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TREATING TYPE 2 DIABETES THROUGH INSULIN
RESISTANCE

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Doctor of Philosophy

ASTON UNIVERSITY

SEPT 2002

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Thesis Summary

Type 2 diabetes is an insidious disorder, with micro and/or macrovascular and nervous damage occurring in many patients before diagnosis. This damage is caused by hyperglycaemia and the diverse effects of insulin resistance. Obesity, in particular central obesity, is a strong pre-disposing factor for type 2 diabetes. Skeletal muscle is the main site of insulin-stimulated glucose disposal and appears to be the first organ that becomes insulin resistant in the diabetic state, with later involvement of adipose tissue and the liver. This study has investigated the use of novel agents to ameliorate insulin-resistance in skeletal muscle as a means of identifying intervention sites against insulin resistance and of improving glucose uptake and metabolism by skeletal muscle. Glucose uptake was measured *in vitro* by cultured L6 myocytes and isolated muscles from normal and obese diabetic *ob/ob* mice, using either the tritiated non-metabolised glucose analogue 2-deoxy-D-glucose or by glucose disposal. Agents studied included lipoic acid, isoferulic acid, bradykinin, lipid mobilising factor (provisionally synonymous with Zinc α 2 glycoprotein) and the trace elements lithium, selenium and chromium. The putative role of TNF α in insulin resistance was also investigated. Lipoic acid improved insulin-stimulated glucose uptake in normal and insulin resistant murine muscles, as well as cultured myocytes. Isoferulic acid, bradykinin and LMF also produced a transient increase in glucose uptake in cultured myocytes. Physiological concentrations of TNF α were found to cause insulin resistance in cultured, but not in excised murine muscles. The effect of the M2 metabolite of the satiety-inducing agent sibutramine on lipolysis in excised murine and human adipocytes was also investigated. M2 increased lipolysis from normal lean and obese *ob/ob* mouse adipocytes. Arguably the most important observation was that M2 also increased the lipolytic rate in adipocytes from catecholamine resistant obese subjects. The studies reported in this thesis indicate that a diversity of agents can improve glucose uptake and ameliorate insulin resistance. It is likely that these agents are acting via different pathways. This thesis has also shown that M2 can induce lipolysis in both rodent and human adipocytes. M2 hence has potential to directly reduce adiposity, in addition to well documented effects via the central nervous system.

Key words or phrases: M2, Lipoic acid, TNF α , Isoferulic acid, Obesity

To Alison, with all my love

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List of abbreviations

2-DG = 2 deoxyglucose
³H2DG = Tritiated 2 deoxyglucose
5-HT = 5 hydroxy tryptamine
ACE = Angiotensin-converting enzyme
ADA = American Diabetes Association
 α HS = Alpha Heremans Scmid glycoprotein
BAT = Brown Adipose Tissue
BMI = Body Mass Index
GAP = GTPase activating protein
G6PDH = Glucose 6 phosphate dehydrogenase
GLUT = Glucose transporter isoform
GRB-2 = Growth factor receptor bound protein 2
GSK 3 = Glycogen synthase kinase 3
HDL = High density lipoproteins
IgG = Immunoglobulin G
IGT = Impaired glucose tolerance
IR = Insulin resistance
IRS = Insulin receptor substrate
LDL = Low density lipoproteins
PEP = Phosphoenol pyruvate
LMF = Lipid mobilising factor
M2 = Sibutramine metabolite 2
MAPK = Mitogen activated protein kinase
MHC = Major histocompatibility complex
NA = Noradrenaline
NEFAs = Non-esterified free fatty acids
PAI -1 = Plasminogen activator inhibitor 1
PEPCK = Phosphoenolpyruvate carboxykinase
PI3K = Phosphoinositide 3 kinase

PIP3 = Phosphatidyl inositol 3, 4, 5 trisphosphate
PKB = Protein kinase B
PKC = Protein kinase C
PC-1 = Plasma membrane glycoprotein 1
PPAR γ = Peroxisome proliferator activated receptor gamma
Rad = Ras associated with diabetes
Ras = Product of gene associated with rat sarcoma
SH = Src homology
Si = Insulin sensitivity
SOS = Guanine nucleotide releasing factor, human homologue of son of sevenless
STZ = Streptozotocin
SYP = Synaptophysin
TG = Triglyceride
TNF α = Tumor necrosis factor alpha
TZDs = Thiazolidinediones
UCP = Uncoupling protein
VLDL = Very low density lipoproteins
WAT = White adipose tissue
ZAG = Zinc alpha 2 glycoprotein

Chapter 1 - Introduction

1.1 - Diabetes Mellitus

Diabetes Mellitus is characterised by a defect of carbohydrate metabolism in which insufficient glucose is oxidised to produce energy. This is due to a lack of, or a reduction in the efficacy of the pancreatic hormone *insulin*. Rising levels of unutilised glucose lead to hyperglycaemia (excess glucose in the blood) and subsequently in the urine. There are two main types of diabetes mellitus (Pickup and Williams, 1997).

Type 1 or Insulin Dependent Diabetes Mellitus (IDDM) usually presents in childhood or adolescence but may emerge in later life in a less acute form. Type 1 diabetic patients have little or no ability to produce insulin due to autoimmune destruction of the insulin producing β cells in the pancreatic islets of Langerhans. Therefore these patients are entirely dependent on the administration of exogenous insulin for survival. Typical type 1 signs and symptoms include thirst, loss of weight and glycosuria (glucose in urine). Acid-base disturbances ensue due to excessive use of fats as an alternative energy source, giving rise to the accumulation of ketone bodies in the bloodstream (ketosis). If untreated, ketosis leads to convulsions proceeding to diabetic coma and death.

Type 2 or Non Insulin Dependent Diabetes Mellitus (NIDDM) usually occurs after the age of 30 and the pancreas usually retains some ability to produce insulin, but this is inadequate to maintain normal glucose homeostasis. These patients may require treatment with oral hypoglycaemic drugs or insulin. In both types of diabetes careful control of the diet is needed, allowing adequate complex carbohydrate, while reducing saturated fats. Type 2 diabetes forms an important focus of this thesis, particularly the relationship between type 2 diabetes and insulin resistance.

1.2 - Insulin Resistance (IR)

IR develops when the biological effectiveness of insulin is reduced, such that it can no longer maintain adequate uptake of glucose into muscle or achieve adequate suppression of glucose output by the liver (Reaven, 1995). Typically, the early stages of the development of insulin resistance are associated with an increase in insulin secretion, which may progress to frank hyperinsulinaemia. The efficacy of insulin action on glucose uptake varies substantially amongst individuals with normal glucose tolerance. One quarter of individuals with normal glucose tolerance show the same degree of IR as patients with type 2 diabetes, whilst almost all patients with impaired glucose tolerance (IGT) and type 2 diabetes manifest IR (Reaven, 1995). There are two key factors that influence the development of glucose intolerance in normal individuals: (1) the extent of loss of *in-vivo* insulin action (i.e. the extent of development of IR), and (2) the ability of the β cells of the pancreas to increase insulin production in a compensatory manner. Thus, if the extent of hyperinsulinaemia is sufficient in IR individuals with normal glucose tolerance, this is thought to offset the IR (Hollenbeck *et al.*, 1987). This is supported by evidence that patients with type 2 diabetes often have higher plasma insulin levels than those with normal glucose tolerance, at least at the time of diagnosis (Reaven, 1976). Moreover, the natural history of type 2 diabetes is associated with increasing hyperglycaemia accompanied by a failure of the β cells to continue increased insulin production (Reaven, 1995). In summary, some individuals are able to maintain a state of compensatory hyperinsulinaemia which may be sufficient to 'ward off' progression to type 2 diabetes. However, others who cannot maintain the required level of hyperinsulinaemia go on to develop severe type 2 diabetes, which may result in severe hyperglycaemia when β cell failure progresses to a state of absolute hypoinsulinaemia (De Fronzo, 1988).

The pathophysiological consequences of chronic hyperinsulinaemia are far from mild and a combination of IR and hyperinsulinaemia is believed to underlie the clustering together of a group of disorders (the so called 'Syndrome X') that predisposes to atherosclerotic cardiovascular disease. These disorders include type 2 diabetes itself or glucose

intolerance, obesity, hypertension, lipid abnormalities, atherosclerosis and a procoagulant state.

1.3 - Insulin resistance and Syndrome X

It is known that insulin resistance with subsequent compensatory hyperinsulinaemia predisposes to a number of abnormalities (Syndrome X). In more detail, these include obesity, IGT or type 2 diabetes, increase in plasma triglyceride, decrease in high density lipoprotein (HDL) cholesterol, increase in the smaller denser sub-fraction of low density lipoprotein (LDL) particles, hypertension, atherosclerosis and increased levels or circulating plasminogen activator inhibitor 1 (Reaven, 1995). These all increase the risk of coronary heart disease associated with Syndrome X and make a large contribution in the pathogenesis of Type 2 diabetes and its clinical complications.

1.4 - Hypertensive state

In comparison with normal non-diabetic normotensive subjects, patients with high blood pressure generally exhibit higher insulin concentrations and are less glucose tolerant (Reaven, 1988; 1991). Up to 50% of an unselected population can show these changes, which persist after successful hypertensive drug therapy (Zavaroni, 1992; Reaven 1988; 1991). It has been established that in these individuals, insulin stimulated glucose uptake has decreased and therefore they are insulin resistant (Reaven, 1988; 1991). Note that these effects are seen in both obese and non-obese hypertensive patients. The occurrence of both insulin resistance and hyperinsulinaemia with high blood pressure strongly suggests that IR and hyperinsulinaemia may play a role in hypertension. Indeed, it is known that hyperinsulinaemia increases renal sodium retention and stimulates sympathetic nervous system activity (Reaven, 1991), both of which would lead to an increase in blood pressure. Also, abnormalities of insulin sensitivity are found in first-degree relatives of patients with high blood pressure who are normotensive themselves (Facchini *et al.*, 1991; Ferrari *et al.*, 1991), but this does not occur in relatives of patients with secondary hypertension (Shamiss *et al.*, 1992). However, acute hyperinsulinaemia

results in vasodilation, which would lower, not raise blood pressure (Anderson & Mark, 1993). Also, dogs infused with insulin for up to 2 weeks showed no increase in blood pressure (Hall *et al.*, 1990). Given that rats injected with insulin *do* show increases in blood pressure (Brands *et al.*, 1991), that a reduction in insulin dose in hypertensive obese type 2 diabetes patients reduces blood pressure (Tedde *et al.*, 1989) and that initiation of insulin therapy in type 2 patients often increases blood pressure, the weight of evidence supports a role for hyperinsulinaemia in hypertension.

1.5 - Hyperuricaemia

Glucose intolerance, dyslipidaemia and hypertension are often associated with increases in serum uric acid (Wyngaarden & Kelly, 1983). It has been found that in normal volunteers a significant correlation exists between insulin resistance and serum uric acid concentration (Facchini *et al.*, 1992). Resistance to insulin-mediated glucose uptake was inversely correlated to urinary excretion of uric acid. Healthy volunteers with asymptomatic hyperuricaemia have been shown to have higher insulin responses to an oral glucose challenge, higher plasma triglyceride, lower HDL cholesterol concentrations and a higher blood pressure than those with normal serum uric acid concentrations (Zavaroni *et al.*, 1993). Whether raised serum uric acid concentrations are causative or a consequence of type 2 diabetes is still unclear.

1.6 - Dyslipidaemia

As well as an increase in the plasma insulin concentration, insulin resistance also results in increased hepatic very-low-density lipoprotein (VLDL) triglyceride (TG) excretion and hypertriglyceridaemia (Reaven, 1995). It has been noted experimentally that modifying insulin action or plasma insulin concentrations results in predictable changes in the rate of secretion of VLDL - TG and plasma TG concentrations. Weight reduction results in a fall in insulin levels, a reduction in VLDL secretion rate and TG levels in the plasma (Olefsky & Reaven, 1974). Conversely, a high carbohydrate diet increases plasma insulin levels and TG concentrations. This increase in the plasma TG levels has

been correlated with the hyperinsulinaemia induced by the carbohydrate diet (Reaven & Miller, 1968). An increase in VLDL –TG pool results in an array of lipoprotein metabolic abnormalities and the ‘possibility exists that at least some of these are directly related to insulin resistance and/or compensatory hyperinsulinaemia’ (Reaven, 1995).

1.7 - HDL cholesterol

There is an inverse relationship between high plasma TG and low HDL-cholesterol concentrations, and both are associated with hyperinsulinaemia (Laws *et al.*, 1991). It is thought that this inverse relationship is due to the enzymatic activity of the cholesteryl ester transfer protein, which promotes the loss of cholesteryl ester from HDL to VLDL (Swenson, 1991). The higher the plasma TG level, the greater the action of cholesteryl ester transferase and the lower the plasma HDL cholesterol level. Low HDL cholesterol levels stimulate an increase in fractional catabolic rate of apoprotein A-1 (Brinton *et al.*, 1994), which is known to be increased in the hyperinsulinaemic state (Chen *et al.*, 1991; Golay *et al.*, 1987). Thus it is possible that IR and/or subsequent compensatory hyperinsulinaemia can affect HDL cholesterol concentrations.

1.8 - LDL particle diameter

It is known that patients with a high proportion of smaller (denser) LDL particles have an increased risk of developing coronary heart disease (Austin *et al.*, 1988-1990, Crouse *et al.*, 1985). There are multiple lipoprotein subclasses but LDL levels in most patients can be divided into two broad categories: those with larger LDL particles (diameter >255Å, subclass A) and those with smaller LDL particles (\leq 255Å, subclass B). Those in subclass B usually have lower plasma HDL cholesterol and higher TG levels, and as these changes are associated with IR and/or compensatory hyperinsulinaemia as mentioned previously, it is probable that subclass B is associated with IR. In fact, Reaven *et al* (1993) have shown normal individuals with subclasses B lipoprotein particles to be hypertriglyceridaemic, hypertensive, hyperinsulinaemic, glucose intolerant and relatively insulin resistant.

1.9 - Postprandial Lipaemia

Upon fasting, the higher the TG level, the larger the degree of post-prandial lipidaemia (Nestel, 1965; Wilson *et al.*, 1985). Due to the high correlation between the development of hypertriglyceridaemia and IR and/or compensatory hyperinsulinaemia (Laws *et al.*, 1991; Nestel, 1964; Olesfsky & Reaven, 1974), we would expect to see increases in post-prandial lipaemia in IR subjects. This seems to be the case in individuals with type 2 diabetes and obesity (Chen *et al.*, 1988; Lewis *et al.*, 1990; 1991) where insulin resistance occurs.

1.10 - Procoagulant state

The concentration of plasminogen activator inhibitor 1 (PAI-1) has been shown to be higher in coronary heart disease patients, and in young males this is thought to be a primary risk factor for myocardial reinfarction (Ho *et al.*, 1990; Mehta *et al.*, 1987; Paramo *et al.*, 1985). As plasma PAI-1 levels and fibrinolysis levels are inversely related, this means that an increase in PAI-1 concentration results in a fall in fibrinolysis and a subsequent increase in infarct risk. It is therefore interesting to note the apparently increased concentrations of PAI-1 in patients with hypertriglyceridemia (Hamsten *et al.*, 1985; Mehta *et al.*, 1987), hypertension (Landin *et al.*, 1990) and type 2 diabetes (Juhan-Vague *et al.*, 1993). In 1993 Potter Van Loon *et al* demonstrated that IR and plasma PAI-1 levels are significantly correlated, but whether this effect of IR acts directly on PAI-1 concentrations or acts via plasma TG levels is unclear.

1.11 - Measuring Insulin Resistance

1.11.1 - Whole Body Measurement

1.11.1.1 - The Euglycaemic Insulin Clamp

The 'gold standard' for measuring total body insulin resistance is the euglycaemic hyperinsulinaemic insulin clamp. Originally developed by Andres *et al* (1966) and De Fronzo *et al* (1979) it involves the primed intravenous (IV) infusion of exogenous insulin in order to maintain plasma levels above fasting, whilst glucose is fixed at a set level by simultaneous IV glucose administration at varying rates. The rate of glucose infusion is determined by measuring plasma glucose levels every five minutes. Thus as plasma glucose levels fall below the set level, more glucose is infused to return it to the set level and vice versa. An index of insulin action is therefore the total amount of glucose infused over the time course of the experiment (M value). The higher the M values per unit time, the greater the subject's insulin sensitivity is. Thus, an insulin resistant individual will require the infusion of far less glucose to maintain the basal glucose levels. This technique has two main advantages: (1) the confounding effects of endogenous insulin secretion, variability of hyperglycaemia and hypoglycaemic counter-regulation are eliminated, (2) additional use of isotopes allows examination of several actions of insulin simultaneously e.g. glucose uptake, glycogenesis and changes in protein metabolism. However, the test does not reproduce physiological conditions, is costly and technically complex. It is therefore unsuitable for routine clinical use.

1.11.1.2 - Minimal Model

Bergman *et al* (1987) developed a procedure in which glucose and insulin are sampled at frequent intervals from an indwelling catheter whilst an intravenous glucose tolerance test is performed. Results are entered into a computer model, which calculates a value for insulin sensitivity (S_i). In addition, this test determines the acute insulin release response to glucose. In subjects with advanced IGT and type 2 diabetes however, there is

a diminished immediate plasma insulin response. As this factor is a major determinant of the analysis, the accuracy of the model is reduced in these subjects. Test results in the minimal model are generally consistent with those of the clamp technique and the procedure is simpler and provides information on both insulin action and secretion.

1.11.1.3 - Plasma insulin concentration

A simple method for making a clinical estimate of insulin resistance can be obtained in certain circumstances by measuring plasma insulin concentrations (American Diabetic Association (ADA), 1998). This type of estimate may be suitable in subjects with normal glucose tolerance and when type 2 is not already well established, i.e. beyond the peak hyperinsulinaemia, which usually occurs during IGT or, at early stages in the pathogenesis of NIDDM. As an estimate of IR, plasma insulin is most informative after overnight fasting, since post-prandial glucose levels are in a constant state of flux and this would confuse the interpretation of insulin levels as an indicator of insulin resistance. High plasma insulin levels together with normal glucose levels are considered to reflect insulin resistance, this would seem to be the ideal test. However, in diabetic individuals plasma insulin levels fall as the beta cell defect takes effect, creating considerable overlap between normal and diabetic insulin resistant subjects, rendering this a very ambiguous type of estimate (ADA, 1998).

1.11.2 - In vivo tissue/cell IR measurement

1.11.2.1 - Glucose Uptake

Skeletal muscle is the main site of insulin-stimulated glucose utilisation by the body, and therefore the main site of insulin resistance contributing to impaired glucose utilisation, energy depletion and fatigue in type 2 diabetics (De Fronzo, 1988). Hence this thesis will focus mainly on skeletal muscle. A widely used technique for assessing tissue and or cellular insulin resistance is the measurement of the uptake of radio labelled 2-deoxyglucose (2-DG). This is a glucose analogue which is taken up via the glucose

transporters in an identical manner to glucose. 2-DG is then phosphorylated by hexokinase (or glucokinase in liver and β - cells) but cannot be metabolised further. The rate of accumulation of the radioactive sugar is linear for several minutes as the transported hexose is largely trapped by phosphorylation. However, there is evidence from adipocytes that 2-DG can be dephosphorylated (Foley, 1986) and that the rate of transport of 2-DG varies according to the concentration of the analogue (Foley, 1980). 2-DG experiments can provide valuable comparative information provided the experiments are well controlled and the limitations of the assay are taken into account. A more expensive alternative is 3-O-methyl glucose (3-O-MG), which is also transported into cells similarly to glucose, but unlike 2-DG is not phosphorylated. 2-DG and 3-O-MG have generally given the same information.

1.11.2.2 - TNF α associated markers

Tumour Necrosis Factor α (TNF α), which is a peptide cytokine involved in the immune response, has been implicated in the IR of human obesity and NIDDM (see later). Upon measuring the plasma concentrations of the soluble fraction of the TNF receptors 1 and 2, Fernandez-Real *et al* (1998) indicated that plasma sTNFR-2 levels, but not sTNFR-1 were an index of insulin resistance. Similarly, Solomon *et al* (1996 and 1997) have reported on the induction of IR in rat hepatoma cells by TNF α and that the level of expression of the calmodulin (CaM) gene in these cells served as a 'sensitive and sophisticated index of insulin action' (Solomon *et al.*, 1997).

1.11.2.3 – Free Fatty acids

Insulin inhibits lipolysis leading to a decrease in free fatty acid release from triacylglycerol in the adipose tissue (and to a much smaller extent in the liver and skeletal muscle). This inhibition is extremely sensitive to insulin and is a potential contributor of dyslipidaemia in insulin resistance. Since obesity frequently accompanies type 2 diabetes and is a recognised insulin resistant state, the effect of insulin on adipocyte lipolysis

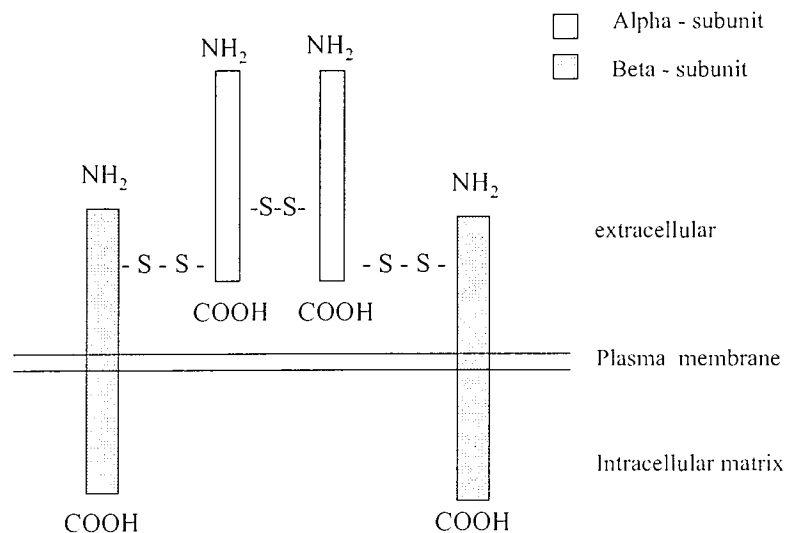
(measured by glycerol release) is a useful indicator of insulin resistance and an important therapeutic target.

1.12 - Normal insulin action

Virtually all mammalian tissues express insulin receptors, including non-classical insulin targets such as erythrocytes, nervous tissue, vascular endothelium, kidney and gonadal cells (Havrankoval *et al.*, 1978; Joeng-Hyok *et al.*, 1983; King *et al.*, 1983 and Saucier *et al.*, 1981). However, the density of the receptors varies considerably between these tissues e.g. 40 per cell on erythrocytes compared with 200,000 per adipocyte and hepatocyte.

Diagram 1.1 - Model of the insulin receptor (adapted from Matthaedi *et al.*, 2000)

The insulin receptor is a 400Kd glycoprotein consisting of two α and two β subunits.

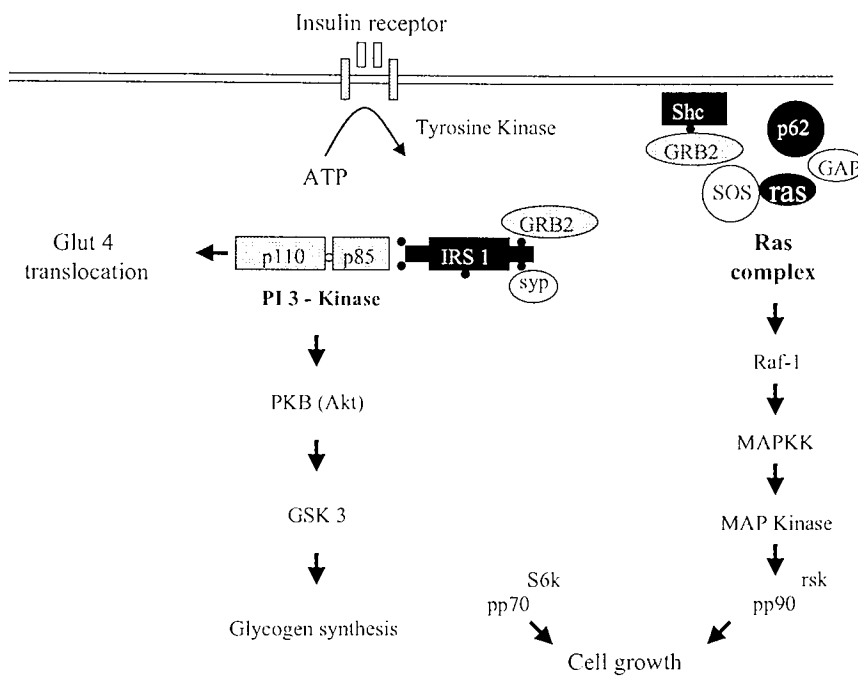


The β subunit spans the plasma membrane and exhibits both intracellular and extracellular domains. The intracellular portion of the β subunit shows tyrosine kinase activity whereas the α subunit is only extracellular and is a repressor of the β subunit tyrosine kinase activity (Kahn, 1980). Binding of insulin to the extracellular portion of the receptor leads to conformational changes, which expose ATP binding sites and

facilitate autophosphorylation of the β subunit on tyrosine residues. This activates the β subunit to function as a tyrosine kinase enzyme. The tyrosine kinase activity is essential for subsequent signalling, and the main substrates for this activity are a family of cytoplasmic proteins, the most researched of which is Insulin Receptor Substrate 1 (IRS-1) (White, 1997). IRS-1 associates to the insulin receptor via a pleckstrin homology domain (Yenush *et al.*, 1996). Upon tyrosine kinase phosphorylation of IRS-1, non-covalent binding occurs between the phosphorylated sites and certain domains on the target proteins (src homology 2 (SH2)). Other known substrates are IRS-2, 3, 4 and Shc (see figure 1.2). Target proteins include GRB-2 and phosphatidylinositol - 3 kinases (PI-3 kinases). The former links the Ras pathway to insulin signalling which eventually results in activation of various enzymes including glycogen synthase, glycogen phosphorylase and glycogen phosphorylase kinase (Kahn, 1997).

Our understanding of insulin post-receptor pathways is evolving daily and the following text outlines the state of knowledge when this program of research was conceived. Recent updates are incorporated into the relevant subsequent chapters of this thesis. However, the key elements of the pathways outlined in diagram 1.2 and the text in this chapter remain accepted intracellular signalling routes between the insulin receptor and its final effectors within the cell (Shepherd and Kahn, 2000; Saitel and Kahn, 2001; Zierath *et al.*, 2000).

Diagram 1.2 - Putative insulin signalling pathway (adapted from Holman & Kasuga, 1997)



1.12.1 - PI-3 Kinase

Phosphatidylinositol-3 kinase (PI-3 kinase) is a heterodimer consisting of two subunits, a 85 Kda (α -p85) and a 110 Kda (p110) subunit. The α -p85 functions as an adaptor with two SH2 domains and one SH3 domain (Otsu *et al.*, 1991; Skolnik *et al.*, 1991; Escobedo *et al.*, 1991) whilst the p110 subunit shows catalytic activity (Hules *et al.*, 1992; Hu *et al.*; 1993). PI-3 Kinase phosphorylates inositol rings at the D-3 position giving PI 3 phosphate from PI, PI 4,5-bisphosphate from PI 4 phosphate and PI 3, 4, 5 trisphosphate from PI 4, 5 bisphosphate (Whitman *et al.*, 1988; Auger *et al.*, 1989). Using a mutant p85 subunit (Δ p85), which cannot bind the p110, Hara *et al.* (1993; 1994) discovered that insulin-stimulated glucose uptake was greatly impaired, whereas Ras activation was not. This suggests that PI-3 kinase coupled with α -p85 is necessary for insulin induced

glucose uptake (Hara *et al.*, 1993; Dhand R *et al.*, 1994). As α - Δ p85 results in inhibition of PI 3, 4, 5 phosphate (PIP-3) production, this lipid may be a mediator of insulin effects further downstream (Holman GD & Kasuga M, 1997). The PI kinases are associated with a number of trafficking processes. The reorganisation of actin, ruffling of the membrane and pinocytic activity observed upon administration of insulin to human epidermoid carcinoma (KB) cells has been associated with PI-3 kinase activity (Kotuni *et al.*, 1994).

1.12.2 - PI-3 Kinase and Glucose Transporters

Insulin stimulates the translocation of the glucose transporter isoform GLUT4 to the plasma membrane up to ten fold over basal levels. GLUT1 translocation is also stimulated by insulin but to a much lesser extent. The fungal metabolite wortmannin, which inhibits PI-3 kinase activity, has been shown to completely abolish the stimulation of GLUT4 and 1 translocation in response to insulin. Immunolocalisation of GLUT4 after wortmannin treatment shows different results depending on whether basal or insulin-induced GLUT4 translocation is inhibited. Treatment of cells in the basal state results in localisation of GLUT4 to the microsomal compartment, whereas treatment after insulin administration shows GLUT4 to be localised in the plasma membrane. Whilst the role of PI-3 kinase activity in the control of GLUT transport is unclear at present, a requirement of PI-3 activity for transfer of GLUT transporters from microsomes to the plasma membrane implies a role in the budding of microsomes and subsequent fusion with the plasma membrane (Holman & Kasuga, 1997).

1.12.3 - PIP 3

PIP 3 is thought to be the physiologically important product of PI-3 kinase activity. It can interact with various isoforms of Protein Kinase C (PKC)(Nakanishi *et al.*, 1993) and Protein Kinase B (PKB) (Franke *et al.*, 1995). PKC stimulators such as phorbol esters can increase glucose transport activity, but the effects are small compared to those produced by insulin (Kirsch *et al.*, 1985; Holman *et al.*, 1990). Whilst it seems unlikely

that PKC isoforms are actually components of the normal stimulatory pathways of insulin, it may be that insulin receptor kinase activity is inhibited by certain isoforms of PKC due to elevations in intracellular glucose, which have a feedback effect on the signalling pathway (Haring *et al.*, 1994). PKB has been identified as a signalling intermediate that is located downstream of PI-3 kinase inhibited by wortmannin (Burgering & Coffey, 1995) and activated by insulin (Kohn *et al.*, 1995). Whether PKB is directly involved in glucose transport regulation is unclear at this time.

1.13 - Defects of insulin action in diabetes and obesity

The hyperinsulinaemia found in obesity and early type 2 diabetes results in down-regulation of the insulin receptors, through increased degradation (Kahn, 1997). In addition, increased insulin levels down-regulate receptor kinase activity, which may be due to altered serine/threonine phosphorylation (Folli *et al.*, 1992; Gavin, 1974). Both effects lead to insulin resistance. Generally, the greater the basal insulin levels the greater the down-regulation of the insulin receptors. Weight loss and prudent diet tend to reduce the insulin levels and correct this receptor defect, thus improving but not totally reinstating insulin action (Kahn, 1980).

1.14 - Insulin resistance and type 2 diabetes: possible molecular mechanisms

In type 2 diabetes there are several potential sites at which defective function could incur insulin resistance (Shepherd and Kahn, 2000; Saitel and Kahn, 2001; Zierath *et al.*, 2000). These include the insulin receptor, IRS-1-4, SH2 domain proteins already identified in the post-receptor insulin signalling pathway and various cellular enzymes which mediate key biological effects of insulin and glucose transporters. Even before the development of type 2 diabetes, people with IR show a reduction in insulin stimulated glycogen synthesis and a fall in glucose-6-phosphate formation (Rothman *et al.*, 1992; Rothman *et al.*, 1995; Shulman *et al.*, 1990). Studies in *ob/ob* mice have shown impairment of insulin receptor and IRS-1 phosphorylation and a reduction in insulin stimulated PI-3 kinase activity. A reduction in insulin receptor kinase activity has also

been seen in type 2 diabetes patients (Caro *et al.*, 1987). The fact that the insulin receptors are structurally and functionally normal before the development of type 2 diabetes and appear to remain structurally normal after the development of type 2 diabetes, and that weight reduction improves kinase activity, shows that the defects are acquired (Friedenburg *et al.*, 1988).

Other studies have indicated a potential inhibitor of insulin action in cells cultured from a type 2 diabetes patient (Maddux *et al.*, 1995) and two agents have been discovered that act as inhibitors of insulin receptor kinase activity; α HS glycoprotein (Srinivas *et al.*, 1993) and a 130 Kda membrane glycoprotein named PC-1 (Maddux *et al.*, 1995). Overexpression of PC-1 in cell lines causes a reduction of insulin mediated phosphorylation of IRS-1 due to decreased autophosphorylation of the β subunit. Liver nucleoside pyrophosphates and PC-1 appear to be the same and can be found in kidney, brain, placenta, skeletal muscle and fat. Another two possible mediators of insulin resistance are TNF α and a Ras related protein called Rad. It is known that TNF α is produced from adipocytes of obese patients and that via a paracrine or endocrine effect it appears to induce insulin resistance by reducing receptor kinase activity (Hotamisligil *et al.*, 1994; Hotamisligil & Spiegelman, 1994; Hofmann *et al.*, 1994). Rad is a 35Kda guanosine triphosphatase (GTPase) (Reynet & Kahn, 1993). It is over-expressed in the skeletal muscle of some individuals with type 2 diabetes, where it appears to work by inhibiting insulin stimulated glucose uptake (Reynet and Kahn, 1993), although the exact mechanism is unclear.

The latest theories suggest that type 2 diabetes and insulin resistance are due to multiple cellular defects and internal concomitant factors. Transgenic mice and cell lines indicate that the IRS proteins (1-4) serve complementary roles in insulin signalling (Saltiel and Kahn, 2001). IRS-1 knockout mice exhibit growth defects and present with impaired glucose tolerance and peripheral insulin resistance (Tamemoto *et al.*, 1994; Araki *et al.*, 1994). Heterozygous IRS-2 knockout mice also present with peripheral insulin resistance but only exhibit growth defects in specific tissues such as the islets, retina and parts of the brain (Kido *et al.*, 2000; Withers *et al.*, 1998). The homozygous double IRS-2 knockout

mouse develops type 2 diabetes (Withers *et al.*, 1998). IRS-3 and IRS-4 knockout mice show little growth retardation or alteration in glucose metabolism (Fantin *et al.*, 2000). Other targeted deletions of insulin signalling components in mice have yielded informative results. Insulin receptor knockout mice die within 3-7 days of birth from diabetic ketoacidosis (Bruning *et al.*, 1997). 'Knocking out' PKB/Akt leads to insulin resistance in the liver and muscle and hence diabetes (Cho *et al.*, 2001). Perhaps surprisingly, GLUT4 and p85 α knockout mice do not become diabetic, in fact p85 α knockout mice exhibit increased insulin sensitivity, indicating the complexity of the insulin signalling cascade (Katz *et al.*, 1995; Fruman *et al.*, 2000; Terauchi *et al.*, 1991).

Pessin and Saltiel (2000) have also noted multifactorial defects in the insulin resistant state, including a decrease in insulin receptor number and kinase activity, decrease in PI3-K activity, defects in translocation of glucose transporters, as well as decreased IRS-1 and 2 concentration and phosphorylation (Pessin and Saltiel, 2000).

1.15 -Type 2 diabetes and treatment

Type 2 diabetes treatment conventionally begins with dietary measures, weight loss in the obese and exercise. Thereafter, oral hypoglycaemic agents and insulin are used as required (Bailey, 1988). As previously mentioned, an energy restrictive diet that causes weight loss improves insulin sensitivity (decreases insulin resistance) and improves monocyte insulin receptor binding indicating up-regulation of insulin receptor action toward normal (Beck-Nielsen *et al.*, 1980). Adipocytes from obese type 2 diabetes patients show improved insulin receptor kinase activity after weight reduction. However, since glucose disposal was improved in obese non-diabetic controls without a corresponding change in receptor kinase activity, this implies that the reduction in insulin resistance is a post receptor phenomenon (Friedenburg *et al.*, 1988). Sulphonylureas mainly act by stimulating insulin secretion by the pancreatic β cells. Controversy exists concerning the clinical significance of any putative 'extrapancreatic activity' of these compounds, and their effects on insulin receptor numbers have been inconsistent and could be indirect due to favourable changes in the metabolic environment. The biguanide

metformin acts as an anti-hyperglycaemic agent i.e. it does not cause overt hypoglycaemia in patients with type 2 diabetes (Bailey, 1988). Metformin reduces hepatic glucose uptake and increases peripheral insulin stimulated glucose uptake as well as reducing lipid oxidation (Bailey & Turner, 1996). Another therapeutic approach is the insulin sensitisers or thiazolidinediones (TZDs) such as troglitazone, rosiglitazone and pioglitazone. These work by increasing the transcription of insulin sensitive genes via binding of the nuclear peroxisome proliferator activated receptor γ (PPAR γ) (Spiegelman, 1998).

1.16 - Aims of present study

The foregoing account has shown that insulin resistance is a key underlying factor in the development of metabolic disturbances such as type 2 diabetes and obesity, and other cardiovascular risk factors such as dyslipidaemia and hypertension. Indeed this collection of disturbances is now recognised as a discreet syndrome.

At the cellular level the causes of insulin resistance are unclear and would appear to be multifactorial, involving an interaction of genetic and environmental influences upon the insulin receptor and post-receptor signalling pathways (Shepherd and Kahn, 2000; Saitel and Kahn, 2001; Zierath *et al.*, 2000). TNF α has emerged as a newly recognised cytokine that contributes to insulin resistance, but the extent to which it is involved is unclear. One aspect of this thesis will be to evaluate the role of TNF α in the development of insulin resistance.

Undoubtedly any intervention to reduce insulin resistance and reinstate normal insulin action would have important potential benefits for the many components of Syndrome X, especially type 2 diabetes and obesity. The possibility of circumventing the defects of insulin resistance on glucose metabolism by directly stimulating glucose uptake and utilisation (or potentiating these effects of insulin) is one potential therapeutic approach of particular advantage for type 2 diabetes and obesity. This is considered in detail in this thesis, looking at novel agents that can either potentially mimic or enhance insulin action

on glucose (and lipid) metabolism. The main agents considered are lipoic acid, sibutramine metabolite 2 (M2), TNF α and bradykinin, along with less detailed considerations of isoferulic acid, lipid mobilising factor (LMF) and the trace elements lithium, selenium and chromium.

The original rationale for the following studies was the conflicting evidence within the literature viz causes of insulin resistance, particularly the effects of TNF α . The principal aims have been to investigate the potential role of agents that partially mimic or enhance actions of glucose as treatments to overcome insulin resistance, notably α -lipoic acid. The investigation of the above compounds has been conducted mainly using isolated muscle preparations and cultured L6 myocytes. Since these aims represent an initial preliminary evaluation of TNF α , lipoic acid, other compounds and other methods have been included.

This thesis therefore sets out to:

- (1) critically review literature pertaining to insulin resistance in type 2 diabetes
- (2) assess methods employed to measure glucose uptake in isolated muscles and cultured muscle cells
- (3) evaluate effect of TNF α on glucose uptake in the systems used
- (4) evaluate effect of lipoic acid alone and in combination with insulin on glucose uptake in the systems used.
- (5) Evaluate effect of other compounds as potential therapeutic interventions against the effects of insulin resistance on glucose uptake.

Chapter 2 - Materials and Methods

2.1 - Introduction

The methods used in the experimental studies described herein have focussed mainly upon the uptake and metabolism of glucose by skeletal muscle and cultured L6 muscle cells. Additional studies have examined the rate of lipolysis in adipose tissue. This approach was adopted as a measure of insulin action (and its reciprocal, insulin resistance) to address the aims of the research programme described previously. The experimental protocols provided a measure for the effect of various agents (e.g. TNF α and lipoic acid) on the control of glucose uptake or lipolysis by insulin, giving an insight into the pathogenesis and potential treatment of insulin resistance in relation to these metabolic processes.

2.1.1 - Chemicals

The chemicals used were of analytical grade. They were obtained from BDH (Poole, Dorset), Sigma-Aldrich (Poole, Dorset) and Fisher Scientific (Loughborough, Leics) unless otherwise stated. Special chemicals such as 2-deoxy-D-glucose, tumour necrosis factor alpha and alpha-D-lipoic acid were from Sigma. The radioisotopes 2-deoxy-D- [^3H]-glucose (7.1632kBq/ml) and U- [^{14}C]-D-glucose (7.4MBq/ml) were from Amersham International (Amersham, Bucks). The glucose oxidase (GOD/PERID) and lactate dehydrogenase kits for measuring glucose and lactate were from Boehringer Mannheim (Lewes, Sussex). All plasticware for cell culture was from Sarstedt (Beaumont Leys, Leics) and culture media and supplements were from GibcoBRL (Paisley, Rens). All solutions were prepared using double distilled water unless otherwise stated.

2.1.2 - Animals

In vivo studies and studies using isolated animal tissues were conducted using obese *ob/ob* mice and homozygous lean *+/+* mice from the Aston colony that carries the *ob* mutation. The mutation is a single base-pair change in the gene encoding leptin, resulting in a stop codon part-way along the gene. Thus the obese *ob/ob* mice have a congenital absence of functional leptin (Zhang *et al.*, 1994). Lack of leptin results in a loss of the leptin satiety signal, causing the mice to overeat and hence become obese. This mutation is autosomal recessive and is transmitted in a Mendelian fashion, the normal homozygous and heterozygous littermates being the 'lean'. Obesity is linked to insulin resistance and the obese-diabetic *ob/ob* mouse provides a very severely insulin resistant animal model of obesity and hyperglycaemia (Zhang *et al.*, 1994, Bailey and Flatt, 1997). The origin of the animals is as follows: in 1949 the obese mice (designated genotype *ob/ob*) were observed in a colony of mice at the Jackson Laboratory, Bar Harbor, Maine, USA. The mutation was transferred to the C57BL/6J background. In 1957 Professor DS Falconer of the Institute of Animal Genetics (Edinburgh University) obtained original C57BL/6J heterozygous (*ob/+*) breeding pairs from Jackson Laboratory. The heterozygous mice were out crossed at Edinburgh to produce two non-inbred local strains: JH with high litter size and CRL with a fast growth rate (Falconer, 1960). Both JH and CRL strains were maintained as closed non-inbred stock for 5 and 10 generations respectively, after which the heterozygous mice were out crossed to two further non-inbred local strains (1966). In the same year *ob/+* breeding pairs from this stock were obtained by the University of Aston (Birmingham). These mice have been used to establish a closed non-inbred colony, which is now in excess of 100 generations. Mice were bred and maintained in an air-conditioned room at $22 \pm 2^{\circ}\text{C}$ with a lighting schedule of 12 hours light (0800-2000h) and 12 hours dark. Standard pellet diet (Economy Rodent Breeder Diet, Lillico, Surrey) and tap water were supplied *ad libitum*.

