3-kinase

Intracellular insulin signalling cascades are a complex series of protein interactions, many of which have not been well characterised. Though proteins such as Shc (307) and CAP-Cbl (23) bind directly to the insulin receptor and confer downstream action it is recognised that binding of insulin receptor substrates (IRS) confers most of the responsibility for glucose transport (56,315). This review is limited to the IRS pathway but acknowledges that alternative or supplementary pathways exist.

There are four members of the IRS protein family but since IRS-3 is not expressed in human tissue and IRS-4 does not appear to be expressed in human muscle or adipose tissue, and if so is non-abundant, the focus will be on IRS-1 and IRS-2 (315). These proteins have a 43% sequence homology and both contain a pleckstrin homology (PH) domain to bind membrane phospholipids, a phosphotyrosine binding domain (PTB) that

binds to the juxtamembrane region of the insulin receptor and a COOH-terminal with potential phosphorylation motifs (315). IRS proteins are phosphorylated by the insulin receptor but do not confer any enzymatic activity. Instead they act as docking proteins that facilitate binding of SH2 domain containing proteins (56). Membrane bound IRS-1 phosphorylates 21 tyrosine residues that facilitate protein-protein interaction and downstream signalling. IRS-1 also contains more than 40 Ser/Thr phosphorylation sites. IRS-2 is more prevalent in the cytosol, unlike IRS-1 which is more abundant at the membrane (170), and has 22 tyrosine phosphorylation sites, though only 13 are conserved with IRS-1 (315). Among the proteins docking with phosphorylated IRS-1 and IRS-2 is the p85 subunit of a heterodimer, phosphatidylinositol 3-kinase (PI3-K).

Akt activity and Glut-4 translocation

PI3-K is a key enzyme in glucose transport, as inhibition of its activity by wortmannin completely blocks GLUT-4 translocation and glucose transport (260). PI3-K is a heterodimer consisting of a p85 regulatory subunit, that binds IRS-1 and IRS-2 via SH2 domains, and a p110 catalytic subunit. Increased PI3-K activity phosphorylates intracellular membrane bound phosphatidylinositol (PI) at the D3 position and synthesizes PI 3,4-P₂ and PI 3,4,5-P₃ (231). However, there are four regulatory subunit isoforms in human skeletal muscle including p85 α , p85 β plus two truncated forms p50 and p55 γ (319). Though not fully understood it is thought that the isoforms may convey specificity of the PI3-K signalling system (318). In addition the catalytic subunit has two isoforms in human skeletal muscle, p110 α and p110 β , though their functional properties appear to be similar (319). Therefore, the PI3-K signalling system is necessary for insulin stimulated glucose transport by increasing intracellular phosphoinositide lipids.

The signalling pathway distal to PI3-K, leading to GLUT-4 translocation, is less well defined. However, it has been shown that the pleckstrin homology domain of Akt/PKB, a 57 kDa mainly cytosolic protein, binds to the PI 3,4,5-P₃ and PI 3,4-P₂ lipid products, generated by PI3-K, and translocates some of the protein to the plasma membrane (340,13). Insulin stimulated Akt/PKB activity appears to be wortmannin-sensitive and therefore PI3-K dependent (13). Akt/PKB has three isoforms but Akt1 accounts for most of the insulin stimulated Akt activity in rat skeletal muscle or hepatocytes and

40% of activity in adipocytes (377). In addition to Akt translocation to the plasma membrane, phosphorylation at Thr³⁰⁸ on the kinase domain and Ser⁴⁷³ on the C-terminal regulatory domain is necessary for full activation (63,143,373). Akt binding to PI 3,4,5-P₃ and PI 3,4-P₂ phospholipids appears to cause a conformational change in the protein, allowing it to be phosphorylated. A 63 kDa protein, 3-phosphoinositide dependent protein kinase 1 (PDK-1) has been shown to phosphorylate Thr³⁰⁸. PDK-1 also has a pleckstrin homology that binds PI3-K generated phospholipids and Akt at the plasma membrane (118). The phosphorylation of Ser⁴⁷³ is less well defined with evidence suggesting the likely candidates to be PDK-1 (373), Akt autophosphorylation (355), or some unknown protein (373). Once Akt has been phosphorylated at both sites it dissociates from the plasma membrane. A direct link between Akt activity and Glut-4 vesicle translocation to the plasma membrane has not been established though constitutively active Akt stimulates glucose uptake and Glut-4 translocation in 3T3-L1 adipocytes (207) and L6 muscle cells (142). However, not all data support these findings as Kitamura et al. (205) found that insulin-stimulated Akt increased protein synthesis but not glucose transport in CHO cells and 3T3-L1 adipocytes. Therefore, the exact role of Akt in Glut-4 translocation is still under investigation and though it has been proposed that Akt may bind to the cytosolic Glut-4 vesicles, the final stage in the translocation of Glut-4 to the plasma membrane remains elusive.

The insulin signalling cascade and Type 2 diabetes

The binding affinity for the insulin receptor is similar but autophosphorylation of the β -subunit is decreased with insulin resistance (415,168). Björnholm et al. (31) also found a decrease in IRS-1 phosphorylation, independent of protein content, and decreased PI3-K activity during a euglycaemic-hyperinsulinemic clamps in a group of patients with Type 2 diabetes. In contrast, age-matched control subjects had a six-fold increase in IRS-1 phosphorylation and a two-fold increase in PI3-K activity. The decrease in IRS-1 phosphorylation and PI3-K activity has also been found in first degree relatives of patients with Type 2 diabetes who had IGT (341). It is possible that IRS-2 tyrosine phosphorylation may compensate for the decreased IRS-1 phosphorylation. IRS-1 null mice develop insulin resistance but not diabetes (14), whereas IRS-2 null mice develop diabetes (391). It is most likely that the decreased tyrosine phosphorylation is more likely due to the decreased insulin receptor phosphorylation or inhibition of binding

sites to the receptor (311,208,188), though polymorphisms in IRS-1 are also associated with impaired insulin signalling (8).

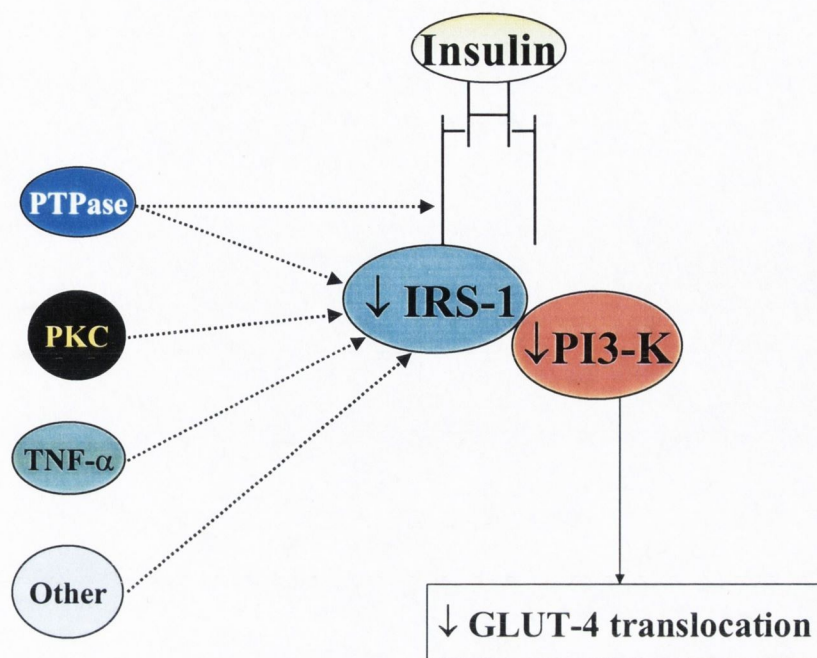


FIGURE 2.8. Impaired insulin-mediated glucose transport is related to increased phosphatase activity and/or serine phosphorylation of the insulin receptor and insulin receptor substrate 1 (IRS-1).

In Type 2 diabetes patients (213) and first degree relatives with IGT (341) Akt kinase activity is reduced in comparison to control subjects. Therefore, the decrease in activity may further contribute to the decrease in Glut-4 translocation and glucose transport. It has also been demonstrated in rat skeletal muscle that the decrease in Akt and glucose transport could be restored by phlorizin, a glucose lowering agent devoid of insulin mimetic properties, supporting the role for a link between Akt kinase activity and Glut-4 translocation (212). However, Kim et al. (200) did not find a decrease in Akt activity in obese non-diabetic or obese diabetic subjects when compared to lean controls under euglycaemic-hyperinsulinemic conditions. As GLUT-4 protein content is similar between control and Type 2 diabetes subjects (91), the overall decrease in insulin receptor substrate binding and phosphorylation appears to be largely responsible for a decrease in Glut-4 translocation associated with Type 2 diabetes (89). This may possibly be due to an increase in tyrosine phosphatase activity (2), or an increase in phosphorylation of serine residues on IRS-1 (208). In particular, proteins such as increased TNF- α and PKC isoforms, may downregulate enzyme activity and attenuate insulin signalling (311,208).

A possible role for protein kinase C

The role of protein kinase C (PKC) in metabolic signalling is poorly understood because the results from glucose transport experiments are equivocal. It appears that membrane bound PKC β_2 and ζ are associated with increased glucose transport (192,109,44,53) while PKC α , δ , θ , and ϵ appear to be related to decreased glucose transport and/or insulin resistance (311,138). This does not exclude a role for other PKC isoforms. Obesity or FFA induced insulin resistance increases membrane bound and decreases cytosolic PKC ϵ and PKC θ (311,138). The translocation may be associated with increased activity and/or synthesis of these proteins (282) but can be reversed with an insulin-sensitiser (312). These data also support the role of IMTG as a key regulator of insulin sensitivity. The cytosolic accumulation of LCFA-CoA associated with decreased CPT-I activity (236) inhibits hexokinase activity (348) and increases diacylglycerol (DAG) accumulation and activation of PKC ϵ and/or θ (236,150). PKC is thought to increase serine phosphorylation of the insulin receptor and/or IRS-1, attenuating intracellular insulin action of skeletal muscle cells. The reduction of IMTG by insulin sensitisers (398), dietary lipid withdrawal or a single bout of exercise increased insulin sensitivity (256).

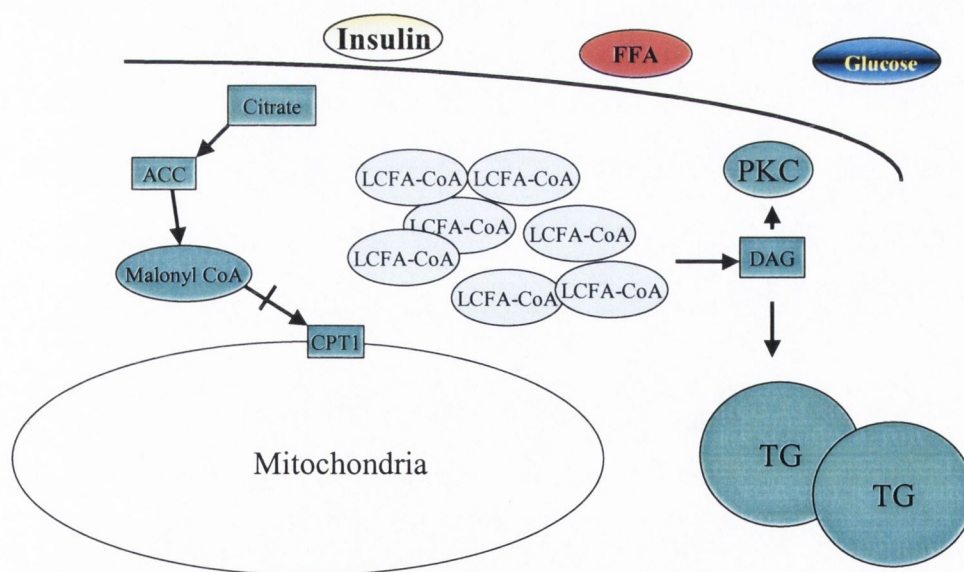


FIGURE 2.9. Free fatty acid mediated inhibition of glucose transport. An accumulation of cytosolic citrate may inhibit mitochondrial fatty acetyl-CoA transport. The resultant accumulation of intra-cellular fatty acetyl-CoA increases PKC activity and inhibits glucose transport.

Conclusion

The regulation of glucose and fat metabolism is dependent on the complex interaction between substrate utilisation, cellular signalling processes and gene expression. The development of insulin resistance and Type 2 diabetes is not fully understood but involves defects in all of the identified areas. It will be necessary to study mechanisms of insulin-independent glucose uptake and characterise the role of insulin signalling inhibitors to gain further insight into the mechanisms of insulin resistance.

Chapter 3

Insulin resistance and GAD antibody status: a case for Type 2 diabetes sub-phenotypes

Introduction

GAD antibody status and β -cell dysfunction

The physiological significance of anti-GAD immunity in Type 1 diabetes or LADA is not fully understood. GAD is one of many antigens that may initiate an autoimmune response in the β -cell. GAD is the rate-limiting enzyme in the conversion of glutamic acid to gamma amino butyric acid (GABA), a major inhibitory neurotransmitter. The exact role of GABA within the pancreatic β -cell is not clear. It may serve as a functional regulator of insulin release, as increased GABA expression has been associated with a decrease in first phase insulin secretion in transgenic mice expressing human GAD65 (321). GABA may also act as a paracrine signalling molecule for communication between β -cells and α -cells. GABA regulates glucagon secretion, so an increase in GABA would result in a failure to suppress glucagon secretion usually associated with insulin secretion and contribute to increased glucose production (296,124).

There are at least two theories related to the aetiology of the immune response to β -cell destruction and GAD plays a key role in both. The first suggests that an increased GAD expression, resulting from environmental stress, alters tolerance to GAD and initiates the response for immune diabetes (276,16). Esposti and Mackay (108) proposed that factors such as nutrient stress, exogenous toxins or genetic defects may influence the GABA network of metabolic pathways leading to an accumulation of glutamate and the overexpression of GAD within β -cells. This may result in deranged secretory vesicle trafficking and overexpression of GAD at the β -cell surface, its subsequent presentation as an antigen, thereby initiating an immune response in those with a genetic predisposition leading to the development of diabetes. Rubi et al. (301) overexpressed GAD in INS-1E cells and found a 37% decrease in glucose stimulated insulin secretion. Similarly, when human GAD65 was overexpressed in mice, there

was a decrease in first phase insulin secretion. In non-obese diabetic (NOD) mice, β -cell suppression of GAD prevented immune diabetes, while expression led to diabetes (400). These data suggest that GAD expression plays a significant role in the development of diabetes.

The second theory is that the immune response to GAD is directly involved in the destruction of pancreatic β -cells (275,230). Kaufman et al. (190) found that GAD, as a key antigen, mediated β -cell destruction via a T-lymphocyte response. Importantly, they found that inactivating GAD-reactive T-cells could prevent the immune response. Tisch et al. (354) found GAD specific reactivity in 4 week old NOD mice, indicating the GAD autoimmune response occurs very early in life but that the T-cell response and diabetes could be prevented by intra-thymic injections of GAD65. In a follow-up paper, Tisch et al. (353) found that inhibiting anti-GAD T cell responses in young NOD mice also prevented diabetes.

The immune response appears to be largely mediated by cytotoxic $CD8^+$ and helper $CD4^+$ T-cells (16,104). $CD8^+$ T cells are involved in the initiation of the immune response while $CD4^+$ T cells are the effector cells, either directly acting on a cell overexpressing an antigen (16), as in the first theory, or indirectly by recruiting cytokines, especially interferon gamma ($IFN-\gamma$) and tumour necrosis factor- α ($TNF-\alpha$), that initiate a series of cellular and molecular processes leading to cell death, as in the second theory (16,104). While there are many potential theories regarding the processes that initiate and result in β -cell destruction (276), it is likely to result from one, or a combination of factors, including antibody dependent cellular toxicity, delayed hypersensitivity, complement activation and cytotoxic concentration of cytokines (16). This process of β -cell destruction is not fully understood but it is thought that both necrosis and apoptosis have a role to play.

Complement activation and insulin secretory dysfunction

The role of complement in the immune response and β -cell destruction has not received a lot of attention. Complement is known to amplify the immune response. Complement activation occurs in response to an antibody-bound antigen or directly by the presence of microorganisms. Activation is a multi-stage process involving at least

20 interacting soluble proteins. Most of these proteins circulate in the blood and extracellular fluid until they are required (6,203). The antibody stimulated, or 'classical' pathway results from the binding of two or more IgG molecules, or one pentameric IgM, to a cell surface antigen. The activation of C3, the main component of complement-mediated action, is dependent on the prior activation of a complexed compound comprising C1 (C1q, C1r, C1s), C2, C4, Factor B, and Factor D (303). Following a sequence of amplifying proteolytic cleavage steps, C3 binds covalently to the surface of the target cell. There, the C3b subunit acts as a conduit for the formation of membrane attack complexes but also acts as a signal for macrophages and neutrophils to phagocytose the target cell. At the same time, the C3a subunit promotes an inflammatory response. The membrane attack complex, also known as the terminal complement complex (TCC), is the main complement action. Through a series of cleavage steps where C3b is loosely bound to C5b, a complex is formed of protein C5b, C6, C7, C8 and C9 (C5b-9). When C9 binds to the complex it causes a conformational change, exposing a hydrophobic region in the target cell membrane, creating a transmembrane channel and altering cell permeability (6,203). The resulting combination of altered cell equilibrium due to increased intracellular calcium $[Ca^{++}]_i$, loss of mitochondrial membrane polarity and loss of ATP along with the inflammatory response due to cytokine production leads to altered functional capacity and/or to cell destruction (203,303).

Complement-mediated cell death occurs by both necrosis and apoptosis. The increase in $[Ca^{++}]_i$, loss of mitochondrial polarity and ATP synthesis, as well as the membrane leakage induce necrosis. However, DNA fragmentation has also been shown to be associated with complement activation (68,50). Caraher et al. (50) incubated rat islet cells with 10% serum from newly diagnosed patients with Type 1 diabetes and found a two-fold increase in DNA strand breaks after 24-hours of incubation in comparison with control human sera. When complement components C1q and C3 were removed, apoptosis was not induced when exposed to rat islets.

Complement activation has also been implicated in decreased functional capacity of β -cells. Conroy et al. (71) found that alanine stimulated insulin secretion was decreased by 48% when newly diagnosed Type 1 diabetes human sera was incubated with the rat pancreatic clonal β -cell line BRIN-BD11 compared to control samples. They also

found that when C1q and C3 were depleted from the IDDM sera, there was no significant difference in insulin secretion compared to control human sera. In a subsequent study, Conroy et al. (71) found that there was a complement-dependent increase in $[Ca^{++}]_i$ when newly diagnosed sera was incubated with BRIN-BD11 clonal β -cells. This was accompanied by a 30% decrease in ATP and was further decreased 40% with a 20-fold increase in DNA fragmentation when incubated with TNF- α , IL-1 β and IFN- γ . Therefore, in sera from newly diagnosed Type 1 diabetes patients the combination of β -cell antibodies and complement components may play a role in insulin secretory dysfunction and β -cell destruction.

The role of soluble C5b-9 (sC5b-9) in human β -cell functioning is uncertain. When comparing sC5b-9 in diabetes patients Bergamaschini et al. (28) found that there was no significant difference in juvenile or adult onset Type 1 diabetes, or Type 2 diabetes patients. They did find an increased C4a in adult onset Type 1 diabetes but could not find any association between complement activation, metabolic control, the presence of antibodies, or duration of diabetes. However, other studies have demonstrated some interesting findings relating complement activation and factors related to Type 2 diabetes. Ebeling et al. (103) found that complement activation and inflammation were associated with Type 2 diabetes. Pasqui et al. (267) found that sC5b-9 was significantly increased in hypercholesterolemic, hypertriglyceridemic and low-HDL subjects compared to a control group. Muscari et al. (246) found that serum C3 was a predictor of risk for myocardial infarction in middle aged men and that insulin was the main covariate. In addition to leptin, TNF- α and IL-6, adipose cells also secrete C3 and the regulatory factors B and D (232). Weyer et al. (385) found serum C3 levels higher in IGT subjects compared to normal glucose tolerant subjects and showed that body fat, glucose disposal, gender and glucose tolerance explained 80% of the fasting C3 variance. However, they conclude that it is not known if the C3 is a cause or a consequence of the changes in insulin action.

In conclusion, complement activation is associated with insulin secretion and insulin resistance. In the pathogenesis of GAD-positive diabetes, complement may play a role in the accelerated rate of β -cell destruction and insulin deficiency, in addition to playing a role, either cause or effect, in insulin resistance. The purpose of this study was to evaluate the clinical and metabolic characteristics of GAD-positive patients with Type 2

diabetes. Secondly, we aimed to correlate markers of immune activity with the key parameters of diabetes pathogenesis, namely insulin resistance and secretion.

Methods

Subjects.