2.2 - Glucose uptake and metabolism in response to α -lipoic acid

2.2.1 - Glucose uptake by muscle, measured by 2-deoxy-D- [3 H]-glucose uptake

Glucose uptake was determined using 2-deoxy-D- [3 H]-glucose, a tritiated glucose analogue characterised in many previous studies (Wilks, 1957; Henriksen *et al.*, 1990; Haber and Weinstein, 1992). 3 H 2DG is taken into cells in an identical fashion to glucose, where it is phosphorylated but not metabolised further (Walker *et al.*, 1989). 3 H 2DG is therefore a good indicator of glucose transport within tissues as measured by the cellular accumulation of 3 H 2DG phosphate. 14 C could be used in place of 3 H, but this is more expensive and does not leave the option of using 14 C interstitial space markers if required (e.g. 14 C sucrose or glucose). Interstitial space appears to be consistent during previous studies reported in this laboratory and hence a specific space marker was not needed in the present study. Incubation time after 3 H 2DG addition was kept to a minimum (20 min at 37°C or longer at lower temperatures), as 3 H 2DG uptake is linear until accumulation of the non-metabolised analogue alters glucose uptake kinetics and osmotic parameters.

Glucose uptake into skeletal muscles isolated from lean and obese mice was measured by the following procedure. Krebs solution (appendix*¹) was supplemented with CaCl₂ at 0.147 μ g ml (1mM), glucose at 1mg ml (5.6mM) and 0.01% Bovine Serum Albumin (BSA)(fraction V). Bovine insulin (Sigma) was dissolved in buffer and used at a final concentration of 10⁻⁶ M, and α -lipoic acid dissolved in dimethyl sulphoxide (DMSO) and used at a working concentration of 10⁻³M as in table 2.1. This concentration was selected as previous studies by Haugaard and Haugaard (1970) and preliminary studies in this laboratory had shown that a concentration of about 10⁻³M was usually required in an *in vitro* preparation to produce a significant metabolic effect. Both lean and obese *ob ob* mice were used. Muscles were isolated from euthanased (cervical dislocation) freely fed mice and placed in unsupplemented gassed (95% O₂: 5% CO₂) Krebs medium at 25-30°C for transport from the Animal Care Unit to the laboratory (5 min).

The following three skeletal muscles were selected for study: diaphragm (mixed type 1 and type 2 fibres), red quadriceps (mainly type 1) and abdominal muscles (mainly type 2). The muscles were rapidly isolated in the following sequence. Firstly the upper thigh was dissected and the vastus intermedius (red quadriceps) muscles adjacent to the femur removed. The lower lateral quadrant of the abdomen was incised and the underlying abdominal muscles removed, then the entire diaphragm excised, trimmed of tendon and cut into two segments (i.e. hemi diaphragms).

Table 2.1 – Protocol design to study the effect of lipoic acid (10^{-3} M) on glucose utilisation by various muscles *in vitro* (insulin 10^{-6} M)

Tube No	Muscle Tissue	Compound added
1	Diaphragm	Insulin
2	Diaphragm	Insulin & Lipoic
3	Diaphragm	Control
4	Diaphragm	Lipoic
5	Red Quadriceps	Insulin
6	Red Quadriceps	Insulin & Lipoic
7	Red Quadriceps	Control
8	Red Quadriceps	Lipoic

The tissues were quickly but carefully isolated, weighed accurately to 0.1mg using a Sartorius (Gottingen, Germany) electronic balance and transferred to 5ml plastic vials. 3ml of the pre-gassed Krebs medium at 37°C, supplemented as described above, was already inserted into each vial and test agents (insulin 10^{-6} M and /or lipoic acid 10^{-3} M) were added as noted in the protocol lists (table 2.1). The vials were stoppered with the lids supplied with the vials and placed in a 37°C shaking (60 reps/min) water bath and gassed with 95% O₂: 5%CO₂ for 1 minute at 30 minute intervals for 2 hours. After 2 hours of incubation the vials were transferred to a water bath at 37°C, gassed and allowed to equilibrate for 5 minutes. The vials were opened and 18.75Kbq (0.5µCi) ³H 2DG was

added (stock 1 μ Ci/ml), after which they were gassed, restoppered, and incubated for 20 minutes at 30°C. The muscle tissue was then removed, thoroughly blotted and placed in a scintillation vial to which 0.5ml of 1M NaOH was added. Vials were then heated at 80°C until all muscle tissue was dissolved. Vials were removed, allowed to cool to room temperature, then 5ml of Optiphase 'HiSafe' II scintillant was added and the vials were counted for ³H using a Packard 1900TR liquid scintillation analyser. For all beta counting a pre-programmed quench correction was undertaken by software installed within the counter. Counts were expressed as disintegrations per minute (DPM) per mg of muscle tissue. In these studies no attempt was made to measure the interstitial compartment with ¹⁴C-sucrose or ¹⁴C-L-glucose since many preliminary studies had not shown any differences in the interstitial compartment with a selection of muscles incubated up to 2h with different concentrations of insulin.

2.2.2 - Glucose uptake by muscle measured by glucose disappearance

2.2.2.1 - Glucose determination

Muscles were isolated and incubated as in section 2.2.1. After two hours the muscle tissue was removed, blotted on tissue paper and re-weighed. The glucose remaining in the medium was assayed using the glucose oxidase method using a GOD/PERID kit (Boehringer Mannheim, protocol in appendix). The difference between the known amount of glucose added and that found at the end of the incubation was expressed as glucose uptake per mg tissue. Control vials were included to check for the effects of evaporation and condensation and the manipulative procedures which had no measurable effect on the glucose concentration after 2 hours provided the vials were vortexed to wash back material attached to the sides of the vessel.

N-ethylmaleimide (0.5mM) was added during the glucose assay at the end of the experiments to prevent the interference of colour development by the thiol groups of lipoic acid (Haugaard and Haugaard, 1970).

2.2.2.2 - Lactate determination

To determine whether lipoic acid increased glucose utilization by anaerobic glycolysis, medium at the end of the 2h incubation was assayed for lactate. This was carried out by a modification of the protocol supplied with the Boehringer Mannheim kit (appendix*²). A sample (1ml) of medium was de-proteinised by adding 0.1ml of uranyl acetate, centrifuging at 5000rpm for 1 min and removing the supernatant. 50µl of the de-proteinised sample was added to 2ml of the reagent (appendix*²), which was then split into two 1ml aliquots. Into one of these 10µl of 3.2 mol/L (42%) ammonium sulphate [(NH₄)₂SO₄] (blank) was pipetted. To the other, 10µl of lactate dehydrogenase/GPT enzyme solution was added. The change in absorbance between the two samples was measured at 340nm using a spectrophotometer (Pharmacia Biotech, Cambridge, Cambs) to determine the lactate content of the test solution. This was undertaken using medium after incubation of muscles from lean and obese *ob/ob* mice, incubated as in section 2.2.1 for 2 hours.

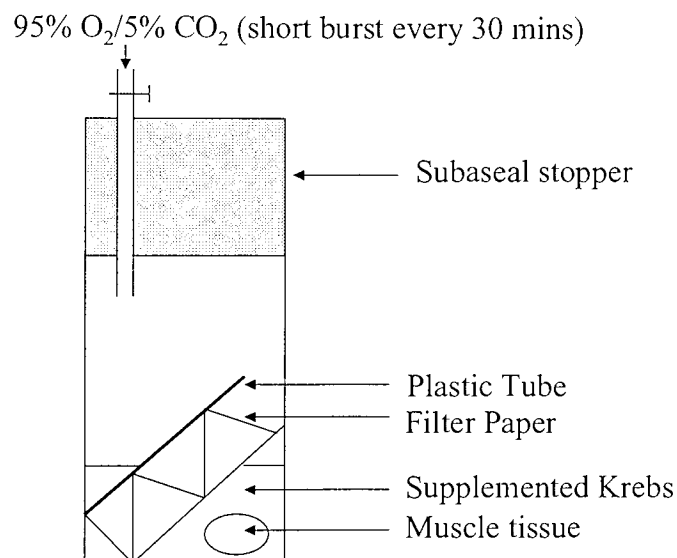
2.2.3 - Glucose oxidation by muscle and glycogen deposition by muscle

2.2.3.1 - Glucose oxidation by CO₂ analysis

To determine whether lipoic acid increased glucose oxidation, the experimental apparatus was set up as in diagram 2.1. The filter paper (2cm², Whatman no 1) was added within a plastic tube (LP3, Sarstedt) within the glass Bijou tube. Muscle tissue and Krebs incubation medium were prepared as in section 2.2.1. U- [¹⁴C]-D-glucose (18.5Kbq/0.5µCi) was added per Bijou tube containing 3ml Krebs medium. Tissue was added at the start of the 2 hour incubation period at 37°C, medium was re-gassed, stoppered and shaken (60 revs/min). The tubes were then removed to ice and 100µl 1M NaOH was injected onto the filter paper through the suba-seal stopper using a long 23g needle. This absorbs the ¹⁴CO₂ from the atmosphere within the tube. After 5 minutes the seal was opened and the muscle tissue removed, blotted, re-weighed and then frozen for

subsequent glycogen analysis (2.2.3.2). After replacing the seal, 0.3ml of 3M perchloric acid was injected through the stopper into the medium to liberate dissolved $^{14}\text{CO}_2$. The filter paper was removed after 30 minutes on ice and transferred to a scintillation vial. 5ml of HiSafe II scintillant was added and the vials were counted for ^{14}C using a Packard counter as per section 2.2.1.

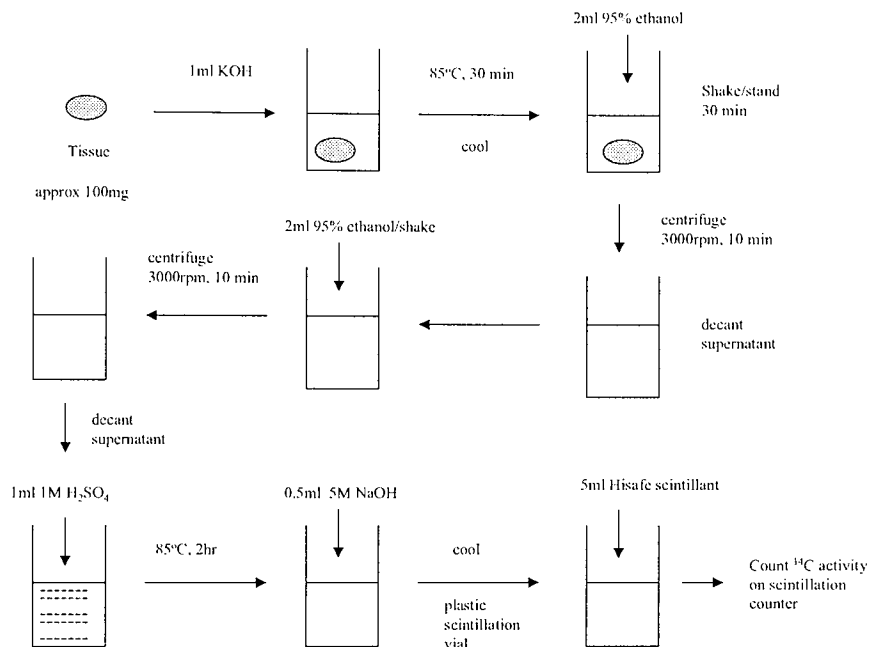
Diagram 2.1 - Apparatus for glucose oxidation measurement



2.2.3.2 – Glycogen analysis

In an initial experiment, frozen tissue samples from section 2.2.3.1 were thawed naturally, tissue was digested in KOH and glycogen was extracted by ethanol precipitation. Labelled ^{14}C glucose incorporated into the glycogen was counted as elaborated in diagram 2.2. This method was not found to give reproducible results and experiments were repeated with the technique described in 2.2.3.3.

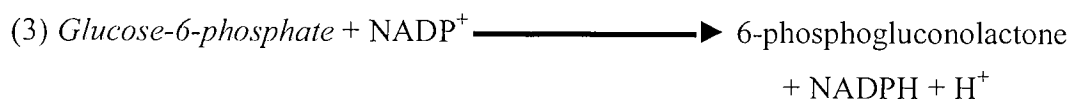
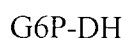
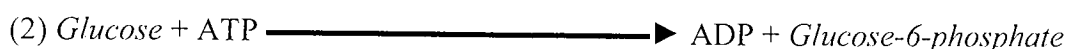
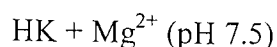
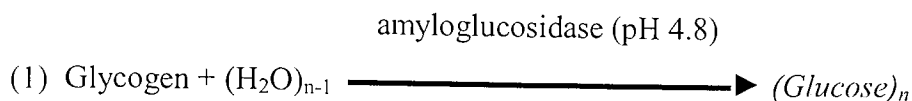
Diagram 2.2 – Methodology flow chart for quantifying ^{14}C -labelled glycogen in muscle tissue



2.2.3.3 – Enzymatic measurement of glycogen deposition in murine muscle

Glycogen is a storage form of glucose found largely in the muscle and liver of most animals. Muscle glycogen is thought to have a particle molecular weight of about 10 million (Orell *et al.*, 1964; Bueding and Reissig, 1964). It is generally thought that for specific and sensitive assays for glycogen, enzymatic hydrolysis is a better method compared to hydrolysis by acid (Keppler and Decker, 1974). The enzyme employed is an amyloglucosidase derived from *Aspergillus Niger*, which hydrolyses glycogen at the α -D- (1-4) and α -D- (1-6) linkages (Fleming and Stone, 1965; Pazur and Ando, 1960). The laborious and inconvenient isolation of glycogen is unnecessary due to the high specificity of the enzyme, which allows hydrolysis even within blood or organ homogenates (Krebs *et al.*, 1963; Johnson and Fusaro, 1966). The glucose formed is then

determined by the use of hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH) as outlined below adapted from Keppler and Decker, 1974).



Glucose liberated upon glycogen hydrolysis is proportional to the increase of NADPH measured via extinction change at 340nm.

2.2.3.3.1 - Hydrolysis of muscle glycogen by amyloglucosidase

The procedure (adapted from Keppler and Decker, 1974) was carried out as follows: Red quadriceps, diaphragm and abdominal muscles were obtained as described in section 2.2.1 and then treated with α lipoic acid as in the same section (10^{-3} M lipoic acid, 2hr incubation at 37°C). The muscles were then homogenised with 5 parts (w/v) ice-cold perchloric acid (0.6M) using a IKA Labortechnik (Staufen, Germany) electric homogeniser. 0.2ml of the resulting homogenate was transferred to a glass tube (on ice) to be used for glycogen hydrolysis. The homogenate plus perchloric acid was then incubated with 0.1ml of potassium hydrogen carbonate (1M) and 2.0ml amyloglucosidase solution (20mg in 20ml acetate buffer) in a stoppered glass tube for 2hr at 40°C.

After 2h the incubation was stopped by the addition of 1ml of perchloric acid (1M) and the tube centrifuged for 5 minutes at 3000rpm. 0.05ml of the acidic supernatant was then taken for the determination of glucose. A homogenate glucose blank was prepared by centrifuging the homogenate in perchloric acid for 15min (3000rpm), neutralising with about 0.5ml of potassium hydrogen carbonate (1M) and taking 0.05ml of the supernatant.

2.2.3.3.2 -Determination of glucose

A Pharmacia Biotech (Cambridge, Cambs) spectrophotometer was used at 340nm, reading against air. 1ml of ATP/NADP/G6P-DH buffer*⁴ was added to the 0.05ml sample of supernatant in a plastic cuvette. This was mixed by inversion and the extinction value noted upon becoming constant (E_1). This took approximately 5 min. 0.01ml hexokinase solution was then added to the same cuvette, mixed and the process repeated until the value was constant (E_2). This took about 5-10min. Subtraction of E_1 from E_2 gives you a value for ΔE , which is used in the following calculation where ΔE_H is the extinction difference from the sample hydrolysate and ΔE_B is the extinction difference of the glucose blank:

$$56 \times \Delta E_H - 3.4 \times \Delta E_B (\mu\text{mole glucosyl units per g. wet. wt tissue})$$

This assumes a liquid content of the muscle to be about 75% and a 6 times dilution (w/v) of tissue sample in the homogenate. After several runs of this experimental procedure very little glycogen was found, so 3mg of rabbit 'carrier' glycogen was added to each vial and the glycogen 'carrier' was subtracted from the final results. This allowed better deposition of the glycogen due to annealing of native glycogen strands to the added carrier glycogen.

Section 2.2.4 - 2-deoxy-D- [³H]-glucose uptake by L6 cells

L6 muscle cells are a well-established cell culture model used to investigate the action of various compounds upon glucose uptake, usually measured by ³H 2DG uptake (Klip *et al.*, 1994). Originally from rat thigh muscle, cloning of the cells has generated a cell line that can be serially passaged whilst retaining the ability to differentiate (Yaffe, 1968). 'Serum starving' (2.2.5.3) is used to induce myotube formation, a prerequisite to the establishment of functional properties that closely parallel skeletal muscle. Personal observation from within this laboratory indicates a loss of action of insulin after twenty plus passages (perhaps due to receptor down regulation), so low passage number cultures have been used in all of the experiments. Insulin-stimulated glucose transport is assumed to be due to GLUT4, but this was not examined by northern or western blots for technical reasons. Certainly there appears to be agreement between these cultures and the isolated muscle preparations (see later). L6 cultures are convenient to use and allow multiple experiments to be conducted simultaneously. However, maintenance is relatively expensive and intensive, and details of procedures are given below.

2.2.5 - Maintenance of L6 cells

2.2.5.1 - Media and sterile solutions

L6 cells were obtained from the European Culture Collection (Porten Down, Devon). The L6 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) to which the following were added per 500ml bottle of DMEM: 25ml foetal calf serum (FCS), 5ml (200mM) glutamate and 5ml of penicillin (100 U /ml) and streptomycin (100µg/ml) antibiotic mix plus 25µg/ml amphotericin B. This is described herein as 5% FCS DMEM. 0.5% FCS DMEM was made by removing 2.5 ml of the FCS and adding to a fresh 500ml bottle of DMEM. Solutions for tissue culture were prepared either in the medium to be used for the study or into 60% DMSO. The solutions in DMSO were customarily prepared at 100 times the required working concentration so that their addition to the culture or incubation medium constituted only one-hundredth of the total

volume (and therefore one hundredth of the concentration of DMSO). Control solutions containing vehicle only were included in all the experimental protocols. Solutions were filter sterilised by Sarstedt 0.2µm micropore filters fitted to 15ml syringes (Terumo, Leuven, Belgium).

2.2.5.2 - Passaging L6 cells

Cells were seeded at 1×10^9 into 200ml flasks containing 20ml of 5% DMEM, then allowed to grow for 2-3 days in a humidified (5% CO₂: 95% air) incubator at 37°C (Hevaeus Instruments, Essex). Upon reaching confluence (approximately 90% coverage of field of view), the flask of cells was removed from the incubator and the 20ml of 5% FCS DMEM removed. 5ml of trypsin/EDTA solution (appendix*³) (GibcoBRL) was added and the flask agitated. Once all of the cells had visibly detached (*c.* 10 min), the mix was transferred to a sterile universal tube and centrifuged at 800rpm for 5 min using an MSE Mistral 2000 centrifuge. The supernatant was decanted, 10ml of fresh medium was added and the cells were re-suspended. 1ml was then added to each flask containing 20ml of fresh DMEM. The flask was placed in the incubator at 37°C. All cell work was carried out in a D-Faster Ultrasafe 48 laminar flow cabinet (Ferrara, Italy) after prior exposure to UV light and liberal spraying of the materials and cabinet with 70% industrial methylated spirit (IMS).

2.2.5.3 - Preparation of L6 cells for glucose uptake

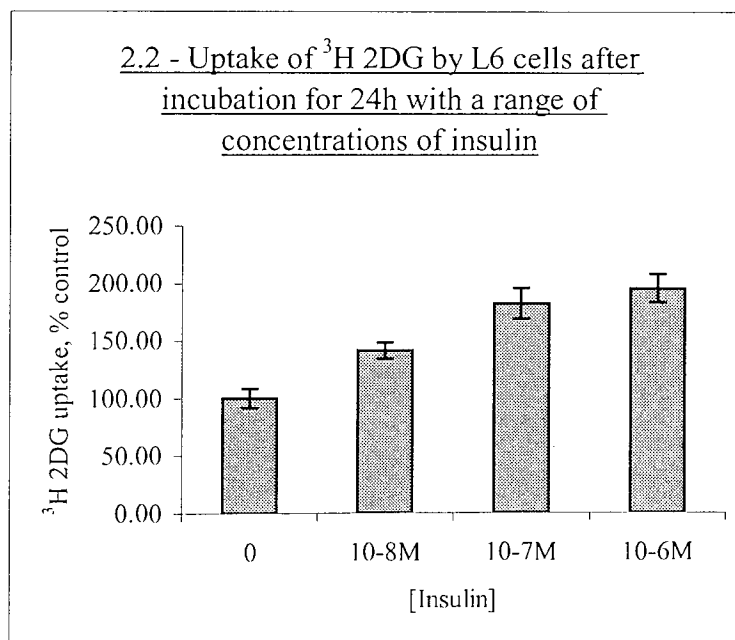
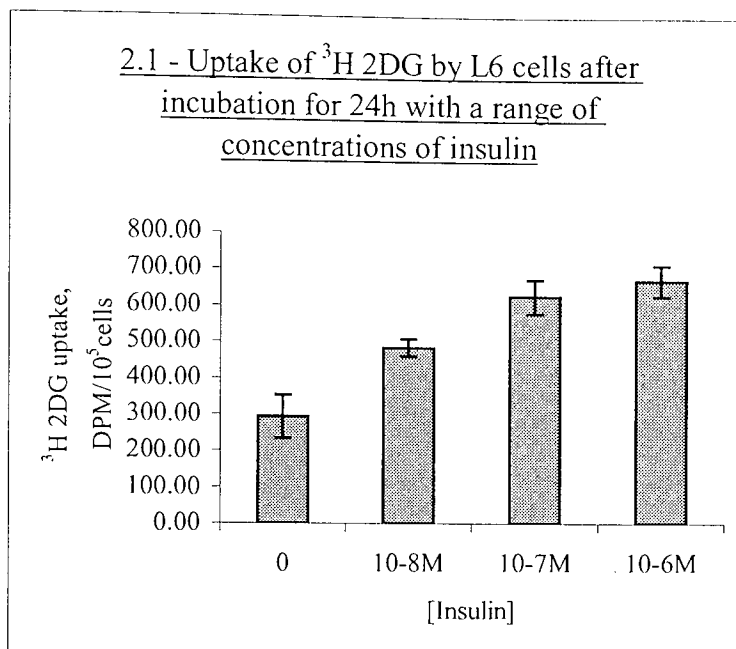
4ml of the re-suspended cell solution from 2.2.5.2 was added to 46ml of fresh 5% FCS DMEM. This was then aliquoted to a 24 well plate at 1ml per well, after which the plates were placed in the incubator. The growth curve of L6 cells under identical conditions to those used for the present studies has been described in detail elsewhere (Bates, 1999). The curve was confirmed in the present studies (approximately 3 days to reach confluence) and standard changes in morphology were observed. Once the cells had reached confluence (section 2.2.5.2) as viewed at 100x magnification on an Olympus CK2 microscope (Olympus Optical Co. Southall, Middlesex), the medium was removed

from each well and replaced with 1ml of 0.5% FCS DMEM (serum starved). 24 hours later the cells were ready for use, having lined up and formed a more cylindrical morphology (multinucleated myotubes). These differentiated cells tend not to divide and hence results can be expressed in relation to number of cells seeded.

2.2.5.4 - 2-deoxy-D- [³H]-glucose uptake by L6 cells

L6 myotubes were incubated with test substances (α lipoic acid at 10^{-2} M – 10^{-7} M and or insulin at 10^{-6} M). After 2, 8, 12, 24 or 48 hours of incubation at 37°C in an atmosphere of 95%O₂: 5%CO₂ the plates were treated as follows: the medium was discarded and the wells washed with room temperature Krebs solution (glucose free). 1ml of Krebs solution with 0.1mM 2-deoxy-glucose containing 7.4Kbq (0.2 μ Ci) 2-deoxy-D- [³H]-glucose was added per well and maintained at 22°C without shaking. After 10 min this was discarded as before and the wells were washed twice with cold Krebs solution (glucose free). 0.5ml of 1M NaOH was then pipetted into the wells and left for 1 hour. The resulting mixture of cells and NaOH was added to 4ml of HiSafe II scintillant in a vial and counted on the scintillation counter for ³H as described previously in section 2.2.1.

Typical values for basal and insulin-stimulated ³H 2DG uptake after 24h incubation of the myotubes are shown as DPM/10⁵ cells in figure 2.1. This confirms the characteristic insulin concentration-response curve for ³H 2DG uptake (Klip *et al.*, 1994). By convention and for the convenience of comparing effects of different treatments in subsequent chapters, ³H 2DG uptake is expressed as % control (value for no added insulin). To illustrate that this provides an accurate representation of the data, the same insulin concentration-response curve is shown in this way in figure 2.2.



Figures 2.1 and 2.2 – Typical values for basal and insulin-stimulated ^3H 2DG uptake after 24h incubation of L6 myotubes shown as DPM/ 10^5 cells (2.1) and as expressed as % control (value for no added insulin)(2.2).

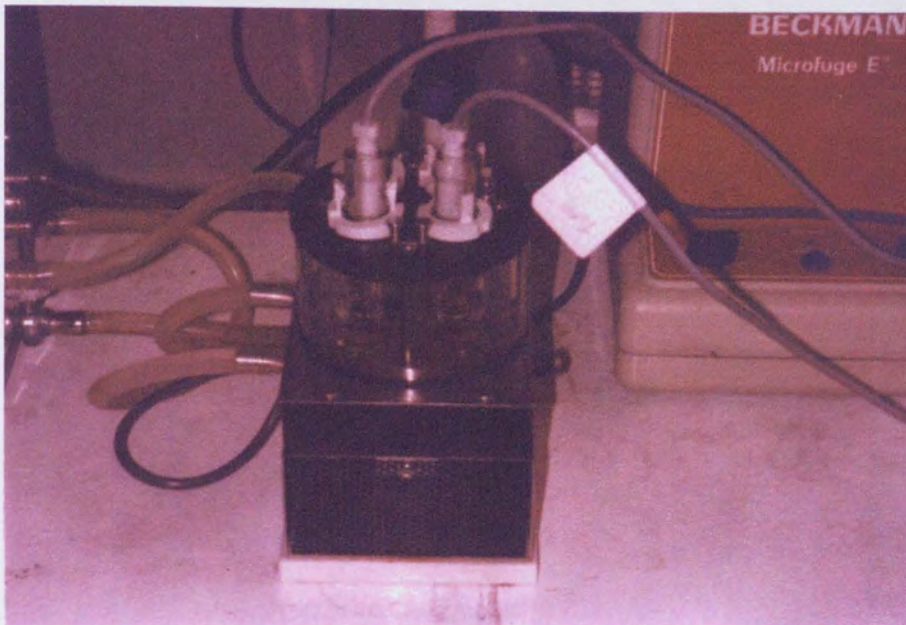
2.2.5.5 – Cytotoxicity testing in L6 cells

A 50% (w/v) solution of trypan blue in phosphate buffered saline (PBS) was prepared at room temperature. Cells were incubated in 24 well plates as described for 2.2.5.4 and the medium was aspirated from the wells and 300µl of the trypan blue solution added to 1ml of medium. The wells were left to stand for 10 min at room temperature without shaking. The trypan blue was then removed and the cells were observed under an inverted microscope at 100x magnification. A single field of vision at about the centre of each well was photographed using an electronic objective lense attachment to the microscope (Videolab Inc, USA). The whole photograph, covering an area of approximately 1mm² was subjected to manual visual counting of all cells to determine the proportion of cells that had excluded the trypan blue dye (uncoloured healthy cells) compared with those which had not excluded the trypan blue dye (blue colouration, deemed to be non-viable cells). Toxicity was expressed as the percentage of cells that did not exclude the dye. A normal healthy well showed <5% trypan blue positive cells.

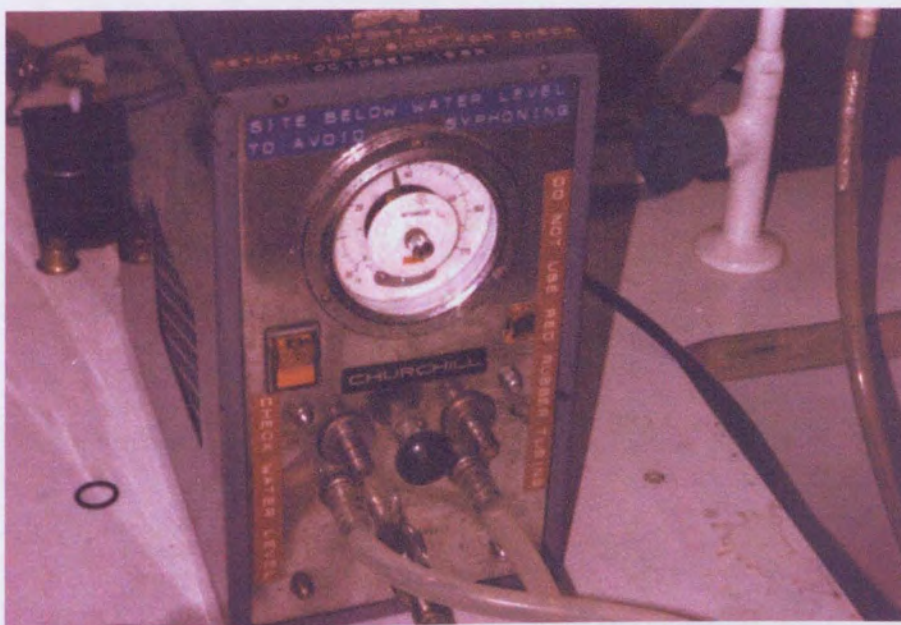
2.2.5.6 - Measurement of oxygen consumption by L6 cells using Clarke O₂ Electrode

L6 cells were grown to confluence in flasks as in 2.2.5.2. The cells were resuspended in 5% FCS DMEM as described in 2.2.5.2 and counted using a haemocytometer. The volume of the suspension was altered by addition of 5% FCS DMEM to give a working suspension of 2×10^5 cells/ml. 3 ml of this suspension was added to the control and test chambers of the Shandon Clarke electrode oxygen monitor (Shandon, YSI, California), the magnetic stirrers were then engaged to aerate the suspension until O₂ saturation in each chamber stopped increasing and the displays were then adjusted to 100%. 10^{-3} M α lipoic acid or vehicle only (control) was added and percentage O₂ saturation noted every 30 sec until the value levelled out. Rate of fall of O₂ tension was expressed as %O₂ decrease per 30 sec.

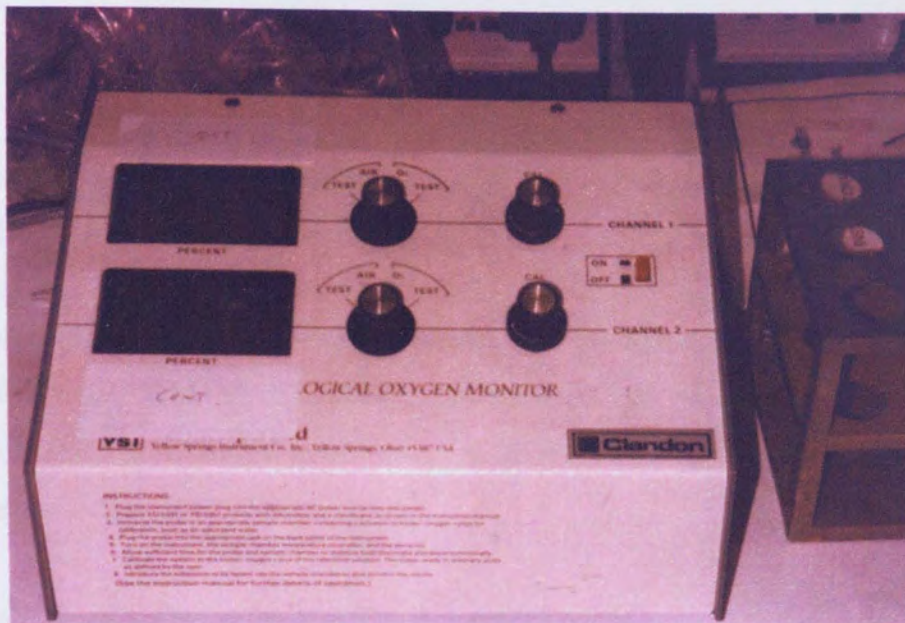
Picture 2.1 - O₂ Electrodes, chambers, 37°C water bath and leads to display for the Shandon oxygen monitor



Picture 2.2 - Churchill thermostatically controlled re-circulation pump



Picture 2.3 - Display unit of Shandon Clarke electrode apparatus



Picture 2.4 - Complete Shandon Clarke electrode apparatus showing chambers



Overleaf: the display is in the foreground (A) with leads off to the chambers (B). In the background is the water-circulating pump (C).

2.3.1-Tumour Necrosis Factor Alpha (TNF α)

2.3.1.1 - Glucose uptake by isolated skeletal muscle, measured by 2-deoxy-D- [3 H]-glucose uptake

Diaphragm and red quadriceps muscles were isolated from freely fed lean mice only (male and female, aged 14 -18 weeks) and incubated as described in section 2.2.1. Insulin was used at a final concentration of 10^{-6} M. TNF α was used at a working concentration of 10^{-11} M.

2.3.1.2 - 2-deoxy-D- [3 H]-glucose uptake by L6 cells

L6 cells from passages 1-20 were differentiated into myotubes and used to measure 3 H 2DG uptake as described in section 2.2.5.4. Insulin was used at 10^{-6} M and TNF α at 10^{-11} M. The incubation period was 2 hours.

2.3.2 -Trace elements

2.3.2.1 *In vivo* study on effects of lithium carbonate on glucose and insulin concentrations in *ob/ob* mice

This study was undertaken in groups of 7-9 *ob/ob* mice (aged 14 -18 weeks). Mice were housed as described earlier (section 2.1.2) in groups of 2-4 per cage. Food and fluid intake was monitored every other day and body weight, plasma glucose and insulin measured weekly throughout the study. Following a one week 'run in' during which no treatment was given, treatment was commenced with the animals being given drinking fluid comprising a solution of lithium in lieu of water (0.5g/l lithium carbonate). Control *ob/ob* mice continued to receive water without added trace element. Blood samples of

50µl were taken for insulin and glucose determination before commencement of treatment and at one and two weeks. Blood samples were taken at about 10am from the tail tip of freely fed mice. Blood was collected on ice into pre-heparinised 250µl eppendorf tubes, centrifuged at 15000g for 10 sec and plasma removed and stored at -20°C for glucose and insulin assay. Insulin was determined using double antibody radioimmunoassay (section 2.5.2) and glucose was measured using the Beckman glucose analyser as described later in section 2.5.1. Weighing the food and fluid containers supplied to each cage and reweighing every other day measured food and fluid intake. The average amount consumed per week was calculated for each cage and divided by the number of mice to give the amount consumed per week per mouse. The spillage of food was minimal and taken as similar for all cages, as shown in earlier studies in this and other laboratories (Flatt and Bailey, 1981).

2.3.2.2 - 2-deoxy-D- [³H]-glucose uptake by L6 cells: effect of trace elements

All trace elements to be investigated were treated identically for the first two experiments. A stock solution of 10⁻¹M was used to create a range of concentrations from 10⁻¹-10⁻⁷M in the experimental medium as in 2.2.5.4. The effect of the trace element studied on glucose uptake in L6 cells was assessed by ³H 2DG uptake after 24 hours (with and without added insulin at 10⁻⁶M). Lithium was in the form of lithium carbonate (Fisons); selenium was sodium selenate (Sigma) and chromium was in the form chromium (III) chloride (Cl₃Cr)(Sigma).

Each agent that showed promise (i.e. evidence of increased glucose uptake) with or without added insulin was then investigated during incubation periods of 2, 4, 8 and 24h in a second series of experiments.

2.3.3 – Isoferulic Acid

This study employed L6 cells as described in section 2.2.5.4. Isoferulic acid (Sigma) was used in these studies at concentrations of 10^{-3} M to 10^{-7} M, with and without insulin at 10^{-6} M. Incubations were undertaken at 37°C for 24, 8 and 4 h.

2.3.4 – Bradykinin

Studies using bradykinin were undertaken with L6 cells following the same procedure as described in section 2.2.5.4. The bradykinin used in these studies was the native murine nanopeptide [Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg] obtained in the lyophilised form from Sigma. The bradykinin was dissolved in PBS at a stock concentration of 10^{-4} M and stored at -20°C in a freezer until use. The bradykinin was thawed naturally and employed at a concentration range of 10^{-6} M - 10^{-9} M, and insulin was used at 10^{-6} M. Glucose uptake was measured by ^3H 2DG uptake over 10 min at 22°C as described in section 2.2.5.4. The experiments were repeated using freshly prepared and once frozen-thawed bradykinin from two different batches due to measurable differences in activity observed after freezing and thawing.

2.3.5 – Zinc α 2 glycoprotein

Zinc α 2 glycoprotein was added to L6 cells as previously described in section 2.2.5.4. The range of concentrations used was 10^{-6} M to 10^{-10} M. Insulin was employed at 10^{-6} M and 10^{-8} M. Professor Michael J Tisdale and Dr Steve Russell from the CRC laboratories at Aston University kindly provided the zinc α 2 glycoprotein. The batch used was originally isolated by preparative HPLC separation of serum proteins from human blood by Miles Laboratories (subsidiary of Bayer Plc) at New Haven CT, USA. The Zn α 2 glycoprotein was chemically characterised as >99% pure by the company and despatched lyophilised at $+1$ to $+4^{\circ}\text{C}$ (on ice). At Aston this material was dissolved in sterile PBS before dilution in the incubation medium.

2.4 - Characterisation of lipolytic activity of adipocytes and adipocyte tissue

Initial feasibility experiments were carried out using normal lean and obese (*ob/ob*) mouse adipose tissue. Subsequently, human subcutaneous and visceral fat samples were obtained from patients undergoing routine surgery for hernia repair (ethical approval obtained). Use of fat pieces from mouse adipose tissue samples was found to be generally less accurate than adipocytes; hence isolated adipocytes were preferred for this study. A detailed characterisation of the mouse and human adipocyte preparations is given in chapter 4. In these studies the main metabolic parameter assessed was lipolysis. This was measured as glycerol release during 2h incubation.

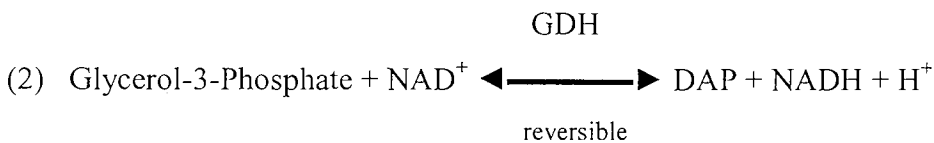
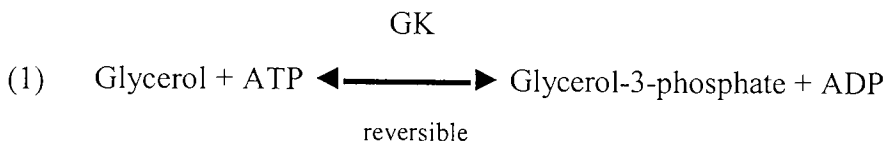
2.4.1-Determination of glycerol release by fat pieces

Epididymal fat pads were excised from freely fed *+/+* or *ob/ob* mice (14-18 weeks) at about 10am after euthanasia by cervical dislocation. Fat pads were placed in 15ml pre-gassed (95% O₂: 5% CO₂) glucose free Krebs buffer supplemented with 1mg/ml BSA at 37°C. The tissue was blotted and cut into smaller pieces weighing about 50mg, and the weight of each piece was recorded. Tissue was rinsed in medium and placed in a plastic experimental vial (capacity 7ml) with 1ml of BSA/Krebs solution at 37°C. 100µl of test agent dissolved in incubation buffer was added (made up at 10x required working concentration). All vials were re-gassed with 95% O₂: 5% CO₂ for 5 seconds, stoppered with the plastic lids supplied, then incubated at 37°C for 2 hours in a shaking water bath at 60 reps/min. Post incubation, tissue was removed from the vials and discarded. A sample of the medium was either stored at -70 °C or assayed immediately for the glycerol concentration.

2.4.2-Assay for glycerol

Glycerol is found in cells in both the free form and as a constituent of more complex lipids. The UV method (Wieland, 1974) is a simple procedure for measuring free glycerol released into the incubation medium. The method removes the necessity for

preliminary purification of the experimental medium and has high specificity. As outlined below, glycerokinase (GK) plus ATP convert glycerol to glycerophosphate. This is then oxidised by NAD-dependent glycerophosphate dehydrogenase (GDH). The formation of NADH by this reaction is measured by change of extinction at 340nm, which is proportional to glycerol concentration in the sample.



Adapted from Wieland, 1974.

200µl of incubation medium taken at the end of the incubation period was transferred into a plastic 1ml cuvette, followed by addition of 830µl of spectrophotometry buffer. (appendix*⁴). The main active components of the spectrophotometry buffer are lactate dehydrogenase and pyruvate kinase. 10µl of glycerokinase was added and the absorbance was read immediately (Abs_{0 min}) at 340nm against distilled H₂O as blank. After 15 minutes the absorbance was read again (Abs_{15 min}). Glycerol release over the 2h incubation period was calculated using the following equation:

$$\text{Abs}_{0 \text{ min}} - \text{Abs}_{15 \text{ min}} \times 1.61 = \text{mM} [\text{glycerol released}] / 2 \text{ hours}$$

The 1.61 in the equation is the NADH extinction coefficient. All values were corrected for tissue weight and expressed as mM glycerol released/2 h/mg tissue.

2.4.3-Preparation of mouse and human adipocytes and measurement of lipolysis

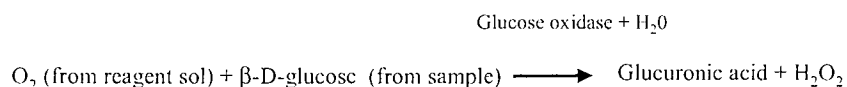
Fat pads were obtained from mice as previously described in section 2.4.1. Human adipose tissue was made available through collaboration with City Hospital, Birmingham. Samples of tissue were transported to the laboratory at Aston in pre-warmed Krebs buffer and isolation of adipocytes commenced as soon as possible after surgical removal of the tissue. Relevant past medical history of the patient was noted, including height, weight, operation carried out and site of fat excision (subcutaneous or visceral) as detailed in chapter 4. Fat tissue (approx 100mg) was placed in vials containing 1ml of collagenase in Krebs buffer (3mg/ml) and minced with a pair of sharp scissors. The mixture was vortexed and gassed (95% O₂: 5% CO₂) then incubated for 1 hour in a shaking water bath (60 reps/min) at 37°C. The adipocyte suspension was removed from the water bath; fresh pre-warmed Krebs (approx 10ml) was added, gently shaken and centrifuged for 1 minute at 1000rpm after which the medium below the floating cell layer was aspirated. This step was repeated 3 times. The surface fat layer was transferred to a beaker containing BSA/Krebs with a magnetic stirrer and 100µl of a representative suspension was removed for cell counting. Careful visual inspection of the preparation was undertaken to confirm apparently healthy intact adipocytes and approximate consistency of size. Extremes of size, such as the very large adipocytes in *ob/ob* mice and very small adipocytes were not captured by this technique as discussed further in chapter 4. The concentration of adipocytes was determined using a Neubauer (Hawksley, England) haemocytometer and then the volume adjusted with BSA/Krebs buffer to give a concentration of 10⁵ adipocytes per ml.

1ml of the adipocyte suspension was transferred to each experimental plastic vial (5ml capacity) and treated as described in the section for fat pieces (2.4.1). After 2 hours the reaction was stopped by adding 0.5ml aspirated medium from below the floating cell layer to 0.5ml perchloric acid. These samples were centrifuged at 1300rpm for 3 minutes. The pellet was discarded and the supernatant assayed immediately for glycerol concentration or stored at -70 °C. To measure the glycerol concentration, 500µl of the supernatant was transferred to a fresh microfuge tube and 100µl of KOH was added. The

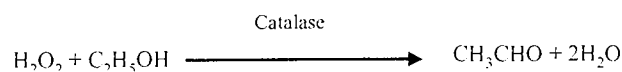
pH was adjusted to 7 with KOH or perchloric acid as necessary after determination with Whatmann narrow range pH paper. Glycerol was then measured as previously described in section 2.4.2.

2.5.1 - Plasma glucose assay

Samples of 5 μ l of plasma were used to measure glucose by an automated glucose oxidase procedure using a Beckman glucose analyser (High Wycombe, Bucks) according to Stevens, 1971. The principle of this method is that the analyser uses oxygen consumption rate to assay for glucose. Rate of oxygen utilisation in the glucose oxidation reaction is directly proportional to the glucose concentration in the test solution. Test sample (5 μ l) is added to the reaction well containing 1ml of glucose oxidase reagent and the following reactions occur:



H₂O₂ undergoes a further reaction with catalase and ethanol which removes the peroxide without yielding oxygen as below:



The disappearance of oxygen is measured by an oxygen electrode in the reaction well and this is calibrated by the machine using standard glucose solutions to give a numerical read-out of the glucose concentration in mmol/l.

2.5.2 - Plasma insulin assay

Plasma insulin radioimmunoassay was conducted by a double antibody procedure validated previously in this laboratory (Bailey and Ahmed-Sorour, 1980) using reagents obtained from Linco (St Louis, M, USA) and ^{125}I -labelled human insulin (Amersham Int, Amersham, Bucks). Radioimmunoassay works on the principle that polypeptide hormones have immunogenic properties. Antibodies are raised in a species by the administration of non-native exogenous insulin, which is sufficiently different from endogenous insulin to elicit an immune response. The assay used is based on competitive binding of a limited number of binding sites of the raised insulin antibody between a variable amount of unlabelled insulin and a constant amount of ^{125}I labelled rat insulin. As fixed amounts of radioactive ligand and antibody are added to the assay, the amount of radioactive ligand bound by the antibody is inversely proportional to the concentration of added non-radioactive ligand. A second antibody bound to the anti-insulin antibody was used to precipitate the insulin bound complex. The radioactivity of the precipitate (antibody-bound insulin) was then measured.

The antibody used was a non-specific anti-insulin antibody raised against human insulin in guinea pig. The antibody showed full cross reactivity to both rat and human insulin. The secondary antibody was raised in sheep against guinea pig gamma-globulin (anti-guinea pig antibody). The assay was carried out as follows: standard concentrations of rat insulin (0.01-2.5ng/tube) were prepared from stock (50ng/ml) rat insulin using a serial dilution method. These were made up in assay buffer consisting of 0.025M phosphate buffer with 0.1%(w/v) sodium azide. Total count (TC) vials were prepared by adding 100 μl of the [^{125}I] human insulin assay tracer diluted in assay buffer. Non-specific binding (NSB) tubes were prepared using 200 μl of assay buffer and zero standard (Bo) tubes with 100 μl of assay buffer. Unknown samples were 10 μl of plasma diluted with assay buffer so as to provide concentrations near to the middle of the standard curve.

Antiserum (100 μl) was added to the all the tubes except TC and NSB, and 100 μl of tracer was added to all tubes. These were then covered, vortexed thoroughly and incubated at

4°C for 4h. After incubation, 250µl of undiluted secondary antibody reagent was added to all the tubes except TC. After being vortexed the tubes were incubated at room temperature for a further 24h. The antibody-bound fraction was separated by centrifugation (2000g for 30 min 4°C) in an MSE refrigerated centrifuge. The supernatant was then discarded and tubes left inverted to drain onto the absorbent paper for 1h. The radioactivity in each tube was determined using a gamma scintillation counter for 1min (LKB Compugamma, Loughborough, Leics). Analysis was carried out in triplicate for the standard curve and duplicate for test samples and results were therefore average counts per minute for each set of three or two tubes.

The best fit standard curve and determination of insulin concentrations was performed automatically by software preloaded into the computer attached to the scintillation counter. The software uses the following three equations to calculate percentage NSB/TC, percentage Bo/TC and percentage bound/Bo for each standard and sample respectively.

$$(1) \quad \%NSB/TC = \frac{NSB \text{ cpm}}{TC \text{ pm}} \times 100$$

$$(2) \quad \% Bo/TC = \frac{(Bo \text{ cpm} - NSB \text{ cpm})}{TC \text{ cpm}} \times 100$$

$$(3) \quad \%B/Bo = \frac{(\text{standard or sample cpm} - NSB \text{ cpm})}{(Bo \text{ cpm} - NSB \text{ cpm})} \times 100$$

The standard curve was produced by plotting percentage B/Bo versus log of the standard rat insulin concentrations. B/Bo was then plotted against ng standard per tube, and ng per sample tube was then read directly from the graph. The assays undertaken in the present study were conducted by Dr CJ Bailey and Dr C Day. The sensitivity of the assay (lowest concentration significantly different from zero) was 0.1 ng/ml, and the intra-assay

coefficient of variation was 3%. All insulin values measured in the present study were measured in a single assay run.

2.6 - Statistical Analyses

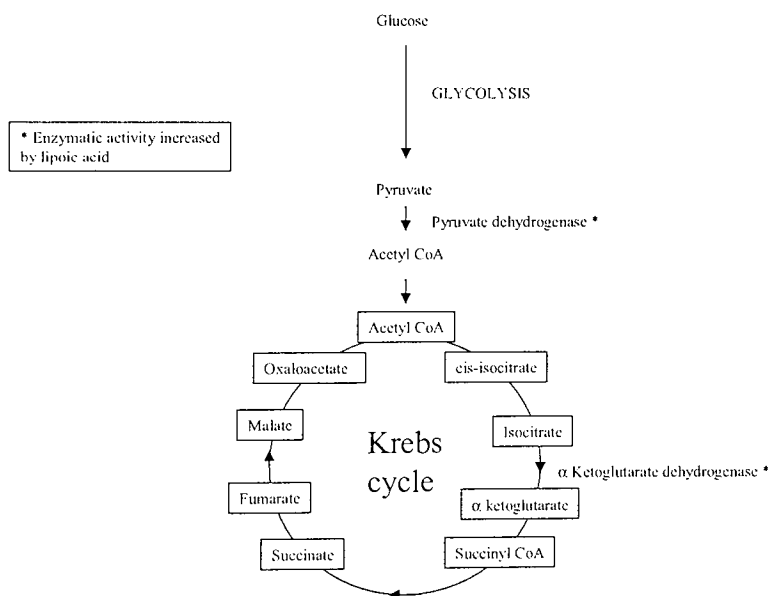
Data are expressed as mean values \pm standard error. Animal experiments were designed using the minimum numbers of animals to provide an amount of tissue estimated to be compatible with discrimination of significance at the 5% level, as suggested by the Scientific Procedures (Animals) legislation text provided by the Home Office. For unpaired analyses repeated measure analysis of variance (ANOVA) was used to compare all test values versus the control value, with a Dunnett's post test if $P < 0.05$. Probability levels of $P < 0.05$ were accepted as significant. For paired analyses a paired Student's t-test was used. All statistical analyses were performed using the Graph pad instat statistical package (Graph pad Software Inc, USA).

Chapter 3 -Lipoic acid

3.1 - Introduction

Lipoic acid or thioctic acid (specifically DL-6-8-thioctic acid) is a natural (dietary) metabolic antioxidant and co-factor in dehydrogenase complexes (Reed, 1962, diagram 3.1).

Diagram 3.1 - Key sites at which lipoic acid serves as an essential co-factor for dehydrogenase complexes



The mechanism through which lipoic acid (as lipoate) forms an integral part of the dehydrogenase reactions is elaborated elsewhere (Stryer, 1988). The putative mechanism through which lipoic acid acts as scavenger of oxygen free radicals is reviewed by Coleman (2001). Lipoic acid is taken into cells and tissues where it is reduced to dihydrolipoate, which it is a more potent antioxidant than lipoic acid itself. This property

has led to the use of lipoic acid in a number of studies involving neurological damage due to oxidative stress. Ziegler & Gries (1997) found that intravenous treatment of diabetic patients with lipoic acid (600mg/day) over three weeks reduced the symptoms of peripheral neuropathy and that oral treatment (800mg/day) over 4 months *may have* improved the cardiac autonomic neuropathy seen in type 2 diabetes. Cameron *et al* (1998) reported the beneficial action of lipoic acid on nerve conduction velocity and blood flow in rodent diabetic models, which is in complete agreement with the findings of several other groups using diabetic rats (Packer & Tritschler 1996; Nagamatsu 1995; Garrett *et al.*, 1997).

The role of lipoic acid as an essential co-factor of dehydrogenase complexes such as α ketoglutarate dehydrogenase and pyruvate dehydrogenase has provided the rationale for a number of experiments on glucose metabolism and transport. As far back as 1970, Haugaard & Haugaard showed that lipoic acid increased the insulin stimulated uptake of glucose in rat diaphragm, by long term *in vitro* incubation measuring glucose disappearance from the incubation medium. This has been borne out by the findings of the two following groups: Jacob *et al* (1996) found that isolated muscle preparations from obese Zucker rats showed increased glycogen synthesis, glucose oxidation and insulin stimulated glucose uptake after a ten-day lipoic acid treatment. Chronic treatment of Streptozotocin (STZ) diabetic rats with lipoic acid reduced blood glucose levels, elevated gastronemius GLUT-4 levels and brought about the normalisation of blood lactate (Khamaisi *et al.*, 1997), all of which were associated with a reversal of the insulin resistance that impairs 2-deoxyglucose uptake in the soleus muscle of these rodents. Lipoic acid is also purported to have an effect on cultured muscle cells, in L6 myocytes it was found to enhance insulin-stimulated glucose uptake due to the stimulation of GLUT 1 and 4 translocation to the plasma membrane from intracellular pools (Klip *et al.*, 1994). In humans, short and long term treatment of type 2 diabetic patients with lipoic acid showed increased insulin-stimulated glucose disposal using the euglycemic hyperinsulinaemic technique (Jacob *et al.*, 1995).

The present study has explored the hypothesis that lipoic acid could have a different potency or effect on glucose uptake and metabolism of different fibre types of muscle with different levels of sensitivity to insulin and different levels of expression of GLUT 1 and 4. The study utilised diaphragm, abdominal and quadriceps muscles from lean +/+ and obese *ob/ob* mice. Whole muscle preparations of the above were employed as outlined in chapter 2 to assess glucose uptake and utilisation. L6 cells were also utilised, with glucose uptake being measured by ^3H 2DG uptake as in chapter 2.

3.1.1 - Whole muscle preparations

Fibre type composition of skeletal muscles varies according to the muscle's role within the organism e.g. postural or locomotor. There are generally considered to be three main types of muscle fibre: type 1 (slow twitch oxidative), type 2a (fast twitch oxidative) and type 2b (fast twitch glycolytic). 'Red' or oxidative muscle has large numbers of GLUT 1 and GLUT 4 glucose transporters (Marette *et al.*, 1992) and hence will have the greatest response to insulin. In muscle, insulin acts to increase uptake and oxidative phosphorylation of glucose, or storage in the form of glycogen. Each muscle group has a unique distribution of fibres and three representative muscles were used in the present study as this allows for the detection of possible differences due to fibre composition. Red quadriceps contains predominantly type 1 fibres, diaphragm has similar proportions of type 1 and type 2 fibres and abdominal muscle is predominantly type 2 fibres (Sherwood 1993; Henricksen 1997; Megeney 1993).

Table 3.1 - Characteristics of the three skeletal muscle fibre types (adapted from Sherwood, 1993)

	Type 1	Type 2a	Type 2b
	Slow twitch Fatigue resistant Oxidative Aerobic	Fast twitch Fatigue resistant Oxidative Aerobic	Fast twitch Fatigable Glycolytic Anaerobic
Characteristics			
Mitochondria	Many	Many	Few
Capillaries	Many	Many	Few
Myoglobin	High content	High content	Low content
Glycogen	Usually low content	Usually Intermediate	Usually high content

3.2 - Results

The results of the experiments are illustrated herein as histograms or numerical tabulations. A description of the data, identifying the key findings of each experiment, is given as an extended legend to each figure or table.

3.2.1 – Effect of α -Lipoic acid on glucose uptake in various lean and obese muscles

Data reported in this section concern the effect of lipoic acid on glucose uptake by muscles of lean mice, measured by ^3H 2DG uptake over 20 minutes after 2 hours incubation at 37°C.

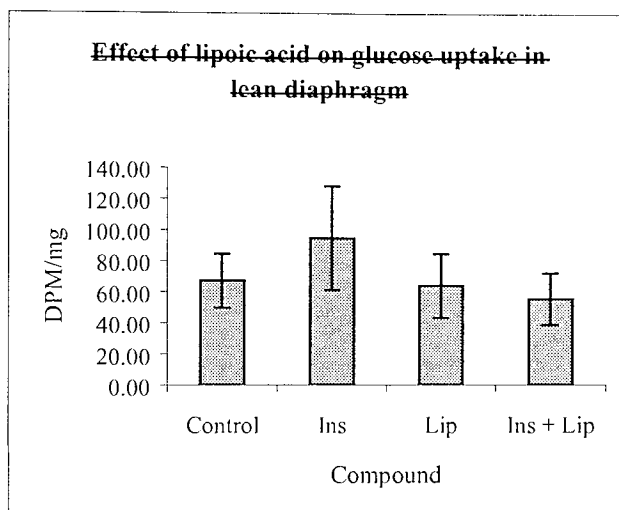


Fig 3.1 - Glucose uptake measured by ^3H 2DG uptake in murine diaphragm muscle was not affected by the addition of 10^{-3}M lipoic acid. Insulin (10^{-6}M) had no effect on glucose uptake. Results are means \pm SE, $n=6$.

Control diaphragm muscles consumed about 70 DPM/mg tissue of ^3H 2DG over the 20-minute period. Insulin (10^{-6}M) caused a 32% increase in ^3H 2DG uptake (NS). The presence of 10^{-3}M lipoic acid had no effect on ^3H 2DG uptake. The combination of lipoic acid and insulin resulted in a 25% reduction in mean ^3H 2DG uptake (NS).

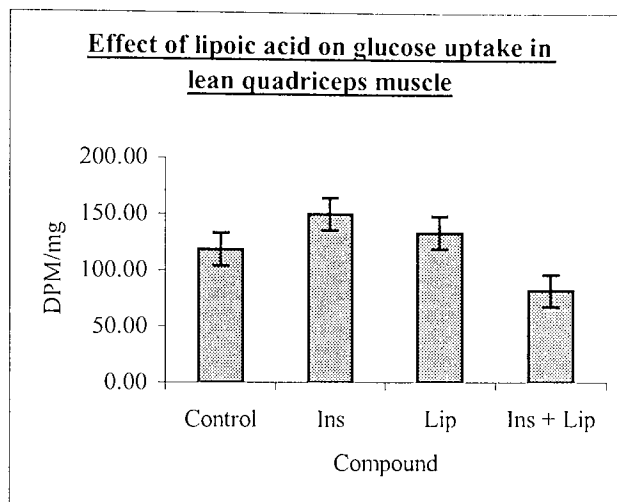


Fig 3.2 - Glucose uptake measured by ^3H 2DG uptake in murine quadriceps muscle was not affected by the addition of 10^{-3} M lipoic acid. Insulin (10^{-6} M) had no effect on glucose uptake. Results are means \pm SE, $n=6$.

Control quadriceps muscles consumed about 120 DPM/mg tissue of ^3H 2DG over the 20-minute period. Insulin (10^{-6} M) caused a 20% increase in ^3H 2DG uptake ($P>0.05$). The presence of 10^{-3} M lipoic acid raised ^3H 2DG uptake by 18%, which was not significant. The combination of lipoic acid and insulin resulted in a 37% reduction in mean ^3H 2DG uptake ($P>0.05$).

3.2.2.1 - The effect of lipoic acid on glucose uptake in lean and obese muscles

Studies described in this section concern the effect of lipoic acid on glucose uptake by muscles of lean and obese mice, measured by the disappearance of glucose from the incubation medium over a period of 2 hours.

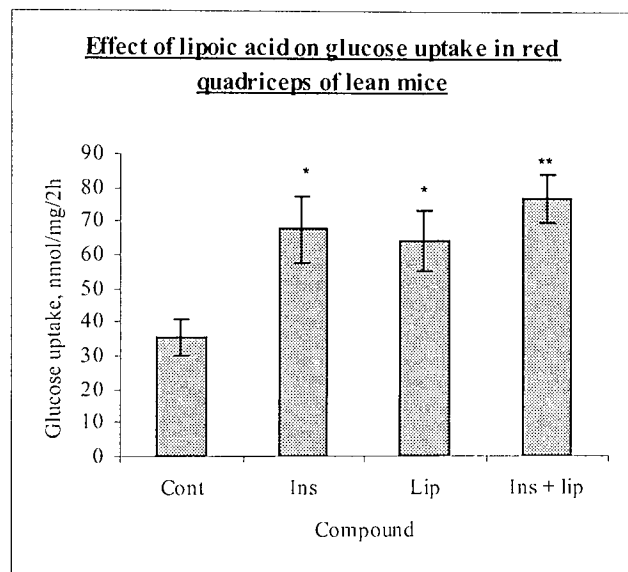


Fig 3.3 - Glucose uptake was enhanced in lean red quadriceps (*vastus intermedius*) muscle upon the addition of $10^{-3}M$ lipoic acid and $10^{-6}M$ insulin. Data expressed as means \pm SE, $n=16$.

Control quadriceps muscles consumed about 35 nmol of glucose per mg tissue over the 2-hour period. Insulin ($10^{-6}M$) increased mean glucose uptake by approximately 100% (* $P<0.05$). In the presence of $10^{-3}M$ lipoic acid glucose uptake was raised by approximately 98% (* $P<0.05$, significant). The mean value for glucose uptake was raised by nearly 110% by lipoic acid and insulin in combination compared to the control, an effect that was also significant (** $P<0.01$).

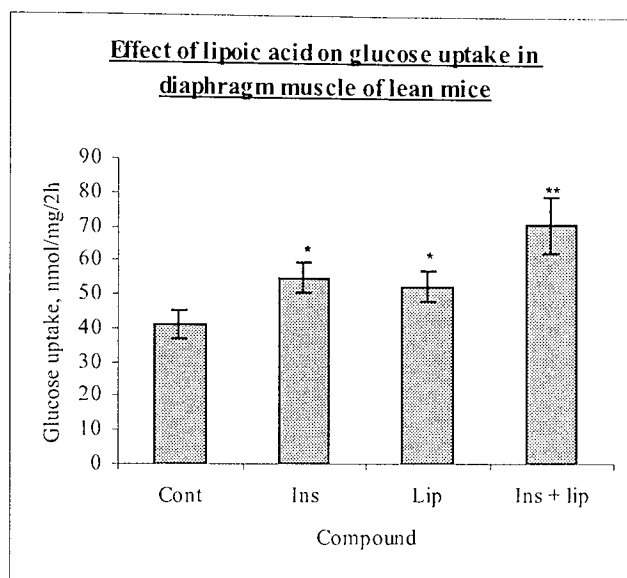


Fig 3.4 - Glucose uptake was enhanced in lean diaphragm muscle upon the addition of 10^{-3} M lipoic acid and 10^{-6} M insulin. Data expressed as means \pm SE, n=16.

Control diaphragm muscles consumed about 40 nmol of glucose per mg tissue over the 2 hour period. Insulin (10^{-6} M) increased mean glucose uptake by approximately 40% (*P<0.05). In the presence of 10^{-3} M lipoic acid glucose uptake was increased by the same amount (approx. 40-45%)(*P<0.05). The mean value for glucose uptake was raised by nearly 90% by lipoic acid and insulin in combination compared to the control, an effect that was also significant (**P<0.01).

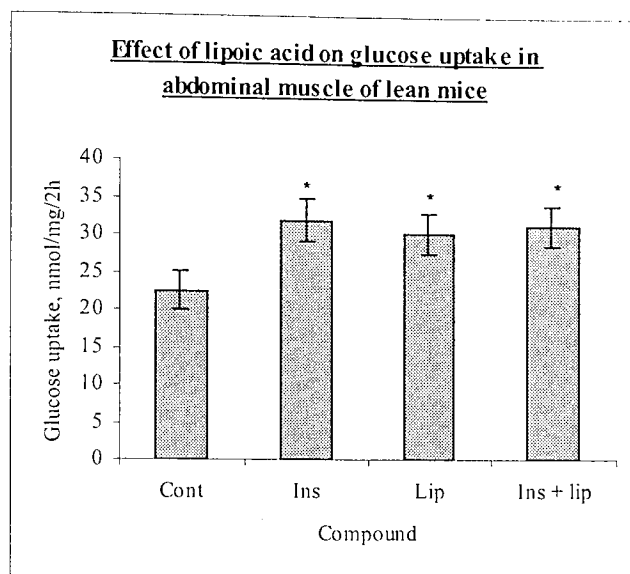


Fig 3.5 - Glucose uptake was enhanced in lean abdominal muscle upon the addition of 10^{-3} M lipoic acid and 10^{-6} M insulin. Results are means \pm SE, $n=16$.

Control abdominal muscles consumed about 22 nmol of glucose per mg tissue over the 2 hour period. Insulin (10^{-6} M) increased mean glucose uptake by 50%, which was statistically significant (* $P<0.05$). In the presence of 10^{-3} M lipoic acid glucose uptake was raised by approximately 48% (* $P<0.05$). The mean value for glucose uptake was also raised to 50% by lipoic acid and insulin in combination compared to the control, an effect that was significant (* $P<0.05$).

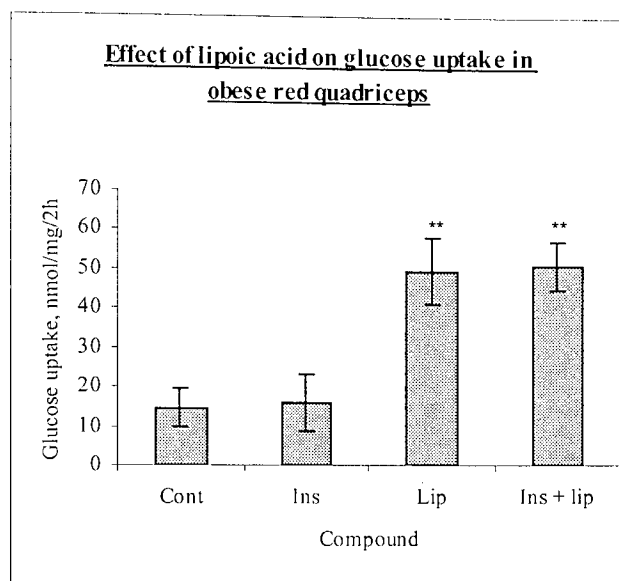


Fig 3.6 - Glucose uptake in obese quadriceps muscle was increased by the addition of 10^{-3} M lipoic acid. Results are means \pm SE, $n=6$.

Control quadriceps muscles consumed about 15 nmol of glucose per mg tissue over the 2 hour period. Insulin (10^{-6} M) had no effect on mean glucose uptake as expected for a tissue that is known to be highly insulin resistant. In the presence of 10^{-3} M lipoic acid glucose uptake was raised by approximately 200% (** $P<0.01$). The mean value for glucose uptake was raised by nearly 210% by lipoic acid and insulin in combination compared to the control (** $P<0.01$).

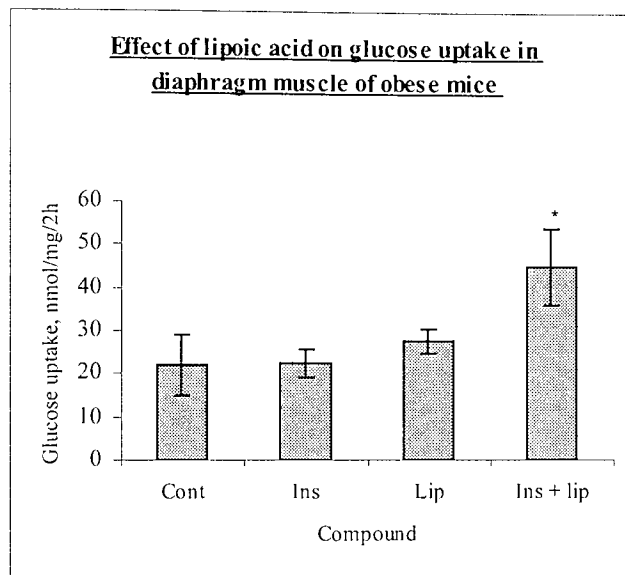


Fig 3.7 - Glucose uptake in obese diaphragm muscle was not significantly affected by the addition of 10^{-3} M lipoic acid alone. Results are means \pm SE, $n=6$.

Control muscles consumed about 20 nmol of glucose per mg tissue over the 2 hour period. Insulin (10^{-6} M) had no effect on mean glucose uptake as expected for a tissue that is known to be highly insulin resistant. In the presence of 10^{-3} M lipoic acid glucose uptake was raised by approximately 15%, which was not statistically significant. The mean value for glucose uptake was raised by nearly 100% by lipoic acid and insulin in combination compared to the control, an effect that was significant (* $P<0.05$).

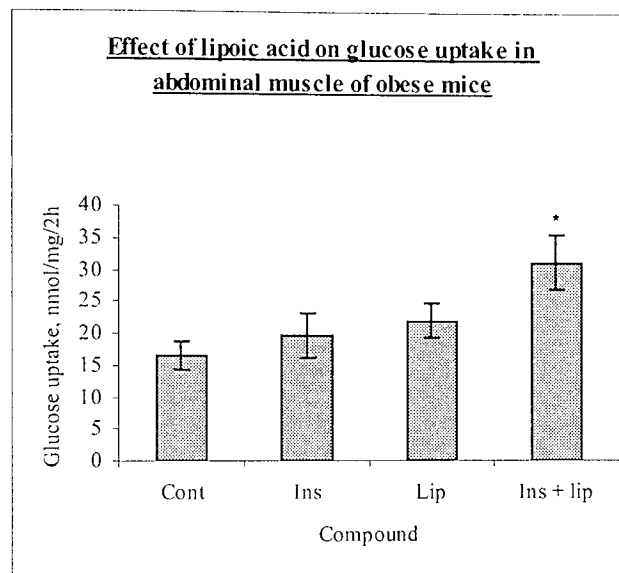


Fig 3.8 - Glucose uptake was enhanced in obese abdominal muscle upon the addition of 10^{-3} M lipoic acid and 10^{-6} M insulin. Results are \pm SE, n=6.

Control abdominal muscles consumed about 16 nmol of glucose per mg tissue over the 2 hour period. Insulin (10^{-6} M) had no effect on mean glucose uptake. In the presence of 10^{-3} M lipoic acid glucose uptake was raised slightly but was not significant. The mean value for glucose uptake was raised by nearly 100% by lipoic acid and insulin in combination compared to the control, an effect that was significant (*P<0.05).

3.2.2.2 - The effect of lipoic acid on lactate production by various muscles *in vitro*

The studies reported in this section show the production of lactate during 2 hour incubations of diaphragm, quadriceps and abdominal muscles of lean and obese (*ob/ob*) mice.

n	<u>Diaphragm</u>				<u>Red Quadriceps</u>				<u>Abdominal</u>			
	C	L	I	L+I	C	L	I	L+I	C	L	I	L+I
1	0.43	-	-	0.58	2.19	-	1.73	1.46	0.20	0.21	0.19	0.21
2	0.72	0.67	0.60	0.60	1.50	0.93	1.51	1.11	0.30	0.24	0.23	0.15
3	0.41	0.41	0.41	0.47	-	0.73	2.35	2.54	0.10	0.18	0.20	0.23
4	0.79	0.65	0.32	0.82	4.90	1.25	1.54	1.39	0.19	0.27	0.21	0.35
5	0.65	0.60	0.69	-	2.45	1.02	1.50	-	0.20	0.27	0.28	0.37
6	0.53	0.37	0.70	-	-	1.18	0.87	1.05	0.25	0.27	0.22	0.36
Mean	0.59	0.54	0.54	0.62	2.74	1.02	1.58	1.51	0.21	0.24	0.22	0.28
P		0.30	0.33	0.37		0.05	0.11	0.09		0.16	0.31	0.08

Table 3.2 - $10^{-3}M$ lipoic acid had no significant effect on lactate production in the various lean muscle tissues tested *in vitro*. C = control, L = lipoic acid and I = insulin. - = failure to isolate tissue. Lactate mg/dl per mg tissue.

Lactate production was within limits at the 5% tolerance level, all P values being non-significant. It could be interesting to note that lipoic acid alone in red quadriceps came very close to significance, as was the case with lipoic acid and insulin in abdominal muscle.

n	Diaphragm				Red Quadriceps				Abdominal			
	C	L	I	L+I	C	L	I	L+I	C	L	I	L+I
1	0.71	0.27	0.08	0.41	1.10	0.79	1.73	0.73	0.19	0.21	0.17	0.17
2	0.41	0.36	0.32	0.54	1.14	0.93	1.51	1.24	0.29	0.42	0.35	0.34
3	0.48	0.68	0.66	0.42	0.59	1.12	2.35	0.79	0.21	0.17	0.14	0.24
4	0.28	0.33	0.26	0.28	1.50	0.54	1.54	0.89	0.14	0.15	0.14	0.17
5	0.55	0.66	0.46	0.57	1.02	1.10	1.50	1.36	0.31	0.34	0.31	0.32
6	0.32	0.30	0.13	0.33	0.68	0.98	0.87	1.67	0.12	0.20	0.12	0.26
Mean	0.46	0.43	0.37	0.43	1.01	0.91	0.79	1.11	0.21	0.25	0.21	0.25
P		0.40	0.21	0.34		0.28	0.16	0.30		0.24	0.46	0.18

Table 3.3 - $10^{-3}M$ lipoic acid had no significant effect on lactate production in various obese muscle tissues in vitro. C = control, L = lipoic acid and I = insulin. Lactate mg/dl per mg tissue.

Lactate production was within limits at the 5% tolerance level, all P values being insignificant. In contrast with the lean mice, lipoic acid in red quadriceps and lipoic acid plus insulin in abdominal muscle was not close to significance. Note the consistently higher lactate production by the control red quadriceps muscles compared with both diaphragm and abdominal control muscles. This is consistent with the rates of glucose uptake seen with these muscles earlier in this chapter.

3.3.1 - Glucose oxidation - $^{14}\text{CO}_2$ analysis (2 hours incubation)

Data reported in this section concern the effect of lipoic acid on glucose oxidation by muscles of obese mice, measured by $^{14}\text{CO}_2$ production over 2 hours incubation at 37°C.

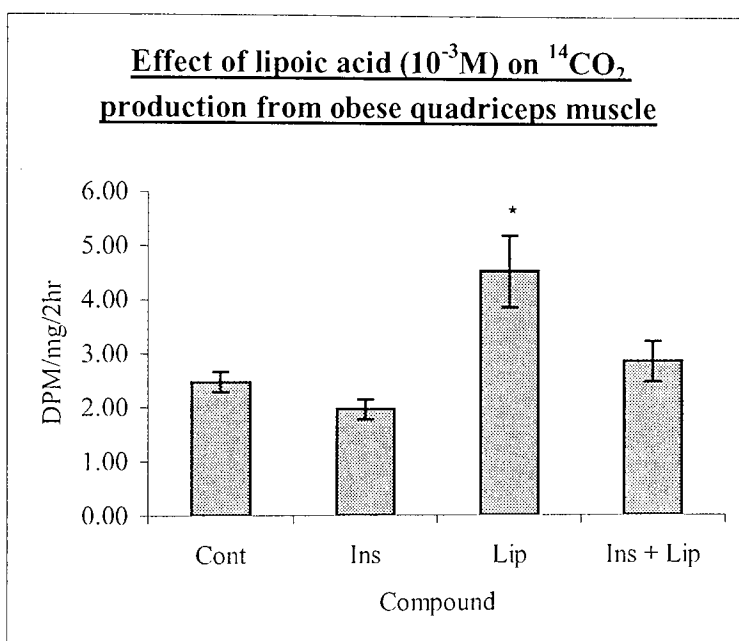


Fig 3.9 – CO_2 production measured by $^{14}\text{CO}_2$ in murine quadriceps muscle was increased by the addition of 10^{-3}M lipoic acid. Results are means \pm SE, $n=6$.

Control quadriceps muscles produced 2.5 DPM/mg tissue of $^{14}\text{CO}_2$ over the 2-hour period. Insulin (10^{-6}M) caused a slight decrease in mean $^{14}\text{CO}_2$ production ($P>0.05$). The presence of 10^{-3}M lipoic acid raised $^{14}\text{CO}_2$ production by 98% ($*P<0.05$). The combination of lipoic acid and insulin resulted in a 20% increase in mean $^{14}\text{CO}_2$ production over the control value ($P<0.05$).

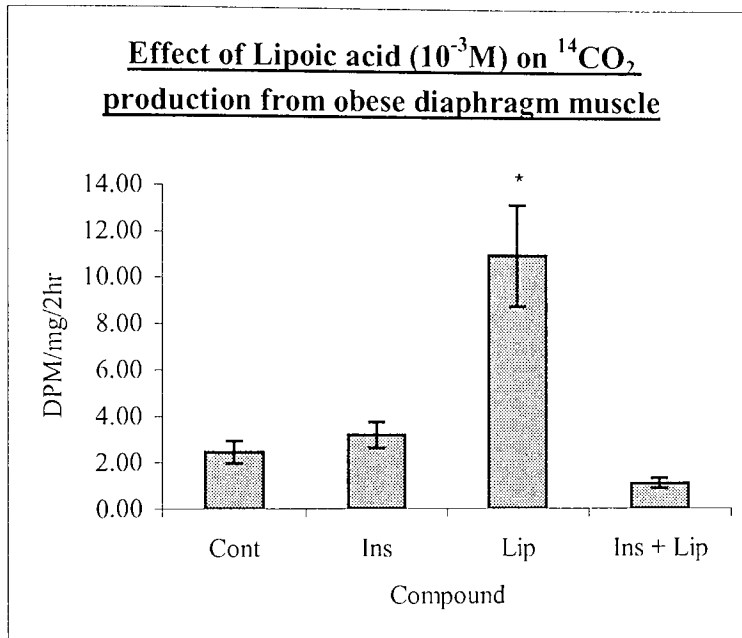


Fig 3.10 – CO_2 production measured by $^{14}\text{CO}_2$ in murine diaphragm muscle was increased by the addition of 10^{-3} M lipoic acid. Results are means \pm SE, $n=6$.

Control diaphragm muscles produced 2.5 DPM/mg tissue of $^{14}\text{CO}_2$ over the 2hr period. Insulin (10^{-6} M) caused a 10% increase in mean $^{14}\text{CO}_2$ production ($P>0.05$). The presence of 10^{-3} M lipoic acid raised $^{14}\text{CO}_2$ production by nearly 300% ($*P<0.05$). The combination of lipoic acid and insulin resulted in a 50% reduction in mean $^{14}\text{CO}_2$ production over the control value ($P>0.05$).

3.3.2 - Glycogen deposition in obese muscles in response to α -lipoic acid analysis with ^{14}C glycogen

n	Soleus				Red Quadriceps				Diaphragm			
	C	I	L	L+I	C	I	L	L+I	C	I	L	L+I
1	0.04	0.50	0.19	0.19	0.50	0.16	0.10	0.19	0.30	0.95	0.17	0.41
2	0.15	0.11	0.37	0.37	0.11	0.03	0.00	0.00	0.31	0.0	0.53	0.21
3	0.54	0.23	0.35	0.35	0.23	0.00	0.00	0.00	0.00	0.13	0.0	0.00
4	0.48	0.35	0.12	0.12	0.35	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.11	0.17	0.26	0.26	0.17	0.06	0.00	0.11	0.00	0.00	0.00	0.00
6	0.10	0.92	0.36	0.36	0.92	0.00	0.00	0.00	0.00	0.45	0.00	0.00
Mean	0.24	0.38	0.27	0.13	0.01	0.04	0.02	0.05	0.10	0.25	0.12	0.10

Table 3.4 - 10^{-3}M Lipoic acid had no significant effect on glycogen production (DPM/g tissue) in lean muscle tissues *in vitro* ($P > 0.10$). C = control, L = lipoic acid and I = insulin. The large number of zero values indicates a void result due to an error in the extraction procedure. See next section on enzymatic measurement of glycogen deposition.

3.3.3 – Enzymatic measurement of glycogen deposition in murine muscle

Lean mice

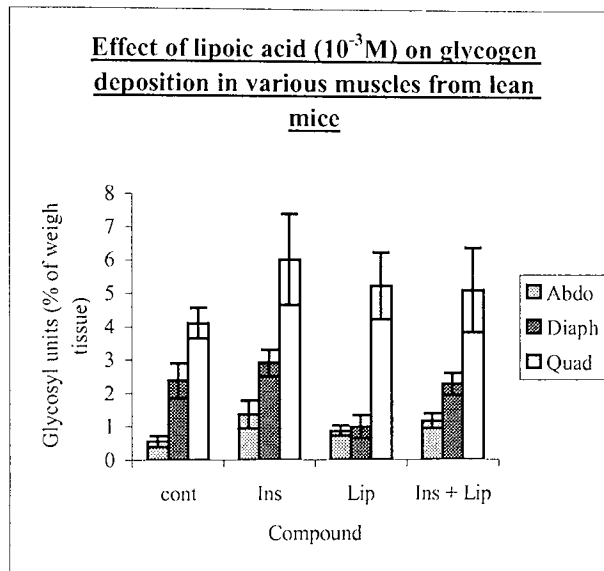


Fig 3.11 – Glycogen deposition measured by enzymatic assay in lean muscles was not affected by the addition of $10^{-3}M$ lipoic acid. Results are means \pm SE, $n=6$.

Control abdominal, diaphragm and quadriceps muscles contained 0.5, 2.5 and 4% glycosyl units per wet weight tissue after the two-hour incubation period. Insulin ($10^{-6}M$) increased glycogen deposition in all muscles studied, though this was not significant. The presence of $10^{-3}M$ lipoic acid reduced glycogen deposition in diaphragm and quadriceps, but caused an apparent slight increase in abdominal muscle (NS). The combination of lipoic acid and insulin caused glycogen deposition similar to that of lipoic acid alone, apparently increased in abdominal muscle but decreased in diaphragm and quadriceps (NS).

Obese mice

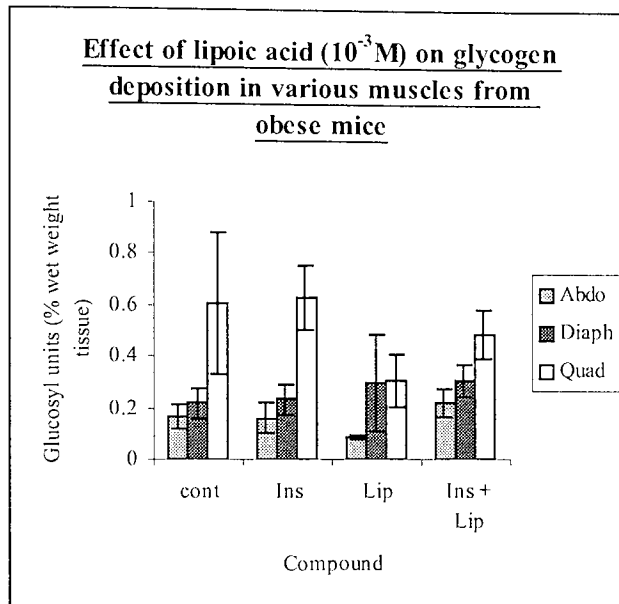


Fig 3.12 – Glycogen deposition measured by enzymatic assay in obese muscles was not affected by the addition of 10^{-3} M lipoic acid. Results are means \pm SE, $n=6$.

Control abdominal, diaphragm and quadriceps muscles contained 0.17, 2 and 0.6 % glucosyl units per wet weight tissue after the two-hour incubation period. Insulin (10^{-6} M) had no effect in all muscles studied ($P>0.05$). The presence of 10^{-3} M lipoic acid reduced glycogen deposition in abdominal and quadriceps, but caused a slight increase in diaphragm muscle ($P>0.05$). The combination of lipoic acid and insulin caused glycogen deposition to increase slightly in abdominal and diaphragm muscle but decrease in quadriceps ($P>0.05$).

3.3.4 – The effect of lipoic acid on glucose uptake in L6 cells

Data reported in this section concern the effect of lipoic acid on glucose uptake by L6 cells, measured by 2-DG uptake over 10 minutes after incubation at 2, 12, 24 and 48 hours incubation at 37°C.

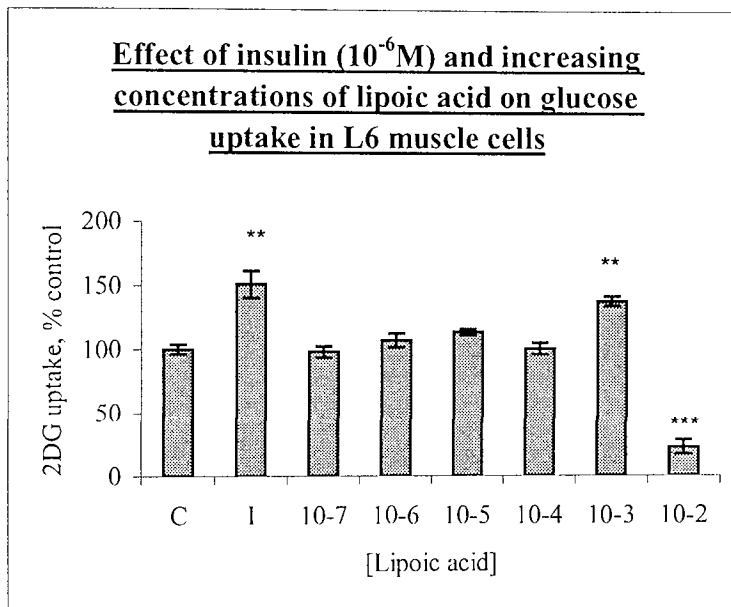


Fig 3.13 – Effect of addition of lipoic acid ($10^{-2} - 10^{-7}$ M) for 2hrs on glucose uptake by L6 cells (** $P < 0.01$). Results are means \pm SE, $n=6$.