A total of 24 patients initially diagnosed with Type 2 diabetes volunteered to participate in this study. GAD antibody positive (n=12) and GAD antibody negative (n=12) subjects with Type 2 diabetes were matched for age, duration of diabetes, gender and glycaemic control. Serum from 10 recently diagnosed (< 1 month) GAD-positive subjects with Type 1 diabetes was used as a control group for the *in vitro* complement activation and insulin secretion experiments. Subjects were identified from our diabetes database and were recruited from the diabetes clinic following detailed explanation of the study. The protocol was approved by the Joint Research Ethics Committee and written informed consent was obtained from all subjects. All subjects completed a medical history and had a physical examination. Body mass index (BMI) was calculated from body weight and height, measured without shoes. Waist and hip circumferences were measured to the nearest 0.1 cm using an anthropometric tape. A 3-hour oral glucose tolerance test (OGTT), using the WHO criteria (5), was used to confirm glucose tolerance. Subject characteristics are presented in Table 3.1.

***In Vivo* Metabolic Response.**

An insulin-modified frequently sampled intra-venous glucose tolerance test (FSIGT) was performed to assess first phase insulin secretion and insulin sensitivity (264). Subjects treated with oral hypoglycaemic agents were withdrawn from these agents for at least 7 days prior to the FSIGT test. Subjects treated with insulin administered half their usual dose of intermediate acting insulin the night before the study and omitted the morning short acting insulin.

Subjects reported to the Metabolic Research Unit at 08:00 after an overnight fast. An antecubital cannula was inserted in each arm following a period of rest. After baseline blood samples for glucose, insulin, C-peptide and lipid profile were taken, a 0.3 g/kg

glucose bolus was administered intravenously over 1-minute. At 20-minutes a 0.05 U/kg insulin bolus (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was administered intravenously and samples collected until 180 min. Minimal Model analysis was used to assess first phase insulin secretion, insulin sensitivity (S_I) and glucose effectiveness (S_G) (289,28,33). Insulin secretion was calculated as the area under the insulin concentration curve (AUC) during the “endogenous” phase, i.e. before the exogenous administration of the hormone. The component of the secretion due to the glucose stimulation was assessed as Δ AUC, the suprabasal AUC, and AIR_G , the average concentration of insulin during the first 8 min. The product $DI = S_I \times AIR_G$, termed disposition index, represents an integrated picture of factors controlling glucose metabolism, i.e. insulin sensitivity and secretion (186). The average S_I and S_G for normal controls from our centre were used for comparison with the parameters obtained in the GAD groups.

***In Vitro* β -cell function**

β -cell culture. Clonal insulin-secreting BRIN-BD11 cells were maintained in RPMI-1640 tissue culture medium with 10% (v/v) fetal calf serum (FCS), 0.1% antibiotics and 11.1mM D-glucose, pH 7.4. Cells were subsequently seeded into 24 or 96 well plates for culture in medium supplemented with 10% human sera. BRIN-BD11 cell viability was determined using a non-radioactive cell proliferation assay - the CellTiter 96 Aqueous Assay. BRIN-BD11 cell viability was similar for all groups, therefore this test is a measure of the function of the β -cell and not cell death.

Insulin secretion measurements from BRIN-BD11 cells. Cells were seeded into 24-well plates at a concentration of 1×10^5 cells per well, supplemented with 10% human serum. Following culture at 37°C the tissue media was removed and replaced with 1ml Kreb's Ringer Bicarbonate buffer. Following a 40-minute pre-incubation at 37°C, the cell monolayers were then incubated in test buffer containing either 1.1mM D-glucose to determine basal, or 1.1mM D-glucose with 10mM L-alanine, to determine stimulated insulin secretion. After 20 minutes incubation at 37°C an aliquot (900 μ l) of buffer was removed for insulin measurement.

Determination of sC5b-9 formation. BRIN-BD11 cells were plated at a density of 7.5×10^5 per ml with 10% (v/v) human sera for 24 hours. Production of TCC (SC5b-9) was determined using the QUIDEL SC5b-9 Enzyme Immunoassay kit. The quantification of SC5b-9 in experimental samples is a three step procedure utilizing: (i) a microassay plate coated with mouse monoclonal antibody which binds specifically to SC5b-9, (ii) HRP-conjugated antibodies to antigens of SC5b-9, and (iii) chromogenic substrate.

Assay methodology.

Serum insulin and C-peptide were measured by the use of commercially available fluoroimmunoassays (Auto-Delfia). Plasma cholesterol and triglycerides were measured using enzymatic methods (Human liquicolor kits/Hitachi Modular). Plasma HDL-cholesterol and LDL-cholesterol were measured directly with enzymatic methods (Randox direct kits/Hitachi Modular). Plasma glucose was measured using a glucose oxidase method (bio Merieux kit/Hitachi Modular) and blood HbA_{1c} measurement was performed using a Hi-Auto A_{1c} analyser (Menarini HA 8140). Glutamic acid decarboxylase antibodies were measured using a direct radioligand assay (Centrak anti-GAD65; Medipan Diagnostica).

Statistics.

Data are presented as mean $\pm$ standard error. An unpaired t-test was used to determine differences between groups. Data sets were log-transformed if not normally distributed. Best subset regression analysis was used to determine the variance associated with insulin secretion. A Pearson correlation was used to detect relationships between variables. Statistical significance was set at $p < 0.05$.

Results

Phenotype

Physical characteristics of the GAD-positive and GAD-negative subjects, matched for age, gender, duration of diabetes and glycaemic control, are presented in Table 3.1. Subjects in both groups were middle aged, had good glycaemic control (HbA_{1c} normal

range (4.9-6.9%)) and had a similar duration of diabetes. The GAD-negative group was more obese than the GAD-positive group. Resting systolic and diastolic blood pressure was similar between groups. GAD-negative subjects were treated by diet (n=4) and oral hypoglycaemic agents (n=8) while three of the GAD-positive subjects were treated with insulin and the others with oral hypoglycaemic agents.

Metabolic Phenotype

Fasting plasma glucose was similar between groups but fasting serum insulin and C-peptide were significantly lower in the GAD-positive group (Table 3.2). Total cholesterol, HDL and LDL cholesterol were similar between groups but fasting triglycerides were significantly greater in the GAD-negative group (p=0.02).

TABLE 3.1. Clinical Characteristics of GAD-positive and GAD-negative groups matched for age, gender, duration of diabetes and glycaemic control.

Variable	GAD-negative	GAD-positive
Age (years)	52.7±1.8	54.5±4.5
Gender (male/female)	7/5	7/5
HbA _{1c} (%)	7.6±0.3	7.8±0.3
Duration of diabetes (years)	3.4±0.6	4.5±1.3
Weight (kg)	91.0±2.7 ^a	77.0±3.2
Height (m)	1.67±0.02	1.70±0.03
BMI (kg/m ²)	32.9±1.3 ^a	26.7±1.2
Waist-to-Hip Ratio (WHR)		
Male	1.01±0.02 ^a	0.92±0.02
Female	0.95±0.03 ^a	0.85±0.03
Systolic BP (mmHg)	134±4	142±6
Diastolic BP (mmHg)	80±3	81±2

Data presented as Mean±SE. ^a significantly different to GAD-positive, p<0.05.

Insulin resistance

The GAD-positive and GAD-negative groups were both severely insulin resistant (Figure 3.1). Their insulin sensitivity indexes were much lower than those of a control population ($6.1 \pm 0.5 \cdot 10^{-4} \text{ min}^{-1}/(\mu\text{U}\cdot\text{ml}^{-1})$; 191), but the S_I from the GAD-negative group was significantly lower than the GAD-positive ($p=0.018$). S_G in both groups was lower ($p=0.0001$) than that of the control population ($0.030 \pm 0.004 \text{ min}^{-1}$), but were not significantly different from each other (0.016 ± 0.001 and $0.015 \pm 0.002 \text{ min}^{-1}$ for GAD-positive and GAD-negative, respectively), indicating that the glucose effectiveness equally contributed to the reduced glucose tolerance of both groups.

TABLE 3.2. Metabolic characteristics of GAD-positive and GAD-negative groups including fasting glucose, insulin, C-peptide and lipids and minimal model calculations of insulin sensitivity and insulin secretion.

	GAD-negative	GAD-positive
Glucose (mmol/l)	9.8 ± 0.6	10.4 ± 1.0
Insulin (pmol/l)	81.3 ± 9.8^a	49.5 ± 10.1
C-peptide (nmol/l)	1.16 ± 0.10^a	0.61 ± 0.11
Triglycerides (mmol/l)	2.73 ± 0.51^a	1.71 ± 0.45
Total cholesterol (mmol/l)	5.2 ± 0.3	4.7 ± 0.4
HDL cholesterol (mmol/l)	1.04 ± 0.04	1.06 ± 0.08
LDL cholesterol (mmol/l)	3.2 ± 0.2	2.8 ± 0.3
S_I ($10^{-4} \text{ min}^{-1}/(\mu\text{U}/\text{ml})$)	0.55 ± 0.11^a	1.23 ± 0.26
AUC (pmol/l · min)	0.90 ± 0.10^a	0.48 ± 0.08
ΔAUC (pmol/l · min)	0.73 ± 0.13^a	0.01 ± 0.05
ΔAIR_G (pmol/l)	34.0 ± 5.2^a	17.4 ± 5.7
DI (10^{-4} min^{-1})	2.73 ± 0.6	2.2 ± 0.9

Data presented as mean $\pm$ SE. ^a significantly different to GAD-positive, $p < 0.05$.

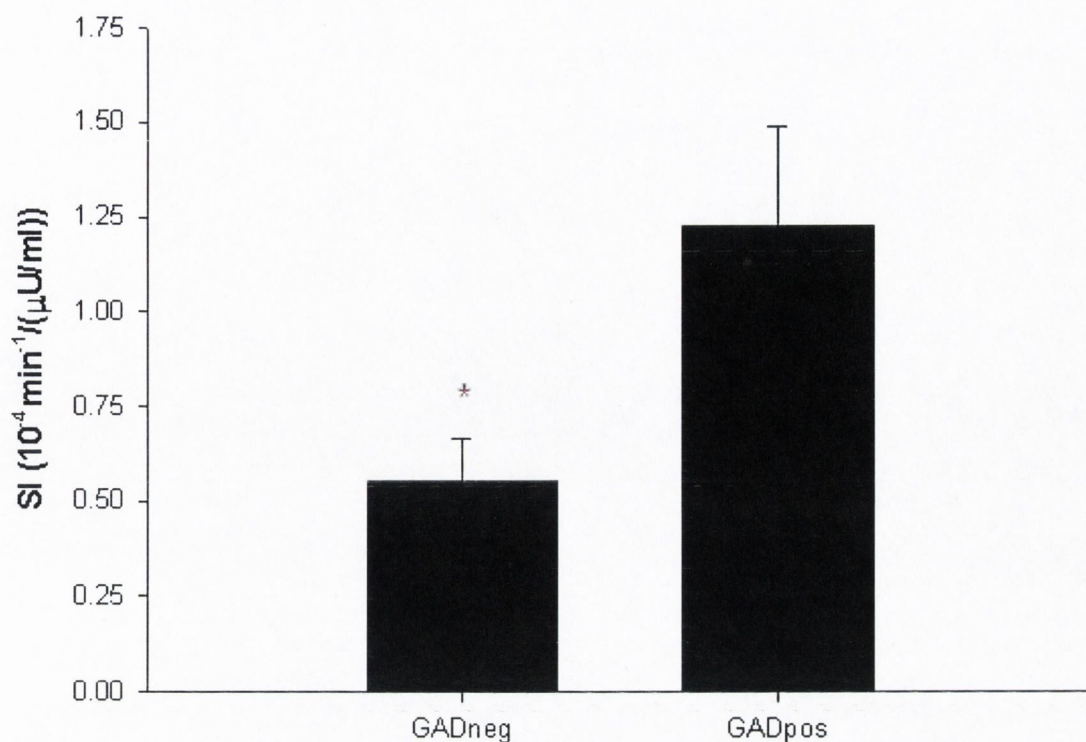


FIGURE 3.1. Insulin sensitivity (S_I) calculated using the Minimal Model for GAD-positive and GAD-negative subjects with Type 2 diabetes. Significance was set at $p < 0.05$. * significantly different to GAD-positive.

Acute-phase insulin secretory response

The first phase insulin and C-peptide response are presented in Figure 3.2. The total insulin area under the curve for insulin from 0-8 mins, the delta insulin area under the curve (ΔAUC), also known as the increase above fasting insulin, and the acute insulin response to glucose (AIRg) were significantly greater in the GADneg group. The disposition index was similar between GAD-positive and GAD-negative groups.

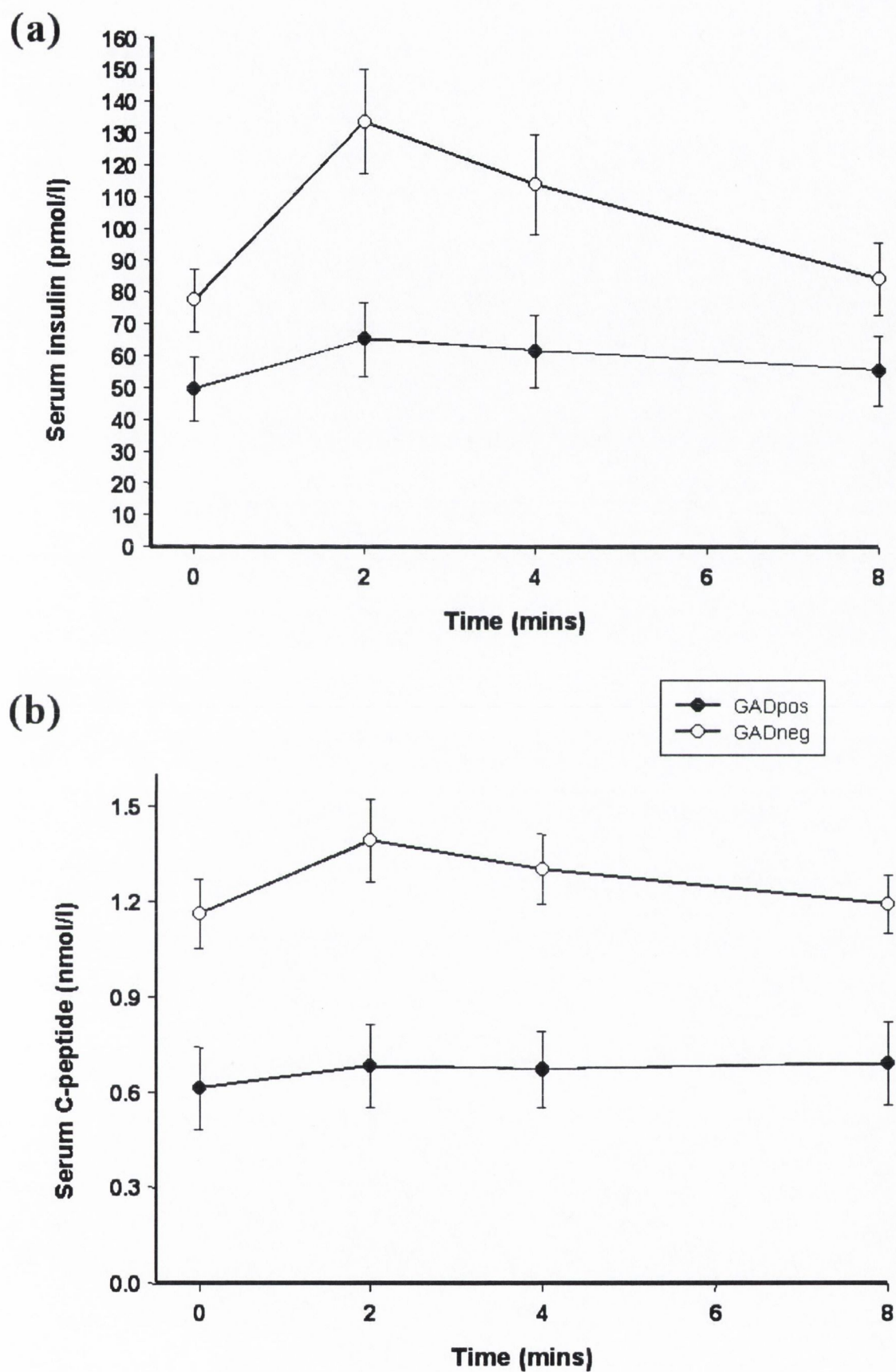


FIGURE 3.2. 1st phase (a) insulin and (b) C-peptide response from 0-8 minutes following a 0.3g/kg body weight intravenous glucose bolus in GAD-positive and GAD-negative groups.

sC5b-9 production on exposure of clonal β -cells to human sera

Levels of TCC, measured as soluble C5b-9, were determined in the media of clonal β -cells supplemented with 10% human serum after 24 hours. TCC was not normally distributed but there was no significant difference in TCC concentrations, either raw data or when log transformed, between media supplemented with GAD-positive (3391 ± 640 ng/ml) or GAD-negative (2761 ± 425 ng/ml) serum. To determine if these values were similar to Type 1 diabetes, sera from 10 recently diagnosed patients with Type 1 diabetes was used (Figure 3.3). TCC was significantly greater in response to Type 1 diabetes sera than either GAD-positive or GAD-negative.

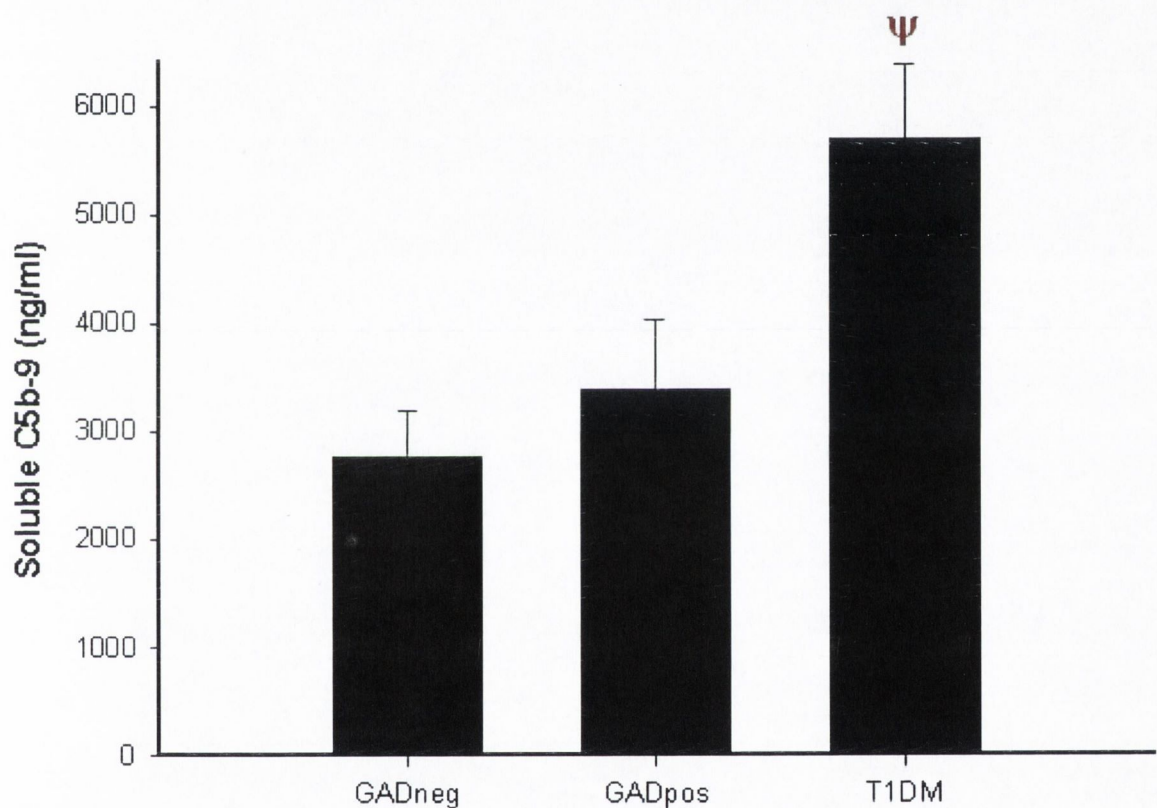


FIGURE 3.3. Terminal complement complex formation in clonal β -cell line BRIN-BD11 following supplementation with human sera for 24-hours. Significance set at $p < 0.05$. Ψ significantly different to GAD-positive and GAD-negative groups.

Effect of sera exposure on basal and alanine-stimulated insulin secretion

Insulin secretion from clonal β -cells supplemented with sera from GAD-negative subjects increased from basal levels of 1.19 ± 0.07 ng/20mins/ 10^6 cells to 2.4 ± 0.1

ng/20mins/ 10^6 cells following alanine stimulation (Figure 3.4). Basal insulin secretion was similar for GAD-positive subjects (1.08 ± 0.06 ng/20mins/ 10^6 cells) but alanine-stimulated insulin secretion was significantly lower (2.05 ± 0.10 ng/20mins/ 10^6 cells, $p < 0.05$). Alanine stimulated insulin secretion was significantly lower in subjects with Type 1 diabetes (1.46 ± 0.14 ng/20mins/ 10^6 cells) compared to GAD-positive ($p = 0.005$) and GAD-negative ($p < 0.001$) subjects, while basal secretion was lower than the GAD-negative subjects.