The effect of lipoic acid ($10^{-2} - 10^{-7}$ M) on glucose uptake by L6 cells was determined during 2hrs incubation at 37°C (95% CO₂: 5% O₂). Insulin alone (10^{-6} M) increased glucose uptake by 50% (** $P < 0.01$). The presence of increasing concentrations of lipoic acid had no significant effect on 2-DG uptake until 10^{-3} M, which increased uptake by 40% (** $P < 0.01$) and 10^{-2} M that caused a decrease of 80% (** $P < 0.01$).

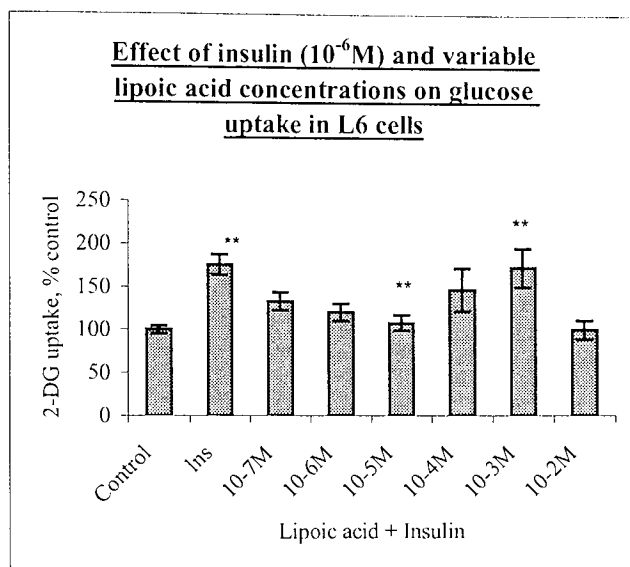


Figure 3.14 – Effect on glucose uptake by L6 cells after 2 hours incubation with 10^{-2} – 10^{-7} M lipoic acid and insulin (10^{-6} M, ** $P < 0.01$). Results are means \pm SE, $n = 6$.

Insulin alone increased 2-DG uptake by 75% (** $P < 0.01$). The presence of increasing concentrations of lipoic acid plus insulin had no significant effect on 2-DG uptake apart from 10^{-3} M, which caused an increase of 75% compared to control (** $P < 0.01$) and 10^{-5} M lipoic acid, which lowered 2 DG uptake compared to insulin alone (** $P < 0.01$).

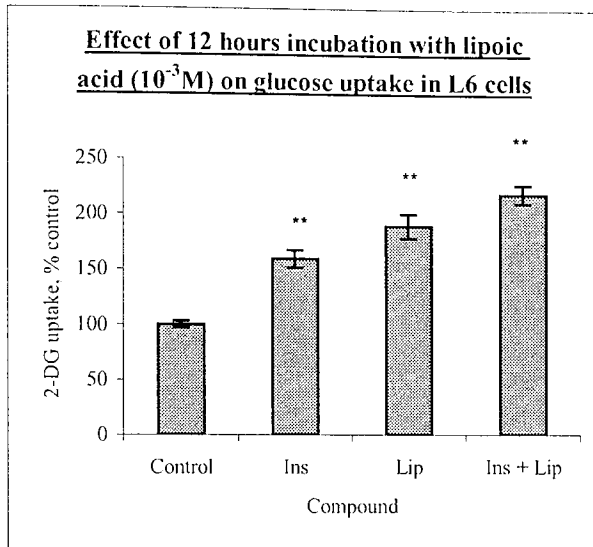


Figure 3.15 - Glucose uptake by L6 cells was increased upon addition of $10^{-3}M$ lipoic acid for 12 hours (** $P < 0.01$). Results are \pm means SE, $n=6$.

Insulin ($10^{-6}M$) increased 2-DG uptake by over 50% versus control (** $P < 0.01$). The presence of $10^{-3}M$ lipoic acid caused an increase of 75% compared to the control (** $P < 0.01$). The combination of lipoic acid and insulin resulted in a 125% increase in 2-DG uptake versus the control (** $P < 0.01$), and a 75% increase compared to insulin alone (** $P < 0.01$).

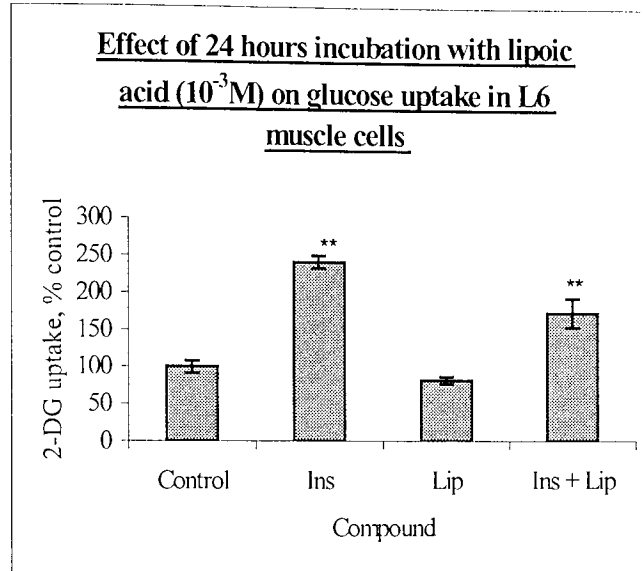


Figure 3.16 - Glucose uptake by L6 cells was increased upon addition of 10^{-3} M lipoic acid for 24 hours compared to control, but decreased compared to insulin alone (** $P < 0.01$, very significant). Results are means \pm SE, $n = 6$.

Insulin (10^{-6} M) increased 2-DG uptake by almost 150% versus control (** $P < 0.01$). The presence of 10^{-3} M lipoic acid caused a slight decrease in 2-DG uptake ($P > 0.05$, not significant). The combination of lipoic acid and insulin resulted in a 80% increase in 2-DG uptake versus the control (** $P < 0.01$), and a 60% decrease compared to insulin alone (** $P < 0.01$).

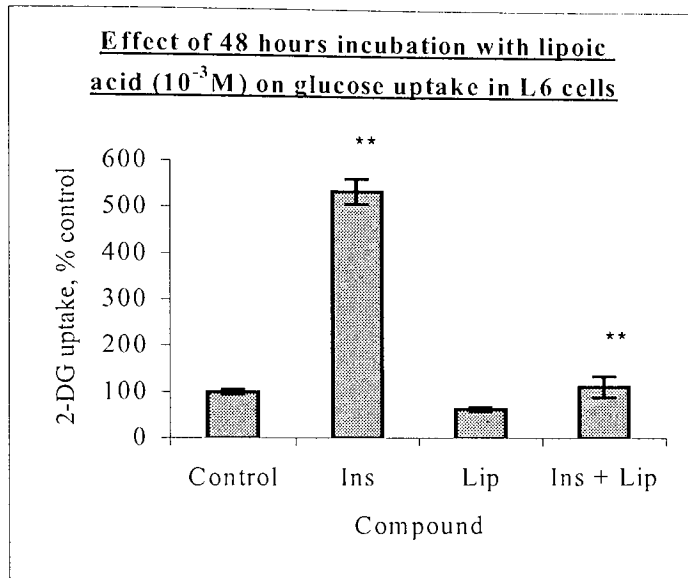


Figure 3.17 - Glucose uptake by L6 cells was decreased upon addition of $10^{-3}M$ lipoic acid and insulin ($10^{-6}M$) for 48 hours (versus insulin alone, $**P < 0.01$) Results are \pm means SE, $n=6$.

Insulin ($10^{-6}M$) increased 2-DG uptake by almost 400% versus control ($**P < 0.01$). The presence of $10^{-3}M$ lipoic acid caused a slight decrease in 2-DG uptake ($P > 0.05$, not significant). The combination of lipoic acid and insulin resulted in a 10% increase in 2-DG uptake versus the control ($P > 0.05$), and a 400% decrease compared to insulin alone ($**P < 0.01$).

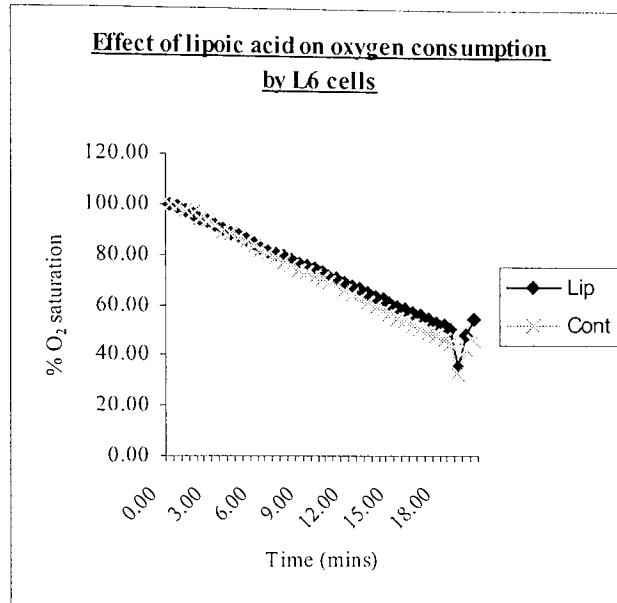


Fig 3.18 - Oxygen consumption by L6 cells (2×10^5 cells/ml) measured by a Clarke-type electrode apparatus was not affected by the addition of 10^{-3} M lipoic acid for 20 minutes ($P > 0.05$, not significant). Results are means \pm SE, $n=6$.

The effect of lipoic acid (10^{-3} M) on oxygen consumption by L6 cells (2×10^5 cells/ml) was determined during 20 minutes incubation at 37°C . Oxygen saturation of the incubation medium fell in a stepwise manner proportional to time and was starting to level out at 40% saturation level. There was no significant difference between the control and lipoic acid values at each time point ($P > 0.05$).

3.4.1 - Effect of α lipoic acid on glucose uptake in various muscles from lean mice (measured by ^3H 2DG uptake)

Variation was seen in basal glucose uptake (control) values when comparing diaphragm (65 DPM/mg/20min) and quadriceps muscles, which showed a glucose uptake value of 175 DPM/mg/20min respectively (figs 3.1 and 3.2). This profile is entirely consistent with the known structure of quadriceps muscles, which are composed of predominantly type 1 fibres compared to diaphragm, which is a mixture of both type 1 and 2 fibres. However, the insulin response was variable within each set of replicates. This could not be attributed to a defect in the batch of insulin used, since the same batch was effective in other studies described in this chapter where it produced the expected effect. It is possible that older mice held under sedentary conditions may develop reduced sensitivity to insulin, which could contribute to the non-significant response of the quadriceps muscles in the present preparation. Other possible factors are: that glucose in the medium saturated glucose transporters within the muscle cells over the two hours incubation time or perhaps most likely, that the insulin had quickly but transiently taken effect and the effect had diminished before the administration of ^3H 2DG (insulin has a relatively short half life in these type of *in vitro* preparations, *c.* 8-10 mins; personal communication, Dr CJ Bailey). Other variables might include stress levels and concomitant release of catecholamines, which could increase basal uptake (Sherwood, 1993). This is unlikely as obviously stressed or morbid animals were excluded from this experiment. The lack of a significant response to insulin seen in this study with lean mice, particularly with quadriceps muscles, negates the validity of the lipoic acid observations seen with this method. Indeed other groups have previously reported an improvement in glucose uptake during incubation of muscle with lipoic acid (Haugaard and Haugaard, 1970; Jacob *et al.*, 1986). Thus another method of assessing glucose uptake was employed, being the assay of glucose disappearance from the medium, to which we will now turn. In addition, abdominal muscle was employed as this provided a muscle with a high type 2 fibre content. This supplied a spectrum of relative fibre composition in the three muscles employed, being quadriceps, diaphragm and abdominal muscles.

3.4.2 - Effect of α lipoic acid on glucose uptake in various muscles from lean mice (measured by glucose assay)

Basal glucose uptake in the quadriceps and diaphragm muscles was very similar, being 35 nmol/mg/2h and 40 nmol/mg/2h respectively (figs 3.3 and 3.4). Basal glucose uptake in abdominal muscle was much lower at 22 nmol/mg/2h (fig 3.5). Basal uptake in muscles with mixed fibre content such as diaphragm was highest of the three muscles tested in this study. Uptake may be more variable in muscles that are composed of an approximately equal mixture of type 1 or type 2 fibres, plus the fact that the diaphragm preparation of the mouse is particularly thin which favours good diffusion. Basal uptake was also higher in quadriceps compared to abdominal muscle as expected. It is when insulin-stimulated glucose uptake is assessed that we see a different pattern emerging: quadriceps glucose uptake was increased by approximately 100% upon insulin administration whereas diaphragm and abdominal glucose uptake increased by 40-50%. An interpretation of this observation is that in this case the type 1 fibres basal uptake in the diaphragm was close to maximal, explaining its relatively poor response to insulin (similar to abdominal) and similarity to that of the quadriceps basal uptake. Lipoic acid produced similar increments in glucose uptake to insulin in all muscles studied (figs 3.3, 3.4 and 3.5). Although the combination of insulin and lipoic acid in diaphragm and quadriceps increased the mean value for glucose uptake to a greater extent than either alone, these were not statistically significant effects (figs 3.3 and 3.4). There was no evidence of an additive effect of the combination of insulin and lipoic acid in quadriceps or abdominal muscle (figs 3.3 and 3.5). These results indicate two possibilities, that lipoic acid is acting via the insulin-signalling pathway in these tissues as this agent effectively mimics the action of insulin, or that insulin has stimulated maximal glucose uptake and that there is no redundancy in GLUT4 numbers to permit lipoic acid stimulated recruitment.

3.4.3 - Effect of α lipoic acid on glucose uptake in various muscles from obese mice (measured by glucose assay)

Basal glucose uptake values were similar in all of the muscles studied, being about 15 nmol/mg/2h in quadriceps, 21 nmol/mg/2h in diaphragm and 16 nmol/mg/2h in abdominal (figs 3.6, 3.7 and 3.8). It is perhaps not surprising that basal glucose uptake values are the same in all three muscles given that the *ob/ob* mouse is known to possess inherent metabolic disturbances which reduce the differences in metabolic activity of these muscle types (Bailey and Flatt, 1997). This is borne out by the fact that all the muscles were typically insulin resistant with insulin causing little variation from basal glucose uptake, being 17 nmol/mg/2h for quadriceps, 22 nmol/mg/2h for diaphragm and 18 nmol/mg/2h for abdominal muscle (c.f. 15, 21 and 16 nmol/mg/2h). Lipoic acid alone increased glucose uptake by approximately 200% in the quadriceps but had no effect in diaphragm or abdominal muscles. However, the combination of lipoic acid and insulin raised glucose uptake above basal by 210% in quadriceps and 100% in both diaphragm and abdominal muscle. These are extremely interesting observations because they suggest that lipoic acid is returning a normal level of glucose metabolism to the type 1 rich quadriceps, re-instating the types of responses one might expect with insulin in normal muscles. An interpretation of these observations (discussed later and supporting the evidence from lean muscles) is that lipoic acid is able to engage the post-receptor insulin-signalling pathway at a site or sites distal to the main blockages responsible for insulin resistance in the *ob/ob* mouse.

3.4.4 - The effect of lipoic acid on lactate production by various muscles *in vitro*

Lipoic acid clearly does not increase lactate production from quadriceps, diaphragm and abdominal muscles of both lean and obese mice (tables 3.2 and 3.3). In fact, in quadriceps muscle from lean mice lipoic acid alone came extremely close to significantly *decreasing* lactate production ($P=0.05$). This would be in agreement with the finding that lipoic acid normalises blood lactate levels in Streptozotocin (STZ) rats (Khamaisi *et al.*, 1997).

Interim comments

This ability of lipoic acid to increase glucose uptake in a manner proportional to insulin in both lean (insulin responsive) and obese (insulin resistant) muscles indicates that this agent works in part via components of the insulin signalling pathway (Yaworsky *et al.*, 2000). These results concur with studies which report that the responsiveness of different muscle types to insulin-stimulated glucose uptake is associated with variations in the expression of glucose transporters (Henricksen *et al.*, 1990; Megeney *et al.*, 1993). As previously mentioned, lipoic acid is a co-factor for both pyruvate and α ketoglutarate dehydrogenases (Reed, 1962). This may mean that in the case of the *ob/ob* mice the activity of these enzymes may be limited by availability of the co-factor. Hence in these circumstances lipoic acid could directly increase glucose oxidation, glycolytic flux and thus glucose uptake. These studies do indicate that glucose uptake is increased in response to lipoic acid and that this glucose is not converted to lactate (tables 3.2 and 3.3). Whether the glucose taken up in response to lipoic acid is stored as glycogen or oxidised in the Krebs cycle I shall endeavour to elucidate in the next section of this chapter. This aside, the ability of lipoic acid alone to surmount the insulin resistance seen in the red quadriceps of *ob/ob* mice indicates a capability to overcome defective insulin signalling. Lean mouse tissue will have been exposed to normal insulin concentrations before incubation, and *ob/ob* mice, which are hyperinsulinemic, will have provided muscles that had been bathed in high insulin concentrations prior to removal. Thus these experiments are unable to establish if lipoic acid is activating insulin signalling or augmenting the effect of endogenous insulin.

3.5.1 - Effect of lipoic acid on glucose oxidation in various muscles from obese mice (measured by $^{14}\text{CO}_2$ production from ^{14}C - D - glucose)

Basal $^{14}\text{CO}_2$ production levels from ^{14}C - D - glucose varied little between the different muscle types examined, being 2.5 DPM/mg/2hr in both quadriceps and abdominal muscle (figs 3.9 and 3.10). The ability of the metabolic disorders inherent to *ob/ob* mice to nullify differences in basal values due to fibre composition of the muscles has already been seen in figs 3.6, 3.7 and 3.8. As expected in these extremely insulin resistant mice the addition of 10^{-6}M insulin did not increase glucose oxidation. Lipoic acid (10^{-3}M) alone increased the production of $^{14}\text{CO}_2$ in both the muscles studied, being diaphragm (11.00 DPM/mg/2hr) and quadriceps muscle (4.5 DPM/mg/2hr). The combination of lipoic acid and insulin had no effect in quadriceps or diaphragm muscles. These findings are in agreement with the study of Jacob *et al* (1996) who found increased rates of glucose oxidation in lipoic acid-treated muscle tissues from obese Zucker rats.

3.5.2 – Effect of lipoic acid on glycogen synthesis in various muscles from lean and obese mice (measured by ¹⁴C glycogen and enzymatic analysis).

3.5.2.1 - ¹⁴C glycogen

Perusal of the data from the ¹⁴C glycogen experiments (3.3.2) clearly indicates that this procedure did not extract the expected amount of glycogen, probably during the ethanol precipitation phase of the protocol as described previously (materials and methods, diagram 2.2). Subsequently, a protocol was used that did not require the isolation of the glycogen or the use of radioisotopes (2.2.3.3).

3.5.2.2 - Enzymatic analysis

Basal glycogen content in the three lean muscles studied varied in a manner inversely related to fibre type proportions (fig 3.11). Quadriceps had the highest basal glycogen at 4 glycosyl units (% wet weight of tissue, henceforth %), followed by diaphragm (2.5%) and abdominal muscles (0.5%). As indicated in table 3.1, type 1 fibres usually possess least glycogen stores whereas type 2b contain large stores of glycogen (Sherwood, 1993). Therefore we would expect quadriceps muscle to have the smallest glycogen stores and abdominal muscle to have the highest as these consist of mostly type 1 and type 2b fibres respectively. However these generalisations are not always borne out by measurements in mice, as noted in the present study. It is unlikely that depletion of glycogen stores has occurred, as incubation conditions were identical to those in section 3.2.2.2, in which no significant concentrations of lactate were produced. This would have indicated respiratory distress in the muscles. Inter-muscle insulin-stimulated glycogen deposition has occurred in a manner consistent with fibre type composition, being highest in the quadriceps (6%), intermediate in the diaphragm (3%) and lowest in the abdominal muscles (1.5%). Glycogen deposition in response to lipoic acid alone did not follow the same pattern, with abdominal and diaphragm being very similar (1% versus 1.2% respectively) and quadriceps being much higher (5.2%). Lipoic acid and insulin in combination produced results very similar to those with insulin alone and it is therefore

unlikely that lipoic acid has had an additive effect in this case. Values for glycogen deposition in the obese *ob/ob* mice (fig 3.12) follow the same basic outline as those for lean mice, with some variation in amplitude of the responses and disruption where lipoic acid is used alone. Unfortunately, differences in responsiveness to insulin, lipoic acid and both agents in combination were not seen in each individual muscle type. The high variability of the data suggests that this method is unsuitable for the required task and would require repetition with inordinately high numbers of animals sacrificed to discriminate whether there is any biologically significant effect. Thus I find myself unable to agree with or refute the claim that lipoic acid increases glycogen synthesis in isolated muscle (Jacob *et al.*, 1996).

3.6 - The effect of lipoic acid on glucose uptake in L6 cells

Insulin caused a typical increase in glucose uptake by L6 muscle cells at 2hr, varying between 50 and 75% (figs 3.13 and 3.14). Lipoic acid alone (10^{-3} M) significantly increased glucose uptake compared to control (3.13), but not in combination with insulin (10^{-6} M) versus insulin alone (3.14). Thus this effect appeared to be independent of exposure to insulin as neither was significantly different to insulin alone in either experiment. These studies at 2hrs (figs 3.13 and 3.14) showing that lipoic acid increased basal glucose uptake by cultured muscle cells are in agreement with the findings of Estrada *et al* (1996) and Tsakiridis *et al* (1995). Upon further study with 10^{-3} M lipoic acid at longer time points a different pattern emerged. At 12hr (fig 3.15) insulin increased glucose uptake by 60%, lipoic acid by 80% and the combination of both by approximately 125% versus control. There appears to be an additive effect of insulin and lipoic acid at this time point as the combination significantly raised mean glucose uptake above that for insulin alone. At 24hr (fig 3.16) insulin raised glucose uptake by 150%, lipoic acid alone had no effect on basal glucose uptake and the combination of both agents significantly decreased glucose uptake compared to insulin alone. This was still significant versus control. At 48hr (fig 3.17) the pattern is similar except that insulin alone raised uptake by over 500% and the combination of insulin and lipoic acid no longer increased basal glucose uptake.

Longer term observations and toxicity studies have not been reported with cultured muscle cells and it should be noted that 10^{-3} M lipoic acid caused cytotoxicity at 24 and 48h, evidenced by sub-normal glucose uptake and impaired trypan blue exclusion (3.8). This apparent long-term cytotoxicity could be due to the strong anti-oxidant profile of the lipoic acid itself (Reed, 1962) or the sequestering of intra-mitochondrial coenzyme A as seen in the liver in response to lipoic acid (Blumenthal, 1984).

3.7 - Effect of lipoic acid on oxygen consumption by L6 cells

Both lipoic acid treated and control L6 cells consume oxygen in a similar linear fashion (fig 3.18), indicating that for the first 20 minutes of exposure to lipoic acid there is no increase in oxidation of glucose. Whilst numerous studies in cells have indicated that lipoic acid induces the transport of glucose, none has indicated the metabolic fate of this increased intracellular glucose (Estrada *et al.*, 1996; Tsakiridis *et al.*, 1995; Yaworsky *et al.*, 2000). Certainly, the present study has shown that within insulin resistant muscles from mice lipoic acid increases glucose oxidation, but this study did not indicate where within the 2h incubation period the $^{14}\text{CO}_2$ was produced. Anecdotal verbal claims at conferences that lipoic acid may increase oxygen consumption *in vivo* have yet to be published in refereed journals. Time limitations with the apparatus used (e.g. oxygen electrode) may have missed possible longer-term effect of lipoic acid on oxygen utilisation by L6 cells. Also, *ob/ob* mice are possibly co-factor deficient and hence their tissues will show an increase in glucose oxidation as seen in my own studies and those of others using these mice.

3.8 - Cytotoxicity studies

Interestingly 10^{-3} M α -lipoic acid caused cytotoxicity in L6 muscle cells at 24h and 48h as indicated by impaired trypan blue exclusion and sub-normal glucose uptake (>50% blue-positive staining cells) compared with control wells (<5% blue-positive staining cells). Moreover, 10^{-2} M α -lipoic acid showed evidence of cytotoxicity at the earlier time points studied. It is possible that sequestration of intra-mitochondrial coenzyme A

(Blumenthal, 1984) or the strong antioxidant properties of α -lipoic acid (Packer *et al.*, 1995) might contribute to the detrimental longer-term effect of a high concentration of this agent on cell viability in culture. Although the cytotoxicity of a high concentration of α -lipoic acid in cell culture does not necessarily imply toxicity *in vivo* where there are opportunities for buffering, metabolism and elimination of an excess of such an agent, it does suggest a restricted useful range of concentrations that could be employed for therapeutic purposes.

3.9 - Overall conclusions

Clearly lipoic acid has potential in the treatment of diabetes as it increases uptake and oxidation of glucose in *ob/ob* muscles, a model for insulin resistance. The L6 cell *in vitro* cytotoxicity results may not necessarily imply *in vivo* toxicity as there is opportunity for buffering, metabolism and elimination of excess lipoic acid *in vivo*. These results do however suggest a narrow therapeutic window for this agent.

The use of lipoic acid as a putative treatment for diabetic neuropathy has provided no reports of impaired glycolytic control, and has indicated improved control from anecdotal comments and non-quantitative reporting (Eason *et al.*, 2001). Where a controlled clinical trial was undertaken, lipoic acid treatment improved glycolytic control in type 2 diabetic patients, associated with improved insulin sensitivity during a euglycaemic hyperinsulinaemic clamp procedure (Jacob *et al.*, 1995). Consistent with the view that lipoic acid could improve insulin action in the insulin resistant state, the present studies found that lipoic acid improved glucose uptake in red quadriceps muscles of insulin resistant *ob/ob* mice. Since the pattern of increased uptake was similar to that shown by normal muscles it is possible that lipoic acid could be engaging a part of one of the post receptor insulin signalling cascades, as indicated by other types of experiments (Estrada *et al.*, 1996; Klip *et al.*, 1996; Khamaisi *et al.*, 1997). A study by Yaworsky *et al.* (2000) has shown that lipoic acid acts to increase translocation of GLUT1 and GLUT 4 glucose transporters in a PI3K dependent manner. Lipoic acid also increased tyrosine phosphorylation of the insulin receptor and IRS - 1 (Yaworsky *et al.*, 2000). Given that

lipoic acid seems to act in a manner similar to insulin, this would explain the pattern of results seen with the three muscle types employed from the lean mice. The greater the content of red fibres the greater the sensitivity to insulin and thus lipoic acid. The ability of lipoic acid to increase glucose uptake in insulin resistant obese mice muscles could be attributed to this compound's ability to engage the signalling pathway in more than one location (Yaworksy *et al.*, 2000), thus alleviating any potential resistance at the insulin receptor level.

The clinical potential of lipoic acid as an antioxidant, possibly an agent to reduce the symptoms of diabetic neuropathy and putatively an agent to reduce insulin resistance cannot be ignored. However, the likely narrow therapeutic index indicated by cytotoxicity *in vitro* would limit the dosage that could be employed. Despite this, no clinical trials to date have reported ill effects upon administration of lipoic acid and the future of insulin resistance treatment with lipoic acid can be looked upon with cautious optimism.

Chapter 4 – Obesity

4.1 - Introduction

Many studies have confirmed a strong association between type 2 diabetes and obesity (Morris *et al.*, 1989; Skarfors *et al.*, 1991; Haffner *et al.*, 1990 and Charles *et al.*, 1991). Indeed, in the UK more than 50% of newly diagnosed type 2 diabetic patients are obese or have previously been obese, and in the USA more than 70% of type 2 diabetic patients are obese at diagnosis (Pi-Sunyer, 1996). Both obesity and diabetes are implicated in the premature development of cardiovascular disease: for example type 2 diabetes increases the incidence of cardiovascular disease by 2-4 fold (Leong & Wilding, 1999). The negative effect on glycaemic control seen with excessive weight gain has been attributed to a marked rise in insulin resistance, particularly associated with visceral adiposity (Scheen *et al.*, 1995). This is thought to be due to a number of factors including the production of certain hormones (e.g. resistin and possibly leptin), cytokines (e.g. TNF α and IL-6) and non-esterified fatty acids (NEFAs), to which we will now turn.

Insulin has a number of important physiological roles in the body, most of which have been discussed in the main introduction. To recap, insulin induces the GLUT4 glucose transporter isoform that mediates the insulin-dependent uptake of glucose into skeletal muscle. Insulin also inhibits glycogen breakdown and gluconeogenesis in the liver. In muscle the glucose is either metabolized to generate ATP via the Krebs cycle or stored in the form of glycogen. In adipose tissue the majority of glucose enters the lipogenic pathway, contributing via glycerol-3-phosphate to the accumulation of NEFA's to form triglycerides. Insulin also antagonises the breakdown of triglycerides present in adipose tissue, thus inhibiting the release of NEFAs. In overweight diabetic subjects there is an elevation in circulating NEFA and glucose levels. The raised NEFA levels decrease insulin-stimulated glucose uptake and utilisation in the skeletal muscle (increasing insulin resistance)(Bonadonna *et al.*, 1990) and provide an alternative energy source for muscle and liver (Randle's glucose-fatty acid cycle). In the liver this energy supply facilitates gluconeogenesis, thus raising hepatic glucose output (Randle *et al.*, 1965). In normal

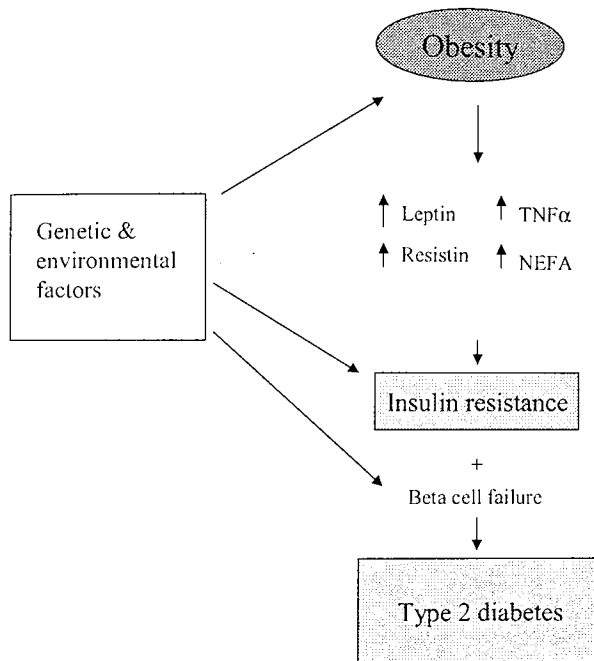
healthy individuals raised NEFAs temporarily stimulate insulin production and secretion but prolonged exposure to raised NEFAs reduces insulin concentrations (Boden, 1997).

Resistin has recently been identified as a peptide hormone produced by adipose tissue (Steppan *et al.*, 2001). In preliminary studies it has been shown to cause insulin resistance in skeletal muscle and liver, and is postulated to provide a mechanism through which excess adiposity impairs insulin action in other tissues. As yet little more is known about resistin, but it is anticipated to play a significant role in the development of insulin resistance in obese individuals.

Leptin is a satiety factor produced by adipocytes that regulates food consumption and calorific expenditure (Shwartz & Seedy, 1997). It is the product of the OB gene first identified by Zhang *et al* in 1994 and acts centrally to reduce food intake and increase sympathetic nervous activity. Leptin is known to be positively linked to insulin resistance (Segal *et al.*, 1996) and TNF α levels (Mantzaros *et al.*, 1997) in man. It is likewise positively linked to BMI and percentage body fat in both man and animals (Maffei *et al.*, 1995; Considine *et al.*, 1996). It has also been shown to impair insulin's hepatic effects (Wang *et al.*, 1997) and reduce insulin production itself (Kulkarni *et al.*, 1997). The overall perception of leptin's effect on insulin action in muscle and adipocytes themselves is less clear (Dagogo-jack, 2001).

TNF α mediated effects on muscle and fat have been extensively discussed in the main introduction. In summary, over-expression of TNF α occurs in several rodent models of obesity (Hotamisligil *et al.*, 1993) and is thought to reduce insulin action via inhibitory serine or threonine phosphorylation of IRS-1 (Hotamisligil *et al.*, 1996). GLUT4 and lipoprotein lipase expression within adipocytes is down regulated by TNF α (Hotamisligil *et al.*, 1993, 1996) and targeted mutation in the TNF α genes of animal obesity models improve insulin sensitivity (Uysal *et al.*, 1997; Ventre, 1997). The interaction of NEFA's, TNF α , resistin and leptin in obesity, insulin resistance and diabetes is summarized in diagram 4.1 overleaf (adapted from Leong & Wilding , 1999).

Diagram 4.1 – Interaction of NEFAs, TNF α , resistin and leptin in obesity, insulin resistance and obesity

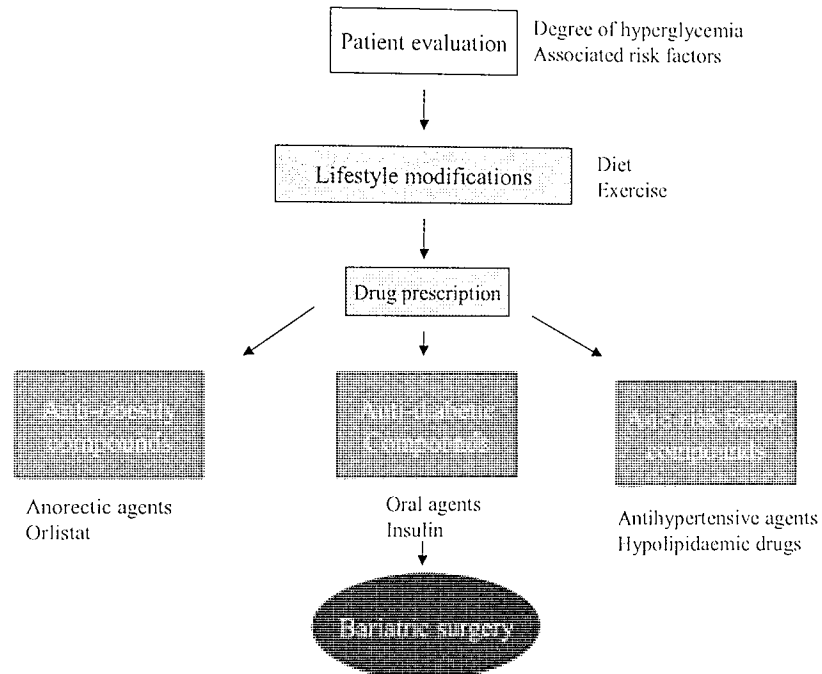


Treatment

The poor glycaemic control seen in most obese diabetic subjects can be improved and sometimes normalized by appropriate weight loss (Brown *et al.*, 1996), which also has the beneficial effect of reducing the impact of weight-associated risks of cardiovascular disease (Pi-Sunyer, 1996). Treatment of the obese diabetic patients involves lifestyle modifications such as a diet designed to restore and maintain normal body weight, increased physical activity and pharmacological intervention which is sometimes used alongside the conventional acute diabetic therapies. Presently only one anti-obesity drug is licensed in the UK, being Orlistat or tetrahydrolipstatin. Orlistat acts by promoting fat malabsorption, binding covalently to pancreatic lipase that is essential for lipid digestion in the intestine (Guerciolini, 1997). In recent years there has been intense interest in the compound sibutramine, a centrally acting noradrenaline and 5-HT reuptake inhibitor. Studies in animals have shown a reduction in food intake after administration of this compound (Ryan *et al.*, 1995), the resulting weight reduction being dose dependent (Bray *et al.*, 1996).

In extreme circumstances, where the patient presents with inordinate obesity despite therapeutic intervention, bariatric surgery may be used. It has been suggested that patients with a BMI over 40 should be considered as candidates for such surgery, and in certain circumstances patients with a lower BMI (35 to 40) might benefit from surgical intervention (NIH, 1992). Included here are patients with severe diabetes mellitus. The various treatment options for primary treatment of the obesity in obese-type 2 diabetic patients are summarized in diagram 4.2 (adapted from Scheen and Lefebvre, 1999).

Diagram 4.2 - Various treatment options for obesity in obese type 2 diabetics



New compounds that could assist weight reduction in obese subjects would be beneficial and remove the potential need for surgical intervention. In principle, early intervention against obesity could prevent or delay the development of type 2 diabetes, and provide a valuable approach to the treatment of type 2 diabetes (Tuomilehto *et al.*, 2001; Pi-Sunyer, 1996). In the following studies human adipocytes and adipose tissue were characterised and tested with a variety of compounds to investigate the potential for future use in the pharmacological treatment of the obese patient.

4.2 - Results

4.2.1 - Characterisation of the lipolytic activity of adipocytes and adipocyte tissue

Data reported in this chapter concern the effect of noradrenaline, the sibutramine metabolite M2, insulin and α -D-lipoic acid on lipolysis by adipose tissue and adipocytes from mice. This was assessed by measuring glycerol release into the medium during incubations for 2 hrs at 37°C. Initial experiments were undertaken to characterise the preparation.

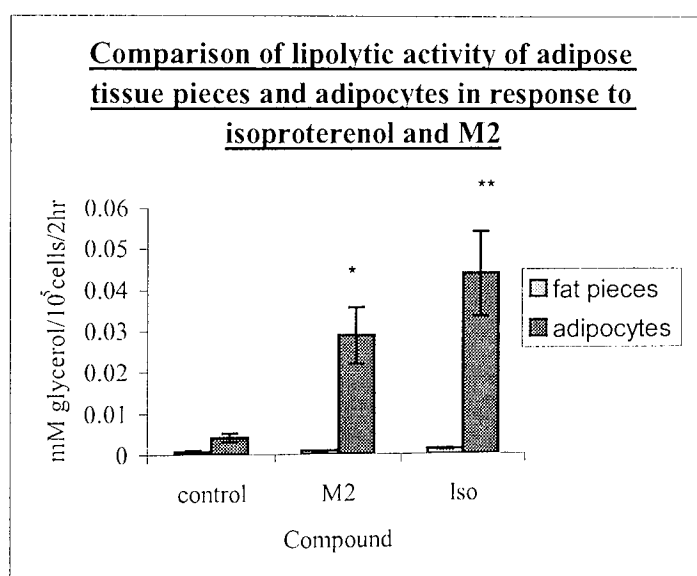


Fig 4.1 - Glycerol release from adipose tissue pieces and adipocytes was increased by addition of $10^{-5}M$ isoproterenol (** $P < 0.01$). The addition of $10^{-6}M$ M2 significantly increased glycerol production from adipocytes (* $P < 0.05$) but not from adipose tissue pieces. Results are means \pm standard error, $n=20$.

The effect of isoproterenol ($10^{-5}M$) and M2 ($10^{-6}M$) on glycerol release from adipose tissue and adipocytes is shown in figure 4.1. Control (unstimulated) adipose tissue pieces released 0.008mM glycerol/10⁵ cells over the 2 hrs compared to 0.05mM from isolated

adipocytes, which is six fold greater ($P < 0.01$). Isoproterenol caused glycerol release from adipose tissue pieces to increase to $0.0013\text{mM}/10^5\text{cells}$ ($P > 0.05$) and that of adipocytes to increase to $0.045\text{mM}/10^5\text{cells}$ ($**P < 0.01$) over the 2 hours. The presence of M2 increased glycerol production by adipocytes to $0.028\text{mM}/10^5\text{cells}$ over the two hours ($*P < 0.05$), but had no effect on pieces of adipose tissue.

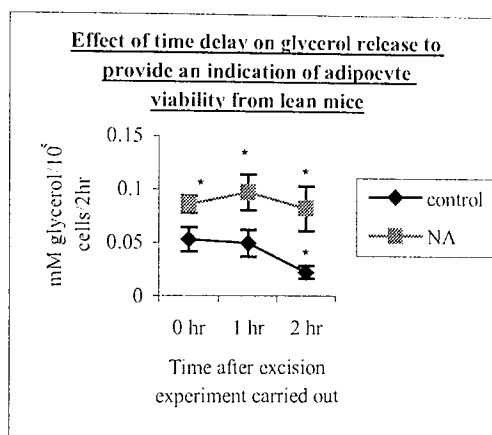


Fig 4.2 - Glycerol release from control and noradrenaline (10^{-6}M) stimulated lean mouse adipocytes was measured at different times after excision for up to 2 hours. Tissue was either processed immediately or maintained in Krebs ringer buffer at 37°C for 1 or 2 hrs before undertaking the standard procedure of adipocyte isolation and incubation. Basal (control) release of glycerol showed a lower mean value at 2hrs compared with 0hrs ($*P < 0.05$). There was no effect of time after excision on NA stimulated glycerol release at 1 and 2h ($P > 0.05$). Results are means \pm standard error, $n=7$.

Figure 4.2 shows the effect of 1 and 2 hours time delay between tissue excision and beginning incubation on glycerol release from lean mouse adipocytes during 2 hours incubation at 37°C . Control (unstimulated) adipocytes released $0.06\text{mM}/10^5\text{cells}$ if incubated immediately or $0.05\text{mM}/10^5\text{cells}$ and $0.02\text{mM}/10^5\text{cells}$ if left for 1 or 2 hrs ($*P < 0.05$) respectively. Noradrenaline-stimulated cells released $0.09\text{mM}/10^5\text{cells}$ if incubated immediately or $0.09\text{mM}/10^5\text{cells}$ and $0.075\text{mM}/10^5\text{cells}$ if left for 1 or 2 hours

respectively ($P>0.05$, not significant). All noradrenaline-stimulated values for glycerol release were significant compared to control (unstimulated) at 0, 1 and 2h ($*P<0.05$).

All experiments in this thesis that involved direct comparison of control and drug-stimulated or drug-inhibited glycerol release used incubations that were started at approximately 10 min after the excision of the tissue for the mouse fat and 30 min after excision for the human adipose tissue. This avoided any small alterations in lipolytic activity that might be associated with the time between excision and beginning of the incubation.

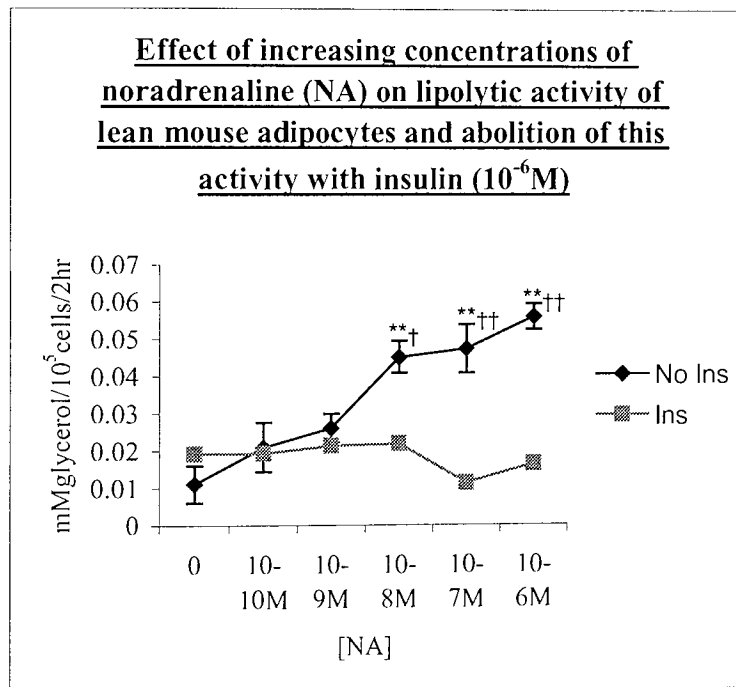


Fig 4.3.1 - Glycerol release from lean mouse adipocytes was increased by addition of (10^{-10} - $10^{-6}M$) noradrenaline ($**P<0.01$ versus control, $††P<0.01$ versus same concentration and added insulin), but this response was prevented by the presence of insulin ($10^{-6}M$). Results are means \pm standard error, $n=7$.

Effect of noradrenaline (10^{-10} - $10^{-6}M$) on glycerol release from lean mouse adipocytes. Control (unstimulated) adipocytes released 0.01mM/ 10^5 cells over the two hours. The

addition of 10^{-10} - 10^{-6} M noradrenaline increased glycerol release in a concentration dependent manner being significant compared to control at 10^{-8} M, 10^{-7} M and 10^{-6} M ($P<0.01$). The addition of insulin prevented this response, causing 10^{-8} M, 10^{-7} M and 10^{-6} M NA without insulin to be significantly higher than the same concentrations in the presence of insulin ($P<0.01$).

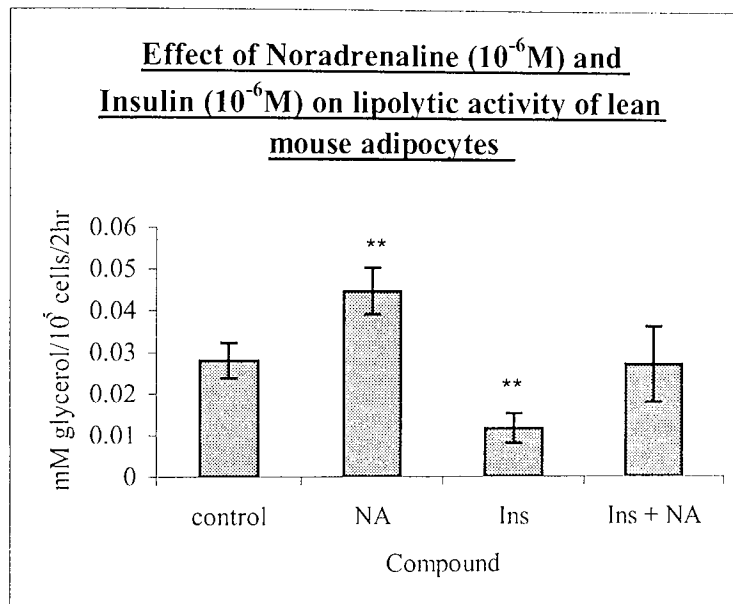


Fig 4.3.2 - Glycerol release from lean mouse adipocytes was increased by addition of noradrenaline (10^{-6} M) and decreased by insulin (10^{-6} M) alone ($*P<0.01$), but had no effect in combination ($P>0.05$). Results are means \pm standard error, $n=6$.

In a separate experiment, the effect of noradrenaline (10^{-6} M) and insulin (10^{-6} M) on glycerol release from lean mouse adipocytes was determined during 2 hours incubation. This was undertaken to reassess the effects NA and insulin in exactly the same protocol as used to test other drugs. Control (unstimulated) adipocytes released approx 0.03mM/10⁵cells over the 2 hours. The addition of noradrenaline caused an increase in glycerol release to almost 0.045mM/10⁵cells (** $P<0.01$). The addition of insulin decreased glycerol release to 0.01mM/10⁵cells (** $P<0.01$) and a combination of both

caused a slight increase in glycerol production versus control, but this was not significant ($P>0.05$).

Murine visceral adipose tissue strongly expresses the β_3 adrenoceptor, and this receptor is activated by NA to mediate a lipolytic effect. The present studies with NA are consistent with an active β_3 adrenoceptor mediated lipolytic pathway in this tissue. Although rodent white adipocytes receive their main lipolytic stimulus via β_3 adrenoceptors, β_1 plays a minor role in lipolysis (Arch *et al.*, 1984; Arch and Wilson, 1996; Harms *et al.*, 1977). A different distribution of expression of β adrenoceptor subtypes is found in the various adipose depots in man (discussed later).

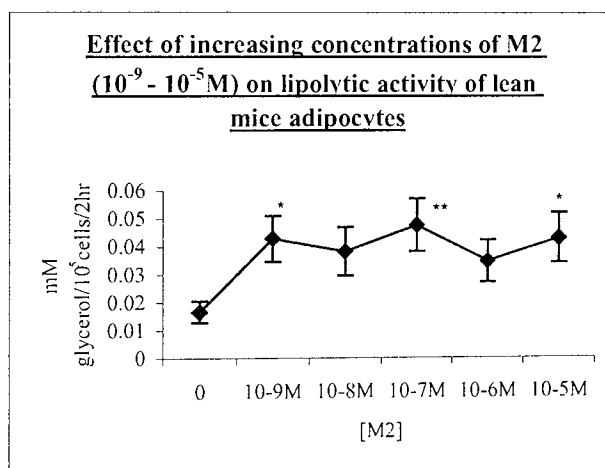


Fig 4.4 - Glycerol release from lean mouse adipocytes was increased by addition of M2 (10^{-9} - 10^{-5} M) (* $P<0.05$, ** $P<0.01$). Results are means \pm standard error, $n=10$.

M2 (10^{-9} - 10^{-5} M) significantly increased glycerol release from lean mouse adipocytes by more than twofold at 10^{-9} M, 10^{-7} M and 10^{-5} M ($P<0.05$, $P<0.01$ and $P<0.05$ respectively). Control (unstimulated) adipocytes released approx 0.015mM/ 10^5 cells of glycerol over the 2 hours in this study.

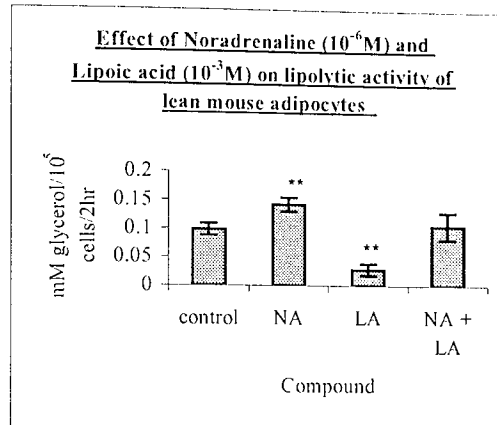


Fig 4.5 - Glycerol release from lean mouse adipocytes was increased by addition of noradrenaline ($10^{-6}M$) and decreased by lipoic acid ($10^{-3}M$) alone ($*P<0.01$), but there was no effect of the two agents in combination ($P>0.05$). Results are means \pm standard error, $n=6$.

The effects of noradrenaline ($10^{-6}M$) and lipoic acid ($10^{-3}M$) were measured alone and in combination. Control (untreated) adipocytes released approx $0.1mM/10^5$ cells over the two hours. The addition of noradrenaline increased glycerol release to almost $0.15mM/10^5$ cells ($P<0.01$). The addition of lipoic acid *decreased* glycerol release to $0.025mM/10^5$ cells ($P<0.01$) and a combination of the two agents slightly increased the mean value for glycerol production versus control, but this was not statistically significant ($P>0.05$).

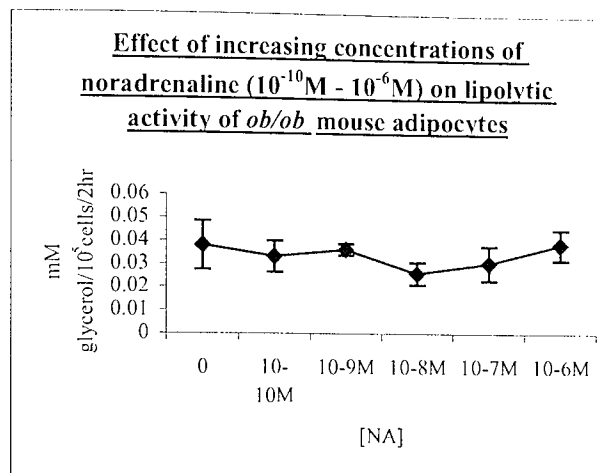


Fig 4.6 - Glycerol release from obese mouse adipocytes was unaffected by addition of (10^{-10} - 10^{-6} M) noradrenaline ($P > 0.05$, not significant). Results are means \pm standard error, $n=6$.

Noradrenaline (10^{-10} - 10^{-6} M) did not significantly affect glycerol release from *ob/ob* mouse adipocytes over the 2hrs incubation period ($P > 0.05$, not significant). Control (unstimulated) adipocytes released 0.04mM/10⁵ cells over the two hours. This basal value was slightly higher than the typical value for basal glycerol release observed in lean mouse adipocytes (see zero value for figures 4.3 and 4.4). However there was no consistent difference in basal release between lean and *ob/ob* mouse adipocytes observed in the present study. This is likely to reflect the similarity in the size distribution of adipocytes isolated by the present protocol whether using lean or *ob/ob* mouse fat. The fat pads from *ob/ob* mice are known to contain a greater proportion of larger adipocytes than lean mice, but large adipocytes are more vulnerable to lysis during the collagenase isolation and are not preserved for the incubations (Bray and York, 1971). This has been noted in previous studies, and has recently been re-affirmed with the present isolation protocol in this laboratory (Richardson, personal communication, 2001).

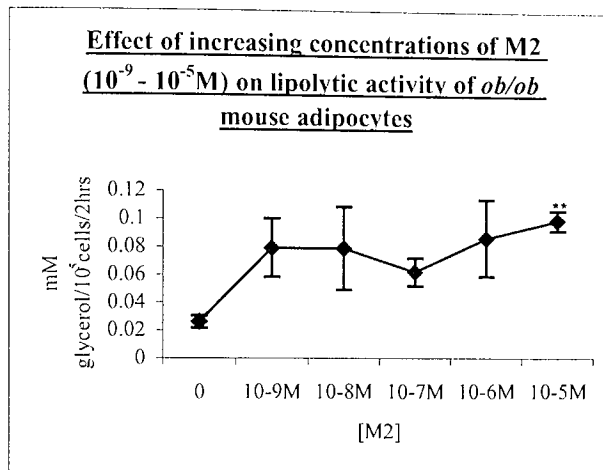


Fig 4.7 - Glycerol release from *ob/ob* mouse adipocytes was increased by M2 (10^{-9} - 10^{-5} M, $**P < 0.01$). Results are \pm standard error, $n=6$.

M2 (10^{-5} M) significantly increased glycerol production from *ob/ob* mouse adipocytes over the incubation period. Control adipocytes released approx 0.02mM/ 10^5 cells over the two hours. Although mean values for glycerol release with M2 at 10^{-9} - 10^{-6} M was higher than control; this did not achieve statistical significance until 10^{-5} M due to the spread of individual data points.

4.2.2 - Human adipocytes

Data reported in this section concern the effect of noradrenaline, M2 and lipoic acid on glycerol release from human visceral and subcutaneous adipocytes. This was measured during incubation for 2 hours at 37°C, as described in the methods section. In humans, β_2 and α_2 adrenoceptors are thought to mediate adipocyte lipolysis, with expression of β_3 being much lower (Enocksson S *et al.*, 1995; Galitzky J *et al.*, 1993). Variability between human adipose depots has also been reported, with higher β and lower α_2 adrenoceptor levels in visceral fat compared to subcutaneous (abdominal) fat (Harmelen *et al.*, 1997). Peripheral subcutaneous fat is thought to have an even lower expression of β adrenoceptors compared to subcutaneous fat from the abdominal region (Arner, 1996). Given that the distribution and levels of expression of β_1 , β_2 and β_3 adrenoceptors appear to vary between rodents and humans (see mouse section) and human and visceral and subcutaneous fat, it would be particularly interesting to examine the effect of M2 in human adipose tissue.

As already shown, M2 stimulates lipolysis in adipocytes from both normal lean and obese-diabetic *ob/ob* mice and this section investigates if this effect is reproducible in human adipocytes. The first step in this process was to characterise briefly the lipolytic response to NA in adipocytes isolated from human subjects. Since the availability of human adipose tissue was limited, the following considerations were made in the selection of tissue to be studied:

- (1) Since M2 would be produced by the degradation of the anti-obesity drug sibutramine in the treatment of obesity, it was relevant to conduct these studies using fat from overweight and/or obese patients.
- (2) The most important fat depots for creating detrimental metabolic effects are the visceral depots – hence the present study focused on the visceral adipocytes of overweight and/or obese individuals.

Results of the initial experiments to provide a preliminary characterisation of the lipolytic response to NA in human subjects and both subcutaneous and visceral adipose tissue are shown in figure 4.8 and 4.9.

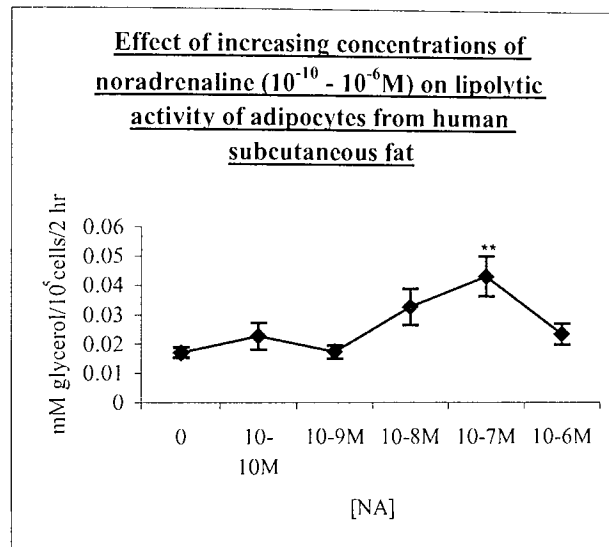


Fig 4.8 - Glycerol release from lean human subcutaneous adipocytes was increased by addition of $10^{-7}M$ noradrenaline (** $P < 0.01$). Mean values for glycerol release were increased at some other concentrations of NA, but did not achieve statistical significance. Results are means \pm standard error, $n=12$.

Despite the constraint of tissue availability from only two lean and obese patients, useful extension of the work on mouse adipocytes was achieved. Control (unstimulated) human subcutaneous adipocytes from a depot overlaying the abdomen released approximately $0.018mM/10^5$ cells over the two hours. Addition of NA to this subcutaneous human adipocyte preparation appeared to increase glycerol release with increasing NA concentration, reaching significance at $10^{-7}M$ NA (** $P < 0.01$).

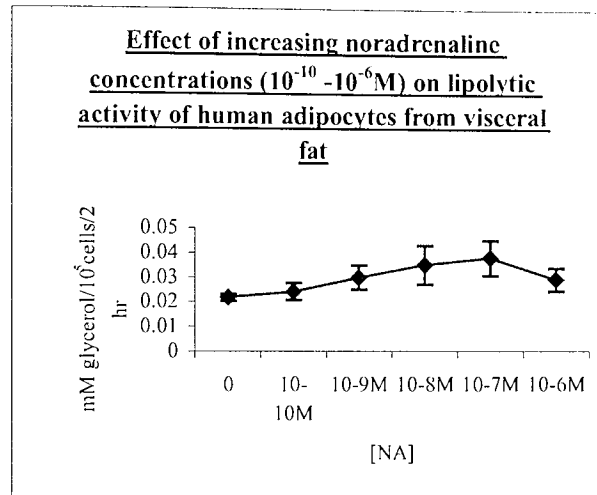


Fig 4.9 - Glycerol release from lean human visceral adipocytes was not significantly altered by noradrenaline (10^{-10} M – 10^{-6} M, $P > 0.05$). Results are means \pm standard error, $n = 12$.

In contrast to the observation in subcutaneous fat, the general increase in glycerol release produced by NA (10^{-10} M – 10^{-6} M) from human visceral adipocytes of a different patient failed to achieve significance ($P > 0.05$). Control (unstimulated) adipocytes released approximately 0.02 mM/ 10^5 cells over the two hours, which was similar to the subcutaneous adipocytes.

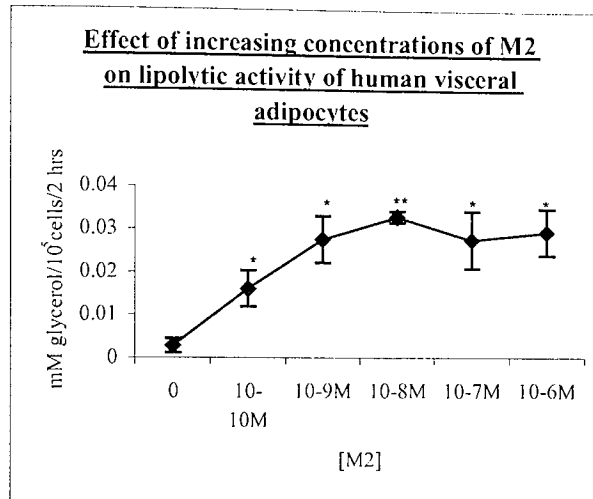


Fig 4.10 - Glycerol release from obese (type 2 diabetic) human visceral adipocytes was increased upon addition of M2 ($10^{-10}M - 10^{-6}M$) (* $P < 0.05$, ** $P < 0.01$). Results are means \pm standard error, $n=6$.

Baseline release of glycerol from visceral adipocytes of an obese human subject was approximately $0.003\text{mM}/10^5\text{cells}$ over the two hours. This was much lower than seen with adipocytes from a lean subject. Visual inspection of the adipocyte preparation indicated that this could not be attributed to a difference in size of the isolated adipocytes, indicating a lower real reduction in lipolytic rate. M2 ($10^{-10}M - 10^{-6}M$) increased glycerol release in a concentration dependent manner, being significant at all concentrations tested (* $P < 0.05$, ** $P < 0.01$).

4.3 - Discussion

The present study has assessed lipolytic activity of fat pieces and isolated adipocytes from lean (+/+) and obese *ob/ob* mice. Preparations from both were used to measure glycerol release under basal conditions and in response to standard agents as a means of characterisation and standardisation. The effect of the M2 metabolite of sibutramine was evaluated as a potential therapeutic agent to enhance lipolysis.

Obese and lean mice

The obese *ob/ob* mice have a congenital absence of functional leptin (Zhang *et al.*, 1994). Lack of leptin results in a loss of the leptin satiety signal, causing the mice to overeat and hence they become obese. This mutation is autosomal recessive and is transmitted in a Mendelian fashion, the normal homozygous and heterozygous littermates being 'lean'. As obesity is linked to insulin resistance, the adipose tissue/adipocytes of these mice provide an ideal model for the study of therapeutic compounds.

4.3.1 - Comparison of adipose tissue pieces and adipocytes

As noted in figure 4.1, there was a difference between the glycerol release in control, M2-treated and isoproterenol-treated values from adipocytes compared to adipose tissue. The control value for glycerol release during 2hr incubation was five-fold greater using adipocytes than adipose tissue, being approximately 0.005mM glycerol/10⁵cells and 0.001mM glycerol/10⁵cells respectively. This difference was more marked after incubation with M2 and isoproterenol, where adipocytes showed values for glycerol release that were approximately twenty and thirty-fold higher than those for adipose tissue. These results are perhaps less surprising than they first appear, due to the nature of the models used, providing better diffusion and accessibility of the test agents to the complete plasma membrane surface of all cells in the isolated adipocyte preparation. The adipose tissue is an intact mass and hence it will be much less penetrable to the experimental compounds compared to isolated adipocytes. Simple volume to surface area ratio comparison would also explain the poor performance of the adipose tissue

pieces, since the cell surface will be the site of drug-receptor interaction. Generally the greater the number of available receptors the larger the magnitude of the pharmacological response in this type of experiment with a limited period of exposure to a substantial amount of agent. In the case of isoproterenol this will be the β adrenergic receptors. As yet the receptor for M2 is unknown. Another factor may be the paracrine antagonistic effects of compounds released from the adipocytes within the fat pieces.

It is evident from the comparison of adipose tissue pieces and collagenase isolated adipocytes that the latter provides a far better model for the study of *in vitro* adipocyte metabolism. This technique was therefore used in subsequent experiments. In addition, NA was substituted in place of isoproterenol in later experiments, as NA is clinically more relevant and was readily available within the laboratory.

4.3.2 - Effect of time delay on adipocyte viability from lean mice

Control values for measurements of glycerol release started at 0, 1 and 2h after cell isolation were 0.06, 0.05 and 0.02mM glycerol/ 10^5 cells respectively (figure 4.2). Whilst the mean values appear to be decreasing with time, noticeably between 1 and 2h, the rate of decrease was not statistically significant. Noradrenaline-stimulated values were 0.08, 0.09 and 0.075mM glycerol/ 10^5 cells for 0, 1 and 2h respectively; again this variation was not statistically significant. The stimulation of lipolytic activity seen with NA at 0, 1 and 2h (each $P < 0.05$ versus no NA) indicates that the cells are functioning in a predictable and consistent manner over this time period.

This study has established that a time delay of up to two hours did not affect adipocyte viability significantly, a useful observation given that the transportation of tissue from City Hospital (Birmingham) to the laboratory at Aston University can take up to three quarters of an hour at times of traffic congestion. However, all comparative studies were undertaken with the incubation of adipocytes starting at about the same time after tissue isolation. Thus later human adipocyte experiments were not affected by differences in time delay during the transportation of tissue from the operating theatre to the laboratory.

4.3.3 - Effect of NA on lipolysis from lean mice adipocytes and abolition of response with insulin

Data presented in figure 4.3.1 and 4.3.2 show that control values for glycerol released into the medium were 0.01mM and approximately 0.02mM glycerol/10⁵cells. The presence of insulin (figure 4.3.2) prevented the stepwise increase in NA-stimulated glycerol release seen in figure 4.3.1, with the normally strongly lipolytic effect of 10⁻⁶M NA being unable to remove the inhibitory effect of the insulin (10⁻⁶M). This result is consistent with previous evidence that insulin is an extremely powerful inhibitor of noradrenaline-stimulated lipolysis, preventing hormone sensitive lipase (HSL) phosphorylation and activation (Stralfors P *et al.*, 1984) and causing a redistribution of HSL itself to the cytosol (Clifford *et al.*, 1997).

In conclusion the isolated adipocyte model of lipolysis provides a reproducible pharmacological preparation that acts in a consistent manner in response to standard agonists and antagonists, and is therefore deemed to be an appropriate model with which to test potential new therapeutic compounds.

4.3.4 - Effect of M2 on lipolysis by adipocytes from lean and *ob/ob* mice

Re-examination of figure 4.4 shows that M2 is an extremely effective stimulator of lipolysis, more than doubling glycerol release from lean mouse adipocytes. The mechanism of action of M2 in nerve cells is via the selective blocking of serotonin and noradrenaline re-uptake (Van der Ploeg, 2000; McNeely and Goa, 1998). However, M2 can increase glucose uptake in muscle cells by another mechanism (Bailey *et al.*, 2001) independent of the inhibitory effect on serotonin and noradrenaline re-uptake. The mechanism responsible for the lipolytic effect of M2 in adipocytes is unknown. One possibility is that M2 could be acting by a similar mode of action as NA e.g. via β adrenergic receptors and hormone sensitive lipase (HSL)(Stralfors *et al.*, 1987). Colleagues at Aston University are investigating this possibility in detail, but time-constraints have prevented my participation in further studies with M2. It is proposed

that initial experiments using adipocytes from lean mice could investigate this hypothesis by incubating adipocytes with both NA and M2 together. If both M2 and NA act on the β adrenergic receptors, we would expect their maximum effect in combination to be the same as either alone, as there is a finite population of receptors for which each compound could be competing. The same maximum effect for each agent alone and the two agents in combination would also be expected if the agents activated the same post-receptor independently of their initial interaction with the cell. However, if the combination proves to be synergistic, this points towards M2 acting on a separate receptor and post-receptor pathway. If the former hypothesis turns out to be the case a selection of β agonists with different specificities could then be used. An observation in *ob/ob* mouse adipocytes discussed below has provided very preliminary evidence that an effect on the adrenoceptor signalling pathway is unlikely to provide a full explanation for the mechanism of M2 on lipolysis. Given that the data have shown that M2 is potentially capable of potently stimulating lipolysis from adipocytes *in vitro*, this could have profound implications for the use of sibutramine in the management of obesity.

Despite the marked effect of M2 on lipolysis by lean mouse adipocytes, M2 had little significant effect on glycerol release from *ob/ob* adipocytes until the highest concentration used (10^{-5} M)(fig 4.7). M2 at 10^{-5} M raised glycerol release approximately five-fold to 0.1mM glycerol/ 10^5 cells compared with the control (0.02mM glycerol/ 10^5 cells). The ability of M2 to overcome the intrinsic metabolic disorders seen within *ob/ob* mice is intriguing. This experiment also raises the possibility that M2 may be acting at least in part via a pathway separate from that of NA (β adrenoceptors) as NA could not overcome the catecholamine resistance seen in these mice (fig 4.6). Further experiments could include isolation of the adipocyte plasma membranes for adenylyl cyclase activity and cAMP production (Belsham *et al.*, 1980). If adenylyl cyclase activity is implicated in the action of M2, a confirmatory study with the adenylyl cyclase inhibitor MDL_{12330A} could then be carried out (Lippe and Aridizzone, 1991). Another possibility exists however to explain the differential effects seen between noradrenaline and M2 in the present study. Recently Hutchinson *et al* (2002) have discovered that two β_3 subtypes utilised different signalling pathways despite being identical pharmacologically.

Obese individuals are commonly hyperlipidaemic, thus the M2-stimulated release of NEFAs from adipocyte stores could initially exacerbate the lipidaemia and transiently increase the risk of cardiovascular complications seen in obesity and type 2 diabetes. Raised NEFAs levels also decrease insulin-stimulated glucose uptake in muscle leading to increased insulin resistance (Bonadonna *et al.*, 1990). Pre-clinical observations in which sibutramine or M2 (up to 10mg/kg i.p.) was administered to *ob/ob* mice have noted an early increase in plasma NEFA concentrations from 4hrs to about 1 week (Day *et al.*, 1998; Day, personal communication). During more chronic periods of sibutramine or M2 administration, plasma NEFA levels are reduced. Perusal of recent clinical data indicates that there is no significant surge in plasma lipid levels. In fact, decreases in lipid levels in sibutramine treated patients have been seen (Lean *et al.*, 1998; Fitchet *et al.*, 1997) but these studies have not looked at NEFA's levels during the first few days of sibutramine administration. In both of the sibutramine clinical trials administration of sibutramine was supplemented with an increase in daily physical effort, reduced calorific intake and advice on eating behaviour. The augmented utilisation of plasma NEFAs liberated by sibutramine (via M2) as an energy source for the increased physical effort, coupled with the well-documented thermogenic effects of M2 (Stock M *et al.*, 1998) could account for the animal and clinical evidence to date.

4.3.5 - Effect of noradrenaline and lipoic acid on lipolysis from lean mouse adipocytes

Figure 4.5 shows that whilst NA significantly increased glycerol above the control, lipoic acid (10^{-3} M) alone *decreased* glycerol release significantly compared to control. This is fully consistent with current theory that lipoic acid acts via the insulin pathway. The fact that the combination of NA and LA removes the stimulatory effect of NA confirms this, as well as telling us that LA is a less potent activator of the insulin-stimulated pathway than insulin *per se* (c.f. figure 4.3.1 and 4.3.2, NA and insulin). If time had been available further experiments would have been carried out on the murine adipocytes themselves and on differentiated 3T3-1 cells. Elucidating whether LA activates the insulin signalling pathway at a proximal or distal location in adipocytes would be approached using the compound Wortmannin as this inhibits PI3 kinase activity. It is

known that within 3T3-L1 pre-adipocytes lipoic acid activates PI3 kinase and protein kinase B or Akt (Yaworsky *et al.*, 2000). In adipocytes, activity of HSL is regulated by two enzymes, cAMP dependent protein kinase A (PKA) and a protein phosphatase, which activate and deactivate HSL respectively (Sztalryd and Kraemer, 1995). Insulin-mediated reduction in PKA activity is thought to occur through activation of another enzyme, phosphodiesterase 3B (PDE 3B) (Degermann *et al.*, 1997). It is therefore interesting to note that a recent study has suggested that it is PKB that activates PDE 3B (Wijkander *et al.*, 1998). This provides a possible mechanism by which lipoic acid could be inhibiting lipolysis in these experiments.

4.3.6 - Effect of NA on lipolysis from visceral *ob/ob* mice adipocytes

NA had no significant effect on glycerol release from abdominal adipocytes of *ob/ob* mice (fig 4.6). Human studies initially indicated that visceral (intra-abdominal) adipocytes had a lower basal lipolysis rate than subcutaneous fat, although this may be due to size difference in the adipocytes from the two areas compared (Goldrick *et al.*, 1970; Ostmann *et al.*, 1979). On a weight for weight basis, visceral adipocytes may show an overall greater rate of lipolysis and greater turnover of fatty acids (Arner, 1997). Using a micro dialysis technique, Jansson *et al* (1992) found overall glycerol release was increased in obese men compared to lean men and that this was irrespective of adipose tissue distribution. If this is also true in obese mice, then the lipolysis rate may already be close to maximal and hence NA stimulation will be unable to increase lipolysis significantly.

As previously noted (chapter 2), the *ob/ob* mouse also has a variety of inherent metabolic disturbances, which may include catecholamine resistance. This is seen in obese human patients and may be due to decreased expression of β_2 adrenoceptors (Reynisdottir *et al.*, 1994). Another group has noted decreased expression and function of HSL in abdominal subcutaneous adipocytes from obese humans, which may also account for the lack of response to NA (Large *et al.*, 1999). Certainly within humans intra-abdominal obesity as seen in *ob/ob* mice is deleterious to health. This is proposed to be predominantly due to

increased NEFA's from the visceral region draining to the liver, facilitating gluconeogenesis and reducing insulin-mediated inhibition of hepatic glucose output (Boden, 1997). Further studies could involve assays of β_2 adrenoceptor binding, activity and mRNA levels as well as HSL expression and activity within the *ob/ob* model.

4.3.7 - Effect of noradrenaline on lipolysis from human adipocytes in subcutaneous fat

The limited availability of human adipose tissue placed constraints on the breadth of the studies that could be undertaken. Although the following experiments form a minor part of the present study they do serve to confirm the activity of M2 in a clinical situation of obesity.

In theory, the results from the present experiments should resemble those of the lean mice as the patient had a Body Mass Index (BMI) of 27. The basal lipolysis level (fig 4.8) compares well with the experiments in lean mouse adipocytes, being 0.0175 mM glycerol/10⁵cells compared to a range of 0.01 - 0.02 mM glycerol/10⁵cells produced from the mouse adipocytes. However the lipolytic response to NA shown by these human subcutaneous adipocytes was not as great as that seen with lean mouse adipocytes (fig 4.3.1 and 4.3.2). Nevertheless, certain similarities are evident, such as a significant response to NA at 10⁻⁷M and the magnitude of the response itself, which was approximately 2 fold above basal. The inability of 10⁻⁶M NA to significantly stimulate lipolysis by the human subcutaneous fat is unlikely to be due to receptor down-regulation due to the acute incubation period used. It may be partially explained by the use of anaesthetics for the procedure (in this case a hernia repair). Our understanding of the effects of local and general anaesthetics on human adipocyte metabolism is somewhat confusing, with some studies indicating inhibition of β agonist-stimulated lipolysis with the general anaesthetic halothane (Bennin *et al.*, 1976) and others reporting no difference (Rosenqvist *et al.*, 1977). In 1997 Large *et al* found that both the local anaesthetic lidocaine and general aesthetic propofol had no effect on agonist-stimulated lipolysis in the isolated adipocytes of obese patients. The use of micro-dialysis may be indicated as this allows the local introduction of test compounds and the determination of their lipolytic effect *in situ* (Arner and Burlow, 1993).

4.3.8 - Effect of noradrenaline on lipolysis from human visceral adipocytes.

Again we see that the basal level of glycerol release is consistent, being approximately 0.02 mM glycerol/ 10^5 cells from human visceral adipocytes (fig 4.9). Due to the relative success of the NA to induce a lipolytic response in subcutaneous fat (fig 4.8) it may be inferred that this lack of response in visceral adipocytes is a genuine phenomenon. On first inspection of the case notes this lack of response is unlikely to be due to the catecholamine resistance seen in obese subjects (see previously) as the patient had a BMI of 16 and is underweight (normal BMI range 18.5-25). However, the patient was being given a colectomy for an unclear underlying condition. This condition itself could well play a part in the lack of response of the adipocytes from the patient's viscera. Certainly such a low BMI would probably alter fat metabolism as most adipocyte stores would have been mobilised. From the overall results, the use of M2 to stimulate lipolysis in adipocytes from visceral fat was warranted, as M2 generated a lipolytic response in the *ob/ob* mice adipocytes after NA had failed to do so (figs 4.6 and 4.7).

4.3.9 - Effect of M2 on lipolysis in human visceral adipocytes

The fact that M2 increased glycerol release from subcutaneous adipocytes at all concentrations tested (10^{-6} M- 10^{-10} M) indicates that this agent is a potent and acutely active lipolytic agent (fig 4.10). The basal (control) value of 0.0025 mM glycerol/ 10^5 cells is extremely low and well below that seen in the mouse adipocytes and other human experiments. This is surprising, given the high basal lipolysis seen in the *ob/ob* mouse, which has a pathophysiology similar to this patient. The patient had a BMI of 29 and type 2 diabetes mellitus.

As discussed in the introduction, type 2 diabetes and obesity are frequently seen together, and active loss of weight often improves glycaemic control in obese diabetic subjects. Therefore, the first part of any treatment for the obese type 2 diabetic patient should encourage weight loss. This might involve the use of compounds that cause the release of glycerol from and hence reduce the size of the fat stores. M2 is clearly able to do this in

isolated adipocytes. Unfortunately time did not allow further studies of this area to identify the mechanism involved or the clinical significance. Having found an intervention such as M2 to liberate fatty acids from stored fat, the next stage in the treatment programme would ideally involve a similar increase in the oxidation of the liberated fatty acids, otherwise triglyceride (TG) would be regenerated via the TG/fatty acid cycle and the excess lipids in the circulation might increase atherogenic risk. Also, high free fatty acid's would damage the β cells and worsen the diabetes. Thus it would be potentially useful to combine use of M2 with an agent to increase metabolic rate.

Indeed, such agents could be the β_3 agonists. As well as inducing an increase in lipolysis via the classic G-protein/cyclic AMP pathway (Arner and Eckel, 1998), β_3 adrenoceptors mediate anti-obesity and anti-diabetic effects in rodents (Weyer *et al.*, 1999). The anti-obesity effect is thought to be due to increased energy expenditure in brown adipose tissue (BAT)(Arch and Wilson, 1996; Himms-Hagen and Danforth, 1996 and Danforth and Himms-Hagen., 1997). This increased energy expenditure is mediated by uncoupling protein 1 (UCP 1), (a protein only expressed in brown adipocytes) which uncouples mitochondrial respiration from ATP generation (Himms-Hagen and Ricquier, 1998). Given that β_3 stimulation also induces lipolysis from white adipose tissue (WAT), β_3 agonists provide a potential treatment where liberated free fatty acids could be utilised in the BAT for thermogenesis (Himms-Hagen and Danforth, 1996). Anti-diabetic effects were thought to be due to increased insulin-stimulated glucose uptake (De Souza *et al.*, 1997; Liu *et al.*, 1998) or reduced plasma FFA concentrations removing the inhibitory effect on glucose uptake (Arch and Wilson, 1996). Recently it has been reported that the β_3 agonist AJ-9677 ameliorates insulin resistance by increasing UCP 1 in both BAT *and* WAT, increasing GLUT 4 mRNA and decreasing nascent TNF α and FFA production (Kato *et al.*, 2001). Unfortunately, the anti-obesity effects have largely been non-reproducible in human studies (Mitchell *et al.*, 1989; Connacher *et al.*, 1992; Haesler *et al.*, 1994 and Goldberg *et al.*, 1995) with two exceptions using the highly selective β_3 agonist CL316,243 (Weyer *et al.*, 1998). These studies also showed anti-diabetic effects.

The compound lipoic acid might have limited use for this purpose as the present experiments found that lipoic acid exerted anabolic effects similar to insulin, at least in the murine model used. However, since lipoic acid did not have inherent lipolytic activity it is probably not a good choice of agent to use alongside M2. A possible candidate that both liberates fatty acids and induces the oxidation of fat is lipid mobilising factor (LMF). Such a protein is produced chronically and morbidly in cancer patients and is thought to be responsible for the cachexia associated with tumours (Beck and Tisdale, 1987). High LMF levels correlate with poor survival rates in cancer patients, particularly those with terminal pancreatic carcinomas (De Wys, 1985), but there is evidence that LMF wasting may be controllable by the fish oil eicosapenatoic acid (Price and Tisdale, 1998). However, the possible use of such a potent wasting factor in any future clinical trials for the treatment of obesity awaits further evaluation of the structure and mode of action of this factor.

4.4 - Conclusion

The clinical adipocyte samples used in the present experimental design pose some limitations e.g. the use of anaesthesia, certain uncontrollable patient variables such as extent and distribution of adiposity, limited numbers of subjects and presence of type 2 diabetes. However these preliminary studies have yielded encouraging results with the use of M2, which should be investigated further. Lipoic acid showed disappointingly little effect on lipolytic activity, which may reflect a different bias of the post-receptor insulin-signalling pathway controlling lipolysis in fat compared with glucose utilisation in muscle. The initial hypothesis that a compound that causes glucose uptake in muscle (see lipoic acid chapter) may have catabolic effects in adipocytes was not borne out by the current investigation. These experiments weigh against the use of lipoic acid to overcome insulin resistance in obese subjects *alongside* the administration of a lipolytic agent such as M2, as lipoic acid may counter this effect. However, initial results with M2 in *ob/ob* mice and an obese diabetic subject were encouraging. Finally, thanks to Mr M Obeid (Surgeon at City Hospital, Birmingham) for making tissue samples available and

collaborating in this project. This work represents the preliminary part of an on-going project, which will be based on the present studies.

Chapter 5 - Isoferulic acid

5.1 - Introduction

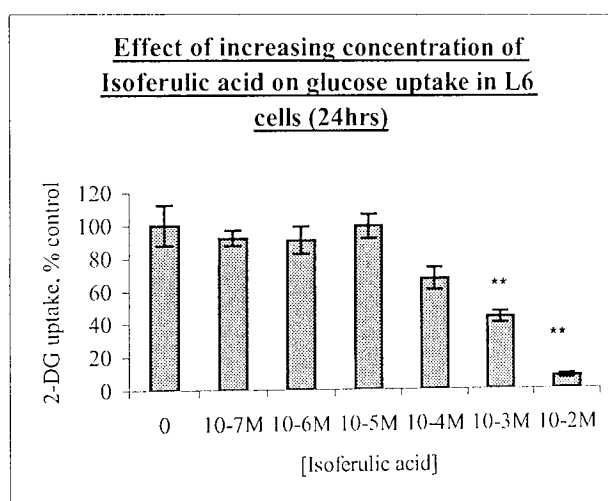
Isoferulic acid (3,4 dimethoxycinnamic acid) is found in a number of plants used in Chinese herbal medicine such as *Cimicifuga species*, *Convolvulus Hystrix* and *Veronicastrum sibiricum* (Sakai *et al.*, 2001; Dawidar *et al.*, 2000; Zhou and Meng, 1992). It is known to have numerous diverse pharmacological effects, which are summarised in table 5.1 below:

Table 5.1 – Biological effects of Isoferulic acid

Biological effect	Author
<i>Anti-inflammatory effect</i> - blocks production of inflammatory proteins in mice and mice cell line.	Sakai <i>et al.</i> , 1999 and 2001 Hirabayashi <i>et al.</i> , 1995
<i>Anti-cancer effect</i> - reduces polyamine levels, which are associated with tumour growth.	Watanabae <i>et al.</i> , 1996
Xanthine oxidase (XO) inhibitor activity - XO is associated with gout, hepatitis and tumours.	Chan <i>et al.</i> , 1995
<i>Analgesic activity</i>	Zhou and Meng, 1992
<i>Antibacterial activity</i> - inhibits growth of <i>Staphylococcus aureus</i> .	Qiao and Chen, 1991
<i>Cholorectic activity</i> - increases bile secretion from liver.	Westendorf and Czok, 1983
<i>Anti-diabetic</i> – lowers blood glucose levels in diabetic rats.	Liu <i>et al.</i> , 1999
<i>Anti-diabetic</i> – increases glucose uptake in mouse myoblast cell line.	Liu <i>et al.</i> , 2001

The anti-diabetic effects of isoferulic acid were considered to be topical and pertinent to this study. It may be that the 1999 study is not as relevant to the aims of this thesis because the rats employed were spontaneously diabetic in a similar way to type 1 diabetics (Liu *et al.*, 1999). The report of increases in glucose uptake in a myoblast cell line provides a rationale for the employment of the L6 cells in an attempt to duplicate these findings.

5.2 - Results



*Fig 5.1 – Effect of isoferulic acid ($10^{-8}M - 10^{-2}M$) on glucose uptake measured by 3H 2DG uptake in L6 cells after 24 hours. Increasing concentrations of isoferulic acid had no positive effect on glucose uptake, but higher concentrations were found to be inhibitory (** $P < 0.01$ versus control, no isoferulic acid). Results are means \pm SE, $n=6$.*

The effect of isoferulic acid ($10^{-2}M - 10^{-7}M$) on glucose uptake by L6 cells was determined during a 24-hour incubation at $37^{\circ}C$. The presence of rising concentrations of

isoferulic acid had no significant effect on glucose utilisation until $10^{-3}M$ and $10^{-2}M$, which lowered 2-DG uptake by 50% and 95% respectively (** $P<0.01$).

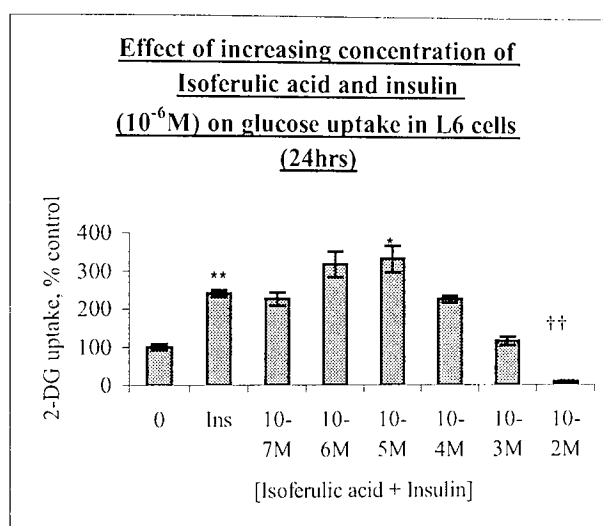


Fig 5.2 – Effect of isoferulic acid ($10^{-7}M - 10^{-2}M$) and $10^{-6}M$ insulin on glucose uptake measured by 3H 2DG uptake in L6 cells after 24hrs incubation. Results are means \pm SE, $n=6$. ** $P<0.01$ versus control (no isoferulic acid); * $P<0.05$ versus insulin alone; †† $P<0.01$ versus insulin alone.