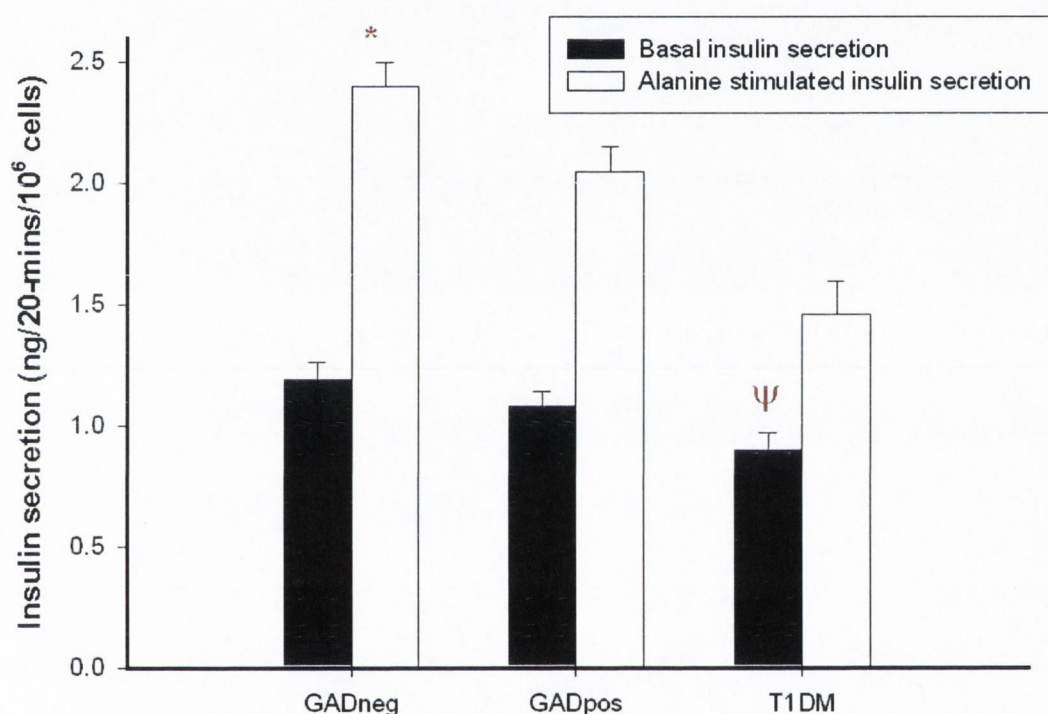


FIGURE 3.4. Insulin secretion from clonal pancreatic β -cells in the presence of 1.1mM glucose and 1.1 mM glucose with 10mM alanine.

Correlation of *in vivo* and *in vitro* measures of insulin secretion

There was a positive relationship between *in vitro* alanine stimulated insulin secretion and the *in vivo* Δ AUC ($r = 0.49$, $p = 0.02$) for all subjects with Type 2 diabetes. TCC formation was inversely correlated with *in vitro* alanine stimulated insulin secretion ($r = -0.71$, $p = 0.0002$) and *in vivo* glucose stimulated Δ AUC ($r = -0.54$, $p = 0.006$). Therefore, complement activation appears to be associated with reduced nutrient stimulated, but

not basal, insulin secretion. Though this relationship was independent of antibody status, TCC formation was positively associated with the duration of diabetes ($r=0.57$, $p=0.003$) and accounted for 39% of the variance in the *in vivo* glucose stimulated Δ AUC for insulin, indicating that complement activation may play a role in determining insulin secretory function in Type 2 diabetes.

Discussion

Results from the present study demonstrate that GAD antibody positive subjects have a phenotype distinct from classical Type 2 diabetes. Of this group, 67% were diagnosed after 40 years of age, 42% had a BMI greater than 29 kg/m^2 , and all were treated initially with diet and/or oral hypoglycaemic agents with only 25% currently insulin requiring. However, the GAD-negative subjects who were matched for age, gender, duration of diabetes and glycaemic control had a significantly greater BMI, and waist-to-hip ratio. Some studies (361,177) have described GAD-positive subjects to be less obese. However, the largest prospective study comparing GAD-positive and GAD-negative subjects with Type 2 diabetes ($n>4000$) reports a similar BMI, waist-to-hip ratio and gender distribution between groups (413). Clinical phenotype alone is insufficient to categorise diabetes subjects as about 40% of the GAD-positive subjects in our study were insulin could easily be mistaken as typical Type 2 diabetes.

Our data indicate that both groups were insulin resistant, compared to a control group ($6.1 \pm 0.5 \times 10^{-4} \text{ min}^{-1}/(\mu\text{U ml}^{-1}; 191)$). Previous studies have also reported a similar degree of insulin resistance in GAD-positive and GAD-negative Type 2 diabetes (358,51). If insulin resistance was the primary cause of diabetes in the GAD-positive group, it would be expected that they would have a similar insulin sensitivity index, as glycaemic control, fasting plasma glucose, age and duration of diabetes were similar between groups. While the GAD-positive subjects were not as insulin resistant, the disposition index, which is the product of insulin sensitivity and insulin secretion, was similar. Therefore, net/overall metabolic function was similar between groups even though the contribution of insulin resistance was different. It is possible that the degree of insulin resistance required for the onset of diabetes in this group may not be as severe because

of their more advanced impairment of insulin secretion. A second possibility is that insulin resistance may be secondary to hyperglycaemia resulting from insulin secretory dysfunction. Insulin secretory function in GAD-positive subjects is critical for determining the classification and treatment of these patients.

Fasting serum C-peptide was 48% lower in the GAD-positive group ($p < 0.05$). Carlsson et al. (51) found LADA subjects had a fasting C-peptide of 0.9 ± 0.4 nmol/l in comparison to 1.1 ± 0.5 nmol/l from Type 2 diabetes and Niskanen et al. (251) found that after 5-years, fasting C-peptide was 0.47 ± 0.24 nmol/l for GAD-positive and 0.97 ± 0.50 nmol/l for GAD-negative. These results are consistent with the present study (0.60 ± 0.15 vs. 1.16 ± 0.10 nmol/l for GAD-positive and GAD-negative) following an average clinical duration of diabetes of 4.5 ± 1.3 years. C-peptide is detectable in GAD-positive subjects between 5 and 10 years after diagnosis (251), in marked contrast to the typical findings in Type 1 diabetes of similar duration where C-peptide is undetectable.

It is important to determine glucose stimulated insulin secretion to evaluate insulin secretory function. First phase insulin secretion was measured in response to a 0.3 g/kg body weight intravenous glucose bolus. The ΔAUC for insulin and the $\Delta AIRg$ refer to glucose stimulated insulin release, which was blunted in both groups, but significantly lower in the GAD-positive group. Niskanen et al. (251) found that in a 10 year follow up, 16.7% of GAD-positive patients at diagnosis became insulin deficient, in comparison with 0.8% of GAD-negative. While insulin deficiency was defined, by these authors, as undetectable C-peptide, they also found that 50% were relatively insulin deficient, failing to have a C-peptide response greater than 0.7 nmol/l in response to glucagon stimulation, compared to 3.3% of GADneg. Using this criteria, none of the GAD-positive subjects in the present study had absolute insulin deficiency, 42% had relative insulin deficiency and the remainder had a C-peptide response > 0.7 nmol/l on at least one time point between 0-8 mins.

It has previously been suggested that the decrease in $AIRg$ and C-peptide was the result of decreased maximal β -cell capacity and not a defect in glucose metabolism (51), or β -cell destruction (358). Basal and glucose-stimulated insulin and C-peptide results from the present study suggest that a functional defect inhibiting glucose stimulated insulin

secretion, either independent of, or in conjunction with β -cell destruction may be responsible for decreased insulin secretion.

Alanine-stimulated insulin secretion was significantly inhibited in the presence of 10% sera from GAD-positive, compared with the GAD-negative group ($p < 0.05$). The alanine stimulated insulin secretion was significantly greater in GAD-negative ($p < 0.001$) and GAD-positive groups ($p = 0.005$) than in Type 1 diabetes, consistent with the *in vivo* data. Furthermore, there was a significant relationship between the *in vitro* alanine stimulated insulin secretion and the *in vivo* Δ AUC from insulin ($r = 0.49$, $p = 0.02$). The strong relationship between *in vitro* and *in vivo* data suggests that a serum-containing factor may be responsible for decreased β -cell function in GAD-positive subjects with accompanying or subsequent β -cell destruction.

Our data show that complement-mediated activation is inversely related to *in vivo* glucose or *in vitro* alanine stimulated insulin secretion, supporting a relationship between insulin secretion and complement activation. This was not associated with antibody status, though only serum soluble, and not membrane-bound, TCC was determined in this study. The *in vitro* formation of TCC accounted for 38% of the variance in Δ insulin AUC following intravenous glucose administration making complement activation a potential serum factor for β -cell dysfunction in all Type 2 diabetes patients. It is possible that complement activation could result in (i) a sustained increase in $[Ca^{2+}]_i$ resulting in a progressive desensitisation and/or (ii) an increase in K_{ATP} resulting from a gradual decrease in β -cell ATP or some other factor. While complement activation is associated with β -cell dysfunction, and the duration of diabetes ($r = 0.57$, $p = 0.003$), it does not fully explain the decreased insulin secretion associated with GAD-positive diabetes. Differentiating between β -cell dysfunction and destruction is very difficult but a continuum may exist whereby complement activation mediates β -cell dysfunction, with the presence of genetic susceptibility and/or GAD positivity initiating β -cell destruction.

The immune response to GAD initiates a T-lymphocyte response, leading directly to β -cell destruction or indirectly by the proinflammatory recruitment of cytokines. Conroy et al. (71) have shown that BRIN-BD11 clonal β -cells supplemented with Type 1

diabetes sera resulted in a decrease in β -cell function but not apoptosis. The addition of TNF-, IFN- γ and IL-1 β resulted in an increased DNA fragmentation and apoptosis. The localised accumulation of cytokines may be a potent stimulus for β -cell destruction in GAD-positive diabetes. DNA fragmentation may interfere with the process of insulin secretion either directly, as the insulin gene may be disrupted, leading to inhibition of transcription and translation, or indirectly, as DNA repair is a process that is energy requiring, thus diverting energy from that required for insulin storage and secretion to that for repair. The latter pathway may be more important in GAD-positive serum-induced DNA damage of β -cells.

Conclusion

The GAD-positive phenotype shares characteristics of both Type 1 and Type 2 diabetes. Complement activation is associated with insulin secretory function but GAD-positive appears to be an indicator of β -cell destruction. We describe a distinct sub-group of insulin resistant subjects who develop Type 2 diabetes because of a failure of insulin secretion to adequately compensate due to GAD antibody-mediated β -cell dysfunction. The degree of insulin resistance required for the onset of Type 2 diabetes in this subgroup is significant but not as severe as classical Type 2 diabetes, because of the more advanced impairment of insulin secretion. We propose that the rate of development and contribution of insulin resistance and secretion could be used to more precisely classify diabetes.

Chapter 4

Early onset insulin resistance and the development Type 2 diabetes

Introduction

The prevalence of Type 2 diabetes has increased dramatically in recent years and has been associated with a decrease in the age of diagnosis. Early onset Type 2 diabetes, where subjects are less than 25 years of age, is associated with a positive family history (10,113,249,273), to a much greater extent than later onset Type 2 diabetes (250). In 1338 adults between 19-37 years, a positive family history of diabetes was strongly associated with an abnormal lipid and glucose profile, high blood pressure and obesity (234). As with typical Type 2 diabetes, obesity is an important factor in early onset Type 2 diabetes (10,113,155,274). Although BMI has limitations in the classification of obesity in children and adolescents, 85% of young Pima Indians are obese (10) and a range of 27-38 kg/m² has been reported for adolescents and young adults diagnosed with Type 2 diabetes (113). Caprio et al. (48) performed a hyperinsulinemic-euglycaemic clamp on preadolescent lean (BMI 18.0±0.9 kg/m²) and obese (BMI 31.0±1.2 kg/m²) children with a mean age of 10.4±0.4 years. In comparison to lean and obese adolescents and adults, the obese pre-adolescent group were similarly insulin resistant with a 45% reduction in insulin-stimulated glucose disposal. Weiss et al. (381) have also demonstrated that the severity of obesity in 4-20 year old children and young adults is associated with an increase in the prevalence of the metabolic syndrome

The role of obesity

An increase in visceral adiposity has been associated with increased basal and glucose stimulated insulin secretion, in addition to insulin resistance in obese adolescent girls (122,49). Some evidence suggests that visceral adiposity is associated with hyperinsulinemia, while total body adiposity has a greater influence on insulin resistance (142). Cruz et al. (75) found that in 8-13 year old children, total fat mass was related to fasting insulin and insulin sensitivity but not the acute insulin response to glucose (AIRg), while visceral fat was independently related to all variables. As the pathogenesis of Type 2 diabetes is not fully understood, it cannot be stated with certainty that body fat accumulation leading to insulin resistance is the primary cause of Type 2 diabetes. It has been suggested that an abnormal insulin pattern in response to a

meal is the earliest detectable change in obese children and that hyperinsulinemia and insulin resistance subsequently followed (218). These studies indicate that patterns of obesity, insulin resistance and insulin secretory dysfunction are similar in childhood, adolescent and adult onset Type 2 diabetes.

Birth weight and early Type 2 diabetes

In addition to the development of obesity, birth weight and childhood growth have been associated with later development of insulin resistance and Type 2 diabetes (10,113). A meta-analysis of 48 papers (249) found an inverse relationship between low birth weight and fasting glucose, 2-hr post glucose, fasting insulin, the prevalence of insulin resistance and Type 2 diabetes. Eriksson et al. (106) found that low birth weight was associated with the development of insulin resistance and Type 2 diabetes. They also found that low maternal BMI, being thin at birth and during childhood and a rapid height increase in childhood were related to insulin resistance while a high maternal BMI and rapid growth in childhood weight and height were related to Type 2 diabetes. Bavdekar et al. (24) also found that Indian children who had a low birth weight but high fat mass at 8 years had the greatest risk of the insulin resistance syndrome.

In Pima Indians there appears to be a U-shaped relationship between birth weight and adult related metabolic abnormalities. Dabelea et al. (80) found that birth weight was negatively associated with fasting insulin, 2-hour insulin and insulin resistance measured using HOMA. While low birth weight individuals were thin, they were more insulin resistant relative to their body size in adulthood and those with a high birth weight were more obese. A U-shaped relationship was also found between birth weight, fat mass and BMI in a group of Caucasian and African American children.

High risk ethnic groups

Most of the research in early onset Type 2 diabetes has been conducted in minority groups (10,113,81), so much so that some regard this form of diabetes as only pertaining to high risk groups such as African American, Mexican Americans, Native Americans and Asians (297). In a review of US research studies including 578 youth diagnosed with Type 2 diabetes, 94% belonged to minority communities (113), while in the only report in the United Kingdom, all 8 girls identified were from Pakistani, Indian or Arabic origin (110). Ethnic groups are characterised by hypertriglyceridemia,

hyperinsulinemia (15,106,271,22), similar insulin mediated glucose disposal but lower insulin sensitivity indices (15,366). The hyperinsulinemia results from increased insulin secretion and decreased insulin clearance (15), and cannot be accounted for by dietary factors alone (223). There may be ethnic differences in the compensatory mechanisms for insulin resistance as African Americans have a higher acute insulin response to glucose than Caucasians or Mexican Americans, associated with decreased hepatic insulin clearance (134).

Further studies are required to determine the pathophysiological differences between ethnic groups. A recent study (136) looking at the genetic factors that contribute to ethnic variation, termed genetic admixture, found that the proportion of an individual's genome associated with a given ancestral origin was independently related with insulin sensitivity and the acute insulin response to glucose. They conclude that a categorisation based on genetic admixture would be more appropriate than broad racial categorisation.

The typical age of diagnosis for adolescents with Type 2 diabetes is between 12-16 years (10,113,81). Very few cases are detected pre-puberty and it is thought that pubertal changes are associated with the onset of Type 2 diabetes. Amiel et al. (11) first reported a decrease in glucose disposal in prepubertal children, with and without Type 1 diabetes. In a large study (n=357) of insulin sensitivity in children across all Tanner stages of development, Tanner stages 2-4 were characterised by significantly lower insulin sensitivity values (18% in girls, 22% in boys), and that Tanner stage 5 was associated with a partial recovery (244). Goran and Gower (132) reported a decrease of 32% as children progressed from Tanner stage 1 to 3, accompanied by a 30% increase in the AIR_G and a rise in fasting glucose and insulin. In a 2 year follow-up, the children who remained in Tanner stage 1 showed a slight increase in insulin sensitivity (6.4 ± 3.1 to $7.4 \pm 3.5 \times 10^{-4} \text{ min}^{-1}/(\mu\text{U/ml})$) indicating that pubertal development and not age is associated with the deterioration in glucose metabolism.

Puberty is associated with physiological flux where the endocrine system is heavily involved in the regulation of physical changes. Travers et al. (357) found that decreased insulin sensitivity in girls progressing from Tanner stage 2 to 3 was related to

increased body fat while boys who decreased body fat, maintained their insulin sensitivity, indicating that these changes may be related to body composition. Not all studies report these gender or body fat relationships with insulin resistance (132,356,244) and indicate that other mechanisms are responsible.

Changes in growth hormone (10,73,243) but not testosterone or estradiol (10,133,357), most likely contribute to insulin resistance. Circulating growth hormone and insulin-like growth hormone (IGF-I), which increase lipolysis and decrease glucose disposal, are greater in puberty, matching the Tanner stages of development (71,243). Though the mechanisms are not fully understood, a combination of factors is likely to contribute to the development of insulin resistance. The binding hormones that regulate the biological function of IGF-I are related to insulin sensitivity (243), hexokinase activity is downregulated which would lead to decreased glycolysis and glycogen synthesis (159), an increased rate of glycerol appearance would increase gluconeogenesis and increased free fatty acid availability would impair glucose uptake (289). Cutfield et al. (78) found that the incidence of Type 2 diabetes in growth hormone-treated children was 6-fold greater than in children not treated with growth hormone, while the incidence of Type 1 diabetes was consistent with the general population. This effect was not reversed when growth hormone treatment ceased indicating that it is not just a transient effect.

Though not definitively known, it appears that increased growth hormone production at the onset of puberty is associated with a decrease in tissue glucose disposal, an increase in gluconeogenesis and free fatty acid availability. Those who are obese, have a family history of Type 2 diabetes or possess risk factors for the development of Type 2 diabetes may fail to compensate for the growth hormone-induced insulin resistance, and develop Type 2 diabetes.

The purposes of this study are to (i) characterise the metabolic and phenotypic traits of early onset Type 2 diabetes in Irish subjects; (ii) assess the relative contribution of insulin resistance and insulin secretory dysfunction in this group compared to an obese, age matched control group and an older, typical group of Type 2 diabetes.

Methods

Subjects.

Subjects were identified from our diabetes database and were recruited from the diabetes clinic following detailed explanation of the study. During the data collection period 11 subjects under 25 years of age were diagnosed with Type 2 diabetes (YT2). This group were compared with 13 age and obesity matched non-diabetic subjects (Young OB) and 14 subjects with typical onset Type 2 diabetes diagnosed after 40 years of age (Old T2). A 3-hour oral glucose tolerance test (OGTT), using the WHO criteria (5), was used to confirm glucose tolerance. The protocol was approved by the Joint Research Ethics Committee and written informed consent was obtained. All subjects completed a medical history and had a physical examination. Body mass index (BMI) was calculated from body weight and height, measured without shoes. Waist and hip circumferences were measured to the nearest 0.1 cm using an anthropometric tape.

Intra-venous glucose tolerance test (FSIGT).

An insulin-modified frequently sampled intra-venous glucose tolerance test (FSIGT) was performed to assess insulin sensitivity and first phase insulin secretion (264). Though this test is usually performed on adult population, it has been validated in children and adolescents (77). Subjects treated with oral hypoglycaemic agents had a 7-day washout period before the test. Subjects treated with insulin administered a short acting insulin the night before the study and omitted the morning short acting insulin. Subjects reported to the Metabolic Research Unit at 08:00 after an overnight fast. An antecubital cannula was inserted in each arm following a period of rest. After baseline blood samples for glucose, insulin, and lipid profile were taken, a 0.3 g/kg glucose bolus was administered intravenously over 1-minute. At 20-minutes a 0.05 U/kg insulin bolus (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was administered intravenously and samples collected until 180 min. Minimal Model analysis was used to assess first phase insulin secretion, insulin sensitivity (SI) and glucose effectiveness (SG) (264,29,263). Insulin secretion was calculated as the acute insulin response to glucose (AIRg) during the "endogenous" phase, i.e. before the exogenous administration of the hormone. The disposition index (DI) is the product of the insulin

sensitivity index and theAIRg, represents an integrated picture of factors controlling glucose metabolism, i.e. insulin sensitivity and secretion (186).

Assay methodology.