The effect of isoferulic acid ($10^{-8}M - 10^{-2}M$) and insulin ($10^{-6}M$) on glucose uptake by L6 cells was determined during a 24-hour incubation at $37^{\circ}C$. Insulin alone raised glucose uptake by 150% compared to control (** $P<0.01$). The presence of $10^{-5}M$ isoferulic acid and insulin caused a 25% increase in 2DG uptake compared to insulin alone (* $P<0.05$). The combination of $10^{-2}M$ isoferulic acid and insulin resulted in a 95% decreased 2-DG uptake versus insulin alone (†† $P<0.01$).

Since incubation with 10^{-5} M isoferulic acid for 24hr increased insulin-stimulated glucose uptake by the L6 cells, studies were undertaken to investigate whether this concentration of isoferulic acid was effective during shorter incubation periods (2,4 and 8 hrs).

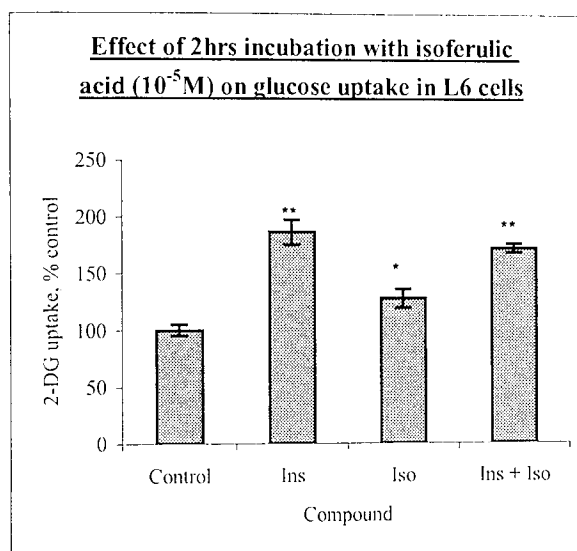


Fig 5.3 – Effect of isoferulic acid (10^{-5} M) and insulin (10^{-6} M) on glucose uptake measured by 3 H 2DG uptake in L6 cells after 2hrs incubation. Results are means \pm SE, $n=6$. * $P<0.05$; ** $P<0.01$ versus control (no isoferulic acid).

Accordingly, the effect of isoferulic acid (10^{-5} M) and insulin (10^{-6} M) on glucose uptake by L6 cells was assessed during a much shorter (2 hrs) incubation at 37°C . Insulin alone raised glucose uptake by 75% compared to control (** $P<0.01$). The presence of 10^{-5} M isoferulic acid caused a small but significant rise in 2DG of approximately 25% (* $P<0.05$). The combination of isoferulic acid and insulin resulted in a 70% increase uptake versus control (** $P<0.01$), but did not increase glucose uptake beyond that produced by insulin alone.

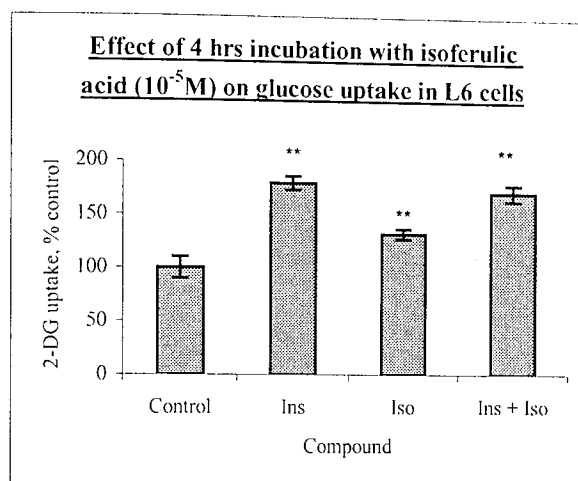


Fig 5.4 - Effect of isoferulic acid ($10^{-5}M$) and insulin ($10^{-6}M$) on glucose uptake measured by 3H 2DG uptake in L6 cells after 4hrs incubation. Results are means \pm SE, $n=6$. ** $P<0.01$ versus control (no isoferulic acid).

After incubation for 4hrs, insulin alone ($10^{-6}M$) raised glucose uptake by 75% compared to control (** $P<0.01$). The presence of $10^{-5}M$ isoferulic acid alone caused a 30% rise in 2DG uptake (** $P<0.01$), while the combination of isoferulic acid and insulin resulted in a 70% increase uptake versus control (** $P<0.01$). Thus, as noted at 2hrs, isoferulic acid alone produced a small but significant increase in glucose uptake, but in the presence of insulin, isoferulic acid did not produce any further increase in glucose uptake than insulin alone.

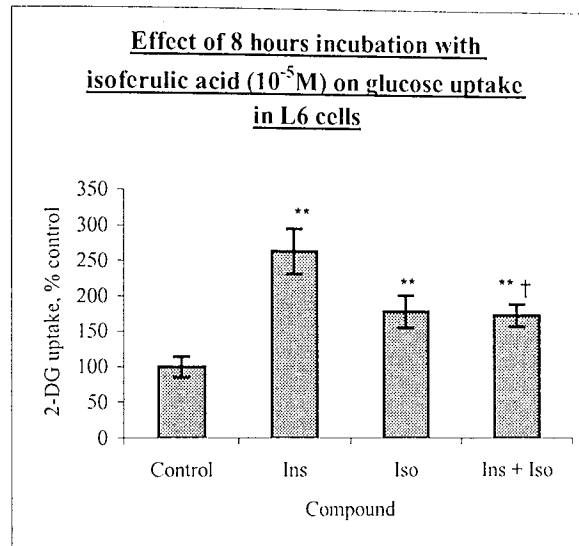


Fig 5.5 – Effect of isoferulic acid ($10^{-5}M$) and insulin ($10^{-6}M$) on glucose uptake measured by 3H 2DG uptake in L6 cells after 8hrs incubation. Results are means \pm SE, $n=6$. ** $P<0.01$ versus control (no isoferulic acid); † $P<0.05$ versus insulin alone.

After incubation for 8hrs, insulin alone ($10^{-6}M$) raised glucose uptake by over 150% compared to control (** $P<0.01$). The presence of $10^{-5}M$ isoferulic acid alone caused a 75% rise in 2DG uptake (** $P<0.01$). This effect was greater ($*P<0.05$) in magnitude than the 25-30% effect seen after 2 and 4hrs incubation. The combination of isoferulic acid and insulin also resulted in a similar (73%) increase in uptake versus control (** $P<0.01$), which represented a decrease in glucose uptake versus insulin alone († $P<0.05$).

5.3 - Discussion

Incubation of L6 cells with isoferulic acid for 24 hours (fig 5.1) had little effect on glucose uptake except at 10^{-3} M and 10^{-2} M concentrations, which decreased glucose uptake (**P<0.01). However, this effect appeared to be directly due to cytotoxicity as evidenced by detachment of the monolayer from the well bases and prolific positive staining during trypan blue exclusion studies (not shown). A repeat experiment with insulin (10^{-6} M) produced similar results (fig 5.2). Insulin alone had a predictable positive effect in glucose uptake, raising uptake by almost 150% versus control. Of note in this figure is the apparent ability of 10^{-5} M isoferulic acid and 10^{-6} M insulin in combination to raise glucose uptake significantly above that of the insulin alone. Thus subsequent experiments employed 10^{-5} M isoferulic acid both with and without 10^{-6} M insulin, and the time course of the effect was investigated.

Figure 5.3 shows the results of a 2-hour incubation. Insulin increased glucose uptake by nearly 100% (**P<0.01), which is typical in these L6 cells. Interestingly, 10^{-5} M isoferulic acid alone increased glucose uptake by 30% (*P<0.01). The combination of isoferulic acid and insulin increased glucose uptake to a similar extent as insulin alone. No such effect of 10^{-5} M isoferulic acid alone was seen in the original concentration (24hr) incubations. This could perhaps be explained by the great difference in time during which isoferulic acid may be degraded and the stimulatory effect in glucose uptake then lost. Indeed, the early (2hr) effect of isoferulic acid was modest in magnitude (25% increase), and the design of the experiments in which glucose uptake is measured after the incubation period would not register an effect if the isoferulic acid was no longer active. Thus the effect seen at 2 hours would be missed by incubation over 24 hours as the phenomenon had already occurred. Hence the importance of the time course studies with isoferulic acid.

Data in fig 5.4 show a similar outcome after a 4hr incubation to that noted after a 2hr incubation. At 4hr we see almost identical glucose uptake with insulin plus isoferulic acid and insulin alone compared with uptake after 2hr. However, after incubation with

isoferulic acid alone for 4hr, glucose uptake was raised by 35% (**P<0.01)(c.f. 10^{-5} M isoferulic acid alone at 2 hours).

As we would perhaps expect, the experiment employing an 8hr incubation period showed an elevated insulin alone response by approximately 150% greater than control (**P<0.01). At this time point we see the largest increase in glucose uptake elicited by isoferulic acid alone at around 75% (**P<0.01). At first glance it may seem that employing this incubation period (8hr) with the combination of isoferulic acid and insulin results in a drop in glucose uptake compared to that seen at 2 and 4hrs. However, this is not the case as uptake is increased approximately 75%, as seen at the previous two points. In summary so far, after 2 and 4hr incubation times insulin alone increased glucose uptake by around 75% then increased to >150% at 8hr. Isoferulic acid (10^{-5} M) increased glucose uptake in a time dependent manner, being 30, 35 and 75% at 2, 4 and 8 hrs respectively. Isoferulic acid and insulin in combination caused a similar increase in glucose uptake to insulin alone at 2, 4 and 8hrs.

The concentration of insulin used in these studies (10^{-6} M) produces a near maximal stimulation of glucose uptake in L6 cells (see methods, chapter 2). Since isoferulic acid was unable to enhance glucose uptake in the presence of this high concentration of insulin (despite increases in glucose uptake in the absence of added insulin) it is possible that isoferulic acid is acting via insulin signalling intermediates. These results suggest that isoferulic acid alone increases glucose uptake in the short term (up to 8hr) in a similar manner to insulin itself, possibly acting via a post-receptor insulin-signalling pathway. This would be consistent with the lack of an additive effect when combining isoferulic acid and insulin at 2, 4 and 8hrs: thus both compounds could not use the same signalling pathway at the same time to increase glucose uptake beyond the maximum capability of that pathway.

As previously mentioned, Liu *et al* (2001) have found that isoferulic acid enhanced glucose uptake into C₂C₁₂ muscle cells. They discovered that a concentration of 10^{-4} M isoferulic acid was maximally effective, similar to my experimental observation of

10^{-5} M. There is a significant difference between the two studies however. Liu *et al* (2001) found that the beneficial effect of isoferulic acid began at 2 minutes and was maximal at 30 minutes. This is obviously a much shorter incubation period than the shortest employed in these experiments (2hr) and hence would have led to the effects reported by Liu *et al* being missed by the current study. However such very short effects would not be likely to be of therapeutic value in the treatment of diabetes, hence the present attention to longer time periods.

Liu *et al* (2001) provided evidence that the isoferulic acid effect on C₂C₁₂ cells was initiated by engagement of the α_{1A} -adenoreceptor. This is the receptor responsible for increased glucose uptake in response to catecholamines in skeletal muscle (Saitoh *et al.*, 1974). Both phospholipase C (PLC) and protein kinase C (PKC) are thought to be activated by α_{1A} -adenoreceptor action (Minneman *et al.*, 1988; Hieble *et al.*, 1995) and both of these signalling intermediates have been linked to the glucose transporter system (Ishizuka *et al.*, 1990; Van Epps-Fung *et al.*, 1997). PLC is thought to contribute to the production of inositol phosphates, which enhance GLUT4 translocation to the membrane (Van Epps-Fung *et al.*, 1997). It is thought that atypical PKCs are needed for insulin-stimulated glucose transport (Saltiel and Kahn, 2001) and this may be the point (down stream of PI3K) that the α_{1A} -adenoreceptor and insulin-signalling pathways cross talk.

Whilst my study strongly suggests that α_{1A} -adenoreceptors must be present and active in the L6 cell line, further work could include receptor binding and/or receptor antagonist studies as used by Liu *et al* (2001). Also, the employment of wortmannin would provide evidence as to the involvement or non-involvement of PI3-K. Overall, isoferulic acid shows potential for alleviating insulin resistance in skeletal muscle, the primary glucose disposal site in response to insulin (De-Fronzo, 1988). However, since the findings of Liu *et al* (2001) only became known towards the end of my research work it was not possible to explore this issue further.

It is conceivable that the report by Liu *et al* (2001), which appeared after I had undertaken the present studies, is compatible with the current investigations. The main

difference concerns the time course. It is possible that isoferulic acid could act initially (over 30 min) by one mechanism (α_{1A} -adenoreceptor) and later (2-8hr) by another mechanism (insulin-like). Alternatively, the temporal sensitivity of C₂C₁₂ cells could be much more rapid than L6 cells. Given the modest magnitude of the increase in glucose uptake caused by isoferulic acid in the present study, it was not deemed a priority for detailed mechanistic analysis.

5.4 - Conclusion

In conclusion therefore, the present studies in L6 muscle cells indicate that isoferulic acid, at a concentration of about 10^{-5} M, provides a small but significant stimulus to glucose uptake in the absence of added insulin. The effect is maximal at about 8hr and lost by 24hr. Isoferulic acid did not increase the glucose uptake effect of a high concentration of insulin (10^{-6} M) and high concentrations of isoferulic acid ($>10^{-4}$ M) were apparently toxic to the cells. The study provides support for claims in the literature that plants rich in isoferulic acid could benefit patients with non-insulin dependent forms of diabetes.

Chapter 6 - Other agents

6.1 - TNF α

6.1.1 - Introduction

TNF α is one of two related cytokines produced by macrophages (TNF α) and some T cells (TNF β). Both are cytotoxic to tumour cells and contribute to the inflammatory response (Kuby, 1997). In more detail, TNF α was first isolated as the causative agent of tumour necrosis in animals infected with bacteria by Penicca *et al* (1984). It was also found to be the mediator of the cachexia and hypertriglyceridemia seen in animals infected with parasites (Beuther *et al.*, 1985). TNF α has a multitude of effects including tumour cell lysis, apoptotic cell death induction, stimulation of IL-2, granulocyte macrophage colony stimulating factor (GM-CSF) production and suppression of lipoprotein lipase action (Smith *et al.*, 1994; Beuther *et al.*, 1989 and Vilcek *et al.*, 1991). TNF α also has effects on lipid metabolism in rats and humans, where *in vivo* administration increases serum triglyceride and very low density lipoprotein concentrations (Fengold & Grunfeld, 1991, 1992). Over-expression of TNF α in adipocytes has been shown to occur in many different genetic rodent models of obesity (Hotamisligil *et al.*, 1993). TNF α works in these systems by affecting insulin receptor signal transduction, more specifically, by an increase in the phosphorylation of IRS-1, making it inhibitory (Hotamisligil *et al.*, 1996). TNF α also down-regulates the expression of GLUT4 and lipoprotein lipase in adipocytes (Hotamisligil *et al.*, 1993, 1996). Moreover, in targeted mutations of TNF α and TNF α receptor genes, insulin sensitivity has been seen to improve (Uysal *et al.*, 1997; Ventre, 1997). Studies on TNF α derived insulin resistance, in tissues other than adipose such as hepatic and skeletal muscle have been undertaken. In 1996 Soloman *et al* demonstrated that TNF α induced insulin resistance in H411E liver cells can be reversed by the anti-diabetic agent Pioglitazone. However, an earlier study by Hotamisligil *et al* (1993) in obese diabetic

rodents using TNF α receptor-specific IgG showed that hepatic glucose output was unaffected, making it unlikely that TNF α induces insulin resistance in the hepatic system.

With regard to skeletal muscle, del Aguila *et al* (1999) found that TNF α impairs both insulin signal transduction and insulin stimulated glucose uptake in cultured C₂C₁₂ myocytes. This was thought to be via the inhibition of IRS-1 and 2 mediated PI3-K activation. This adds weight to an earlier study in pini28 mouse myocytes which also showed TNF α mediated down-regulation of insulin stimulated insulin receptor activity (Storz *et al.*, 1998). In contrast, Nolte *et al* (1998) found that acute *in vitro* exposure of soleus and epitrochlearis muscles to TNF α had no such inhibitory action on the insulin stimulated signal transduction mechanisms of the insulin receptor. They concluded that 'in contrast to Fao hepatoma cells and 3T3-L1 fibroblasts, skeletal muscle *does not* develop insulin resistance in response to short term exposure to TNF'.

In insulin resistant cancer patients (in which the wasting is believed to be TNF α mediated) it has been discovered that there was a significant increase of TNF α mRNA and down regulation of GLUT 4 in peripheral tissues (Noguchi *et al.*, 1998). In another study in humans however, cultured muscle cells from patients with type 2 diabetes were found to show *increased* glucose uptake upon prolonged and acute treatment with TNF α (Ciaraldi *et al.*, 1998). They concluded that in type 2 subjects increased glucose uptake in conditions of excess TNF might have a compensatory role. In the face of such contradictory studies, there is obviously a requirement for further studies on TNF α in whole muscle preparations and myocyte cultures.

The present study has utilised diaphragm and quadriceps muscles from lean +/+ mice. Whole muscle preparations of the above were employed as outlined in chapter 2 to assess glucose uptake and utilisation. L6 cells were also utilised, with glucose uptake being measured by ³H 2DG uptake as described in chapter 2.

6.1.2 – Results

6.1.2.1 - Effect of TNF α on glucose uptake in two lean muscles

Data reported in this section concern the effect of TNF α on glucose uptake by muscles of lean mice, measured by ^3H 2DG uptake over 20 minutes after 2 hours incubation at 37°C.

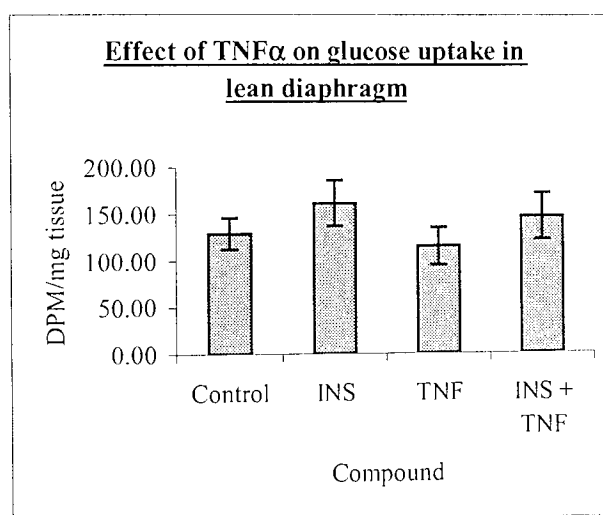


Fig 6.1.1 - Glucose uptake measured by ^3H 2DG uptake in murine diaphragm muscle was not affected by the addition of 10^{-11} M TNF α . Results are means \pm SE, $n=6$.

Control diaphragm muscles took up about 125 DPM/mg tissue of ^3H 2DG over the 2-hour period. Insulin (10^{-6} M) caused a small increase in mean ^3H 2DG uptake by approximately 17%, which was not significant. In the presence of 10^{-11} M TNF α ^3H 2DG uptake was reduced by 20%, however this decrease was not significant. The mean value for ^3H 2DG uptake was increased by approximately 5% by TNF α and insulin in combination compared to TNF α alone ($P>0.05$). Insulin (10^{-6} M) had no effect on mean ^3H 2DG uptake in the present study. As mentioned earlier, it is possible that the prior 2-

hour incubation may have somewhat saturated glucose accumulation by the tissue. This may have compromised the ability of the tissue to show a subsequent insulin stimulated increase in ^3H 2DG uptake. This issue pertains to each of the studies in these tissues previously incubated for 2 hours, and this potential limitation of the method will be reviewed in the discussion section.

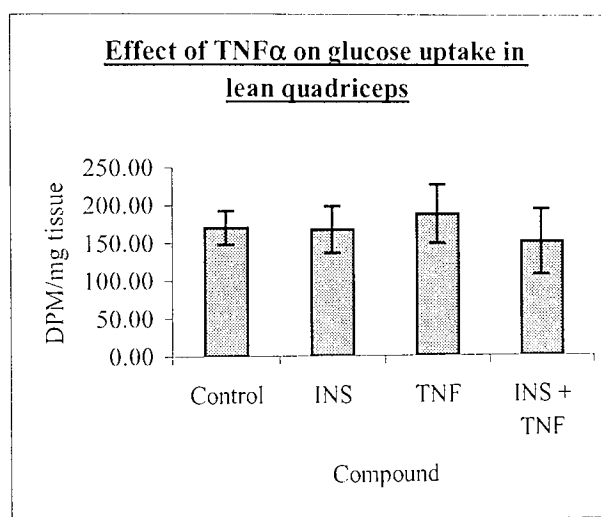


Fig 6.1.2 - Glucose uptake measured by ^3H 2DG uptake in murine quadriceps muscle was not affected by the addition of 10^{-11} M $\text{TNF}\alpha$. Results are means \pm SE, $n=6$.

Control quadriceps muscles took up about 175 DPM/mg tissue of ^3H 2DG over the 2 hour period. Insulin (10^{-6} M) had no effect on ^3H 2DG uptake. In the presence of 10^{-11} M $\text{TNF}\alpha$ ^3H 2DG uptake was slightly increased by 5%, however this increase was not significant. The mean value for ^3H 2DG uptake was reduced by approximately 14% by $\text{TNF}\alpha$ and insulin in combination compared to $\text{TNF}\alpha$ alone ($P>0.05$).

6.1.2.2 - TNF α experiment (^3H 2 deoxyglucose)

This graph shows an initial preliminary study into the effect of TNF α on glucose uptake in L6 cells, measured by ^3H 2DG uptake over 10 minutes after a 2-hour incubation at 37°C.

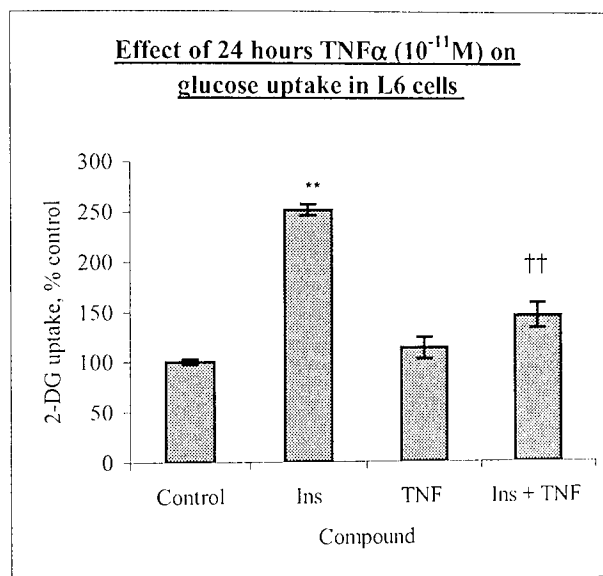


Fig 6.1.3 - Glucose uptake in L6 cells was affected by the addition of 10^{-11}M TNF α for 24 hours. Results are means \pm SE, $n=12$.

The effect of TNF α (10^{-11}M) on glucose uptake by L6 cells was determined during a 2 hour incubation at 37°C (95% $\text{CO}_2/5\%$ O_2). Insulin (10^{-6}M) caused a 150% increase in ^3H 2DG uptake (** $P<0.01$). The presence of 10^{-11}M TNF α increased ^3H 2DG uptake by 10% ($P>0.01$). The combination of 10^{-11}M TNF α and insulin had no effect on mean ^3H 2DG uptake compared to control ($P>0.05$). However, this combination *decreased* uptake by 80% compared to insulin alone (†† $P<0.01$)

6.1.3 – Discussion

6.1.3.1 - Effect of TNF α on glucose uptake in various lean muscles

Control values for each muscle studied were fairly similar, being 125 DPM/mg in the diaphragm and approximately 165 DPM/mg in the quadriceps (figs 6.1.1 and 6.1.2). From previous comments in the lipoic acid chapter (3) we would expect quadriceps to have a higher DPM/mg at basal glucose uptake. This is because the quadriceps consists of predominantly type 1 fibres whereas diaphragm is a mixture of type 1 and 2 fibres. This could perhaps be due to the presence of endogenous insulin prior to excision stimulating a slight increase in basal uptake in the diaphragm. Insulin response in all the muscle studied was poor and as these experiments were carried out in parallel to those with lipoic acid this poor result was possibly due to factors outlined in the lipoic acid chapter (3). These were saturation of the muscle with glucose prior to ^3H 2DG addition and the relatively low half-life of insulin (*c.* 8-10 mins). In the case of lipoic acid a glucose assay was subsequently employed successfully. However, the high price of TNF α along with the fact that the use of more lean and perhaps obese mice would have been unjustified without a positive result led to the use of L6 cultured cells, to which we will now turn.

6.1.3.2 - Effect of TNF α on glucose uptake in L6 muscle cells (measured by ^3H 2DG uptake)

Insulin caused a typical increase in glucose uptake over 24hrs, being approximately 150% above basal (fig 6.1.3). The presence of TNF α alone had no significant effect on basal uptake, though there was a slight (*c.* 10%) increase. The combination of insulin (10^{-6}M) and TNF α (10^{-11}M) decreased uptake by 80% compared to insulin alone. It is likely that in this study TNF α is modulating insulin-mediated signal transduction mechanisms as basal uptake was not reduced and thus this inhibitory effect is only seen in the presence of insulin signalling. Current understanding of TNF α mediated insulin resistance *in vitro* indicates that this is the case (Hotamisligil and Spiegelman, 1994;

Hotamisligil *et al.*, 1994; Hauner *et al.*, 1995; Kellerer and Haring, 1995). Tartaglia and Goeddel (1992) reported that a TNF α induced insulin resistant state occurs due to the action of TNF α on the p55 or p75 receptors. Demonstrably TNF α induces phosphorylation of serine residues on the insulin receptor substrate IRS-1, which is thought to inactivate or attenuate insulin signalling (Hotamisligil and Spiegelman, 1994; Guo and Donner, 1996). This inactivation is mediated through neutral sphingomyelinase (SMase)(Peraldi *et al.*, 1996). SMase hydrolyses sphingomyelin within the plasma membrane to ceramide, which in turn activates ceramide activated protein kinase (Liu *et al.*, 1994). It is this kinase that is thought to induce the serine phosphorylation of IRS-1, leading to inhibition (Hotamisligil *et al.*, 1996). Ceramide is therefore thought to mediate the effects of TNF α on insulin-stimulated cell signalling and it has been reported that GLUT4 gene down-regulation occurs in 3T3-L1 adipocytes in response to intracellular ceramide signals (Long and Pekala, 1996). A study in 1995 by Begum and Ragolia in rat skeletal muscle cells has shown that TNF α 's inhibition of glycogen synthesis acts via ceramide production. TNF α also induces dephosphorylation of mitogen-activated protein kinase (MAPK) inhibiting activation of MAPK by insulin. (Begum and Ragolia, 1996). It has been shown that the TNF receptor 1 (TNFR1) subtype has a large contribution in TNF-mediated insulin resistance (Sethi *et al.*, 2000). Whilst the overall mechanisms are unclear this TNF-mediated insulin resistance is now thought to involve PLC γ , PKC ζ and PKB (Storz *et al.*, 1998 and Ravichandran *et al.*, 2001). Given PLC and PKC's putative roles in insulin signalling as outlined in the discussion for chapter 5, these two insulin-signalling intermediates are likely candidates for TNF α mediated insulin resistance (Ishizuka *et al.*, 1990; Van Epps-Fung *et al.*, 1997). PKB/Akt is pivotal in signalling downstream of PI3K (see introduction) and hence again would have a profound effect on glucose transport.

Given that skeletal muscle TNF α expression is high in insulin resistant obese and/or diabetic subjects (Sagizadeh *et al.*, 1996) this indicates a possible mechanism by which TNF α could induce insulin resistance as skeletal muscle is the main site of impaired glucose metabolism in diabetes (Haring and Mehnert, 1993). Any agent that could overcome TNF α induced insulin resistance in skeletal muscle would be of immense

clinical importance in the treatment of type 2 diabetes. Thiazolidinediones could be such agents, as this group of drugs can block TNF α action within cells and reduce TNF α expression in animal models of obesity (Hofmann *et al.*, 1994; Ohsumi *et al.*, 1995; Peraldi *et al.*, 1997).

6.2 - Bradykinin

6.2.1 - Introduction

Regular physical exercise is known to reduce future risk of developing type 2 diabetes (Centre for Disease Control USA, 1996; Pickup & Williams, 1997) and is prescribed to patients with type 2 diabetes as this reduces blood glucose concentrations (American Diabetes Association, 1998). The mechanism by which physical exercise (and hence muscle contraction) increases glucose uptake is not known at this time. Since muscle exercise is invariably associated with localised hyperaemia due to increased production of local vasodilator signals, these signals might themselves directly increase tissue uptake of glucose. One such candidate mediator molecule has been postulated in the form of bradykinin. Bradykinin is a natural polypeptide consisting of nine amino acids, which is a potent vasodilator and contractor of smooth muscle. The kinins are not normally found in the blood, but are formed by the action of proteolytic enzymes (kalikreins) on specific plasma globulins called kininogens (Martin, 1994). Conditions that stimulate kinin production include tissue damage, changes in blood pH and temperature, all of which occur during exercise. Release of bradykinin is known to occur when exercise intensity increases above normal (Stebbins *et al.*, 1990).

As indicated previously, insulin-stimulated glucose uptake in muscle is due mainly to the increased translocation of GLUT4 glucose transporters from intra-cellular microsomally localised pools to the plasma membrane (Cushman & Wardzala, 1980; Suzuki & Kono, 1980). It is thought that similar mechanisms are involved in the enhanced glucose uptake seen during exercise (Douen *et al.*, 1990; Goodyear *et al.*, 1991) and that this effect is mediated via an insulin-independent pathway (Lund *et al.*, 1995). Since the effect of

exercise to increase muscle glucose uptake appears to be insulin independent, or at least requires only the presence of a nominal insulin concentration, exercise provides the opportunity to elucidate a novel mechanism for 'physiologically active' increased uptake of glucose by muscle.

Bradykinin receptors are classified into two main subtypes, B₁ and B₂ (Regoli & Barabe, 1980). Most of the *in vivo* actions of bradykinin are thought to be mediated via B₂ receptors and it is known that this receptor subtype is expressed on the surface of skeletal muscle tissues (Figuroa *et al.*, 1994). At the time the present work was under consideration no published papers were found in the literature concerning the possibility of a direct effect of bradykinin on muscle glucose uptake. Thus an initial experiment was undertaken to ascertain if bradykinin could induce an increase in glucose uptake in cultured L6 muscle cells.

Short-term incubations of 1-8 hours were selected for bradykinin because this peptide is known to be relatively unstable in physiological conditions. It has a plasma half-life of less than 5 minutes. It is quickly metabolised by two carboxypeptidases present in plasma known as Kininases I and II (Kininase II is believed to be the same as angiotensin converting enzyme or ACE). Another reason for selecting a short period of incubation is that the effect of exercise to increase glucose uptake is evident within minutes of beginning the exercise. The beneficial effect is usually measurable for several hours after exercise has stopped (Pickup & Williams, 1997).

6.2.2 - Results

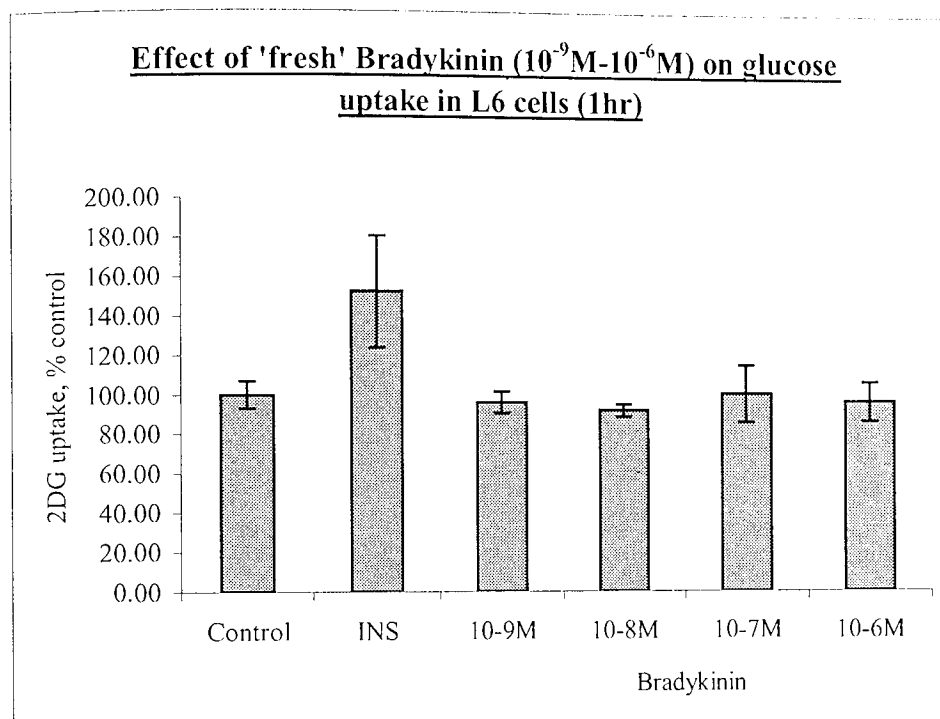


Fig 6.2.1 - 3H 2DG uptake in L6 cells was not affected by the addition of $10^{-9}M - 10^{-6}M$ bradykinin for 1 hour. Results are means \pm SE, $n=4$.

The effect of bradykinin ($10^{-9}M - 10^{-6}M$) on glucose uptake by L6 cells was determined during a 1-hour incubation at $37^{\circ}C$ (95% $CO_2/5\% O_2$). Insulin ($10^{-6}M$) alone raised glucose uptake by 55% ($P>0.05$) in this study. The addition of rising concentrations of bradykinin ($10^{-9}M - 10^{-6}M$) had no significant effect on glucose uptake ($P>0.05$). In case a 1-hour incubation period was not long enough to generate an effect of bradykinin in the cultured muscle cells, longer periods of incubation were tested. A time of 4 hours was selected for the next study.

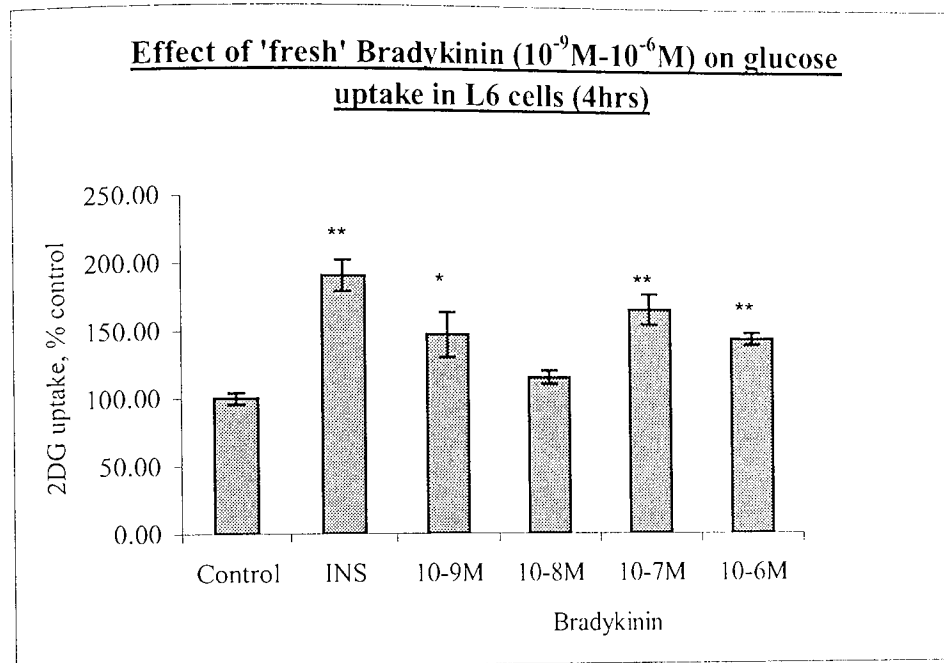


Fig 6.2.2 - 3H 2DG uptake in L6 cells was increased by the addition of $10^{-9}M - 10^{-6}M$ bradykinin and insulin ($10^{-6}M$) for 4 hours. Results are means \pm SE, $n=4$.

After a 4-hour incubation, bradykinin ($10^{-9}M - 10^{-6}M$) increased glucose uptake by L6 cells. Insulin ($10^{-6}M$) alone raised glucose uptake by 90% (** $P < 0.01$). Bradykinin at concentrations of $10^{-9}M$, $10^{-7}M$ and $10^{-6}M$ increased glucose uptake in this study (* $P < 0.05$, ** $P < 0.01$ and ** $P < 0.01$ respectively). This experiment was subsequently repeated at 4 and 8 hours with a larger sample size ($n=6$) to both extend the study and verify these results (figure 6.2.3 and 6.2.4 respectively).

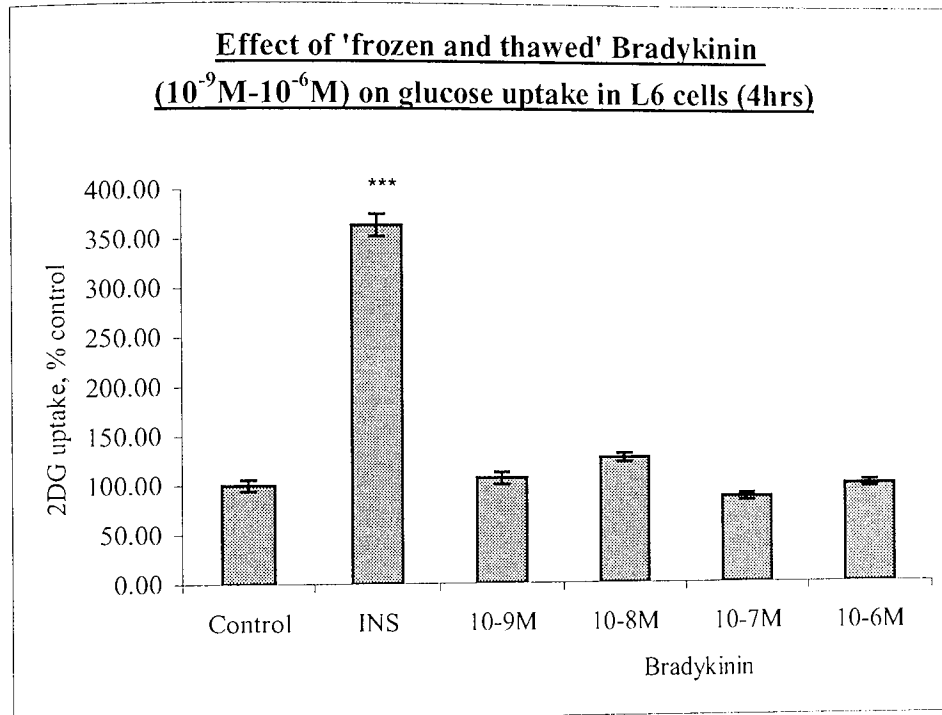


Fig 6.2.3 - ^3H 2DG uptake in L6 cells was unaffected by the addition of $10^{-9}\text{M} - 10^{-6}\text{M}$ bradykinin for 4 hours. Results are means \pm SE, $n=6$.

When the study was repeated with the same bradykinin frozen and thawed, there was no effect of bradykinin concentrations of $10^{-9}\text{M} - 10^{-6}\text{M}$ on glucose uptake. However, insulin alone was still effective, raising glucose uptake by 250% (***) $P < 0.001$.

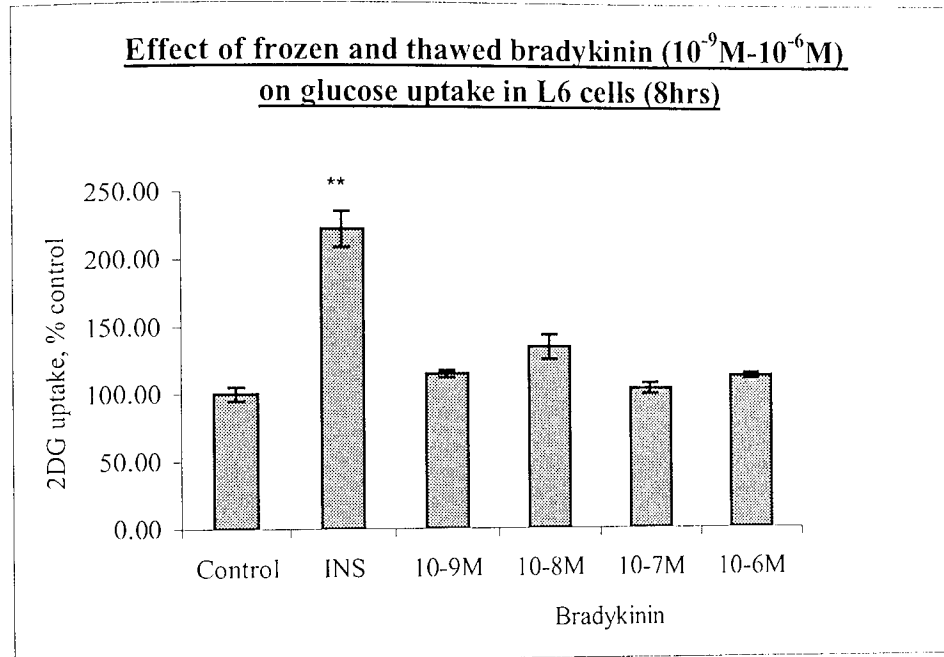


Fig 6.2.4 - 3H 2DG uptake in L6 cells was unaffected by the addition of $10^{-6}M$ – $10^{-9}M$ bradykinin for 8 hours. Results are means \pm SE, $n=6$.

The addition of rising concentrations of bradykinin ($10^{-6}M$ – $10^{-9}M$) had no effect on glucose uptake after incubation for 8 hours ($P>0.05$). As in figure 6.2.3, insulin alone was effective, raising glucose uptake by 225% (** $P<0.01$). In order to investigate bradykinin's apparent loss of efficacy when frozen and thawed, a comparative study was undertaken with 'old' (frozen and thawed) versus newly ordered ('fresh') bradykinin at 4 hours (figures 6.2.5 and 6.2.6 respectively).

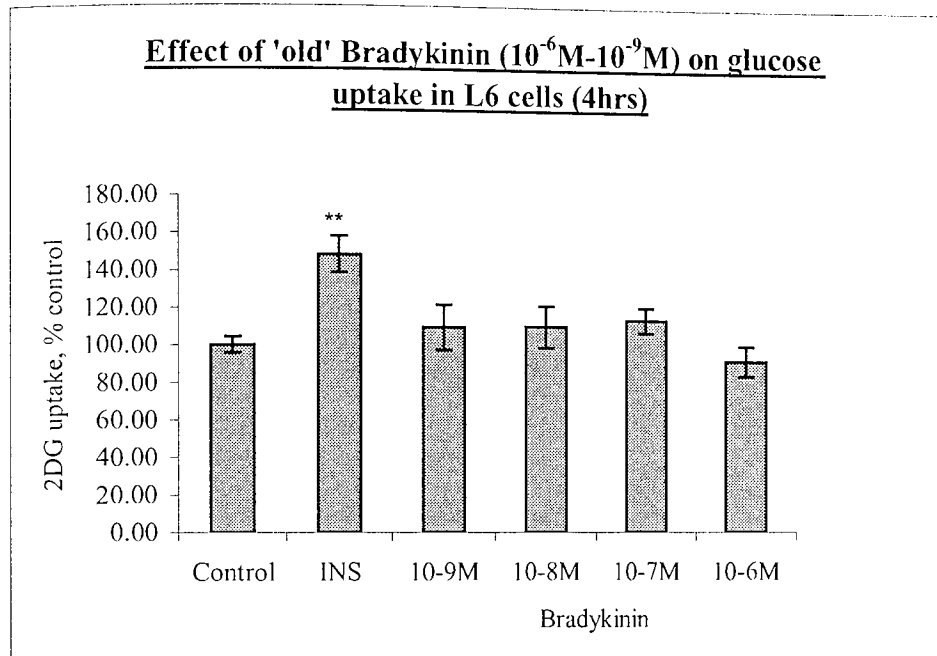


Fig 6.2.5 - 3H 2DG uptake in L6 cells was unaffected by the addition of $10^{-6}M$ – $10^{-9}M$ old batch bradykinin for 4 hours. Results are means \pm SE, $n=6$.

As previously noted, 'old' (freeze-thawed) bradykinin at concentrations ranging from $10^{-9}M$ – $10^{-6}M$ had no effect on glucose uptake by L6 cells during 4 hours incubation at $37^{\circ}C$ ($P>0.05$). Insulin alone raised glucose uptake by 150% (** $P<0.01$).

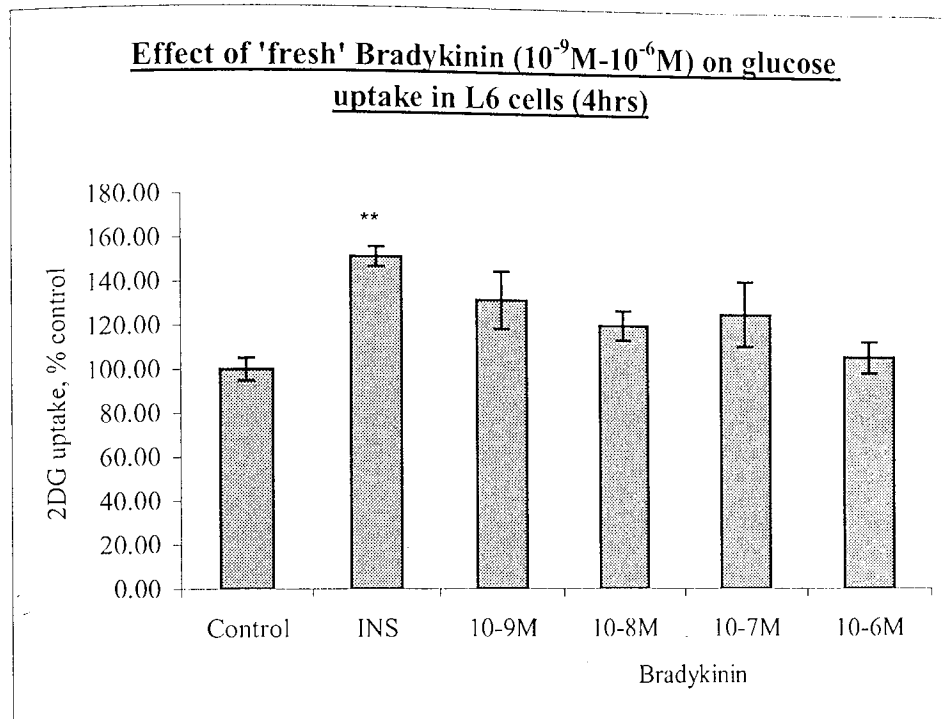


Fig 6.2.6 - ^3H 2DG uptake in L6 cells was unaffected by the addition of 10^{-6} M – 10^{-9} M new batch bradykinin for 4 hours. Results are means \pm SE, $n=6$.

Despite previously increasing glucose uptake (figure 6.2.2), bradykinin (10^{-9} M – 10^{-6} M) did not significantly increase glucose uptake in this study that employed 'fresh' bradykinin during 4 hours incubation at 37°C . Insulin alone raised glucose uptake by 150% (** $P < 0.01$). The final experiment in this series would investigate any possible synergy between bradykinin (fresh) and insulin (10^{-6} M) at 4 hours.

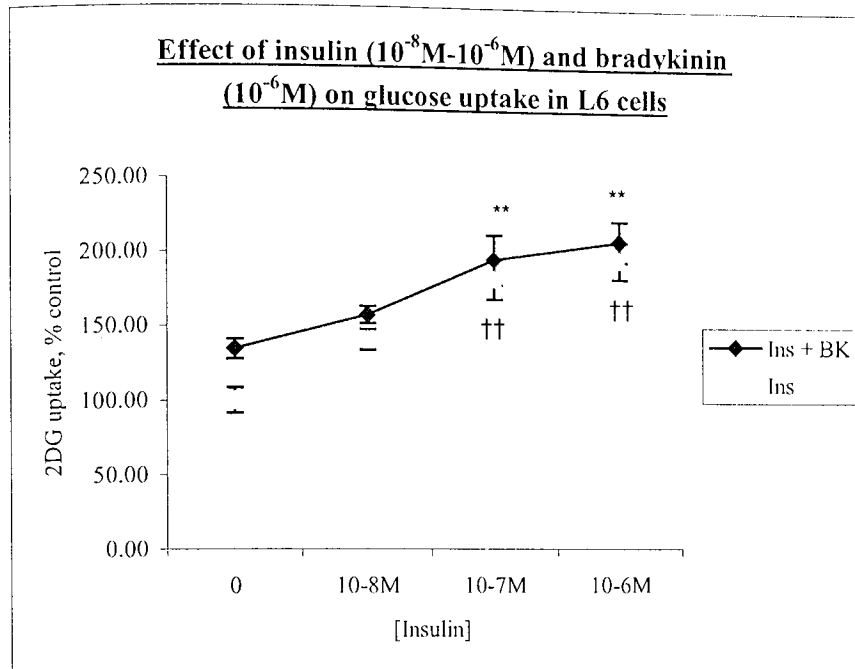


Fig 6.2.7 - 3H 2DG uptake in L6 cells was unaffected by the presence of bradykinin ($10^{-6}M$) and insulin ($10^{-8}M$ - $10^{-6}M$) 4 hours. Results are means \pm SE, $n=6$.

The effect of insulin ($10^{-8}M$ - $10^{-6}M$) and bradykinin ($10^{-6}M$) on glucose uptake by L6 cells was investigated during a 4-hour incubation at $37^{\circ}C$. Insulin alone raised glucose uptake in a concentration related manner, significantly at $10^{-7}M$ and $10^{-6}M$ versus control ($++P<0.01$). The addition of rising concentrations of insulin and bradykinin also increased glucose utilisation, significant at $10^{-7}M$ and $10^{-6}M$ ($**P<0.01$). There was no significant difference between the two curves at the same insulin concentrations ($P>0.05$).

6.2.3 - Discussion

The first in this series of experiments was carried out for an incubation period of 1 hour (fig 6.2.1). Insulin stimulated glucose uptake in these L6 cells in a typical manner, though perhaps slightly on the low side of normal (*c.* 60% increase compared with studies in other passages of these cells). This response was relatively poor and personal experience with this cell line indicates that less than or equal to 1 hr incubation periods can sometimes be insufficient to raise a satisfactory insulin-stimulated increase in glucose uptake. All concentrations of bradykinin employed (10^{-9} M – 10^{-6} M) failed to stimulate glucose uptake. In contrast, Kishi *et al* (1998) have reported increases in 3 H 2DG uptake in L6 cells after 10 min incubations with 10^{-10} M- 10^{-6} M bradykinin. However, the L6 cells in that study had been transfected with bradykinin B₂ receptors (BK₂Rs), sensitising those cells to the exogenous bradykinin that was administered.

The second experiment (fig 6.2.2), which involved a 4 hr incubation shows a better response to insulin, approximately 85% compared to 60% at 1 hr. At this time bradykinin increased glucose uptake at three of the four concentrations studied, 10^{-9} M, 10^{-7} M and 10^{-6} M (but not 10^{-8} M). In the light of subsequent evidence about the lack of expression of bradykinin receptors in L6 cells (Kishi *et al.*, 1998) this may be unexpected. This was a surprising result as the rationale of transfecting BK₂Rs in the study by Kishi *et al* (1998) was that they found that L6 cells expressed few or no bradykinin receptors as estimated by mRNA analysis. At this time, my hypothesis was that the small population of bradykinin receptors present could indeed induce an increase in glucose uptake if sufficient incubation time was allowed. This experiment was confirmed in quadruplicate to allow for more powerful statistical analysis. After this mechanistic investigations were to be performed.

As can be seen (fig 6.2.3), upon repetition (*n*=6) the apparent bradykinin-induced increases in glucose uptake seen at 4 hr had disappeared. The large insulin response indicates that the cells are functional. The same pattern is seen when incubating for 8 h (fig 6.2.4). As bradykinin is a peptide, perhaps this lack of response was due to freeze

thawing, a process that can affect the biological activity of other peptides such as insulin and hence may have reduced the bradykinin's potency. More bradykinin was ordered and this 'fresh' batch was compared to the bradykinin employed previously, which was subsequently referred to as the 'old' batch (frozen and thawed, figs 6.2.5 and 6.2.6). These results indicated that whilst there was no statistical difference between the bradykinin concentration curves and the controls of each batch, the 'fresh' bradykinin seemed to increase glucose uptake to a greater extent versus control compared to the 'old' batch. One final experiment was undertaken using a similar design to those undertaken with lipoic acid (chapter 3). The highest apparently effective concentration of bradykinin from figure 6.2.2 (10^{-6} M) was employed with varying concentrations of insulin (10^{-8} M- 10^{-6} M). The addition of bradykinin raised the mean value for glucose uptake by 45% compared to control cells without insulin, but this was not statistically significant. The combination of insulin and bradykinin did not significantly increase glucose uptake above that of insulin alone.

6.2.4 - Conclusions

Using the L6 cell model fresh bradykinin appears to stimulate a modest increase in glucose uptake. The mechanism for this increase may be via a very small number of bradykinin receptors or due to another receptor and signal transduction pathway. Though this agrees to a certain extent with the findings of Kishi *et al* in 1998, the large effect seen by that group could be due to the use of L6 myotubes transfected with BK₂Rs upgrading the bradykinin response. Perhaps this work is not transferable to the whole animal, as muscle cells release bradykinin during exercise (Stebbins *et al.*, 1990) and muscle cells are known to express bradykinin receptors *in vivo* (Figueroa *et al.*, 1996). It is thought that stimulation of glucose uptake is insulin-independent during physical activity (Hayashi *et al.*, 1997). However, Carvahlo *et al* (1997) have shown that short-term bradykinin administration to insulin-resistant rats increased insulin receptor and IRS-1 phosphorylation in rat muscle. This effect was not seen in normal rats. These results imply a connection between the insulin and bradykinin signalling systems and this has been postulated by Haring *et al* (1996). Despite this, as recently as 1998 Hiyashi *et*

al have claimed that ‘there is no known cell-surface “exercise receptor” that mediates exercise-stimulated intracellular signalling molecules’. They then postulated that it may be mechanical and/or chemical stimuli within the contracting muscle that stimulates an increase in glucose uptake during exercise, such as changes in intercellular pH, variations in intra-cellular calcium concentrations and changes in the AMP:ATP ratio (Hiyashi *et al.*, 1998).

Present results indicate the fragility of bradykinin preparations, as evidenced by the removal of effect once the bradykinin was frozen and then subsequently thawed for use in further experiments. This fragility could mean that bradykinin is short lived *in vivo*, but as it is constantly replaced in exercising muscle this could explain the difference between *in vivo* and *in vitro* results from bradykinin studies. It is likely that bradykinin provides a partial explanation for the exercise-induced increase in glucose uptake effect, perhaps working independently of or with other vasodilator molecules such as K^+ and CO_2 during exercise. Such relationships would provide a deserving avenue for future investigation.

6.3 - Zinc- α 2-glycoprotein (ZAG) or Lipid Mobilising Factor (LMF)

6.3.1 - Introduction

Patients with neoplastic diseases frequently exhibit body wasting or cachexia. It is thought that this loss in body mass is not a direct result *per se* of decreased food intake due to the nausea and sickness that often accompany such illnesses (Garattinni *et al.*, 1980). Tumour type plays an important role in the incidence of cachexia, e.g. 8% of patients with oesophageal carcinoma exhibit cachexia compared to 70-80% of patients with gastric or pancreatic cancers (Strain, 1979). The reasons for these differences are uncertain, but may be due to tissue-specific production of cachetic factors discussed below. Unfortunately, progressive cachexia is associated with a poor prognosis and resistance to cancer chemotherapy and is often a major contributory factor to the premature mortality of these patients (De Wys *et al.*, 1985; Fearon, 2001).

In 1985 Heymsfield and McManus reported that the primary energy sources in cachexia were skeletal muscle protein and adipose tissue, in agreement with a study in 1980 by Watson and Sammon who found that body fat depletion was a major component in weight loss accompanying cachexia-causing cancers. Tumours require substrates for oxidative phosphorylation, and neoplasms utilise fatty acids for this purpose as well as for structural roles in membrane lipids (Mulligan and Tisdale, 1991). The necessary fatty acids are thought to be host derived (Spector, 1975). Cachetic cancer patients have elevated circulating non-esterified fatty acid (NEFA) concentrations and increased glycerol turnover rates compared to normal individuals or non-cachetic cancer patients (Shaw and Wolfe, 1987). Thompson *et al* (1993) confirmed the presence of raised NEFA levels and also noted elevated HSL mRNA in cancer patient-derived adipose tissue, consistent with the view that cancer cachexia is associated with increased adipocyte lipolysis (relative to lipogenesis) causing depletion of adipose depots.

The presence of a biologically active tumour-associated lipid mobilising factor (LMF) in cancer cachexia has been known for some time (Costa and Holland, 1962; Kitada *et al.*,

1980). In 1998, Hirai *et al* reported the isolation and characterisation of an LMF from the urine of cachectic cancer patients. They found that the amino acid sequence and molecular weight of the isolated and purified LMF was identical to that of Zinc- α 2-glycoprotein (ZAG), a plasma protein that has been detected in many body fluids (Portmanns and Schmid, 1968). ZAG has a molecular weight of *c.* 40 KDa, consists of a single polypeptide chain and has a degree of sequence and domain structure homology with major histocompatibility complex (MHC) molecules (Araki *et al.*, 1988). The function of ZAG in healthy subjects is unknown, however in neoplastic disease ZAG levels appear to correlate with tumour differentiation in breast cancer (Diez Itza *et al.*, 1993) and oral tumors (Brysk *et al.*, 1999). Perhaps more importantly, ZAG also shares biological activity with LMF, raising the possibility that the two molecules are in fact one and the same (Penio *et al.*, 1998; Portmanns and Schmid, 1968).

Tumours often exhibit alterations in glucose metabolism, with over-expression of glucose transporter genes resulting in intracellular accumulation of glucose (Yamamoto *et al.*, 1990). Alterations in the enzymes of glycolysis (Weber *et al.*, 1961) as well as increased hexokinase gene transcription are also seen (Johansson *et al.*, 1985). This up-regulation of glucose transport is insulin-independent as hypoglycemia occurs in tumour-bearing animals and humans with normal or decreased insulin levels (Bibby *et al.*, 1987; Heber *et al.*, 1985). This suggests that a tumour-secreted plasma borne factor may be involved. It has been found that cancer cell-conditioned media increases glucose uptake into L6 myoblasts (Li and Adrian, 1999) and that LMF isolated from the urine of patients with cancer cachexia can increase glucose uptake into C₂C₁₂ myoblasts over 24h (Al-Alami, 2001). These observations provide a rationale for investigating whether ZAG can increase glucose transport by muscle cells and offer a template for a new approach to the treatment of the hyperglycaemia of obese patients with type 2 diabetes. Thus the present study was undertaken to evaluate further effects of ZAG on glucose transport in L6 cultured muscle cells, with a view to gaining preliminary information about the possible mechanism involved.

6.3.2 - Results

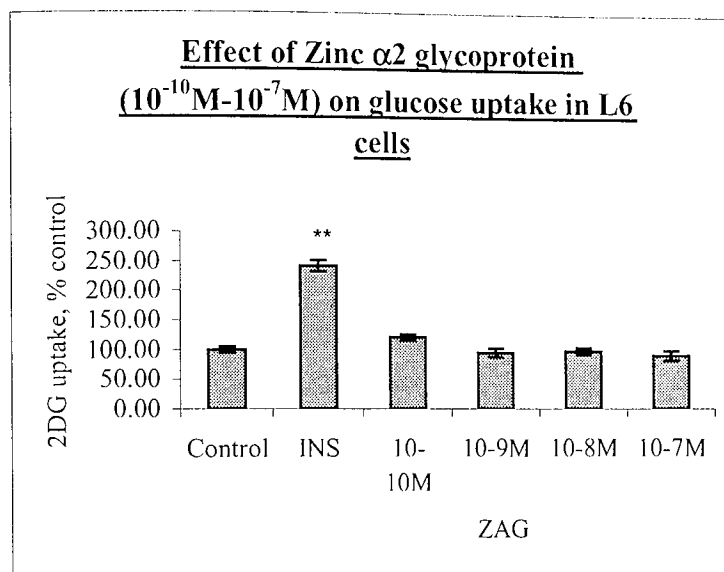


Fig 6.3.1 - Zinc α 2 glycoprotein (10^{-10} M – 10^{-7} M) or ZAG had no effect on glucose uptake (measured by 3 H 2DG uptake) in L6 cells after incubation for 24hrs. Results are means \pm SE, n=6. **P<0.01 versus control (no ZAG).

The effect of ZAG (10^{-10} M – 10^{-7} M) on glucose uptake by L6 cells was determined during 24 hours incubation at 37°C. Insulin (10^{-6} M) alone raised glucose uptake by 150% (**P<0.01). The addition of rising concentrations of ZAG (10^{-10} M – 10^{-7} M) had no significant effect on glucose uptake (P>0.05).

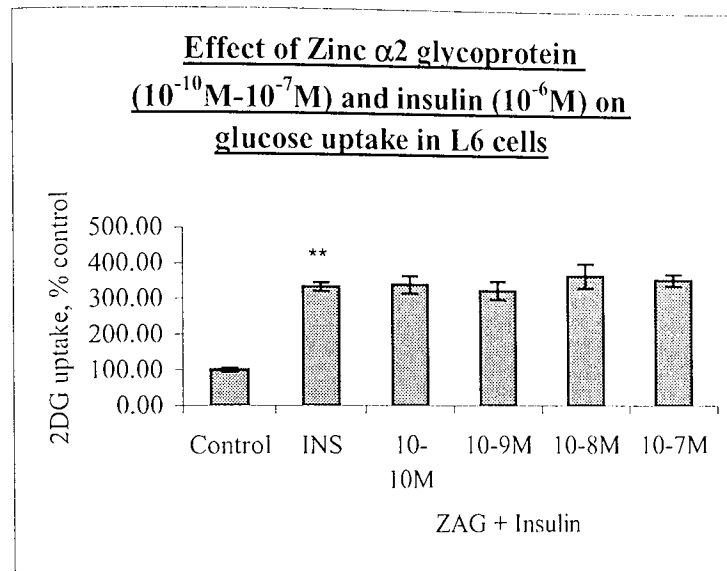


Fig 6.3.2 - Glucose uptake measured by ^3H 2DG uptake in L6 cells was not affected by the addition of 10^{-6}M insulin plus ZAG ($10^{-6}\text{M} - 10^{-10}\text{M}$) for 24 hour above that of insulin alone (10^{-6}M). Results are means \pm SE, $n=6$. ** $P<0.01$ versus control (no insulin or ZAG).

The effect of ZAG ($10^{-10}\text{M} - 10^{-7}\text{M}$) on glucose uptake by L6 cells was determined in the presence of insulin during 24 hours incubation at 37°C . Insulin (10^{-6}M) alone raised glucose uptake by 250% (** $P<0.01$). The addition of rising concentrations of ZAG ($10^{-10}\text{M} - 10^{-7}\text{M}$) had no significant effect on insulin-stimulated glucose uptake ($P>0.05$).

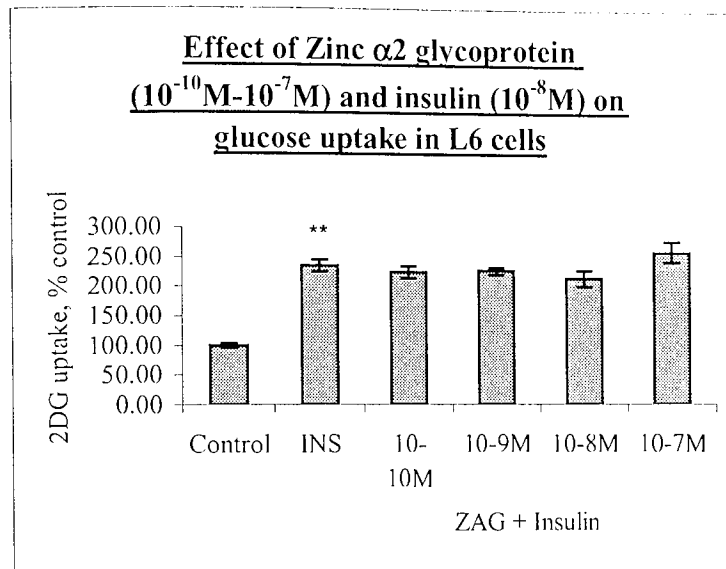


Fig 6.3.3 - Glucose uptake measured by ^3H 2DG uptake in L6 cells was not affected by the addition of 10^{-8}M insulin plus $10^{-10}\text{M} - 10^{-7}\text{M}$ ZAG for 24 hours above that for insulin alone (10^{-8}M). Results are means \pm SE, $n=6$. ** $P<0.01$ versus control (no added ZAG or insulin).

The effect of ZAG ($10^{-10}\text{M} - 10^{-7}\text{M}$) on glucose uptake by L6 cells was determined during 24 hours incubation at 37°C with a sub-maximally stimulating concentration of insulin (10^{-8}M). Insulin alone raised glucose uptake by almost 150% (** $P<0.01$). The addition of rising concentrations of ZAG had no significant effect on insulin-stimulated glucose uptake ($P>0.05$).

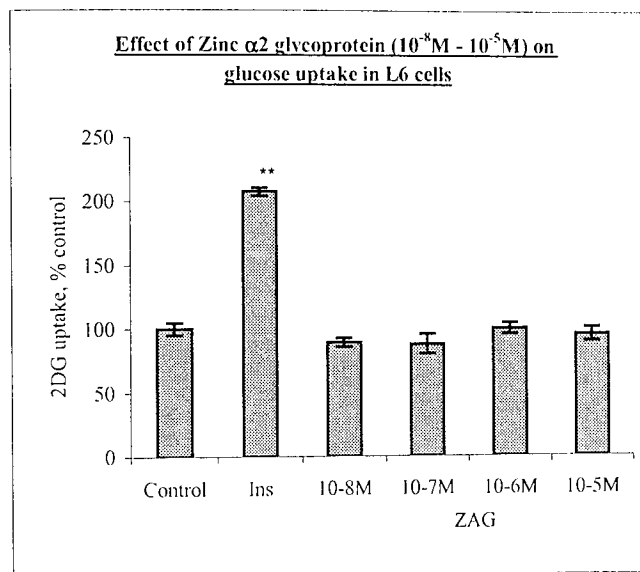


Fig 6.3.4 - ³H 2DG uptake in L6 cells was affected by the addition of 10⁻⁶M insulin but not 10⁻⁵M – 10⁻⁸M ZAG for 24 hours. Results are means ± SE, n=6. **P<0.01 versus control (no added ZAG or insulin).

Since low concentrations of ZAG were ineffective both alone and in combination with insulin, glucose uptake was now measured after incubation for 24hrs with high concentrations of ZAG (10⁻⁸M-10⁻⁵M) at 37°C. Insulin (10⁻⁶M) alone raised glucose uptake by almost 100% (**P<0.01). The addition of rising concentrations of ZAG had no significant effect on glucose uptake (P>0.05).

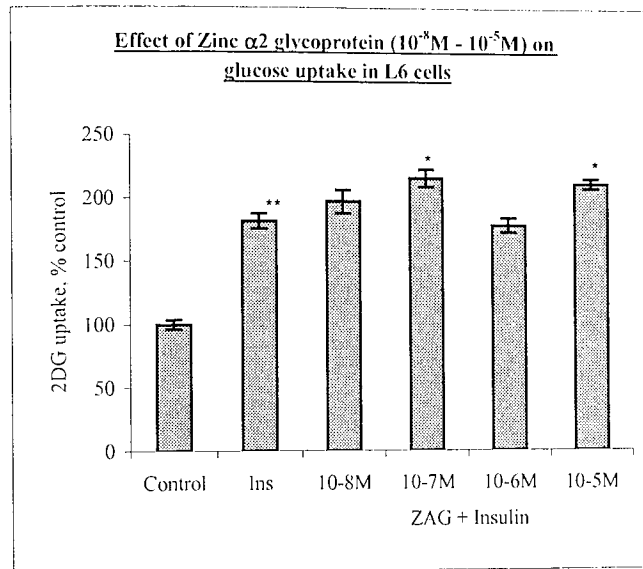


Fig 6.3.5 - Glucose uptake measured by ^3H 2DG uptake in L6 cells was affected by the addition of 10^{-6} M insulin plus 10^{-8} M - 10^{-5} M ZAG for 24 hours. Results are means \pm SE, $n=6$. ** $P<0.01$ versus control (no added insulin or ZAG), * $P<0.05$ versus insulin alone.

The effect of ZAG (10^{-8} M - 10^{-5} M) on glucose uptake by L6 cells was determined during a 24 hours incubation at 37°C . Insulin (10^{-6} M) alone raised glucose uptake by almost 90% (** $P<0.01$). The addition of rising concentrations of ZAG plus insulin significantly increased uptake at 10^{-7} M and 10^{-5} M. (* $P<0.05$).

6.3.3 - Discussion

Due to the limited availability of ZAG, which is prepared in small quantities only by a complex extraction process from human plasma by Bayer Pharmaceuticals (New Haven, CT, USA), a range of low concentrations of ZAG (10^{-10}M – 10^{-7}M) was selected for the initial experimentation. As seen in figure 6.3.1, exposure to insulin (10^{-6}M for 24hrs) increased glucose uptake in L6 cells by nearly 150% (see methods and other chapters involving L6 cells), an increase typically seen in this model. The presence of increasing concentrations of ZAG (10^{-10}M – 10^{-7}M) had no significant effect on glucose uptake. This may be contrasted with the stimulatory concentration of LMF observed in the studies by Islam-Ali (2001), which were in the range 200 – 600 nM (approximately 10^{-1}M – 1M). However, stock solutions of ZAG supplied by Bayer and obtained from Professor Mike Tisdale and Dr Steve Russell were unavoidably of low concentration due to extraction from human plasma. Allowing for $n=6$ the maximal concentration of ZAG that could be employed in the present studies with L6 cells was only 10^{-7}M and this consumed the remaining supply of material. Therefore it is quite possible that this experiment may have used insufficiently high concentrations of ZAG and thus no effect was observed. Acquisition of larger amounts of ZAG was not possible at the time that these experiments were conducted. *In vivo* studies measuring food intake effects of ZAG have shown that lyophilized and subsequently re-constituted ZAG loses much of its biological activity (Professor Mike Tisdale, personal communication). Therefore it is necessary to use freshly isolated ZAG that is transported from the USA to the UK on ice at 4°C . This provided a further restriction on the amount of ZAG available for study.

It was postulated at the time these studies were conducted that ZAG *per se* may not increase glucose uptake at the concentrations studied (10^{-10}M – 10^{-7}M), but may act synergistically with insulin (10^{-6}M). Figure 6.3.2 shows this experiment and clearly indicates that any increase in glucose uptake was due to the addition of the insulin and not any hypothesised biological activity of ZAG under these conditions. There was no significant difference between ZAG (10^{-10}M – 10^{-7}M) plus insulin and insulin alone. Stimulation of glucose uptake in L6 cells with 10^{-6}M insulin is maximal (personal

observations within the laboratory) and hence may have been masking any possible additive effect of ZAG, so this experiment was repeated with a sub-maximally stimulatory concentration of insulin (10^{-8}M) to see if this was the case (fig 6.3.3). Using a 24h incubation period 10^{-8}M insulin increased glucose uptake by almost 150%. Although 10^{-7}M ZAG plus insulin (10^{-8}M) raised the mean value for glucose uptake above insulin alone this was not statistically significant. In conclusion, it is likely that low concentrations ($10^{-10}\text{M} - 10^{-7}\text{M}$) of ZAG were insufficient to generate any significant effects on glucose uptake in L6 cells, whereas high (about $10^{-4}\text{M} - 10^{-3}\text{M}$) concentrations of LMF were reported to increase glucose uptake in C_2C_{12} myoblasts (Al-Alami, 2001).

Before concluding these experiments, it was decided to use the remaining available ZAG at higher concentration. Given the limited amount of ZAG, this could only be undertaken by reducing the number of replicates to 4 (e.g. $n=4$) in order to approach potentially biologically active higher concentrations. This alteration in experimental design allowed ZAG to be employed up to 10^{-5}M and the results of these experiments can be seen in figures 6.3.4 and 6.3.5. Again, ZAG alone failed to increase glucose uptake (fig 6.3.4) despite the increase in concentration administered. However, we appear to finally see an additive effect when insulin (10^{-6}M) is employed in conjunction with ZAG at 10^{-7}M and 10^{-5}M . Caution should be employed in interpreting these results, as the number of replicates is limited. In this experiment 10^{-6}M ZAG did not significantly increase glucose uptake whereas the preceding (10^{-7}M) and following (10^{-5}M) concentrations in the curve did. However, this may indicate that ZAG exerts a biphasic action rather than a statistical inconsistency due to the restricted number of replicates.

In the study by Li and Adrian (1999) they estimated that the molecular size of the molecule responsible for increasing glucose uptake was *c.* 5000, which is much smaller than ZAG. However, ZAG is glycosylated whereas the factor of Li and Adrian's is not, and speculation within the Cancer Research laboratory at Aston University is that this may be a tryptic digest of ZAG, which still retains biological activity (Professor M Tisdale, unpublished results). If this is true, then the action of this factor utilises GLUT-4 transporters, since cytochalasin B inhibits the stimulatory effects on glucose uptake.

Protein synthesis was also involved as cyclohexamide abolished the effects of this factor (Li and Adrian, 1999).

6.3.4 - Conclusion

It is important to consider that it has not been conclusively established that ZAG and LMF are the same molecule. Supplies of both ZAG and LMF are very limited due to the necessity of extraction from human plasma, and may be marginally contaminated by other molecules during extraction, despite rigorous efforts to exclude such materials. It is not known what the normal circulatory concentration of ZAG is, despite the high concentrations seen in the plasma of cachectic cancer patients, making it difficult to decide on a realistic experimental concentration to employ. The availability of the extracts used in the present study was not certain and ZAG's sensitivity to freeze thawing limited the amounts available for study. However, most of the evidence to date indicates that ZAG and LMF are likely to be the same biological molecule (Portmanns and Schmid, 1968; Penio *et al.*, 1998) and the present study suggests that high concentrations of ZAG/LMF can increase glucose uptake into muscle. Why this should be accompanied by muscle wasting in cachexia is probably explained by a concomitant rise in Protein Mobilising Factor (PMF) derived from the tumour that causes muscle protein catabolism (Islam-Ali, 2001).

6.4 -Trace elements

6.4.1-Introduction

For several decades there have been recurrent accounts in the literature that deficiencies of various trace elements and metals can precipitate or exacerbate diabetic conditions (Bailey, 2000). There have also been isolated reports that dietary supplementation with trace elements improves glycaemic control in diabetic patients, although much controversy exists in this area, as detailed below. Some *in vitro* studies have presented evidence that trace elements can both improve and impair insulin action, without conclusive outcomes. The concept of rectifying a simple trace element deficiency to improve insulin action in certain individuals provides an attractive prospect.

Lithium

Lithium, in the form lithium carbonate, is administered to patients to prevent mania and manic-depressive psychoses (Gershon, 1970; Mendals *et al.*, 1972). Thyroid function can be interfered with in patients on lithium as well as changes in renal function, which can lead to diabetes insipidus.

In 1977 Johnston reported two cases in which lithium therapy was associated with the onset of diabetes mellitus of an acute nature. Both patients rapidly became hyperglycemic (21.3 mmol/L and 107 mmol/L) and the later patient had marked glycosuria and ketonuria. Despite medical intervention, rapid degeneration of the patient's conditions ensued, quickly proving fatal. Johnston concluded that thirst, polyuria and fatigue in lithium treated patients should not be treated as acceptable consequences of lithium administration. At this time another case of this nature was reported, this was attributed to lithium toxicity until blood glucose tests were performed (Van der Velde and Gordon, 1969). Other accounts had provided contradictory findings that indicated both insulin-mimetic and non-insulin-mimetic effects of lithium therapy (Shopsin *et al.*, 1972; Henniger and Mueller, 1970).

In addition to these case studies, many scientific studies have reported apparent insulin-mimetic properties of lithium *ex vivo* (Bosch *et al.*, 1986; Haugaard *et al.*, 1974 and Bhattachanrya, 1964). However, *in vivo* data have again been contradictory, with the glucose tolerance of normal rats declining (Shah and Pishdad, 1980) or improving (Vendsborg 1979) upon lithium dosage.

Lithium and its potential effects in the diabetic state first came to my attention whilst reading an article in the Wellcome News (1999). In the article, it was stated that lithium potentially acts in depression by inhibiting glycogen synthase kinase three (GSK-3) and inositol monophosphatase (Klein *et al.*, 1989; Melton *et al.*, 1989; Berridge *et al.*, 1989). Both of these enzymes are elements in the insulin receptor signal transduction mechanism and could therefore be implicated in insulin resistance. This provided a compelling rationale for embarking on preliminary experiments with L6 cells, and for re-analysing unpublished results from a previous study carried out by the Aston diabetes research group on lithium supplementation of *ob/ob* mice.

Selenium

Selenium has been recognised as an essential trace element for many years (Chen *et al.*, 1980). However, this element's exact role in the body is still not clear, but it does appear to have anti-oxidant properties (Mc-Keehan *et al.*, 1976) as well as acting as a co-factor for guathanine peroxidase (Rotruck *et al.*, 1973). Pertinent to this study, selenium or selenate has been shown to possess insulino-mimetic properties in rat adipocytes (Ezaki *et al.*, 1990). A profound elevation in glucose transport was noted, and elucidation of mechanism indicated that stimulation of insulin-sensitive cAMP phosphodiesterase occurred in this cell line. This leads to increased production of GLUT1 and GLUT2 and their translocation to the plasma membrane. However, insulin-receptor kinase and phosphotyrosine phosphatase activity was not increased (Ezaki *et al.*, 1990). In 1991, McNeil *et al.*, found that in STZ induced diabetic rats sodium selenate restored

euglycaemia. This was also noted in comparable studies by Ghosh *et al.*, (1994) and Berg *et al* (1995).

In 1996 Furnsinn *et al.*, studied isolated rat soleus muscle and discovered that selenium/selenate increased glucose uptake into this tissue in a dose dependent manner. They also found that this elevation in uptake was accompanied by a corresponding rise in glycolysis, both aerobic and anaerobic. Selenate has been shown to have 'insulin-like' effects on the metabolic processes of glycolysis, gluconeogenesis and the pentose-phosphate pathway (Stapleton, 2000). In diabetic rats, oral selenate was found to almost completely reverse the abnormal liver enzyme profile seen in these animals. This included increased activity of several glycolytic enzymes including glucokinase and pyruvate kinase as well reduced activity of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK)(Becker *et al.*, 1996). Selenate also restored the expression of fatty acid synthase and glucose-6-phosphate dehydrogenase (G6PDH) to normal in diabetic animals (Ghosh *et al.*, 1994) and cultured rat hepatocytes (Stapleton *et al.*, 1994).

Treatment of 3T3 adipocytes with selenate induced phosphorylation of the insulin receptor (Pillay and Makoba, 1992). Five years later, selenium was found to induce an increase in phosphorylation of IRS-1 and the β subunit of the insulin receptor when incubated with primary rat hepatocytes or 3T3-L1 cells (Stapleton *et al.*, 1997). PI-3 kinase is associated with a number of the actions of insulin (see introduction) including GLUT-4 translocation to the membrane, stimulation of DNA synthesis via Ras and the regulation of key glycolytic and glycogenic enzymes. Selenate has been shown to increase PI-3 kinase activity in cultured rat hepatocytes. Downstream from PI-3 kinase is the protein pp70 S6 kinase, which is involved in protein synthesis. This has been shown to exhibit increased phosphorylation when exposed to selenate (Ezaki *et al.*, 1990). Insulin mediates cell growth and protein synthesis via the signal transduction component mitogen activated protein kinase (MAPK). Selenate can activate MAPK (Stapleton *et al.*, 1997; Hei *et al.*, 1998) in 3T3-L1 adipocytes and cultured hepatocytes. This activation was measured by a marked increase in phosphorylation of MAPK. Hei *et al.*, (1998)

found that the increase in MAPK activity with selenate was similar to that of insulin in isolated rat adipocytes.

Clearly, selenate has potential as an insulino-mimetic in the diabetic state. However, most of the previous studies employed hepatocytes or adipocytes. Given that muscle is the quantitatively largest effector tissue for insulin (in terms of regulation of blood glucose levels) then it would be desirable to investigate the effects of selenium in glucose uptake in cultured muscle cells.

Chromium

As a dietary factor, chromium (valency state III) has been shown to be involved in the control of type 2 diabetes in a recent clinical trial (Anderson, 1999). Chromium supplementation resulted in a reversal of diabetic symptoms, including normalisation of blood glucose in a patient who was insulin resistant (Jeejeebhoy *et al.*, 1977). Earlier evidence for the involvement of chromium in the treatment of diabetes is generally consistent, provided chromium III is used. This observation has been supported by the work of several laboratories, which have shown improved glucose tolerance and lower levels of circulating insulin upon chromium supplementation (Anderson, 1988). Cefalu *et al.*, (1999) found that chromium picolinate (1000µg per day) has an effect on the insulin sensitivity of obese subjects with a familial type 2 diabetic link. They concluded that such improvements in sensitivity to insulin, coupled with a non-significant change on body fat in these subjects, implies that insulin action within the muscle has been directly enhanced by the chromium (Cefalu *et al.*, 1999).

Chromium picolinate as a dietary supplement given to type 2 diabetic patients has been reported to have beneficial effects, such as significantly lower blood glucose and insulin values after two months compared to controls. Total plasma cholesterol also decreased, but this was after four months (Ravina *et al.*, 1995; Evans, 1989; Lee and Reasner, 1994 and Anderson *et al.*, 1997). The effects appeared to be dose dependent (Anderson *et al.*, 1997). A chromium chloride (which has a lower bio-availability than picolinate)

supplement study has also shown this form of chromium to be a positive influence on diabetic parameters (Glinsmann and Mertz, 1966). In a follow up study the improvement in blood glucose levels was seen again and eighty percent of patients reported marked alleviation of their diabetic symptoms such as diuresis, excessive thirst and fatigue (Cheng *et al.*, 1999). However, some studies have failed to find an improvement in diabetic patients on chromium (Sherman *et al.*, 1968; Rabinowitz *et al.*, 1983 and Trow *et al.*, 2000), though this may have been due to the low doses employed and the type of chromium used (Anderson, 2000).

Chromium is thought to potentiate insulin action. In 1987 Anderson *et al.*, found that an increase in insulin sensitivity upon chromium supplementation was due at least in part to a rise in insulin receptor numbers. Chromium has also been shown to activate insulin receptor kinase (Davis, 1997 and Vincent, 1999), which would lead to insulin signalling in the cell. This effect was found to occur only in the presence of insulin and seemed to be induced by the presence of a low molecular weight chromium-binding compound. Some controversy surrounds this binding compound. Another aspect to the mode of action of chromium is the ability of the element to inhibit phosphorylation of phosphotyrosine phosphatase (PTP-1). This is a rat homologue of the human PTP-1B, which inhibits insulin receptor action (Imparl-Radosevich *et al.*, 1998). Removal of insulin receptor inhibitory influences and the addition of insulin receptor stimulatory factors would lead to insulin signal-transduction mechanisms being fully activated.

There is a large body of positive evidence for a beneficial role of chromium in the diabetic state. No trace element study would be complete without an investigation of chromium as a potential alleviator of insulin resistance. As before, potential effects were examined in L6 cells.

6.4.2 - Results

6.4.2.1 - In vivo study on effects of lithium carbonate on glucose and insulin levels in *ob/ob* mice

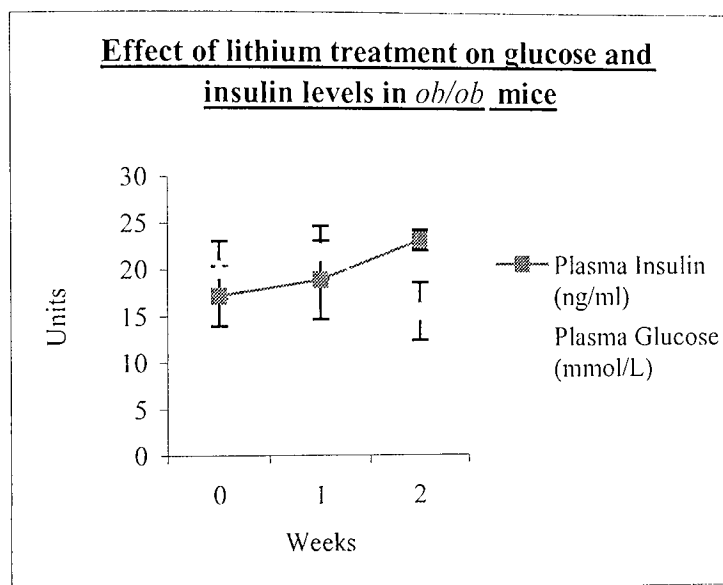


Fig 6.4.1 - Plasma glucose and insulin levels in *ob/ob* mice were not affected by the addition of 0.5g/L lithium carbonate for 2 weeks. Results are means \pm SE, $n=8$. Units on X-axis are ng/ml for insulin and mmol/l for glucose.

The effect of lithium carbonate (0.5g/L in the drinking water) on glucose and insulin levels in *ob/ob* mice was determined during a 2 week *in vivo* study. Insulin levels showed a general increase over the two weeks ($P>0.05$). Initially plasma glucose levels appear to rise and then fall off rapidly after 2 weeks but this was not significant ($P>0.05$).