Serum insulin and C-peptide were measured by the use of commercially available fluoroimmunoassays (Auto-Delfia). Plasma cholesterol and triglycerides were measured using enzymatic methods (Human liquicolor kits/Hitachi Modular). Plasma HDL-cholesterol and LDL-cholesterol were measured directly with enzymatic methods (Randox direct kits/Hitachi Modular). Plasma glucose was measured using a glucose oxidase method (bio Merieux kit/Hitachi Modular) and blood HbA_{1c} measurement was performed using a Hi-Auto A_{1c} analyser (Menarini HA 8140). GAD antibody status was determined using by ELISA using soluble rhGAD (DiamydTM). Serum concentrations of intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), E-selectin and P-selectin were measured using a commercially available monoclonal antibody ELISA assay (R & D Systems). High sensitivity C-reactive protein (hs-CRP) was measured by a commercially available ultrasensitive competitive immunoassay (Roche Diagnostics).

Statistics.

Data are presented as mean±standard error of the mean. An unpaired t-test and a one-way ANOVA were used to determine differences between groups. Data that were not normally distributed were log-transformed before analysis. A Pearson correlation was used to detect relationships between variables. Statistical significance was set at $p<0.05$.

Results

Physical characteristics.

The subject characteristics are presented in Table 4.1. The YT2 group were younger at diagnosis ($p<0.001$) but had a similar duration of diabetes ($p=0.48$) compared to the Old T2 group. All groups were similarly obese though the young T2 group were taller ($p=0.002$) and about 20 kg heavier ($p=0.005$) than the older T2 group. Waist-to-hip

ratio and blood pressure were similar between all groups and both diabetes groups had the same duration of disease of about 3 years. All subjects were negative for glutamic acid decarboxylase (GAD) antibodies.

All but one of the young T2 group had at least a two generation family history of Type 2 diabetes. One subject in this group was adopted and therefore the family history was unknown. The Young OB group and the older T2 group had a 54% and 50% family history of Type 2 diabetes, respectively. All of the older T2 and all but one of the young T2 group were receiving oral hypoglycaemic agents with one YT2 subject treated with a combination of metformin and insulin. Acanthosis nigricans was present in 3 YT2 and 3 Young OB subjects, but not in the Old T2.

TABLE 4.1. Physical characteristics of Young Type 2 diabetes (n=11), Young obese (n=13) and Old Type 2 diabetes (n=14)

	Young OB	YT2	Old T2
Age (yrs)	19.0±1.2	22.5±1.2	53.7±1.5*
Age at diagnosis (yrs)	-	19.5±1.2	50.5±1.5
Duration of diabetes (yrs)	-	2.5±0.8	3.2±0.6
Weight (kg)	104.7±4.8	110.3±5.9	91.1±2.9 ^ψ *
Height (m)	1.72±0.02	1.79±0.03	1.66±0.02 ^ψ
BMI (kg/m ²)	35.1±1.3	34.2±1.5	33.2±1.0
Gender (Male/Female)	3M/10F	8M/3F	8M6F
Family History (%)	54%	100%	50%
Systolic BP (mmHg)	121±4	125±4	130±4
Diastolic BP (mmHg)	77±2	78±2	77±2
Waist-to-Hip ratio - male	0.95±0.01	1.03±0.02	1.01±0.01
female	0.94±0.03	0.97±0.01	0.93±0.03

Data presented as mean±SE. ^ψ significantly different to young T2; * significantly different to Young OB; p<0.05.

Fasting glucose, insulin and glycaemic control

Fasting plasma glucose was significantly greater in the diabetes groups than the Young OB. The YT2 and Young OB groups had fasting hyperinsulinemia but despite the similar duration of diabetes fasting insulin was significantly lower in the Old T2 group ($p=0.02$). Glycemic control was similar between the young T2 and older T2 diabetes groups and significantly greater than the Young OB subjects.

The YT2 group displayed a similar lipid profile to the Old T2 group with elevated triglycerides and LDL-cholesterol in comparison to the Young OB. HDL-cholesterol was significantly lower in the YT2 group than either the Young OB or the Old T2 group while total cholesterol was similar between groups.

Insulin sensitivity

All groups were insulin resistant in comparison to a reference normal population of $6.1 \pm 0.5 \times 10^{-4} \text{ min}^{-1}/(\mu\text{U ml}^{-1})$ (191). Both diabetes groups were significantly more insulin resistant than the Young OB. Glucose effectiveness (S_G) was similar between both diabetes groups and significantly lower than the Young OB.

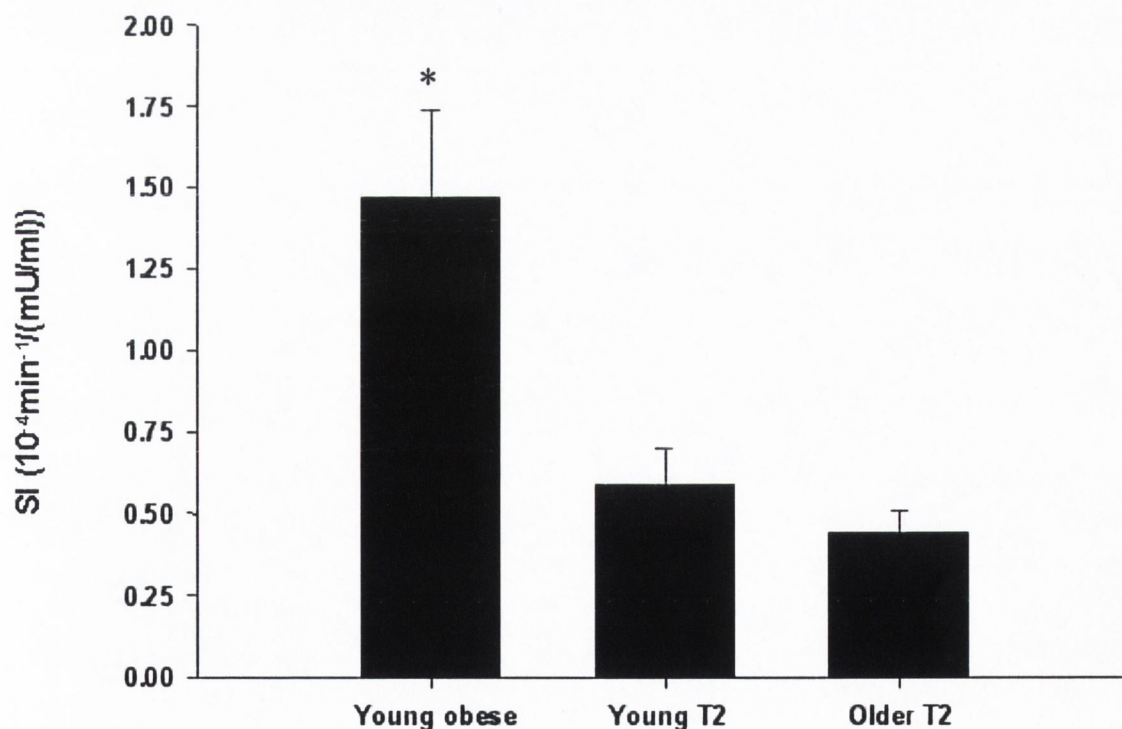


FIGURE 4.1. Insulin sensitivity index measured with minimal model analysis following an intra-venous glucose tolerance test. * significantly greater than YT2 and Old T2, $p<0.05$.

TABLE 4.2. Metabolic and inflammatory marker data of Young Type 2 diabetes (n=11), Young obese (n=13) and Old Type 2 diabetes (n=14)

	Young OB	YT2	Old T2
Plasma glucose (mmol/l)	4.8±0.2	11.2±1.1*	11.2±0.6*
Serum insulin (pmol/l)	139±20	159±39	83±10 ^ψ *
HbA _{1c} (%)	5.5±0.1	8.9±0.6	8.7±0.5
Total cholesterol (mmol/l)	4.1±0.2	4.5±0.2	5.3±0.3
HDL cholesterol (mmol/l)	1.06±0.06 ^ψ	0.96±0.04	1.09±0.04 ^ψ
LDL cholesterol (mmol/l)	2.5±0.2	3.2±0.3	3.4±0.3*
Triglycerides (mmol/l)	1.26±0.09	1.9±0.2*	2.4±0.5*
S _I (10 ⁻⁴ min ⁻¹ (μU/ml))	1.47±0.27	0.59±0.11*	0.44±0.07*
S _G (min ⁻¹)	0.022±0.001	0.017±0.002*	0.016±0.001*
AIR _G (pmol/l)	1094±204	162±46*	108±12 ^ψ *
Disposition Index (10 ⁻⁴ min ⁻¹)	242.0±47.4	18.2±4.9*	8.0±1.7 ^ψ *
hs-CRP (mg/L)	5.3±1.4	13.6±4.6*	
ICAM (ng/ml)	354±29	363±28	403±34
VCAM (ng/ml)	504±73	689±131	716±49 ^ψ
E-selectin (ng/ml)	76±10	93±8	100±11
P-selectin (ng/ml)	118±7	122±8	83±5 ^ψ *

Data presented as mean±SE. *significantly different to Young OB. ^ψsignificantly different to young T2.

Acute phase insulin secretory response

The AIR_G was significantly blunted (p=0.002) in the YT2 and Old T2 groups in comparison to the Young OB. The Young OB had an average 6-fold and 9-fold greater insulin response than the YT2 and Old T2 groups, respectively. Though severely blunted, the AIR_G and the disposition index were significantly greater in the YT2 compared with the Old T2 group. The disposition index of the Young OB was 12-fold

greater than the YT2 and 25-fold greater than the Old T2 group. The relationship between insulin sensitivity and the AIR_G is presented in Figure 4.2. The YT2 and Old T2 groups were more insulin resistant with a negligible β -cell response, and are displaced within the oval. The insulin secretory function of the Young OB compensates for insulin resistance, with an inverse relationship between AIR_G and insulin sensitivity.

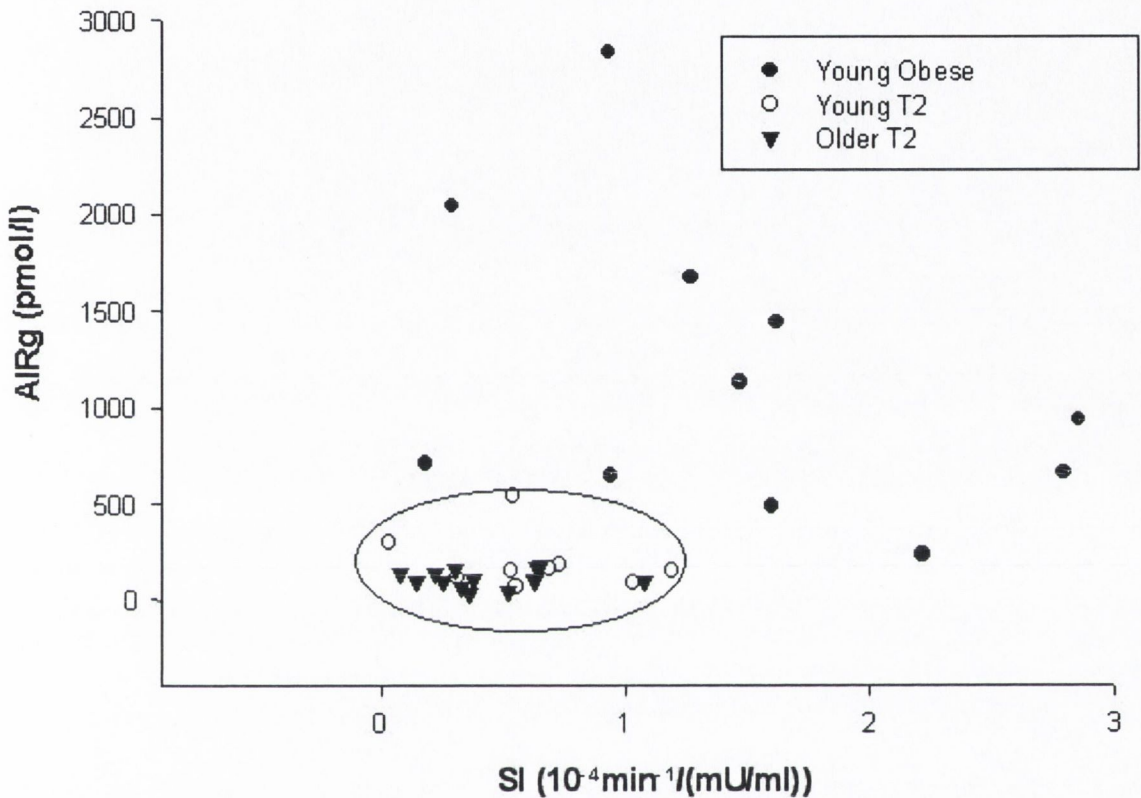
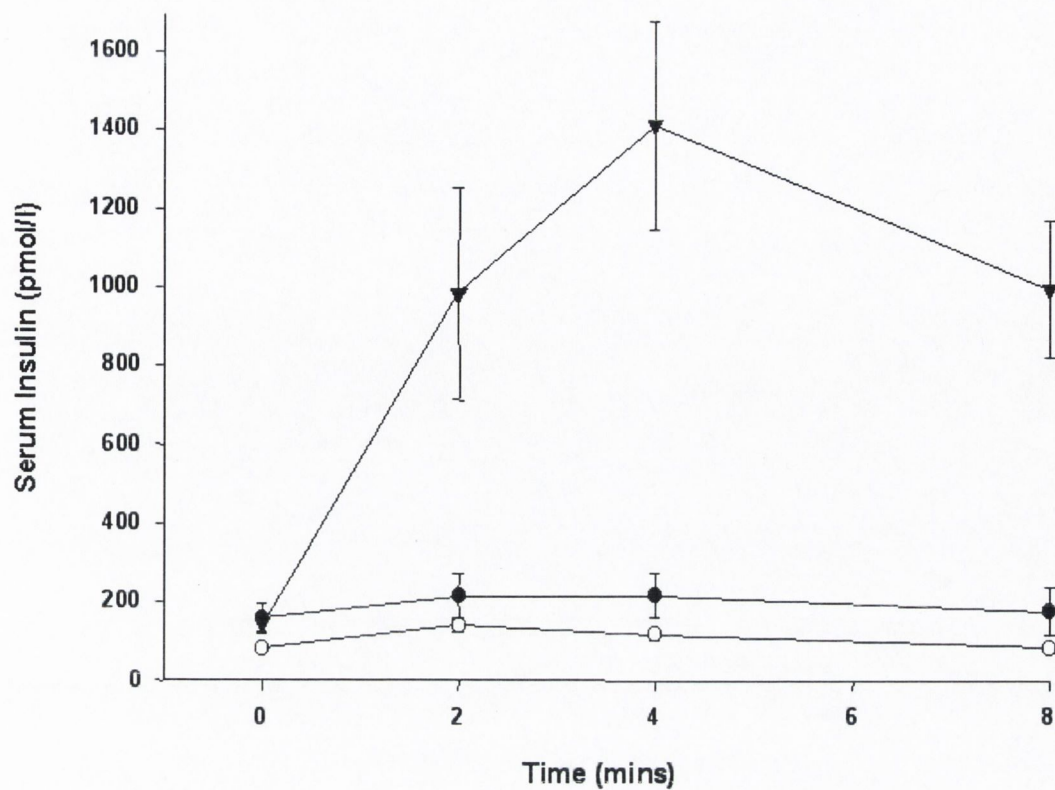


FIGURE 4.2. The relationship between the insulin sensitivity index and the AIR_G for the Young OB (n=13), the YT2 (n=11) and the Old T2 (n=14). The enclosed circle encompasses all of the diabetes subjects

Inflammatory and soluble cell adhesion markers

The concentrations of soluble cell adhesion molecules, presented in Table 4.2, were elevated in Young OB and YT2 but there was no significant difference between these groups. The inflammatory marker CRP was significantly greater in the YT2 group, though it was markedly raised in both young groups. In the YT2 group soluble cell adhesion molecules E-selectin, ICAM and VCAM were positively associated with fasting serum insulin ($r=0.84$, $p=0.002$; $r=0.79$, $p=0.007$; and $r=0.85$, $p=0.002$) and the first phase insulin secretory response ($r=0.84$, $p=0.002$; $r=0.77$, $p=0.01$; $r=0.86$, $p=0.001$). P-selectin was associated with fasting plasma glucose ($r=0.65$, $p=0.04$) and there was a positive trend with HbA_{1c} ($r=0.56$, $p=0.07$).

(a)



(b)

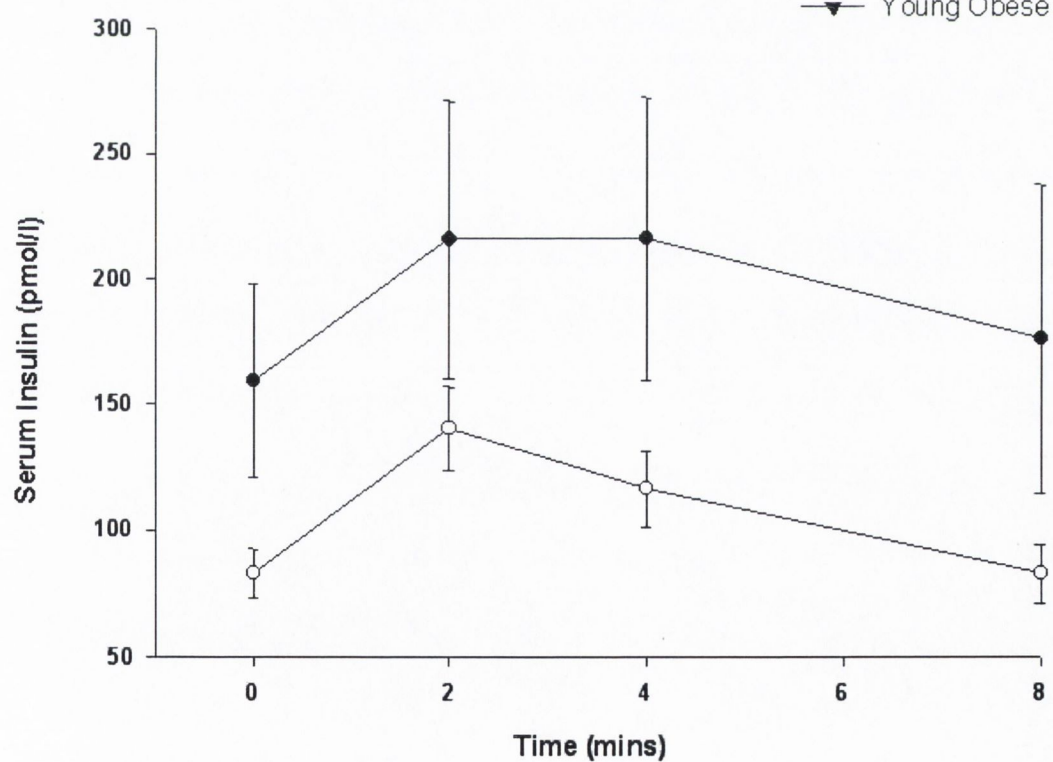


FIGURE 4.3. First phase insulin secretory response to 0.3 g/kg intravenous glucose for (a) Young OB (n=13), YT2 (n=11) and Old T2 (n=14) and (b) the two groups with Type 2 diabetes.

Discussion

In this study, we have compared the clinical and metabolic characteristics of three groups of obese Irish, Caucasian subjects: a group with early onset insulin resistant diabetes, a group with later onset typical Type 2 diabetes and a group of young obese non-diabetic subjects. We have confirmed that the early onset group have an insulin resistant form of diabetes, associated with fasting hyperinsulinemia, loss of first phase insulin secretion and a variety of adverse cardiovascular risk markers including elevated serum inflammatory markers. Therefore, based on their phenotype, and according to recent ADA guidelines on classification of diabetes, the early onset group have Type 2 diabetes exactly the same as to the older cohort from the same clinic.

Until recently it was thought Type 2 diabetes was adult onset and that all diabetes presenting in adolescence or young adulthood was Type 1 diabetes. The YT2 subjects in this study were diagnosed based on the classification outlined by the American Diabetes Association (10) where antibody status, obesity, fasting insulin/C-peptide and OGTT values were considered. These data clearly demonstrate that early onset Type 2 diabetes is not only prevalent in high risk or minority ethnic groups and is a growing worldwide phenomenon. All of the YT2 subjects, but only 50% of the Old T2 and 54% of the Young OB, had a family history of Type 2 diabetes, supporting previous data (10,113,249,273). In a large group of obese Italian children, with a similar BMI to the present study, 54% had a family history of Type 2 diabetes (173) but in normal weight, pre-pubertal children, family history was not a contributing factor to fasting glucose or insulin, insulin sensitivity or AIR_G (133). These findings support the notion that young people with a family history who are chronically exposed to an environment that results in weight gain are particularly at risk of developing Type 2 diabetes.

There has been a 67% increase in obesity in the Irish population in the past decade (253), and particularly in the past four to five years (248). In an international comparison Ireland has obesity rates second only to the US for 13- and 15-year females and third only to the US and Greece for boys (224). This has been associated with documented adverse changes in diet and a reduction in physical activity in all age groups, but particularly in young people (248). The YT2 group were obese with a mean

BMI of $34.2 \pm 1.5 \text{ kg/m}^2$, supporting the established relationship between obesity and early onset Type 2 diabetes. The failure to compensate for insulin resistance has resulted in the development of diabetes as the obese controls were similarly obese. There was a negative relationship between BMI and AIR_G ($r = -0.69$, $p = 0.02$) for the obese controls but not the diabetes groups suggesting that obesity is contributing to insulin secretory dysfunction, and may develop later in this group.

Acanthosis nigricans, skin pigmentation related to insulin resistance, is a prominent characteristic of early onset Type 2 diabetes. Of the case based studies in the United States the prevalence of acanthosis nigricans ranged from 56-92% for those with Type 2 diabetes (10). The prevalence is significantly lower in our data with 27% of the YT2 and 23% of the obese controls identified with acanthosis nigricans. The aetiology of Type 2 diabetes in young Caucasians may differ to minority ethnic groups, as our subjects were severely insulin resistant independent of the presence of acanthosis nigricans.

In prepubertal and adolescent boys and girls, Goran et al. (134) found that the insulin sensitivity index following minimal model analysis was $6.3 \pm 0.6 \times 10^{-4} \text{ min}^{-1}/(\mu\text{U/ml})$ for Caucasians, $4.1 \pm 0.6 \times 10^{-4} \text{ min}^{-1}/(\mu\text{U/ml})$ for African Americans and $4.5 \pm 0.5 \times 10^{-4} \text{ min}^{-1}/(\mu\text{U/ml})$ for Mexican Americans. In obese pubertal adolescents progressing from Tanner stage 1 to stage 3 the insulin sensitivity index decreased from 2.6 ± 0.8 to $1.7 \pm 0.14 \times 10^{-4} \text{ min}^{-1}/(\mu\text{U/ml})$ (132). Though this group are younger than the Young OB subjects in the present study, they closely represent the characteristics of this group. There is very little reference data on insulin sensitivity for the young Type 2 diabetes.

The early onset of diabetes is thought to correspond to puberty and an increase in the growth hormone/IGF-I axis during Tanner stages 2-4 resulting in decreased insulin sensitivity (73,243). The average age of diagnosis in this study was 19.5 ± 1.2 years, older than studies in ethnic minorities and may reflect a failure to diagnose patients because it is a relatively new phenomenon. It is also possible that growth hormone related insulin resistance might not be the main predisposing factor in this group. This young group with Type 2 diabetes have an identical metabolic phenotype as typical or older Type 2 diabetes subjects and are more insulin resistant than an age and obesity

matched group. The onset of Type 2 diabetes seems more closely related to β -cell failure to compensate for insulin resistance, whether obesity or growth hormone induced.

It is clear that glucose stimulated insulin secretion compensated adequately in the obese controls but not in the diabetes groups. The young obese and the YT2 groups had fasting hyperinsulinemia, which was adequate to maintain fasting glucose in the young obese but not the YT2 group. The Old T2 group was not hyperinsulinemic, indicating a greater degree of insulin secretory dysfunction. The reason for increased fasting insulin cannot be explained directly by the results of the current study. It may be that there was a longer timespan between the onset of diabetes and diagnosis in the Old T2 group, therefore insulin secretory function had deteriorated further. The YT2 group were also heavier than the Old T2 group suggesting that the observed difference may be a feature of body size.

The AIR_G was significantly blunted in both diabetes groups, contributing to hyperglycaemia in these subjects. The small but significantly greater β -cell response to glucose in the young Type 2 diabetes group may reflect the higher fasting glucose, as the increase above baseline is comparable to the older Type 2 diabetes subjects. The insulin secretory dysfunction associated with Type 2 diabetes is thought to occur in conjunction with or subsequent to insulin resistance (382). Our results suggest that the β -cell response is better in young subjects with a similar duration of diabetes. These results support the ADA consensus statement that insulin resistance is the primary defect, with progression to diabetes occurring in those who fail to compensate with increased β -cell insulin secretion.

Cardiovascular disease usually presents in adulthood but can develop in childhood if obesity, insulin resistance or diabetes are present (27,339). The association between Type 2 diabetes and cardiovascular disease is well established (45,128). The low HDL, high triglycerides and the elevated inflammatory markers in the YT2 group indicate an increased risk of cardiovascular disease in this group. The soluble adhesion molecules are similar or greater than other studies in Type 2 diabetes (221,17,345) but the hs-CRP results in the obese controls and YT2 are greater than other studies in Type 2 diabetes (221,117,308). A hs-CRP greater than 1.6 mg/l increases the risk of cardiovascular

events (332), greater than 2.1 mg/l increases the risk of cardiovascular disease 2.5-fold and values greater than 4.18 mg/l increases the risk of developing diabetes 3-fold (347). Based on these findings, the YT2 group are more at risk of cardiovascular disease than the Old T2 group but the obese controls have an increased risk of developing diabetes within 5 years.

Of grave concern is the advanced degree of endothelial dysfunction in both young groups and the implications for diabetes treatment and prevention. The YT2 group also has evidence of other diabetes-related complications including hypertension, hyperlipidemia, proteinuria, and retinopathy. Although these are preliminary data, it appears that the development of complications is occurring at an accelerated rate in the YT2 group. This has major implications for future treatment and either the severity of insulin resistance, the failure to respond to treatment or the lifestyle adherence of the subjects has resulted in a rapid onset of diabetes related complications. Of greatest concern is the fact that once β -cell function is further diminished, it is likely that glycaemia will be more difficult to control, the rate of complications will increase and treatment options will be limited for this group.

Conclusion

Early onset Type 2 diabetes in Caucasian subjects is characterised by insulin resistance and insulin secretory dysfunction, similar to older Type 2 diabetes subjects. The early development of diabetes further increases the risk of cardiovascular disease with lower HDL-cholesterol and greater soluble adhesion molecules and acute-phase proteins. The treatment of these patients could be difficult if glycaemic control is not improved and a key public health focus must be to prevent the development of diabetes in similarly obese, high risk individuals.

Chapter 5

Exercise-mediated enhancement of insulin sensitivity in obese and Type 2 diabetes subjects is independent of IRS-1 associated PI3-K.

Introduction

Metabolic response to exercise

Exercise is a metabolic stress that acutely increases the demand for energy production. The neuro-endocrine response facilitates fuel mobilisation and energy production, preferably by the oxidation of carbohydrate and lipid substrates in skeletal muscle mitochondria. While oxidation of lipid and carbohydrate are both increased during exercise, the limitation of oxygen availability favours carbohydrate as the preferential substrate. Skeletal muscle glucose uptake is increased during exercise and blood glucose concentrations are maintained by the breakdown of liver glycogen, gluconeogenesis and indirectly by increased muscle glycogen utilisation. Prolonged or high intensity exercise has been shown to deplete both liver (47) and muscle glycogen (281), resulting in reduced carbohydrate oxidation and blood glucose concentration. The post-exercise period is characterised by an increased rate of glucose uptake to replenish muscle glycogen concentrations (137). Therefore, exercise is associated with metabolic flux and accelerated rates of substrate utilisation.

Accumulated bouts of exercise, or exercise training, enhance metabolic efficiency. Increased oxygen uptake, delivery and utilisation, in addition to the increased size, number and function of mitochondria facilitate greater rates of energy production and the relative contribution of lipid in substrate utilisation (157). Exercise training, independent of age, increases insulin sensitivity (204,282) and results in lower fasting insulin concentrations (409). Studies using the hyperinsulinemic-euglycaemic clamp demonstrate that for the same circulating insulin concentration, glucose disposal rates are higher and endogenous glucose production rates are lower in exercise trained subjects (90,204,241,201). In younger subjects with normal glucose tolerance (NGT), training does not alter the glucose area under the curve in response to an oral glucose tolerance test (OGTT) but results in a significantly lower insulin response (313,178). In

older subjects with NGT, training also results in a lower insulin response to an OGTT with (313,314) or without (69) an improved glucose tolerance. Seals et al. (313) found that masters athletes had a similar glucose and insulin response to an OGTT as young athletes, a better insulin response than young sedentary subjects and a better glucose and insulin response than older sedentary subjects. Therefore, exercise training has a positive impact on insulin sensitivity, glucose disposal and insulin secretion in NGT subjects.

Exercise, insulin sensitivity and Type 2 diabetes

Holloszy et al. (158) was among the first to demonstrate that exercise training improved glucose tolerance in subjects with IGT or Type 2 diabetes. Following a 12-month exercise training programme, subjects with Type 2 diabetes who had an adequate capacity to secrete insulin, had normal or improved glucose tolerance while all subjects with IGT had normal glucose tolerance. Subsequent research using the hyperinsulinemic-euglycaemic clamp demonstrates improved insulin sensitivity in those patients with Type 2 diabetes who exercise regularly (408,378). The impact of exercise on day to day glucose control is similarly positive. Though there have been some conflicting results, a meta-analysis of studies has confirmed that regular exercise does result in better glucose control (42).

While regular exercise has positive effects on oxygen consumption, resting and exercise heart rate and stroke volume, blood lipid, and glucose control, short term exercise training appears to be beneficial to insulin sensitivity. Perseghin et al. (269) have shown improved insulin sensitivity in the insulin resistant offspring of Type 2 diabetes parents after a six week aerobic training programme, while others have shown that 7-days (69,158,408,378,294) or even a single bout of exercise (40,408,378,293,95,96) have improved glucose tolerance and insulin sensitivity. Bogardus et al. (40) conducted a hyperinsulinemic-euglycaemic clamp 16-hours after an acute bout of exercise and found a significantly greater glucose disposal rate in healthy males. Perseghin et al. (269) found similar results in insulin resistant offspring while Cusi et al. (76) showed a similar response in Type 2 diabetes patients.

The improved insulin sensitivity following an acute bout of exercise lasts between 12 and 40 hours (149,401), outlining the importance of regular exercise to maintain the

improved insulin sensitivity and leading many to believe that exercise training was largely accounted for by the last exercise bout. Acute exercise is also associated with lower blood glucose concentrations (408). It does appear that exercise training results in greater improvements in insulin sensitivity than just the last exercise session (401). However, even these effects are relatively short lived as Hortobagyi et al. (163) reported decreased insulin sensitivity in exercise trained endurance athletes following 14 days of training cessation. Heath et al. (149) found a similar result in active men and women following 10 days of detraining but found that glucose tolerance could be restored following an acute bout of exercise. These changes, especially in response to acute or short term exercise are independent of improvements in oxygen consumption, blood flow or weight reduction indicating that the local changes in the skeletal muscle result in the improvements in glucose uptake and utilisation.

Regulation of cellular metabolism in response to exercise

Exercise-mediated regulation of glycogen synthesis

The mechanism responsible for enhanced exercise-mediated glucose disposal is not fully understood but cellular flux following exercise is partly responsible. Prolonged or high intensity exercise significantly depletes muscle and liver glycogen stores. The immediate post-exercise response favours skeletal muscle glycogen resynthesis over splanchnic glucose disposal or glucose oxidation (228), though plasma insulin is low and epinephrine is elevated. While the hormonal milieu does influence metabolic regulation, it appears that local factors influence the rate of muscle glycogen synthesis (288,281). The regulation of glycogen synthase activity plays a very important role in post-exercise glycogen synthesis and glucose uptake. The activation of glycogen synthase post-exercise is immediate (255) but the rate of glycogen synthesis is dependent on both carbohydrate availability and muscle glycogen concentration. Young et al (400) demonstrated that glycogen synthase activity remained elevated for at least 18-hrs post-exercise when fed a high fat diet, but had returned to baseline in carbohydrate fed rats. In humans, Bogardus et al. (40) found that insulin-stimulated glycogen synthase activity was increased 3-fold 16-hrs after glycogen depleting exercise, but 2-fold when 100g of carbohydrate was provided after exercise. The activity of glycogen synthase decreases in proportion to muscle glycogen synthesis,

indicating that glycogen concentration may be central to the regulation of glycogen synthesis following exercise (280).

Glycogen synthase activity is inversely proportional to the rate of glycogen depletion (397,242) while the rate of glycogen resynthesis is controlled by the remaining glycogen (280). Animal perfusion studies have shown that glucose disposal and glycogen synthesis occur in rat skeletal muscle immediately post-exercise, even when insulin is not provided. Price et al. (281) found that immediately following exercise, glycogen synthesis was regulated by an insulin-independent, muscle glycogen-dependent, mechanism while insulin-mediated glucose disposal was more effective once glycogen concentrations exceeded 35mmol/l. Therefore, insulin-stimulation may not play a prominent role in the immediate post-exercise period when a separate contraction-mediated mechanism may be responsible. The distinct mechanisms for glucose disposal following insulin-stimulation and exercise may act sequentially in the resynthesis of glycogen following exercise. Even though the insulin response is critical to the regulation of carbohydrate homeostasis, it also appears that exercise is a potent stimulus for glycogen synthase activation and an important factor in improving insulin sensitivity.

Exercise, glycogen synthesis and Type 2 diabetes

Endurance exercise training increases the ability to accumulate glycogen after exercise (153,137). Ebeling et al. (102) have shown that trained athletes have greater blood flow, GLUT-4 protein content and glycogen synthase activity than untrained individuals. Therefore, if exercise has an insulin-independent mechanism for enhancing glucose uptake and disposal it may alleviate many of the defects associated with insulin resistance and Type 2 diabetes. Perseghin et al. (269) have shown increased muscle glycogen synthesis in the insulin resistant offspring of Type 2 diabetes parents after a six week aerobic training programme. It has also been shown that insulin-stimulated muscle glycogen synthase activity can be increased following exercise and that acutely glycogen levels can be normalised (89). The potential residual effects of a single bout of exercise appear to offer many of the exercise training adaptations and therefore, provides an interesting model to study. Devlin et al. (96) examined insulin sensitivity in Type 2 diabetes subjects 12-hours following glycogen-depleting exercise. They found that total glucose disposal increased during a $40\text{mU}\cdot\text{m}^{-2}\cdot\text{min}^{-1}$ insulin infusion, the

result of greater non-oxidative glucose disposal. Activation of insulin-stimulated glycogen synthase after exercise was similar to lean controls and basal endogenous glucose production was significantly decreased. The overall effect was a decrease in fasting plasma glucose concentration (197 ± 12 vs. 164 ± 9 mg/dl). The metabolic clearance rate for glucose was still markedly decreased and though insulin resistance could be decreased by one bout of exercise it could not be completely reversed. Glycogen concentrations alone do not regulate glucose disposal, as improvements in insulin sensitivity persist after muscle glycogen resynthesis has occurred (395).

Regulation of substrate oxidation during, and in response to, exercise

Substrate oxidation also plays an important role in the regulation of cellular metabolism, and as previously mentioned, substrate competition may impair glucose uptake and/or fatty acid oxidation and contribute to insulin resistance. The characterisation of substrate utilisation during, and in response to, exercise is complicated by a number of factors including the relative contribution of plasma FFA, plasma triglycerides, and intra-muscular triglycerides (IMTG); the methodologies used; the intensity and duration of exercise; individual muscle mass; the endocrine and vascular response to exercise and gender (331). In addition there appear to be different responses in lean, obese and Type 2 diabetes subjects. The somewhat conflicting results in the literature and the lack of consensus on the response to exercise limit the interpretation of this data.

In lean controls, carbohydrate and fat oxidation rates increase during exercise. There is a preference for carbohydrate oxidation during moderate to intense exercise but when carbohydrate stores decrease during prolonged exercise, there will be a greater reliance on fat oxidation (334,331). Muscle glycogen resynthesis is the primary goal after exercise and insulin stimulated carbohydrate oxidation rates are still decreased 16-hrs post exercise when carbohydrate is not provided (40). Exercise training results in a shift toward greater fat oxidation during submaximal exercise and in recovery from exercise, while the relative contribution of carbohydrate oxidation is decreased (206,241,161,169). There are conflicting results related to basal glucose and fat oxidation rates following exercise training with some (241,295,206), but not all (90), studies showing increased rates. In order to further explain the adaptations associated

with training, lipolysis and the relative contribution of plasma FFA, triglycerides and IMTG to total fat oxidation rates was explored.

During prolonged low intensity exercise Klein et al. (206) did not find any difference in lipolysis between trained and untrained men while 10- or 12-weeks of training did not result in any change in lipolysis (90,161). Although Stallknecht et al. (346) have found an increased insulin sensitivity of lipolysis in adipose tissue following exercise training and Romijn et al. (295) reported increased glycerol and FFA rates of appearance (Ra) at rest, the Ra of FFA exceeds fat oxidation during exercise and is unlikely to be the rate limiting explanation for enhanced lipid oxidation (331). The improvements in whole body fat oxidation during submaximal exercise cannot be accounted for by increased plasma FFA oxidation (161) suggesting that the greater utilisation of FFA following exercise training is due to IMTG (216,169,206). Larson-Meyer et al. (216) found that IMTG decreased 25% during a 2-hour run in female athletes. The close proximity of IMTG to the mitochondria make it an ideal substrate, especially in the post-exercise state when carbohydrate availability has decreased. While endurance training appears to result in a greater accumulation of IMTG, the rate of turnover is also accelerated (331).

Exercise, substrate oxidation and Type 2 diabetes

The enhanced utilisation of IMTG could be potentially important as a training adaptation but the exercise intensity and analysis techniques influence the interpretation. Increasing exercise intensity results in a decrease in whole body fat oxidation with plasma FFA used predominant during low intensity exercise and a greater reliance on IMTG during moderate to high intensity exercise (162). It has also been suggested that IMTG increases during high intensity exercise, reflective of the continued cellular FFA uptake but failure to oxidise (331). Recent work on the mechanisms responsible for enhanced FFA oxidation suggest that exercise-mediated upregulation of gene expression associated with peroxisome proliferator-activated receptor alpha and gamma (PPAR α/γ) and PPAR γ co-activator-1 (PGC-1) may be responsible (359,162). In particular, Tunstall et al. (359) found that the increased fat oxidation following 9 days of exercise training was associated with an increased expression of FAT/CD36, regulating FFA uptake across the plasma membrane, and CPT-1, regulating mitochondrial FFA uptake.

The exercise-mediated enhancement of fat oxidation has a potential positive impact for obesity and Type 2 diabetes, where oxidation rates are lower. However, when Hargreaves et al. (148) measured leg glucose uptake and leg RQ, with or without intralipid infusion, they found decreased glucose uptake before (64%), during (33%) and after exercise (42%) exercise with no change in leg RQ measured at 40-mins of exercise. These results suggested that fat oxidation rates may not increase following exercise training and that an upper limit of fat oxidation may exist. In support of this, van Aggel-Leijssen et al. (371) found that total fat oxidation did not change at rest in obese subjects after training at 40%- or 70% $\text{VO}_{2\text{max}}$ for 12-weeks. They did notice an increase in fat oxidation during exercise at the lower intensity, due to non-plasma fatty acid, but not at the higher intensity. The basal RER was lower for the high intensity training group, giving a high basal fat oxidation but the absolute rate of total fat oxidation after training was similar for the low and high intensity groups, supporting the view of an upper limit of fat oxidation during exercise. Energy restriction and exercise training have not been successful at reducing the elevated IMTG in obese subjects (229) though IMTG turnover has not yet been addressed. Contrary to the expected benefits of exercise on fat oxidation in obese subjects it appears that while carbohydrate oxidation may be increased, there is a lack of evidence supporting a significant training-related increase in fat oxidation (32).

If excess lipid availability alters substrate utilisation in obese subjects, the combination of elevated glucose and lipid in Type 2 diabetes subjects should result in further metabolic stress. Kang et al. (189) found that carbohydrate oxidation, when expressed as a percentage of total energy expenditure, was similar in control and Type 2 diabetes subjects exercising at 50% $\text{VO}_{2\text{peak}}$ (54% vs 57%) and 70% $\text{VO}_{2\text{peak}}$ (74% vs 74%). While the overall rates of carbohydrate oxidation were similar the Type 2 diabetes group had a greater overall rate of glucose utilisation, as opposed to muscle glycogen, in comparison to a control group, contributing to decreased plasma glucose during exercise. Giacca et al. (127) also found that there was no difference in carbohydrate oxidation rates between lean control, obese or Type 2 diabetes subjects. Though not significantly different the Type 2 diabetes group had lower fat oxidation during exercise than the lean control group. Following exercise, lipid oxidation rates were similar between all three groups but the Type 2 diabetes subjects have significantly greater rates of carbohydrate oxidation than either the lean controls or obese subjects.

Therefore, while fat oxidation does not appear to be impaired during or after exercise in Type 2 diabetes, plasma glucose oxidation is increased to lower circulating glucose concentrations.

Following one-legged aerobic exercise training in subjects with Type 2 diabetes, Dela et al. (89) found that while there was no significant difference in basal glucose or fat oxidation, there was a trend toward decreased glucose oxidation in both groups and an increase in fat oxidation in the control group. During a euglycaemic-hyperinsulinemic clamp 16-hrs after the last training bout, the rate of carbohydrate oxidation had increased in both groups, but significantly more in the controls and while fat oxidation was lower, the findings were not significant. However, in normo-glycaemic insulin resistant offspring of Type 2 diabetes, Perseghin et al. (269) found that while carbohydrate oxidation rates were proportionally decreased in the offspring, there was no increase in response to an acute bout or 6-weeks of exercise training. There is a lack of evidence to draw conclusions regarding substrate utilisation in obese and Type 2 diabetes subjects but the elevated fasting glucose and/or lipids result in different metabolic responses than lean controls. These changes may result in differential regulation of insulin signalling mechanisms and GLUT-4 translocation.