6.4.2.2 - ^3H 2-deoxyglucose uptake by L6 cells

Lithium

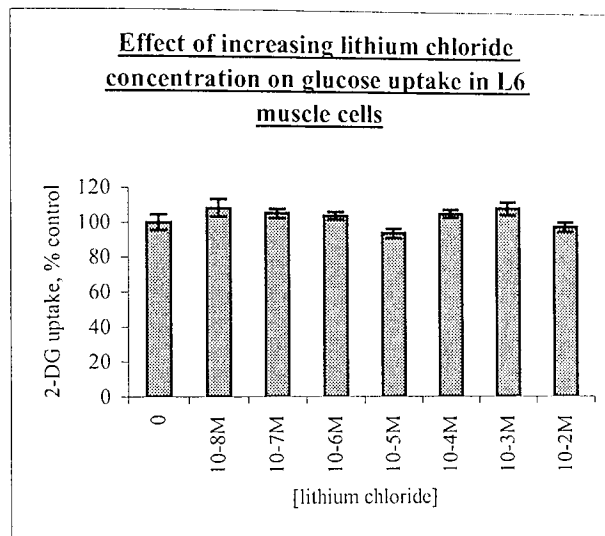


Fig 6.4.2 - Glucose uptake measured by ^3H 2DG uptake in L6 cells was not affected by the addition of $10^{-2}\text{M} - 10^{-8}\text{M}$ lithium chloride for 24 hours. Results are means \pm SE, $n=6$.

The effect of lithium chloride ($10^{-8}\text{M} - 10^{-2}\text{M}$) on glucose uptake by L6 cells was determined during 24-hour incubation at 37°C . The addition of rising concentrations of lithium chloride had no significant effect on 2DG uptake ($P>0.05$).

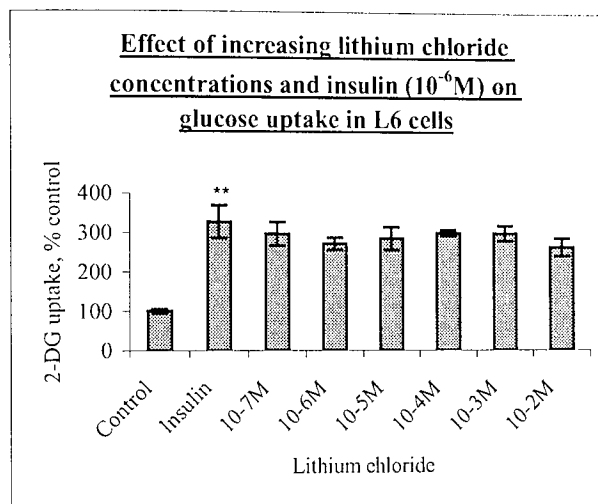


Fig 6.4.3 - Glucose uptake measured by 3H 2DG uptake in L6 cells was not affected by the addition of $10^{-7}M - 10^{-2}M$ lithium chloride and $10^{-6}M$ insulin in combination. Insulin alone significantly increased glucose uptake (** $P < 0.01$ versus control). Results are means \pm SE, $n=6$.

The effect of lithium chloride ($10^{-2}M - 10^{-8}M$) and insulin ($10^{-6}M$) on glucose uptake by L6 cells was also determined during 24-hour incubations. Insulin alone raised glucose uptake by over 200% versus control (** $P < 0.01$). The addition of rising concentrations of lithium chloride plus insulin had no significant effect on 2DG uptake compared with insulin alone ($P > 0.05$).

Selenium

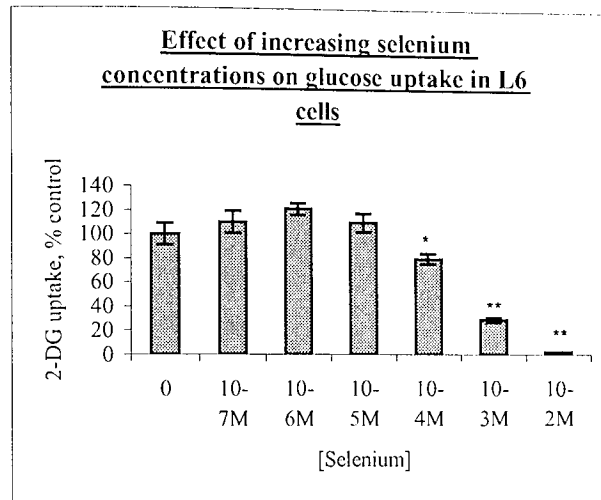


Fig 6.4.4 – Effect of sodium selenate ($10^{-7}M$ – $10^{-2}M$) on glucose uptake measured by 3H 2DG uptake in L6 cells after incubation for 24 hours. Results are means \pm SE, $n=6$. ** $P<0.01$ versus control (no sodium selenate).

The effect of sodium selenate ($10^{-2}M$ – $10^{-7}M$) on glucose uptake by L6 cells was determined during 24-hour incubation at $37^{\circ}C$. Rising concentrations of sodium selenate had no significant effect on 2DG uptake until $10^{-4}M$, $10^{-3}M$ and $10^{-2}M$ which lowered 2-DG uptake by 20%, 70% and 100% respectively (* $P<0.05$, ** $P<0.01$).

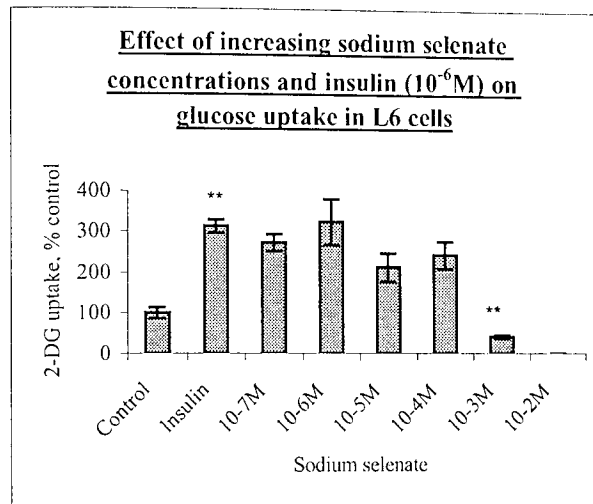


Fig 6.4.5 – Effect of sodium selenate ($10^{-8}M - 10^{-2}M$) with insulin ($10^{-6}M$) on glucose uptake in L6 cells after incubation for 24hrs. Results are means \pm SE, $n=6$ ** $P<0.01$ versus control (no insulin or sodium selenate).

The effect of sodium selenate ($10^{-2}M - 10^{-8}M$) with insulin ($10^{-6}M$) on glucose uptake by L6 cells was determined during 24-hour incubations. Insulin alone raised glucose by 200% versus control (** $P<0.01$). Rising concentrations of sodium selenate plus insulin had no significant effect on 2DG uptake until $10^{-2}M$, which decreased uptake by 80% versus insulin alone (** $P<0.01$).

Chromium

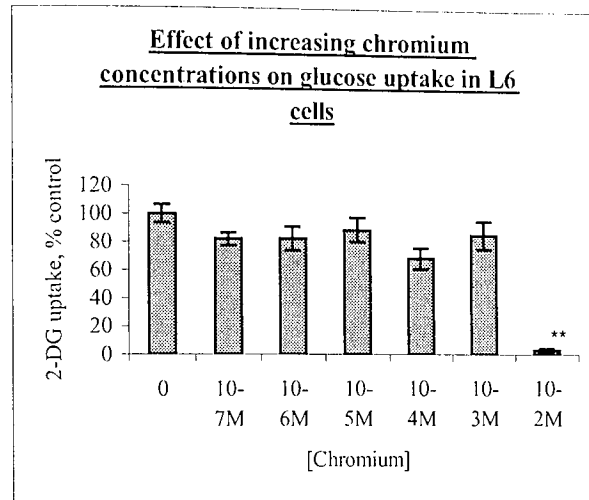


Fig 6.4.6 – Effect of chromium chloride ($10^{-5}M - 10^{-2}M$) on glucose uptake measured by 3H 2DG uptake in L6 cells after incubation for 24 hours. Results are means \pm SE, $n=6$. ** $P < 0.01$ versus control (no added chromium).

The effect of chromium chloride ($10^{-7}M - 10^{-2}M$) on glucose uptake by L6 cells was determined after 24-hour incubations at $37^{\circ}C$. The addition of rising concentrations of chromium chloride had no significant effect on 2DG uptake until $10^{-2}M$, which completely inhibited 2-DG uptake by 100% (** $P < 0.01$).

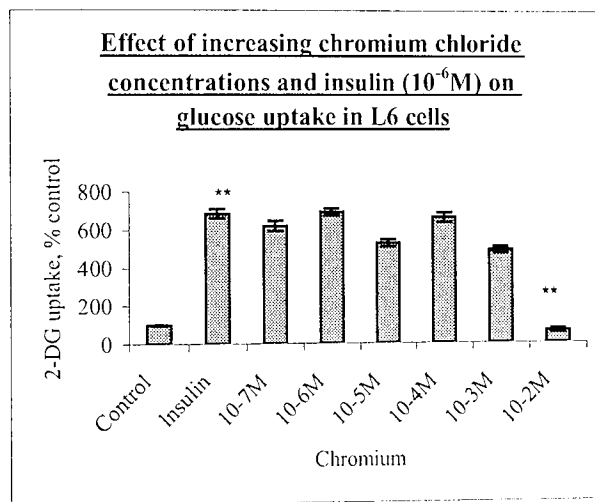


Fig 6.4.7 – Effect of chromium chloride ($10^{-7}M - 10^{-2}M$) and insulin ($10^{-6}M$) on glucose uptake measured by 3H 2DG uptake in L6 cells after incubation for 24hrs. Results are means \pm SE, $n=6$. ** $P<0.01$ versus control (no added insulin or chromium).

The effect of chromium chloride ($10^{-2}M - 10^{-8}M$) and insulin ($10^{-6}M$) on glucose uptake by L6 cells was determined after a 24 hour incubation. Insulin alone raised glucose by 600% compared to control (** $P<0.01$). Rising concentrations of chromium chloride plus insulin had no significant effect on 2DG uptake compared to insulin alone until $10^{-2}M$ chromium chloride, which decreased uptake by 80% versus insulin alone (** $P<0.01$).

6.4.3 - Discussion

Lithium

As can be seen in figure 6.4.2 increasing concentrations of lithium chloride (10^{-8}M - 10^{-2}M) had no effect on glucose uptake in L6 cells incubated for 24 hours. Similarly, a lack of effect was obtained when employing the same concentrations of lithium with 10^{-6}M insulin (fig 6.4.3). Insulin alone increased glucose uptake by nearly 200% (** $P < 0.01$). However, the combination of insulin and various concentrations of lithium chloride did not result in an increase in glucose uptake above that elicited by insulin alone. Therefore in this cell culture model lithium and insulin are non-synergistic. An effect of lithium on glucose uptake should be measurable in cell lines, as they are likely to be trace element deficient. This would also be true of the *ob/ob* mice used in the *in vivo* study (fig. 6.4.1). Unfortunately, lithium carbonate was shown to have no effect on glucose or insulin levels when administered to the *ob/ob* mice. Thus the *in vivo* results represent a third possibility, with previous studies showing that glucose tolerance of normal rats declined or improved upon lithium treatment (Shah and Pishdad, 1980 & Vendsborg, 1979 respectively).

To attempt to provide some explanation for the variable effects of lithium on glucose homeostasis reported in the literature and the lack of effect seen in the present study, it is appropriate to consider some additional actions of lithium suggested in earlier literature. Whilst lithium has been reported in some studies to improve glucose uptake and utilisation, other studies have noted the reverse, and there are various case studies (Johnston 1977; Van der Velde and Gordon, 1969) implicating lithium as a precipitating factor for diabetes in some patients treated for psychoses. It has been suggested that lithium can suppress insulin secretion (Fontela *et al.*, 1990). Thus in individuals on the brink of diabetes with a deficiency of β -cell function or incipient β -cell failure, lithium-induced suppression of insulin secretion could impair glucose homeostasis and precipitate diabetes.

It has also been suggested, as noted in the rationale for the present study, that lithium can improve insulin action. Thus in a diabetic individual with substantial insulin resistance but considerable reserve of β -cell function, the improvement of insulin action by lithium could out-weigh a modest suppression of β -cell function. This would have the overall outcome of an improvement in glycaemic control.

Thus it has been suggested that lithium acts *in vivo* with two opposing actions on glucose homeostasis, namely to reduce insulin release and improve insulin action. Depending upon which effect predominates in an individual diabetic patient due to their prevailing level of insulin secretion and insulin resistance, it would be possible for lithium to either improve or impair glycaemic control. The present study in *ob/ob* mice was unable to shed further light on this situation. The lack of effect of lithium in these mice could reflect the extreme severity of both the insulin resistance and the hyperinsulinaemia, such that small or modest changes in either parameter might not be expected to have any substantive effect on the level of hyperglycaemia. Indeed lithium treatment produced a small rise in insulin levels, but the lowering of mean glucose levels was not statistically significant. This leaves open the verdict on the actions of lithium on glucose homeostasis, and may indicate that further unrecognised actions of lithium are responsible for the various reports of effects in diabetic states. In L6 cells no apparent effect of lithium on basal or insulin-stimulated glucose uptake suggests that lithium does not have a direct action on muscle that alters normal glucose handling.

Selenium

At high concentrations of 10^{-3} M and 10^{-2} M selenium impaired insulin-induced glucose uptake by L6 cells (**P<0.01). However, as seen with previous studies, such a negative impact on glucose uptake was due to cytotoxicity, as evidenced by detachment of the cellular monolayer and extensive positive staining during trypan blue exclusion studies (see appendix). The same pattern is also seen with a combination of sodium selenate and insulin (10^{-6} M). Sodium selenate at 10^{-3} M and 10^{-2} M reduced uptake of glucose by 50% and 80% respectively (**P<0.01). Again this can be attributed to cytotoxicity.

Literature to date indicates that selenate and selenium have an effect on glucose utilisation in cell lines. However, as stated in the introduction these cellular studies employed adipocytes and hepatocytes. None has employed a skeletal muscle cell line, although Furnsinn *et al* 1996 used rat soleus muscle and found increased glucose uptake in response to selenate. It may be that conditions in terms of the availability of trace elements and anatomical organisation *ex vivo* are very different to those seen *in vitro*. Whether the cell lines lack a fundamental component necessary for the action of selenium remains conjectural. Alternatively the effect of selenium could involve other cell types (endothelial etc) present in whole muscle but absent in cell culture. It could be that the beneficial effects of selenium are not seen in skeletal muscle cell lines at all, only in hepatocytes or adipocytes. Indeed, if selenium acts mainly on the liver to reduce glucose production and output, this could explain most of the *in vivo* blood glucose lowering effects of selenium reported in the literature (McNeil *et al.*, 1991; Ghosh *et al.*, 1994; Berg *et al.*, 1995).

Chromium

Chromium (valency 3) of increasing concentration (10^{-7}M - 10^{-2}M) for 24 hours had no effect on glucose uptake in L6 cells except at 10^{-2}M (fig 6.4.6) at which point the uptake of glucose was completely inhibited (** $P < 0.01$). This reduction can be attributed to cytotoxicity as seen by positive trypan blue staining (see appendix) and detachment of the cellular monolayer. As shown in figure 6.4.7 the addition of insulin alone (10^{-6}M) increased glucose uptake by 600% (** $P < 0.01$). The combination of insulin and 10^{-7}M - 10^{-2}M chromium chloride had no additional effect and reduced glucose uptake at toxic concentrations of chromium.

Extensive work has been carried out on chromium in the diabetic state in humans (Jeejeebhoy *et al.*, 1977; Anderson, 1988; Cefulu *et al.*, 1999). Once again as per selenium we find a number of 'mode of action' studies, none of which has been carried out in skeletal muscle tissue or cells. Activation of insulin receptor kinase as discovered

by Davis in 1997 and confirmed by Vincent in 1999 only occurred in the presence of a low molecular weight chromium-binding compound. Given the lack of chromium in the supplemental media used, it would have been expected that if chromium is important for a direct effect on basal glucose or insulin-stimulated glucose uptake in muscle this would have been evident in the present experiments. Thus the present studies suggest that the insulin-like effects, or insulin-enhancing effects of chromium indicated from *in vivo* studies in diabetic patients must be due predominantly to either effects on other tissues such as liver, or indirect effects on muscle that require the presence of other tissues or organs. It must also be noted that chromium supplementation of the diet is believed to improve glucose homeostasis when there is dietary chromium deficiency i.e. the supplement is reinstating normal chromium levels (Anderson, 1997). There is no evidence that chromium hyper-supplementation can produce additional benefits in patients who already have adequate chromium levels. The present studies suggest that chromium is unlikely to have a direct effect on glucose uptake in muscle, but it cannot be discounted that the putative binding compound or other co-factors are necessary for the insulin-like or insulin-enhancing effects of chromium in muscle, and these compounds or co-factors are limiting (or present in inadequate amounts) in the L6 muscle cell line.

6.4.4 - Conclusion

Despite previous studies raising the possibility that lithium, selenium and chromium can influence whole body glucose metabolism and exert insulin-like effects *in vitro*, the present study did not observe these elements to have any significant effect on basal or insulin-stimulated glucose uptake in L6 cells. This could be due to the lack of additional (unestablished) factors required for the elements' insulin mimetic activity in the cells. Whole muscle preparations or whole animal studies (in normal animals) treated with the trace elements and followed by studies in isolated myocytes, adipocytes or hepatocytes could provide additional information that might accommodate the limitations of the cell model with regard to additional factors that may be necessary for the effectiveness of the trace elements. However, the present studies provide the first evidence that these

elements on their own do not have direct effects on isolated muscle cells to significantly alter glucose uptake.

Chapter 7 - Conclusion

This thesis has considered a number of potential intervention targets to further investigate and treat the lesions responsible for insulin resistance. As a basis for the work, techniques were employed and evaluated to quantify the effects of insulin on primary adipocytes, whole muscle preparations and muscle cells. The major focus was on skeletal muscle because this is the main site of insulin-stimulated glucose uptake and the main tissue responsible for abnormal glucose homeostasis in type 2 diabetes (De Fronzo, 1988).

Adipose tissue is normally highly responsive to insulin, where this hormone prevents the release of non-esterified fatty acids (NEFAs). In overweight diabetic subjects there is an elevation of plasma NEFAs and glucose levels, at least partly as a result of insulin resistance in that tissue (Boden, 1997). These increased plasma NEFA levels bring about a decrease in insulin-stimulated glucose uptake in the skeletal muscle via the glucose-fatty acid (Randle) cycle and hence induce further insulin resistance (Randle *et al.*, 1963). Many studies have been carried out in rodent models of obesity with the intention of increasing the release of FAs from adipose tissue by increasing catecholamines-mediated lipolysis via the β_3 adrenoceptor (Arch *et al.*, 1996; Himms-Hagen and Danforth, 1996 and Danforth *et al.*, 1997). In principle, if the FFAs are utilised in liver and muscle as a source of energy, the reduction in adipose tissue mass would alleviate obesity and reset the glucose-fatty acid cycle to reduce both hyperglycaemia and insulin resistance. As there is a known difference between the β_3 regulation of lipolysis in rodents compared to humans (Arch *et al.*, 1984; Arch and Wilson, 1996; Harms *et al.*, 1977), clinical tissue was employed for many of the present studies on adipocyte lipolysis.

A particular focus of this thesis was the effects of the anti-oxidant compound lipoic acid on glucose uptake in isolated murine muscles and L6 myocytes (chapter 3). This compound was found to increase glucose uptake in both of the preparations studied, but also possessed a narrow therapeutic window. Of particular interest was the ability of lipoic acid to increase glucose uptake into the muscles of insulin-resistant obese *ob/ob*

mice, which are very unresponsive to insulin. Lipoic acid was found to act in a similar manner to insulin and recent studies indicate that this compound does indeed utilise post-receptor components of the insulin signal-transduction pathway (Yaworsky *et al.*, 2000). Isoferulic acid (chapter 5) increased glucose uptake into L6 muscle cells in an apparently similar manner to lipoic acid, although current studies indicate that isoferulic acid also acts via adrenergic mechanisms to generate its metabolic effects in cells (Liu *et al.*, 2001).

Studies described in this thesis were unable to confirm TNF α -induced insulin resistance in isolated whole muscle preparations as suggested by Nolte *et al* (1998). However, a clear inhibitory effect of TNF α on glucose uptake was observed in L6 muscle cells. The difference between the isolated tissue and cell preparations was attributed in part to differences in sensitivity of the preparations to insulin and other agents (considered later in this conclusion).

Lipolysis experiments showed that lipoic acid inhibits the release of NEFAs from adipocytes and, in this respect therefore acts in a similar way to insulin in this tissue. Given the ability of lipoic acid to engage the insulin-signalling pathway this is not an unexpected result. M2, the secondary metabolite of sibutramine, was shown to increase the release of glycerol from adipocytes. This is an important observation, which suggests that M2 may have a non-central nervous system weight reducing effect and might be worthy of consideration as a drug in its own right.

This chapter considers each of the main findings of this thesis and tentatively draws conclusions. An evaluation of the relevance of the present findings is also presented to shed light on current concepts of insulin resistance mechanisms and the clinical potential for novel intervention.

Lipoic acid clearly has potential as an agent for overcoming insulin resistance. The ability of lipoic acid to induce glucose uptake in *ob/ob* mouse muscles indicates that it can activate insulin signalling in a severely insulin resistant state (chapter 3). This action

would be expected to lower blood glucose levels in insulin resistant diabetic individuals, offering the potential to reduce the morbidity effects associated with hyperglycaemia in type 2 diabetes. However, this thesis indicates that in L6 muscle cells lipoic acid had a narrow therapeutic window. Clinical trials of lipoic acid have employed large doses with no ill effect, probably due to buffering, metabolism and excretion of lipoic acid in the liver (the first pass effect) as well as partitioning in the plasma and interstitial fluid compartments which would reduce the effective dose reaching the skeletal muscles. This study has indicated that lipoic acid can induce glucose uptake and therefore possesses an anti-diabetic effect. There is the added bonus that lipoic acid has been shown to reduce or alleviate diabetic symptoms such as neuropathy, for which it is used as a treatment in countries such as Germany (Coleman *et al.*, 2001).

The initial lipolysis experiments in both *ob/ob* and lean mice were encouraging (chapter 4). Given that the *ob/ob* mouse is an accepted model for obesity-linked insulin resistance and type 2 diabetes (Flatt and Bailey, 1997), the evaluation of the isolated adipocyte system gave clinically valid results. This was shown by the catecholamine resistance evident in noradrenaline-stimulated adipocytes of both the *ob/ob* mice and human obese subjects. Lipoic acid had no effect on lipolysis in the preliminary mouse studies and was therefore not employed on the human tissue as these samples were limited in availability. The targeting of visceral human tissue samples was valid as this is the most important fat depot when considering the deleterious metabolic effects of obesity. The sibutramine metabolite M2 has been shown in these preliminary experiments to be effective at inducing lipolysis in isolated adipocytes from both *ob/ob* mice and human visceral fat from an obese type 2 diabetic patient. Initiating the release of glycerol and/or free fatty acids from the adipose depots is only half the clinical battle. Dual therapy would be needed to induce the metabolism and other means of elimination of the liberated fatty acids, which would result in a reduction in visceral fat depots and a consequential reduction in morbidity associated with central visceral obesity. Whilst this thesis did not experimentally identify such an agent, the lipid mobilising factor (LMF) seen in cachectic patients with certain cancers was suggested, and pre-clinical trials would need to be undertaken to explore this possibility (chapter 6).

This thesis has identified the ability of isoferulic acid to increase glucose uptake in L6 skeletal muscle cells (chapter 5). As with lipoic acid this increase in glucose uptake should decrease glucose levels within the blood, reducing the hyperglycaemia in type 2 diabetes. Given that the magnitude of hyperglycaemia in diabetic patients corresponds to micro-vascular morbidity, isoferulic acid therapy could reduce the level of illness associated with type 2 diabetes. Isoferulic acid is thought to act via the α_1 adrenoceptor and not the insulin-signalling pathway, though it may be possible that there is cross talk between the two processes due to the sharing of common signalling intermediates (Liu *et al.*, 2001).

At the time of initiation of the studies with TNF α (section 6.1), this molecule was thought to be a candidate paracrine factor from adipose tissue inducing insulin resistance in skeletal muscle. Experiments with isolated muscles outlined in this thesis did not show such an effect, but the study with L6 muscle cells clearly indicated that TNF α was causing a lack of response to insulin. Should TNF α be conclusively implicated in skeletal-muscle insulin resistance, it may be clinically possible to block TNF α with antibodies and hence remove this molecule's inhibitory effect on glucose uptake. Another option would be the use of thiazolidinediones, which are anti-hyperglycaemic agents but have also been found to ameliorate TNF α action in cells and even down-regulate TNF α expression (Hofmann *et al.*, 1994; Ohsumi *et al.*, 1995; Peraldi *et al.*, 1997).

Clinically, the experimental findings with bradykinin are unlikely to have a large impact as exercise is already prescribed to obese and/or type 2 diabetic patients (Centre for Disease Control USA, 1996; Pickup and Williams, 1997). Given that type 2 diabetes is not usually immediately life threatening, exogenous bradykinin therapy would be an unusual clinical choice. This is especially true when contrasted with type 1 diabetes, in which insulin is administered. Indeed, during exercise the contracting muscles will be producing bradykinin and this could make exercise prescription the best option (Stebbins *et al.*, 1990).

Present studies with ZAG/LMF outlined in this thesis indicate that this peptide alone is not capable of increasing glucose uptake into L6 muscle cells (section 6.3). However, once high concentrations were reached, a synergistic effect was seen with insulin. This is an extremely promising development. Given the link between type 2 diabetes and obesity it is clear that ZAG/LMF has the potential to combat type 2 diabetes on two fronts. ZAG/LMF causes liberation of fatty acids from adipocytes and increases the uptake of glucose into the muscles. If ZAG/LMF is able to stimulate the uptake and metabolism of the fatty acids (liberated from the adipose depots) in the skeletal muscle then ZAG/LMF could be of immense clinical significance.

Mobilisation of fat depots would reduce the weight of the patient and cause a corresponding decrease in the risk of morbidity associated with type 2 diabetes. The stimulation of glucose uptake into skeletal muscle should reduce or correct the hyperglycaemia seen in type 2 diabetes, also alleviating the complications associated with increased plasma glucose concentrations. These include micro and macro-vascular damage, insulin resistance itself and other components of Syndrome X. Should ZAG/LMF be able to bring about the metabolism of liberated fatty acids from the fat depots then this peptide would provide a safe method for reducing both the weight and severity of symptoms of obese type 2 diabetics whilst avoiding the deleterious cardiovascular consequences of elevated plasma NEFA levels. Before clinical trials of ZAG/LMF two factors would have to be considered: an improvement in the isolation or synthesis of the protein would be needed and a tight controlling factor and/or precise dosage studies would need to be carried out.

Investigations with lithium, selenium and chromium in L6 muscle cells showed no positive effect on glucose uptake at all the concentrations studied (section 6.4). Whilst lithium has been reported to improve glucose metabolism (Bosch *et al.*, 1986; Haugaard *et al.*, 1974; Bhattachanrya, 1964) there is also evidence that lithium can reduce glucose uptake (Shah and Pishdad, 1980), perhaps precipitating diabetes in some individuals. No cytotoxicity was seen with this trace element, which is important as lithium compounds

are used extensively clinically. However, no direct benefit on glucose metabolism was found, and no beneficial effect in insulin resistant *ob/ob* mice. Both selenium and chromium had no effect on glucose uptake in L6 muscle cells, making it likely that these two trace elements do not alter diabetic parameters via changes in skeletal muscle uptake and/or metabolism. Selenium studies with regard to any putative hepatic effect (Becker *et al.*, 1996) might be a more fruitful future avenue of investigation. Evidence to date seems to suggest that chromium acts via a low molecular weight binding compound (Davis, 1997; Vincent, 1999) which is probably not present in the L6 cells but likely to be active *in vivo*. Of concern was the cytotoxic effect evident at high concentrations of both selenium and chromium. Further investigations of this effect would be necessary before hyper-supplementation with either trace element could be considered. Had the trace elements studied been found to increase glucose uptake *in vitro*, then this would have opened up the possibility of a cheap supplementation of diabetic patients with these trace elements.

This thesis has generated novel insights into the investigation and possible alleviation of insulin resistance, a major component of the pathology of type 2 diabetes. These studies have also validated the use of most of the experimental methods employed, whilst providing a basis for further work using these validated methods. Given the annual increase in incidence of type 2 diabetes an improved understanding of the mechanisms involved, along with the discovery of compounds to combat the underlying causes will be essential. The studies reported herein demonstrate that TNF α probably does not have a sufficiently potent role in the development of insulin resistance to make it a key therapeutic target. However, the present studies have encouragingly shown that isoferulic acid and more particularly lipoic acid can alleviate the insulin resistant state. In addition, M2 can stimulate lipolysis in adipocytes from obese individuals indicating a possible future clinical use of this sibutramine metabolite. Transient increases in glucose uptake observed with bradykinin and LMF should be further investigated for clinical potential.

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Appendix

A.1 – Chemicals and solutions

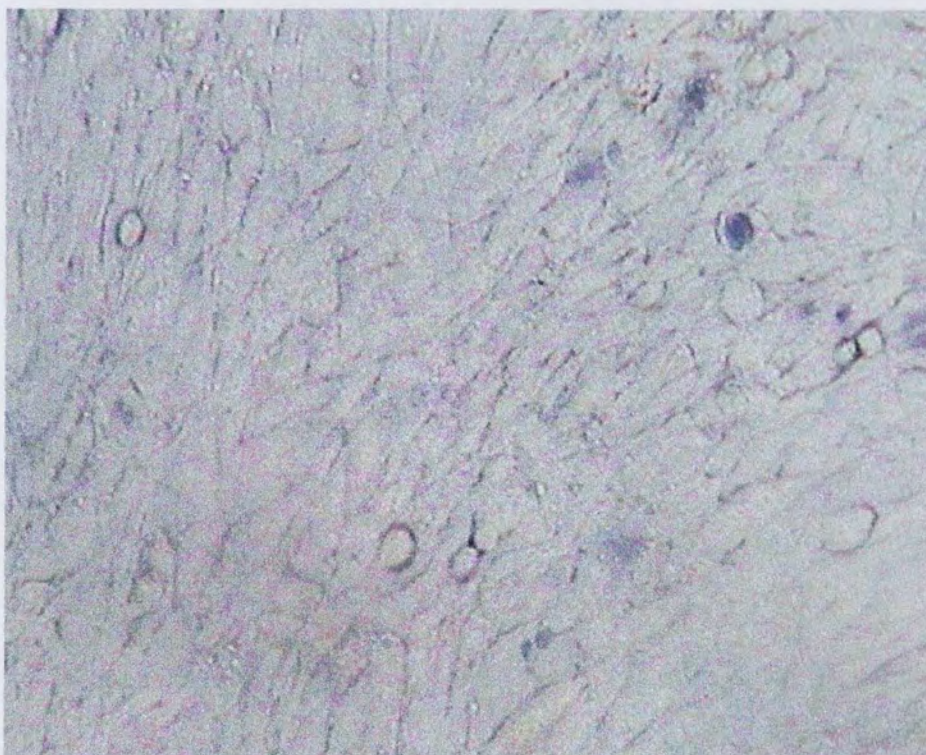
*¹ - Krebs contains: 118 mM NaCl, 5mM KCl, 25mM NaHCO₃, 1.18 mM MgSO₄ 7H₂O and 1.16 mM KH₂PO₄. This was gassed with 5%CO₂/95%O₂ for 20 minutes.

*² - Preparation of reagent: This is a solution of 0.5M carbonate containing 63mM glutamate and 4.6 mM NAD. To prepare 100ml of the reagent dissolve 0.927g of L-glutamic acid in 50ml of 1M NAHCO₃ solution. Add 1M NaOH until pH reaches 10, making up the remaining volume with distilled water. Finally dissolve 0.322g of NAD in this solution and store on ice until use. The preparation without NAD is stable if stored at 5°C, but once NAD is added it should be used within 24 hours. Hence the solution is made up daily.

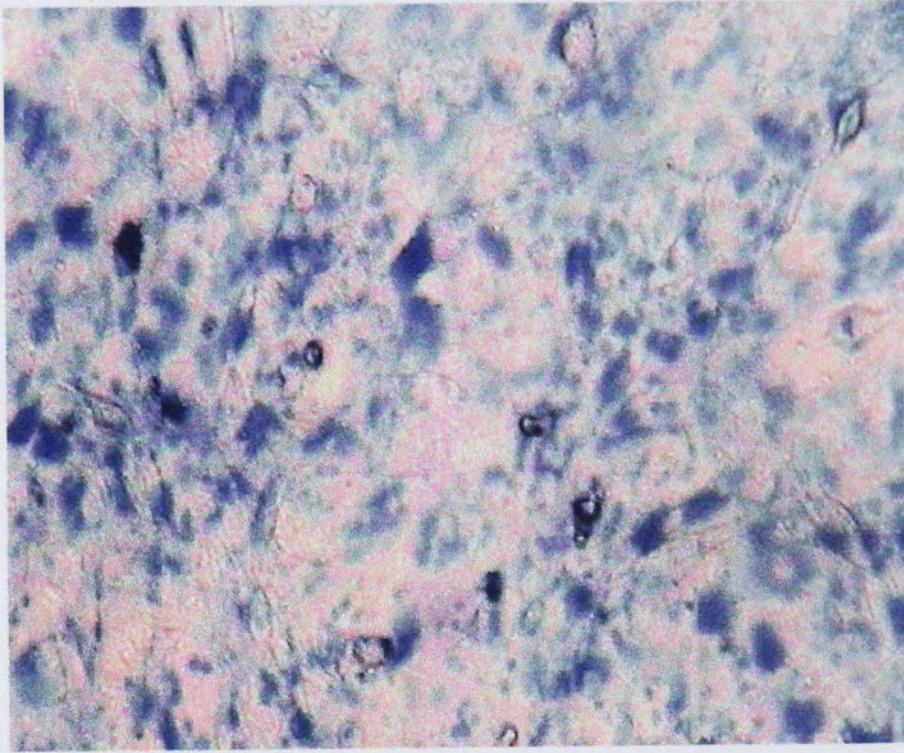
*³ - 500ml containing: 0.5g porcine trypsin and 0.2g EDTA.

*⁴ - Spectrophotometry buffer contains: MgSO₄ (0.0493g); Triethanolamine (1.5g); PEP (0.0183g); ATP (0.06068g) and NADH (0.0177g). Dissolve in deionised water then pH with 10% perchloric acid (make up to 100ml). The following can then be added just before use: Lactate dehydrogenase (73µl) and Pyruvate kinase (53µl).

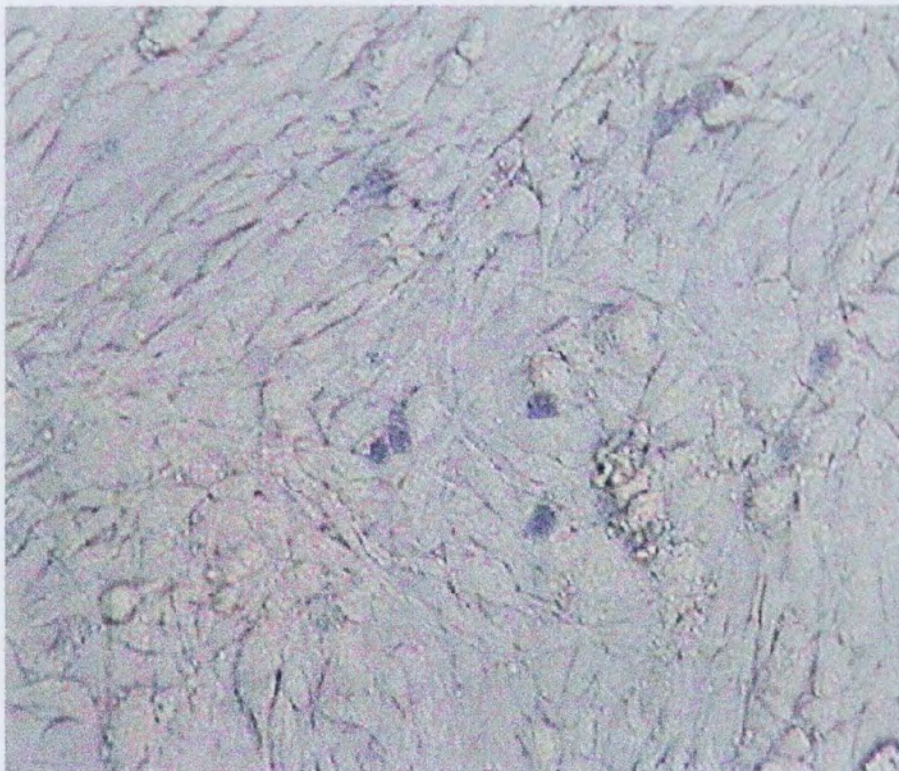
A.2 - Cytotoxicity staining



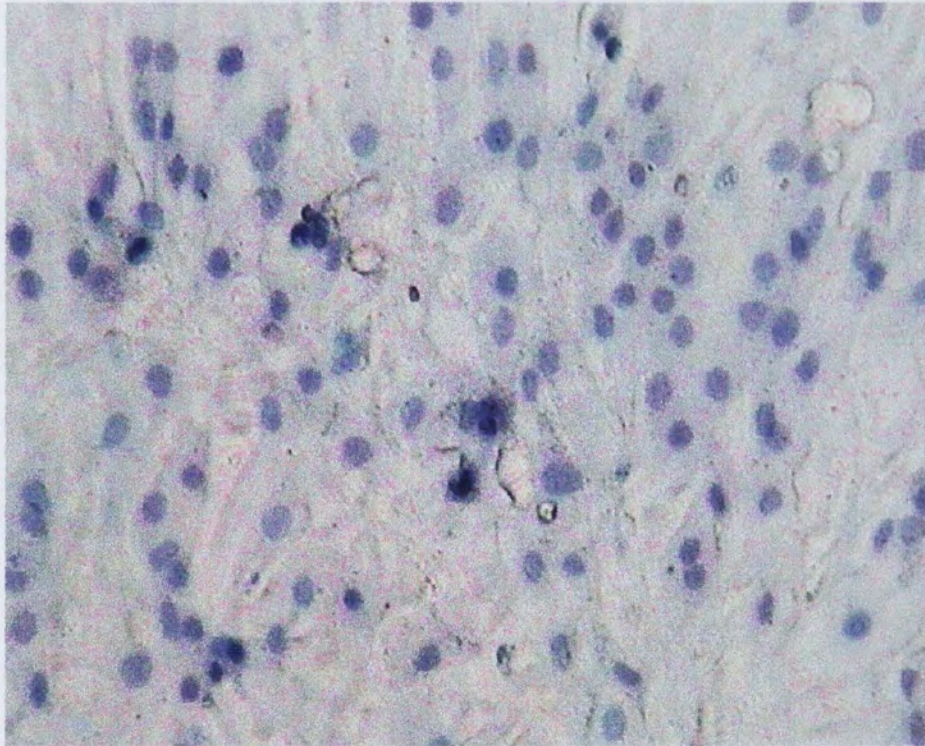
Picture A.1 - Plate showing control well for chromium chloride (none present). Note less than 5% positive staining with trypan blue (x400).



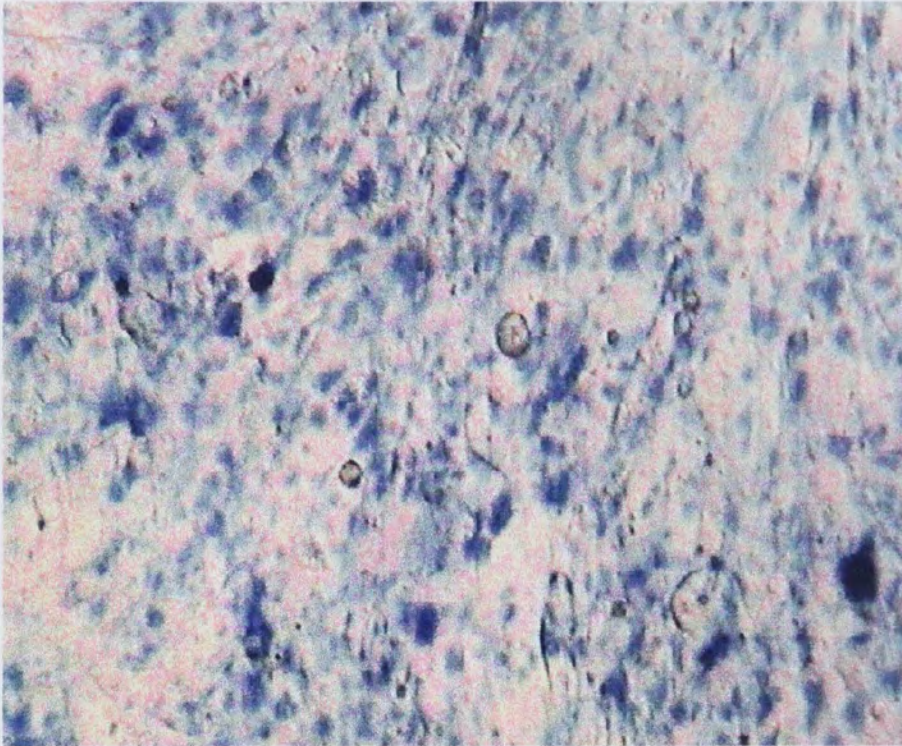
Picture A.2 - Plate showing cytotoxicity of 10^{-2} M chromium chloride. Note extensive positive staining with trypan blue (x400).



Picture A.3 - Plate showing control well for sodium selenate (none present). Note less than 5% positive staining with trypan blue (x400).



Picture A.4 - Plate showing cytotoxicity of 10^{-3} M sodium selenate. Note *c.* 40% positive staining with trypan blue (x400).



Picture A.5 - Plate showing cytotoxicity of 10^{-2} M sodium selenate. Note *c.* 90% positive staining with trypan blue (x400).

Lipoic acid increases glucose uptake by skeletal muscles of obese-diabetic *ob/ob* mice

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Aim: Alpha-lipoic acid has been reported to increase glucose disposal in diabetic states. This study has examined the effect of alpha-lipoic acid on glucose uptake by cultured L6 muscle cells and different types of skeletal muscles in normal



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Treatment with α -lipoic acid has been reported to improve insulin-stimulated glucose metabolism in several [7-10], but not all [11], studies with diabetic and insulin-resistant animal models. α -Lipoic acid has also been reported to improve insulin-stimulated glucose disposal in patients with type 2 diabetes [12,13]. There is also evidence that α -lipoic acid can increase glucose uptake by

that contribute to the insulin resistance in *ob/ob* mice [21,22]. Differences may exist between muscle types, as some are normally more insulin-sensitive (high proportion of red type 1 fibres) and others are less insulin sensitive (high proportion of white type 2 fibres) [23,24].

To investigate whether α -lipoic acid can improve insulin sensitivity by a direct effect on insulin resistant

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The therapeutic use of lipoic acid in diabetes: a current perspective

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Dedicated to the memory of M.J. Winn, Ph.D.

Abstract

Lipoic acid attenuates lipophilic acid accumulation through cellular



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agents, which are potent antioxidants, effective in diabetes (GSSG), lipoic acid, a condition which is prone to insulin resistance

reasons

the agent.

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has not found in the world. This review discusses the use of lipoic acid and antioxidant systems and the potential for lipoic acid in the treatment of diabetes.