Exercise and the insulin signalling cascade

Insulin-independent GLUT-4 translocation during exercise

The synergistic effect of exercise and insulin on whole body glucose disposal (85) led to the speculation that exercise and insulin may have distinct mechanisms promoting glucose transport. Despite this, GLUT-4 translocation, the primary mechanism for insulin-mediated glucose transport, also occurs following muscle contraction in animal (287,128) and in human subjects (351). Thorell et al. (351) measured plasma membrane bound GLUT-4 following a $0.8 \text{ mU kg}^{-1} \text{ min}^{-1}$ hyperinsulinemic-euglycaemic clamp and 60-minutes of exercise at 70% $\text{VO}_{2\text{max}}$ in 9 healthy subjects and found a similar response to hyperinsulinemia (32% above baseline) and exercise (35% above baseline). While GLUT-4 translocation accounts for exercise-mediated glucose transport, animal studies indicate that there may be distinct insulin and exercise responsive subcellular pools of GLUT-4 containing vesicles (99,62,46). Whether this is also the case in human skeletal muscle has not been determined.

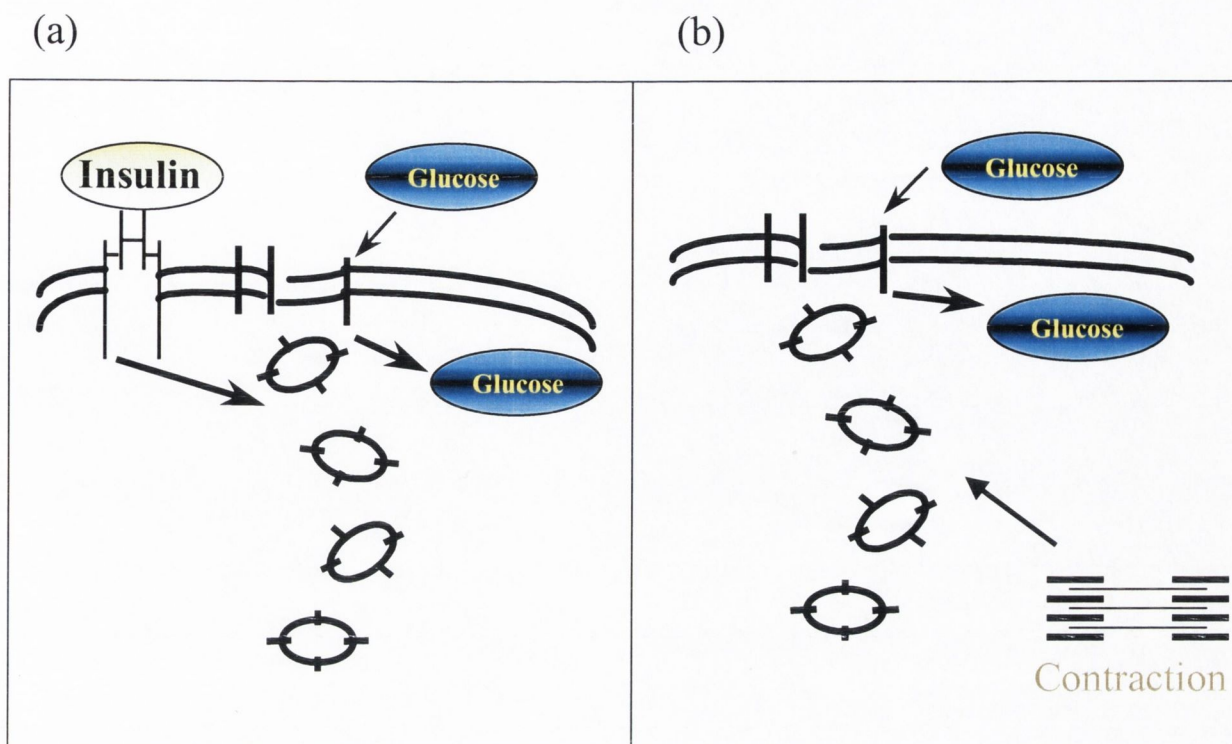


FIGURE 5.1 (a) insulin and (b) contraction-mediated glucose transport in skeletal muscle both translocate GLUT-4 containing vesicles to the plasma membrane.

While it is accepted that GLUT-4 translocation accounts for glucose transport during exercise, it does appear that a distinct intracellular signalling cascade is responsible. Data from animal studies (130,388), subsequently confirmed in humans (255,392,393,209), demonstrate that IRS-1 associated PI3-K activity is decreased immediately after exercise with no change or decreased insulin receptor (393) and IRS-1 phosphorylation (392). We have previously measured IRS-1 associated PI3-K activity immediately and 30-minutes following glycogen depleting exercise, with or without post-exercise carbohydrate, in a group of seven healthy subjects. PI3-K activity was below baseline immediately after the exercise session and remained suppressed for at least 30-minutes in the absence of carbohydrate. When carbohydrate was provided after exercise, PI3-K activity increased but was still significantly lower than when carbohydrate was ingested without exercise (255). Exercise-mediated glucose transport occurs via a GLUT-4 dependent, PI3-K independent mechanism.

Insulin sensitivity and intracellular signalling following exercise

Functional changes in insulin signalling

In response to exercise, paradoxically, there is an improvement in insulin-mediated glucose disposal. Exercise training has a positive impact on IRS-1 associated PI3-K activity in young healthy subjects (164,204). We conducted a hyperinsulinemic-euglycaemic clamp on a group of trained and untrained individuals. Insulin-stimulated whole body glucose disposal and PI3-K activity were significantly elevated in the trained compared to the untrained group with a positive correlation between glucose disposal and PI3-K activity (204). Houmard et al. (164) also found increased insulin-mediated glucose disposal and PI3-K activity following 7-days of exercise in young healthy subjects. However, in older subjects who exercise trained for 7-days there was no increase in PI3-K activity despite increased insulin-mediated glucose disposal (343). Therefore, a more detailed analysis of the insulin signalling mechanisms is necessary.

The two factors that contribute to enhanced GLUT-4 translocation are increased protein expression and/or increased function. In human studies, total skeletal muscle GLUT-4 protein content (91,74,140,272,406) is greater following exercise training. A 10 weeks single leg exercise training programme reported a $26 \pm 11\%$ increase in total GLUT-4 protein content (91) while other studies have shown that exercise training for as little as 5-7 days has increased GLUT-4 content (140,74,272). The increase in GLUT-4 content correlates with the improved insulin-mediated glucose disposal (74,210). Cox et al. (74) found a 3.1 ± 0.7 fold increase in GLUT-4 content and a 2.2 ± 0.3 fold increase in glucose disposal following 7-days of training in young and old males and females. In animal studies, training also leads to an increase in GLUT-4 protein content (131,330,345) but not always to an increase in glucose disposal for older rats (403). Therefore, while it is possible that increased insulin-mediated glucose disposal may be accounted for by increased GLUT-4 protein content, the translocation of GLUT-4 is dependent on the insulin-signalling cascade.

Exercise, protein expression and metabolic adaptation

There have not been many studies of protein expression in human skeletal muscle. Wadley et al. (376) found that there was a transitory effect on IRS-2 and p85 gene expression following 9 days of training, but no effect on the insulin receptor or IRS-1, concluding that the improvements in insulin action were not associated with transcriptional activation. In a comparison of trained and untrained subjects Yu et al. (406) found that training resulted in greater GLUT-4 protein content but decreased insulin receptor (44%), IRS-1 (57%) and IRS-2 (77%) protein. Some (58) but not all (200) animal studies help to support some of these findings. Chibilan et al. (58) exercise trained a group of female Wistar rats for 1-day or 5-days. Following 5-days of exercise, GLUT-4 expression was elevated, insulin receptor expression increased, while IRS-2 was unchanged and IRS-1 expression decreased, similar to the human study. These results were different to an acute bout of exercise that resulted in increased expression of GLUT-4 and IRS-2, with no change in the insulin receptor or IRS-1. Taken together these results indicate that there may be transient changes in protein expression, depending on the intensity and duration of exercise, in addition to the time of measurement.

On the other hand, Kim et al. (199) found an increase in GLUT-4, PI3-K, IRS-1 and insulin receptor mRNA following 9 weeks of training in male Sprague Dawley rats. While studies consistently demonstrate an increase in GLUT-4 protein following exercise training, conclusive findings cannot be drawn about protein expression within the insulin signalling pathway. It is possible that while mRNA levels increase following training, protein translation does not, and the decrease in protein content of the insulin receptor, IRS-1 and IRS-2 in human skeletal muscle may be reflective of increased protein function or degradation.

While insulin receptor tyrosine phosphorylation is not increased after exercise (393,392), 7 days of training increases insulin receptor autophosphorylation in young sedentary subjects (404). However, Dela et al. (88) found no increase in insulin receptor kinase activity following 10 weeks of training. The improvements in IRS-1 associated PI3-K activity following training has previously been covered but little information is available regarding IRS-1 phosphorylation or IRS-2. In female Wistar rats exercised for 5 days, insulin receptor and IRS-1 phosphorylation were increased and

while IRS-1 associated PI3-K was increased, there was no change in IRS-2 associated PI3-K activity (58). The increase in IRS-1 phosphorylation was contrary to the decrease in IRS-1 expression indicating an increased function despite lower protein content. Even after 1-bout of exercise insulin receptor and IRS-1 phosphorylation were increased, as was IRS-1 and IRS-2 associated PI3-K, indicating a specialised rather than redundant role for these proteins.

Akt kinase

Finally, the results regarding Akt kinase, which is also involved in GLUT-4 translocation, are controversial. Human studies have shown no change in the phosphorylation or activity of Akt immediately following exercise (393,389) or after exercise training (406). Animal studies have been used to get further insight into the role and response of Akt to exercise training. Some studies support the human data and report no change in Akt activity or phosphorylation following exercise (395,388,320), while others have shown increased function immediately (307) or after 5 days training (58). Of the three characterised isoforms Turnisky et al. (363) found that exercise increases the function of Akt1 but not Akt2/3. Sakamoto et al. (306) found exercise to activate all three Akt isoforms but the transient effect is short lived, returning to baseline at 15-minutes post. In healthy subjects the consistent data indicate that exercise training leads to increased insulin-mediated glucose disposal, accompanied by an increase in GLUT-4 protein content. There is still controversy regarding the role of the insulin signalling cascade in this population but increased IRS-1 associated PI3-K activity is evident, despite possible decreases in IRS-1 expression.

Insulin signalling and Type 2 diabetes

GLUT-4 protein and mRNA also increase in response to exercise training in subjects with Type 2 diabetes. Dela et al. (91) found that following 9 weeks of single leg exercise training skeletal muscle GLUT-4 protein content and mRNA had increased similarly in Type 2 diabetes subjects and age-matched controls. While protein concentration was similar, mRNA was always lower in the Type 2 diabetes group. Following 7 consecutive days of exercise, insulin resistant men aged 50-70 years improved insulin-mediated glucose disposal 33%, without a corresponding increase in phosphotyrosine associated PI3-K or Akt activity (343). Cusi et al. (76) found that an acute bout of exercise did not increase insulin-stimulated glucose disposal in Type 2

diabetes subjects but normalised obese subjects to values similar to lean controls. Insulin receptor and IRS-1 phosphorylation in addition to IRS-1 associated PI3-K were reduced in obese subjects and negligible in the Type 2 diabetes group, strongly indicating that factors independent of insulin signalling, or distal to PI3-K were responsible for the increased glucose disposal. There is a lack of conclusive evidence regarding the mechanism responsible for insulin-mediated glucose disposal in Type 2 diabetes subjects. The relatively few studies suggest that GLUT-4 protein content may account for the increased glucose disposal, though the signalling mechanism regulating the translocation is not fully characterised.

The purpose of the current study is to evaluate the impact of an acute bout of exercise and 7 consecutive days of exercise on glucose disposal and the insulin signalling cascade in obese and Type 2 diabetes subjects.

Methods

Subjects

8 subjects with Type 2 diabetes and 7 weight matched obese male and females volunteered to participate in the study. After the nature and risks of the study were explained in detail, written informed consent was obtained. Subjects were excluded if they had a positive resting 12-lead ECG or stress test. Maximal oxygen uptake (VO_{2peak}) was determined by indirect calorimetry (SensorMedicsTM) using an incremental bicycle ergometer protocol. A 3-hour oral glucose tolerance test (OGTT), using the WHO criteria (5), was used to confirm glucose tolerance. The protocol was approved by the Joint Research Ethics Committee and written informed consent was obtained. All subjects completed a medical history and had a physical examination. Body mass index (BMI) was calculated from body weight and height, measured without shoes. Waist and hip circumferences were measured to the nearest 0.1 cm using an anthropometric tape.

Experimental Design:

The study design is presented in schematic format (Figure 5.2.). On day 1, baseline insulin sensitivity was determined with a 2-hr euglycaemic-hyperinsulinemic clamp.

The following afternoon (day 2) subjects reported to the Metabolic Research Unit and exercised on a stationary bicycle ergometer for 60-minutes at 75% $\text{VO}_{2\text{peak}}$. To assess the effects of the acute bout of exercise a euglycaemic-hyperinsulinemic clamp was repeated 16-hours later (day 3). Subjects exercised on 7 consecutive days for 1-hr at 75% $\text{VO}_{2\text{peak}}$ (days 4-10). Sixteen hours after the last training session (day 11), subjects had a euglycaemic-hyperglycaemic clamp to determine insulin sensitivity.

Euglycaemic-hyperinsulinemic clamp technique

Dietary advice was provided to the subjects to ensure they consumed a balanced, calorie sufficient diet for two days prior to the clamp. Subjects reported to the Metabolic Research Unit at 8 am following a 10-hr overnight fast. A polyethylene cannula was inserted retrograde into a dorsal hand vein for blood sampling and an antecubital vein for infusion of insulin and glucose. Baseline blood samples were taken and a muscle biopsy was taken from the mid-vastus lateralis muscle. The sampling hand was heated to about 50°C to measure arterialed venous blood. A primed continuous 40 $\text{mU}\cdot\text{m}^{-2}\cdot\text{min}^{-1}$ insulin infusion was started and plasma glucose was clamped at 5.0 ± 0.5 mM with a variable glucose infusion (20% dextrose) for 2-hrs. Blood samples for glucose and insulin were drawn at 5- and 15-minute intervals, respectively. A final muscle biopsy was taken after 2-hrs.

Exercise training protocol

All exercise sessions were supervised in the Metabolic Research Unit. Subjects were weighed and then remained seated for 5-minutes before blood pressure was monitored. After completing a stretching routine subjects exercised at $\sim 75\%$ $\text{VO}_{2\text{peak}}$ for 1-hour. The first 5-minutes and the last 5-minutes were used as a warm-up and cool-down. The exercise intensity was determined from the heart rate (± 5 beats per minute) corresponding to 75% $\text{VO}_{2\text{peak}}$, as measured by indirect calorimetry. Blood pressure was monitored every 10-minutes and continuous radiotelemetry heart rate during exercise and for 10-minutes following each session.

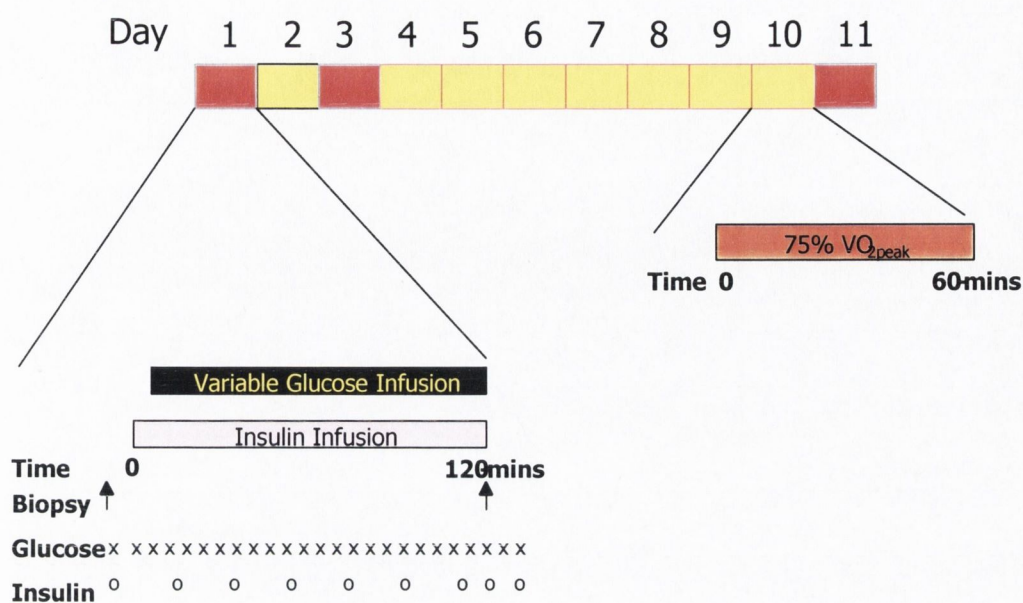


FIGURE 5.2. Schematic representation of the study design for the exercise protocol.

Laboratory analysis

Serum insulin was measured by the use of commercially available fluoroimmunoassays (Auto-Delfia). Plasma glucose was measured using a glucose oxidase method (Beckman) and blood HbA_{1c} measurement was performed using a Hi-Auto A_{1c} analyser (Menarini HA 8140). Plasma cholesterol and triglycerides were measured using enzymatic methods (Human liquicolor kits/Hitachi Modular). Plasma HDL-cholesterol and LDL-cholesterol were measured directly with enzymatic methods (Randox direct kits/Hitachi Modular). Serum concentrations of intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), E-selectin and P-selectin were measured using a commercially available monoclonal antibody ELISA assay (R & D Systems). High sensitivity C-reactive protein (hs-CRP) was measured by a commercially available ultrasensitive competitive immunoassay (Roche Diagnostics).

Muscle protein extraction: A muscle biopsy was taken from the mid-vastus lateralis muscle using the procedure described previously (30,101). In brief, an area of skin was

anaesthetised and a small (0.5 cm) incision made. A sterilised Bergstrom skeletal muscle biopsy needle was inserted into the thigh muscle and 50-200 mg of tissue removed. Pressure was maintained on the wound for 10 minutes and the incision was closed with steristrip tape and wrapped tightly in a crepe bandage. Muscle samples were snap frozen in liquid nitrogen and stored at -80°C .

Muscle analysis

Approximately 50mg of muscle tissue weighed and freeze dried overnight. Samples were dissected free of fat cells, weighed and homogenised using a motorised pestle in buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2 , 0.5 mM Na_3VO_4 , 1% Triton X-100, 10% Glycerol, 20 mM Tris pH 7.8, 1 $\mu\text{g}/\text{ml}$ Leupeptin, 0.2 mM PMSF, 10 mM NaF, 1 $\mu\text{g}/\text{ml}$ Aprotinin, 1 mM EDTA, 5 mM Na pyrophosphate, 1 mM benzamidine). Samples were rotated at 4° for 30-minutes and then centrifuged at 12000g for 15-minutes. A commercially available colorimetric assay using a modified Lowry protocol was used to determine protein content and measured on a plate reader set at 595nm (Bio-Rad DC Protein assay).

PI3-kinase activity

600 μg of protein was brought to a final volume of 500 μl with homogenate buffer and immunoprecipitated with 80 μl of 1:1 protein A sepharose slurry and 6 μl of anti-IRS-1, anti-IRS-2 or phosphotyrosine antibodies (Upstate Biotechnology). Samples were incubated overnight, rotating at 4°C . The beads were pelleted by 30-seconds centrifugation at 14000 rpm and washed 3 times in 500 μl homogenate buffer, twice in 500 μl of 500mM LiCl, 100mM Tris pH 8.0 and once in 500 μl of 150mM NaCl, 1mM EDTA, 10mM Tris pH7.6. Samples were resuspended in 20mM Hepes pH7.3, 1mM DTT and 5 mM MgCl_2 , split into two equi-volume samples and washed. The beads were resuspended in 20 μl kinase assay buffer (20mM Hepes pH7.3, 20mM β -glycerophosphate pH 7.2, 5mM Na pyrophosphate, 30mM NaCl, 1mM DTT). 20 $\mu\text{g}/\text{sample}$ of phosphatidylinositol (Avanti Polar) was solubilised in chloroform, dried down in a nitrogen stream and reconstituted in kinase assay buffer (2 $\mu\text{g}/\mu\text{l}$) containing cholic acid (10mg/ml). The reaction buffer was completed with the addition of 20 $\mu\text{Ci}/\text{sample}$ radiolabelled ATP and 12 $\mu\text{M}/\text{sample}$ unlabelled ATP. After 15-minutes the reaction was stopped with 20 μl of 8N hydrochloric acid and the phosphatidylinositol 3-phosphate extracted with 160 μl of 1:1 methanol-to-chloroform.

The phases were separated by centrifugation and the lower organic phase separated by thin layer chromatography (TLC). The radioactivity incorporated into PI3-kinase was determined by Phosphor Imaging and results normalised to a liver standard.

GLUT-4 Protein content

60µg protein was mixed with 4x Laemmli loading buffer pH 6.8 (20% glycerol, 62.5 mM Tris-HCl, 2% SDS, 0.00125% bromophenol blue, 2% β-mercaptoethanol). The samples were loaded in a 4% stacking gel (9 ml DDW, 3 ml 30% acrylagel, 1.25 ml 0.8% Bis-acrylagel, 4.5 ml 0.5M Tris pH 6.8, 180 µl 10% SDS, 200µl 10% ammonium persulfate and 17.5 µl Temed) and run on a 10% gel (12 ml DDW, 15 ml stabilizing 30% acrylagel, 6 ml of 0.8% Bis-acrylagel, 11.25 ml 1.5M Tris pH 1.5, 460 µl 10% SDS, 300µl 10% ammonium persulfate and 35µl Temed). A prestained Biorad high range standard was loaded to visualize the rate at which the protein separated in the gel. The protein was transferred to polyvinylidene difluoride membrane using semi-dry Western Blot for 45 minutes at 300mA. Non-specific binding sites on the membrane were blocked in a 5% milk/TBS-t buffer (10mM Tris pH 7.5, 100mM NaCl, 0.1% Tween 20) overnight with agitation at 4°C and incubated for 12-hours at 4°C with anti-GLUT4 antibody (NAME) diluted 1:2,000 in 3%BSA/TBS-t. The membrane was washed for 60-minutes with agitation in TBS-t, incubated for 5-minutes in enhanced chemiluminescence reagents (Amersham Life Science) and exposed to autoradiographic film (Kodak X-OMAT™).

Statistical analysis

Data are presented as mean ± standard error. Data not normally distributed were log-transformed. A Student t-test and a paired t-test were used to determine significant differences in descriptive and baseline data. A two-way analysis of variance (ANOVA) and a two-way repeated measures ANOVA were used to determine significant main effects and interactions. A Student Newman-Keuls post-hoc test was used to identify specific mean differences. A Pearson product moment correlation was used to determine relationships between variables. Statistical significance was accepted at the $p < 0.05$ level of confidence for all variables.

Results

Subject characteristics

Physical characteristics of the subjects are presented in Table 5.1. Subjects were similar in age, body mass index, and body surface area. There was no change in body weight following the exercise protocol. Maximal oxygen consumption was below age predicted values but similar between groups. There was a negative relationship between BMI and VO_{2peak} ($r=-0.77$, $p<0.05$) in both groups. Subjects cycled at an intensity corresponding to 75% VO_{2peak} and heart rate was monitored continuously using radiotelemetry. There was no significant difference in the exercise intensity as a percentage of peak heart rate (79 ± 2 vs. $81\pm3\%$ for the T2DM and Obese groups, NS) or the exercising heart rate (130 ± 4 vs. 132 ± 6 bpm for the T2DM and Obese groups, NS).

TABLE 5.1. Physical and metabolic descriptive characteristics of the Obese and Type 2 diabetes groups.

	Obese (n=7)	T2DM (n=8)
Age (years)	47.7 ± 3.9	44.6 ± 2.2
Weight (kg)	100.9 ± 5.5	106.7 ± 4.0
Height (m)	1.75±0.01	1.73±0.04
Body mass index (kg/m ²)	32.9 ± 2.1	35.8 ± 1.9
Body surface area (m ²)	2.23 ± 0.06	2.29 ± 0.06
Systolic blood pressure (mmHg)	129±6	138±2
Diastolic blood pressure (mmHg)	87±3	84±5
Fasting glucose (mmol/l)	5.5±0.2	10.1±1.1*
Fasting insulin (pmol/l)	62.4±18.0	63.0±7.2
HbA _{1c} (%)	5.6±0.2	7.2±0.5*
Peak heart rate (bpm)	163±6	162±5
VO _{2peak} (l/min)	2.43 ± 0.13	2.25 ± 0.24

Data presented as mean±SE. * significantly different to obese subjects, $p<0.05$.

Lipids and inflammatory markers

Blood lipid and inflammatory markers were measured fasting before the baseline hyperinsulinemic clamp (Table 5.2.). Total cholesterol, HDL- and LDL-cholesterol were within the normal range and similar between groups. Serum triglycerides were significantly greater in the T2DM ($p<0.05$). Soluble VCAM and ICAM concentrations were within the normal range for the obese and T2DM groups. E-selectin concentrations were normal for the obese group but outside the normal range for the T2DM. The results between groups were not statistically different. P-selectin was significantly greater in the obese group and outside the normal range. C-reactive protein (CRP) was elevated to a similar extent in both groups (2.53 ± 0.55 vs. 2.58 ± 0.93 mg/l for obese and T2DM groups). There was a significant negative relationship between VO_{2peak} and CRP ($r=-0.74$, $p=0.01$)

TABLE 5.2. Lipid and inflammatory markers in obese and Type 2 diabetes groups.

	Obese (n=7)	T2DM (n=8)
Triglycerides (mmol/l)	1.3±0.3	2.7±0.4*
Total cholesterol (mmol/l)	4.6±0.3	5.1±0.2
HDL-cholesterol (mmol/l)	1.15±0.09	1.10±0.09
LDL-cholesterol	2.82±0.37	2.63±0.09
E-sel (ng/ml)	62.8±12.8	77.9±4.8
P-sel (ng/ml)	172.9±25.6	101.8±14.3*
ICAM (ng/ml)	271.9±25.0	285.5±12.8
VCAM (ng/ml)	412±48	407±56
hs-CRP (mg/l)	2.53±0.55	2.58±0.93

Data presented as mean±SE. * significantly different to obese subjects, $p<0.05$.

Glucose metabolism and whole body glucose disposal

Subjects with Type 2 diabetes had a significantly greater HbA_{1c} ($7.2\pm0.5\%$ vs. $5.6\pm0.2\%$, $p<0.05$). Fasting glucose was significantly greater in the T2DM group but decreased following the training protocol (10.1 ± 1.1 vs. 8.6 ± 0.7 mmol/l, $p<0.05$). The

exercise protocol did not result in any changes in fasting glucose in the obese group (5.5 ± 0.2 vs 5.7 ± 0.1 mmol/l). Fasting insulin was similar between groups and did not change following exercise.

Glucose infusion rate data are presented in Figure 5.3. The glucose infusion rate increased following 1-day and 7 consecutive days of exercise in the obese ($16 \pm 5\%$ and $57 \pm 23\%$ for 1- and 7-days exercise, respectively) and T2DM groups ($54 \pm 17\%$ and $113 \pm 29\%$ for 1- and 7-days of exercise, respectively). There was a significantly greater percent increase in the T2DM group following 7 days of exercise ($p < 0.05$). In obese subjects baseline glucose infusion rates were 4.5 ± 1.4 mg/kg/min, and improved to 5.2 ± 1.5 mg/kg/min after a single bout of exercise and 6.6 ± 1.4 mg/kg/min following 7 days of exercise. These results were not significantly different. Glucose infusion rates for the T2DM subjects increased significantly after 7 days of exercise, but not after the single bout (2.6 ± 0.7 vs. 3.2 ± 0.8 vs. 4.1 ± 1.0 mg/kg/min, for baseline, acute and 7 days of exercise). The baseline glucose infusion rate for both groups was positively correlated with VO_{2peak} ($r = 0.56$, $p < 0.05$) and negatively related to BMI ($r = -0.69$, $p < 0.01$).

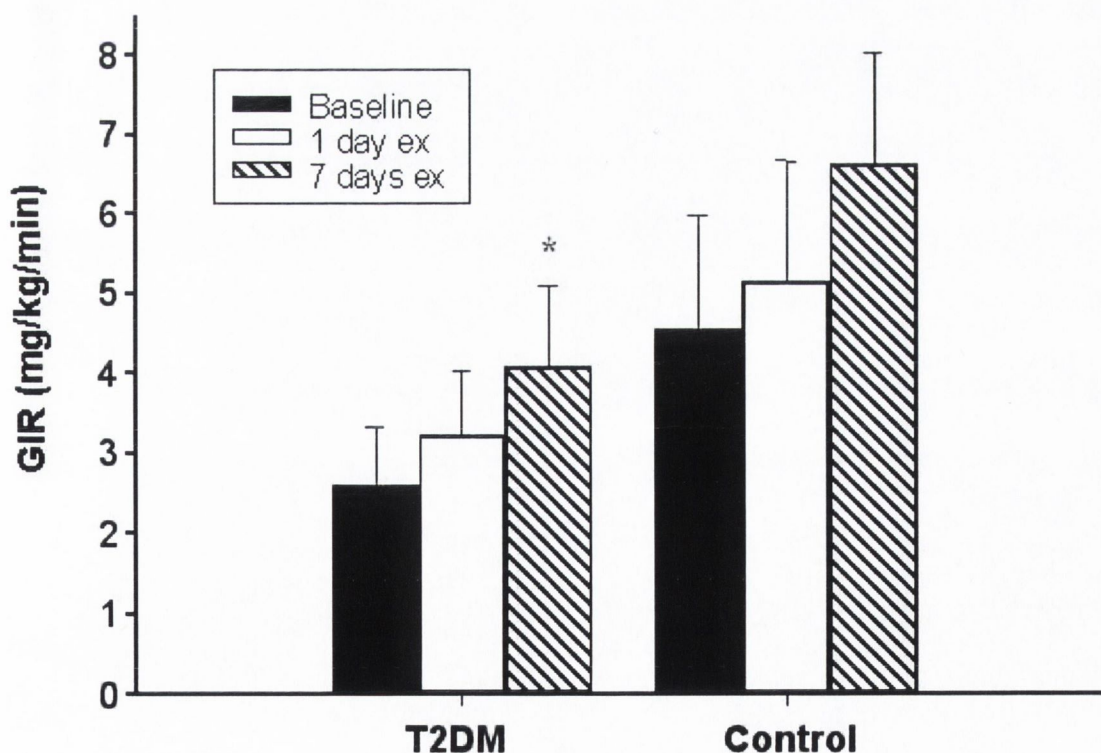


FIGURE 5.3. Glucose infusion rate for the Obese and T2DM groups at baseline and following 1-day and 7-days of exercise. Data expressed as mean $\pm$ SE. * significantly different to baseline, $p < 0.05$.

IRS-1 associated PI3-kinase activity

Basal and insulin stimulated IRS-1 associated PI3-K activity are presented in Table 5.3. Insulin significantly increased IRS-1 associated PI3-K activity in the obese and T2DM groups at baseline and following 1-day of exercise. After 7-days of exercise insulin did increase IRS-1:PI3K activity above baseline in the obese subjects (0.82 ± 0.13 vs. 1.54 ± 0.30 arbitrary units, $p<0.05$) but not the T2DM (0.97 ± 0.17 vs. 1.34 ± 0.21 arbitrary units, NS). IRS-1 associated PI3-K was also expressed as the degree of activation over fasting by dividing the measured activity during insulin stimulation by the fasting activity (IRS1:PI3K) and presented in Figure 5.4. Baseline IRS1:PI3K activation was greater in the T2DM group than the obese group (2.7 ± 0.4 vs. 1.9 ± 0.2 fold increase, $p<0.05$). There was no significant change in the activation of IRS1:PI3K following exercise in the obese group but it was significantly blunted in the T2DM following 1-day (1.9 ± 0.2 fold, $p<0.05$) and 7-days of exercise (1.5 ± 0.4 fold, $p<0.05$).

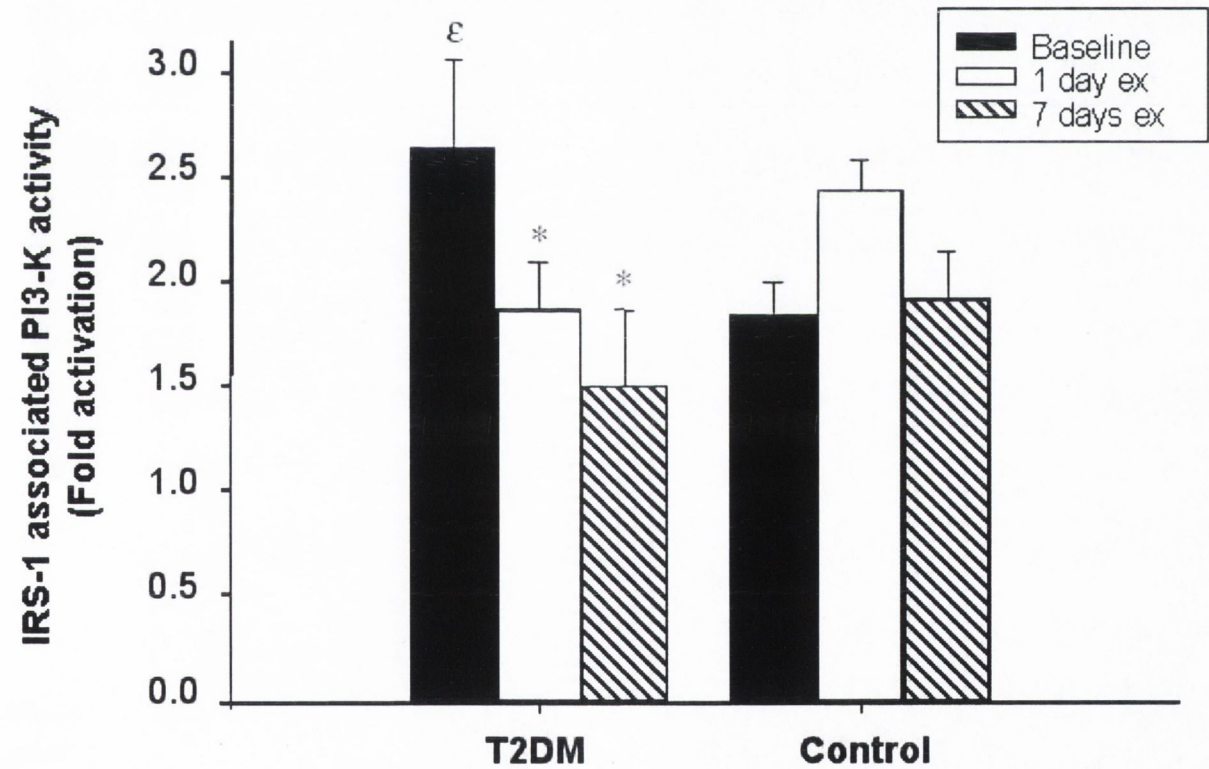


FIGURE 5.4. IRS-1 associated PI3-K activity expressed as the insulin stimulated activity divided by the fasting activity. Data presented as mean±SE. * significantly different to baseline, $p<0.05$; ε significantly lower than T2DM, $p<0.05$.

IRS-2 associated PI3-K activity

Insulin infusion did not significantly increase IRS-2 associated PI3-K activity at baseline in the obese group (0.44 ± 0.07 vs. 0.43 ± 0.07 arbitrary units, NS) or the T2DM group (0.33 ± 0.04 vs. 0.45 ± 0.07 arbitrary units, NS). Neither 1-day of exercise nor 7-days of exercise subsequently resulted in a change in basal or insulin stimulated IRS-2 associated PI3-K activity (Table 5.3). When expressed as a proportional change over fasting activity (Figure 5.5), IRS2:PI3K activation was not significantly different between obese and T2DM subjects at baseline (1.50 ± 0.30 vs. 1.00 ± 0.04 fold, NS). Similarly, there was no significant difference in the activation during insulin infusion following 1-day or 7-days of exercise in either group.

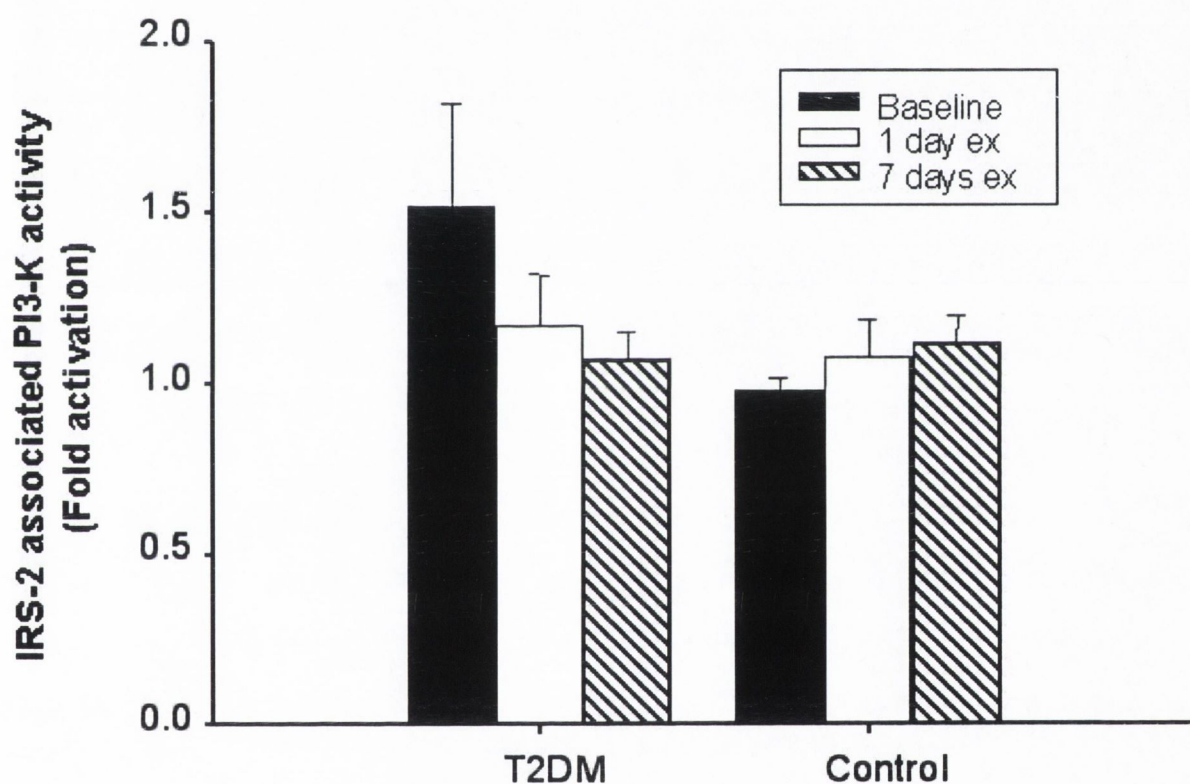


FIGURE 5.5. IRS-2 associated PI3-K activity expressed as the insulin stimulated activity divided by the fasting activity. Data presented as mean $\pm$ SE.

Phosphotyrosine associated PI3-K

Phosphotyrosine associated PI3-K activity (Ptyr:PI3K) is reported in 5/8 T2DM subjects and 5/7 obese subjects. The mean IRS-1:PI3-K and IRS-2:PI3-K activity and the pattern of activation for these subgroups was similar to the completed groups.

Insulin stimulation did not significantly increase Ptyr:PI3K activity in the obese or T2DM groups at baseline or following 1-day or 7-days of exercise (Table 5.3). The activation of Ptyr:PI3K activity over fasting activity (Figure 5.6.) was significantly greater in the obese group (0.87 ± 0.07 vs. 1.35 ± 0.23 fold, $p<0.05$) at baseline. There was a tendency toward improved Ptyr:PI3K activation following 1-day of exercise (1.16 ± 0.04 fold, $p=0.09$) in the T2DM group but not after 7-days of exercise (1.17 ± 0.08 fold, NS). In the obese group Ptyr:PI3K activation decreased significantly after 1-day and 7-days of exercise (1.35 ± 0.23 vs. 0.95 ± 0.07 vs. 0.96 ± 0.08 fold, $p<0.05$).

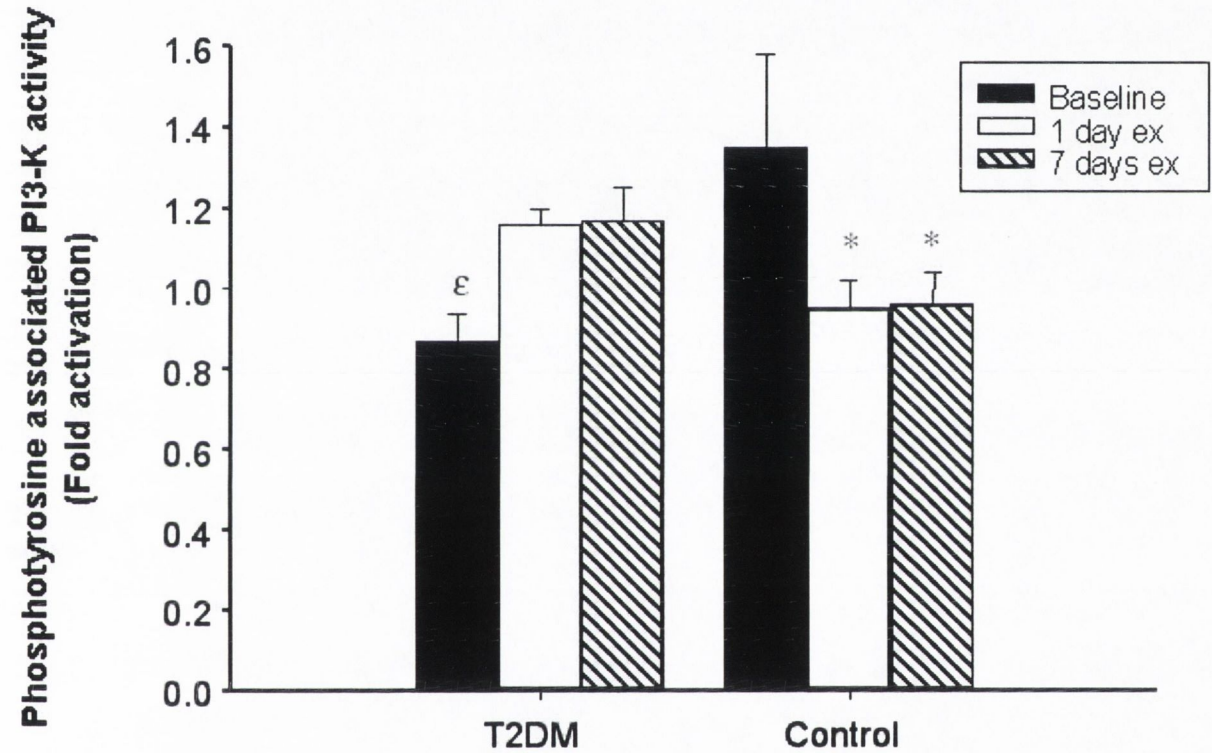


FIGURE 5.6. Phosphotyrosine associated PI3-K activity expressed as the insulin stimulated activity divided by the fasting activity. Data presented as mean $\pm$ SE. * significantly different to baseline, $p<0.05$; ε significantly lower than obese group, $p<0.05$.

GLUT-4 protein content

Total GLUT-4 protein content was determined from biopsy samples taken prior to insulin infusion (Figure 5.7). There was no significant difference in GLUT-4 protein content from baseline following 1-day or 7-days of exercise in the T2DM group (0.90 ± 0.06 vs. 0.91 ± 0.05 vs. 0.93 ± 0.04 arbitrary units, NS) or the obese group (0.85 ± 0.06 vs. 0.82 ± 0.05 vs. 0.84 ± 0.04 arbitrary units, NS). There was no difference in total GLUT-4 protein content between groups.

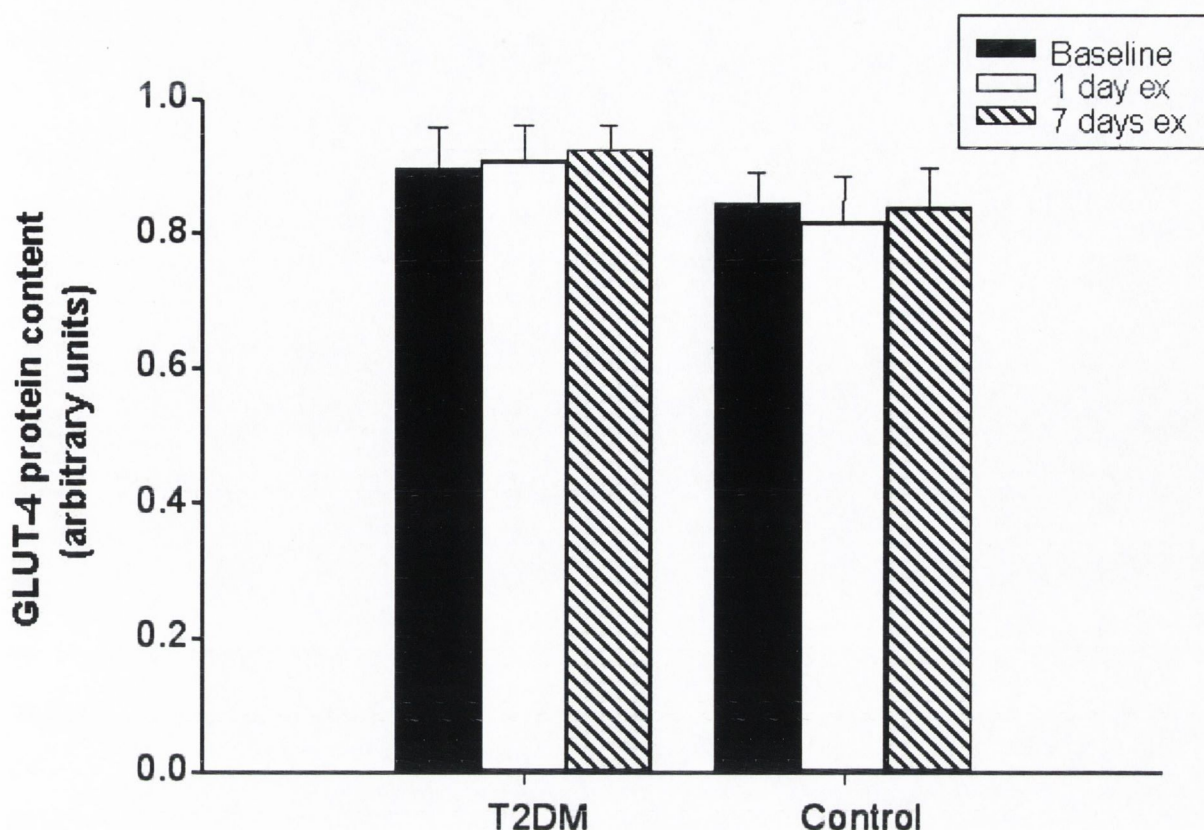


FIGURE 5.7. Total GLUT-4 protein content in T2DM and obese groups at baseline and following 1-day and 7-days of exercise training. Data expressed as mean $\pm$ SE.

TABLE 5.3. IRS-1-, IRS-2- and phosphotyrosine-associated PI3-K activity before and after insulin infusion at baseline, and following 1-day and 7-days of exercise.

		IRS-1:PI3-K		IRS-2:PI3-K		PTyr:PI3-K	
		Pre	Post	Pre	Post	Pre	Post
Baseline	Obese	0.76 $\pm$ 0.06	1.39 $\pm$ 0.11*	0.44 $\pm$ 0.07	0.43 $\pm$ 0.07	0.76 $\pm$ 0.11	1.09 $\pm$ 0.28
	T2DM	0.84 $\pm$ 0.15	2.20 $\pm$ 0.55*	0.33 $\pm$ 0.04	0.45 $\pm$ 0.07	1.05 $\pm$ 0.26	0.86 $\pm$ 0.15
1-day ex	Obese	0.70 $\pm$ 0.07	1.60 $\pm$ 0.12*	0.45 $\pm$ 0.08	0.49 $\pm$ 0.06	1.06 $\pm$ 0.23	1.11 $\pm$ 0.26
	T2DM	0.93 $\pm$ 0.16	1.61 $\pm$ 0.19*	0.36 $\pm$ 0.06	0.41 $\pm$ 0.07	0.95 $\pm$ 0.16	1.08 $\pm$ 0.15
7-days ex	Obese	0.82 $\pm$ 0.13	1.54 $\pm$ 0.30*	0.44 $\pm$ 0.07	0.48 $\pm$ 0.08	1.00 $\pm$ 0.17	0.99 $\pm$ 0.23
	T2DM	0.97 $\pm$ 0.17	1.34 $\pm$ 0.21	0.41 $\pm$ 0.04	0.40 $\pm$ 0.04	1.01 $\pm$ 0.18	1.12 $\pm$ 0.34

Data presented as mean $\pm$ SE. * significantly different to baseline, $p < 0.05$.

Discussion

Whole body glucose disposal

The present study confirms that exercise has an important role in the treatment of Type 2 diabetes. Both fasting glucose and insulin sensitivity were improved following 8 days of exercise training, independent of changes in body weight or aerobic endurance. These results are consistent with protocols of a similar, or longer duration, in normoglycaemic (204,164) and Type 2 diabetes subjects (158,408,378). Baseline glucose infusion rates correlated with aerobic endurance and BMI indicating that those who are least fit and most overweight have the lowest insulin sensitivity. These results demonstrate that treatment of Type 2 diabetes with exercise is the most effective means of addressing one of the primary defects of the disease, insulin resistance. Sulfonylureas and metformin control blood glucose, without any sustained effect on the pathophysiology of diabetes. The glitazones, newer oral agents, which target insulin resistance are promising and 3 months treatment with troglitazone led to a similar degree of improvement in insulin sensitivity as the current exercise protocol (174).

While 7 consecutive days of exercise was effective, the acute bout of exercise did not significantly improve insulin sensitivity. An acute bout of exercise has previously been shown to improve glucose disposal (269) and many of the benefits of exercise training have been attributed to the effect of the last bout of exercise, the effects of which are short lived. Results from the current study are consistent with those of Cusi et al. (76) who found no difference in insulin stimulated glucose disposal (2.39 ± 0.34 vs. 2.41 ± 0.45 ml/kg/min) 24-hours following a bout of exercise at 65% $\text{VO}_{2\text{peak}}$ for 60-mins. However, while not statistically significant, the acute bout of exercise in our study did result in a $54 \pm 17\%$ increase in glucose infusion rates, which is a clinically relevant outcome and accounts for a large proportion of the overall improvement.

While the Type 2 diabetes subjects had improved insulin sensitivity, the obese group were not significantly different. However, they still demonstrated a $57 \pm 23\%$ improvement following exercise training and were within the expected range of 6-8 mg/kg/min at the end of the study. Tanner et al. (343) exercise trained insulin resistant men for 7-days at 70% $\text{VO}_{2\text{peak}}$ and found a 33% ($p < 0.001$) increase in glucose infusion

rates. Thus, while the obese group did not improve to the same magnitude as the Type 2 diabetes group, they were starting from a much healthier baseline.

Impact of exercise on the insulin signalling cascade

IRSI-1 and IRS-2 associated PI3-kinase activity

Insulin-mediated glucose disposal is facilitated by GLUT-4 translocation to the plasma membrane, and subsequent insertion and activation. Increased PI3-K activity has been shown to be a necessary step in the translocation process, as inhibition of this enzyme with wortmannin completely blocks glucose transport (260). IRS-1 associated PI3-K activity is regarded as the primary signalling pathway with IRS-2 associated PI3-K possibly providing a complementary or substitute pathway. Our results demonstrate a progressive decrease in fold-activation of IRS-1 associated PI3-K activity following exercise in the Type 2 diabetes subjects, despite increased insulin mediated glucose infusion rates. IRS-2 associated PI3-K activity did not compensate and showed a similar declining pattern. In contrast, the obese group had similar IRS-1 and IRS-2 associated PI3-K before and after exercise training.

These results are at variance with the exercise response in insulin sensitive human (204), and animal (58), studies but consistent with insulin resistant (343), obese and Type 2 diabetes (76) responses. Cusi et al. (76) found that 24-hours following an acute bout of exercise in obese and Type 2 diabetes subjects, insulin receptor and IRS-1 phosphorylation were increased but not IRS-1 associated PI3-K activity or glucose disposal. In middle-aged insulin resistant men who followed a 7-day training programme, glucose infusion rates were increased, despite no change in phosphotyrosine associated PI3-K activity (343). Therefore, the regulation of glucose disposal following exercise in normal and insulin resistant subjects appears to be different.

One possible explanation for these findings may be the different isoforms of PI3-K. There are at least 7 different isoforms documented in insulin responsive tissue, 5 of the regulatory subunit (p85 α , p85 β , p55 α , p50 α , p55^{PIK}) and 2 of the catalytic subunit (p110 α , p110 β). Insulin differentially regulates the PI3-K isoforms in human skeletal muscle (88) and in obese diabetic (*ob/ob*) mice (197). Therefore, these isoforms may

have specific roles in signal transduction depending on the individual tyrosine kinase receptor (172). It is possible that exercise may augment the activity of a particular isoform of PI3-K or that an isoform specific difference may exist in the response between insulin sensitive and insulin resistant subjects.

Another explanation relates to changes in the expression and function of IRS-1 and IRS-2. Expression of the insulin receptor, IRS-1 and IRS-2 were 44%, 57% and 77% lower in endurance trained runners (406), despite the fact that glucose disposal rates and IRS-1 associated PI3-K activity are greater in trained individuals (204). Following 9 days of exercise training, glucose disposal was increased but there was no change in the expression of the insulin receptor or IRS-1 in young healthy subjects (376). In the same subjects an acute bout of exercise resulted in a transitory increase in IRS-2 and p85 expression, this effect was lost following training. In female Wistar rats IRS-1 expression was decreased after 5 days of exercise but PI3-K activity was increased (58), collectively suggesting that exercise training results in either increased rates of IRS-1 and IRS-2 protein degradation or decreased rates of protein synthesis. These changes in expression may influence the adaptive response to exercise in obese or Type 2 diabetes subjects, who may not compensate for altered expression of insulin signalling proteins.

Functional changes in IRS-1 and IRS-2 in response to exercise are not always coupled with PI3-K activity either. Cusi et al. (76) found that while insulin-stimulated glucose disposal and IRS-1 associated PI3-K activity were not increased 24-hrs after an acute bout of exercise, insulin receptor and IRS-1 phosphorylation were increased in obese and Type 2 diabetes subjects. Despite the decrease in IRS-1 associated PI3-K activity, due to changes in expression or function, IRS-2 does not appear to be a compensatory mechanism. Results from the present study clearly indicate that IRS-2 associated PI3-K activity did not contribute significantly to either the obese or Type 2 diabetes groups. An alternative pathway for PI3-K activation was first proposed by Zhou and Dohm (407) who found increased phosphotyrosine associated PI3-K activity, despite no change in IRS-1 phosphorylation following 60-minutes of treadmill running in male Sprague Dawley rats. This is supported by data from IRS-1 knockout mice that stimulate insulin mediated glucose disposal, though to a lesser extent (14). While IRS-2 was the most likely candidate to compensate for decreased IRS-1 associated PI3-K activity, results from IRS-2 knockout mice show that IRS-2 is not necessary for insulin

or exercise stimulated glucose transport in skeletal muscle (154). Howlett et al. (165) found that IRS-2 associated PI3-K activity may partially account for the increase in phosphotyrosine associated PI3-K activity after exercise. The IRS-2 associated PI3-K data from the present study do not support the role of IRS-2 as a viable compensatory pathway.

Therefore, IRS-independent activation of PI3-K or alternative pathways may be responsible for the changes. In a sub-group of the obese and Type 2 diabetes groups we determined phosphotyrosine associated PI3-K activity. The IRS-1 and IRS-2 associated PI3-K activation was identical to the whole group but phosphotyrosine associated PI3-K was markedly different. In response to acute exercise or 7 days of exercise training, phosphotyrosine associated PI3-K increased, though not significantly, in the Type 2 diabetes group while it decreased significantly in the obese group. These results further support the theory of differential regulation of PI3-K isoforms and suggest that changes in glucose disposal for the Type 2 diabetes subjects may result from IRS-independent activation of PI3-K but independently of PI3-K for the obese group.

Phosphotyrosine associated PI3-kinase activity

Phosphotyrosine associated PI3-K activity accounts for approximately 95% of all tyrosine proteins activating PI3-K (261), though the length of time for immunoprecipitation influences the proteins isolated (388), while some isoforms are bound with greater affinity (318). Following acute exercise, normal rats (407), muscle insulin receptor knockout (MIRKO) mice (393) and IRS-2 knockout mice (165) exhibit increased phosphotyrosine associated PI3-K activity, despite decreased IRS-1 associated PI3-K, indicating that another protein may activate PI3-K activity. In response to short term exercise training in humans and animals, insulin sensitive subjects increase (164), while insulin resistant subjects do not increase phosphotyrosine associated PI3-K activity (343,60). The present results in the Type 2 diabetes subjects indicate that while IRS-1 and IRS-2 associated PI3-K activity decrease, there is a trend toward increased phosphotyrosine associated PI3-K activity, leaving the possibility that proteins, other than IRS, activate specific isoforms of PI3-K.

While a number of studies have speculated about the role of additional proteins to activate PI3-K, there is no conclusive evidence to date (394,393,165). A 200-kDa

protein has been co-precipitated with p85 α from isolated cardiomyocytes in response to insulin and contraction (352), though other studies have failed to reproduce the results in skeletal muscle (165). Howlett et al. (165) also found that phosphotyrosine associated PI3-K activity was increased following *in vivo* but not *in vitro* contraction, indicating that a systemic factor may be responsible, a theory supported by other studies (123,175). Isakoff et al. (175) found that other growth factors were capable of activating PI3-K but not affecting glucose transport demonstrating that while PI3-K may be necessary, it is not sufficient to activate PI3-K.

In this context the contribution of PI3-K to increased glucose disposal following exercise should be questioned. When PI3-K activity was decreased by pre-treatment with PDGF (337) or overexpressing IRS-1 interacting domains (316) in 3T3-L1 adipocytes, glucose disposal rates were not affected. Morris et al. (245) also found that competitive inhibition of the insulin receptor and IRS-1 had no effect on glucose disposal. Though these studies have not been performed in response to muscle contraction, they demonstrate that glucose disposal rates can be maintained in the absence of effective PI3-K signalling.

In the obese group IRS-1 and IRS-2 associated PI3-K activity were unchanged and phosphotyrosine associated PI3-K decreased though glucose infusion rates were not impaired. This suggests that exercise was unable to reverse the inhibitory effects of protein phosphatases or serine phosphorylation, known to downregulate IRS-1 and PI3-K (2,208). It is also possible that PI3-K independent pathways or changes in the subunit regulation of PI3-K may influence the activity of the enzyme.

Akt kinase

Akt is distal to PI3-K and is thought to be involved with GLUT-4 translocation. The role of Akt in insulin resistant tissue and following exercise is inconclusive as the change in phosphorylation or activity do not match changes in PI3-K activity. Immediately after exercise when PI3-K activity is decreased, Akt activation is increased in rats and MIRKO mice (306,388,393). Akt appears to increase following exercise in healthy insulin sensitive animals and humans (406,58) but not insulin resistant subjects (60,343), similar to PI3-K.

Impact of exercise on GLUT-4 protein expression

PI3-K activity could not account for changes in glucose infusion rates therefore GLUT-4 protein was determined. In contrast to other studies that have shown an increase in GLUT-4 expression following exercise of a similar duration (140,272), results from the present study did not significantly change. Even in Type 2 diabetes subjects, exercise training has been shown to increase GLUT-4 protein content (91). While the protein content may not have changed, the translocation or activation of GLUT-4 may have improved to increase glucose disposal. Yu et al. (406) found that insulin-regulated aminopeptidase (IRAP), a key protein in the vesicle translocation and docking of GLUT-4, was 4.7-fold greater in aerobically trained subjects compared with sedentary controls. GLUT-4 protein expression only increased 2-fold placing importance on the activation of GLUT-4 once docked in the plasma membrane.

The stress activated mitogen activated protein kinase p38 has been implicated in the activation of GLUT-4. Sweeney et al. (342) demonstrated that an inhibitor of p38 blocked insulin mediated glucose transport, but did not have any effect on the translocation or docking of the GLUT-4 vesicle at the plasma membrane. They proposed that a separate insulin stimulated pathway might regulate the translocation/docking and the activation of GLUT-4, with phospho-p38 being responsible for the activation phase.

The role of alternative mechanisms to explain improved insulin sensitivity

In addition to the insulin signalling cascade, there are a number of other factors that may account for increased glucose disposal following 7 days of exercise training. Caloric restriction alone increases 3-O-methylglucose transport without increasing IRS-1, IRS-2 or phosphotyrosine associated PI3-K activity (82). Weight loss in subjects with Type 2 diabetes also results in improved insulin sensitivity (194). Weight loss was not a desired outcome of the present study but these studies demonstrate alternate mechanisms of improved glucose disposal.

The biochemistry of exercise adaptation has shown that the size and number of mitochondria increase with 7-10 days of exercise training, increasing the capacity and effectiveness of glucose oxidation (157). The activity of Krebs cycle enzymes, such as citrate synthase, also increase in a short period of time, as do enzymes of β -oxidation.

The increased capacity to oxidise carbohydrate, in addition to the increased glycogen storage capacity may account for the changes observed.

The turnover of intramuscular triglycerides (IMTG) also increases in response to exercise training, a factor thought to account for changes in glucose disposal. Accumulation of intramuscular triglycerides is thought to result from glucose-mediated inhibition of carnitine palmitotransferase I activity, decreasing FFA transport into the mitochondria for oxidation. The subsequent accumulation of cytosolic FFA increase PKC activity and decrease glucose transport. Greater IMTG turnover and oxidation could alleviate the resistance on FFA oxidation and glucose transport, increasing insulin-mediated glucose disposal.

Conclusion

Exercise training leads to increased insulin-mediated glucose disposal in obese and Type 2 diabetes subjects through a mechanism independent of IRS-1 associated PI3-K. The data from the present study indicate that the beneficial effect of exercise on insulin mediated glucose disposal is distal to PI3-K, either through alternative protein activation of PI3-K, or GLUT-4 activation or by some other physiological adaptations to exercise. Further characterisation of the mitochondrial adaptations, including the role of PGC-1 and its relationship with PPAR α/γ is necessary as there may be a direct link between insulin sensitivity, substrate oxidation and gene expression.

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