INVESTIGATION OF POTENTIAL INTERVENTION TARGETS TO IMPROVE INSULIN ACTION:

A THERAPEUTIC APPROACH

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Investigation of Potential Intervention Targets to Improve Insulin Action: A Therapeutic Approach

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Impaired insulin action (insulin resistance) is a key factor in the pathogenesis of diabetes mellitus. To investigate therapeutic targets against insulin resistance, this thesis explores the mechanism of action of pharmacological agents and exogenous peptides known or suspected to modify insulin action. These included leptin, a hormone primarily involved in the regulation of body weight; sibutramine, an antiobesity agent; plant-derived compounds (pinitol and chamaemeloside) and agents known to affect insulin sensitivity, e.g. metformin, tolbutamide, thiazolidinediones, vanadyl sulphate and thioctic acid. Models used for investigation included the L6 skeletal muscle cell line and isolated skeletal muscles. *In vivo* studies were undertaken to investigate glycaemia, insulinaemia, satiety and body weight in streptozotocin-induced diabetic mice and obese (*ob/ob*) mice.

Leptin acutely altered insulin action in skeletal muscle cells via the short form of the leptin receptor. This direct action of leptin was mediated via a pathway involving PI 3-kinase but not Jak2. The active metabolites of sibutramine had antidiabetic properties *in vivo* and directly improved insulin sensitivity *in vitro*. This effect appeared to be conducted via a non-PI 3-kinase-mediated increase in protein synthesis with facilitated glucose transport, and was independent of the serotonin and noradrenaline reuptake inhibition produced by sibutramine. Pinitol (a methyl inositol) had an insulin mimetic effect and was an effective glucose-lowering agent in insulinopenic states, acting directly on skeletal muscle. Conversely chamaemeloside appeared to improve glucose tolerance without directly altering glucose transport. Metformin directly increased basal glucose uptake independently of PI 3-kinase, possibly via an increase in the intrinsic activity of glucose transporters. Neither tolbutamide nor thiazolidinediones directly altered insulin sensitivity in L6 skeletal muscle cells: however vanadyl sulphate and thioctic acid increased glucose transport but appeared to exert toxic effects at therapeutic concentrations.

Examination of glucose transport in skeletal muscle in this thesis has identified various components of post-receptor insulin signalling pathways which may be targeted to ameliorate insulin resistance.

Type 2 Diabetes Mellitus
Obesity
L6 Skeletal Muscle Cells
Glucose Transport
Insulin Signalling

For Mum, Dad and Robin,
With love

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2DG	2-deoxy-[^{3}H]-glucose
5HT	5-hydroxytryptamine (serotonin)
AGE	Advanced glycation end product
AgRP	Agouti-related protein
ANOVA	Analysis of variance
ATB-BMPA	2-N-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannose-4-yloxy)-2-propylamine
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMOV	bis(maltolato)oxovanadium
Bo	Zero Standard
BSA	Bovine serum albumin
CAD	Coronary artery disease
cAMP	Adenosine 3',5'-cyclic monophosphate
CART	Cocaine and amphetamine-regulated transcript
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene related peptide
CH	Cycloheximide
CHO	Chinese hamster ovary
CNS	Central nervous system
Crk	v-crk avian sarcome virus CT-10 oncogene homolog
CVS	Cardiovascular system
Cyt B	Cytochalasin B
DMEM	Dulbecco's modification of eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDL	Extensor digitorum longus
EDTA	Ethylene diaminetetraacetic acid
FCS	Foetal calf serum
FDA	Food and drug administration
FFA	Free fatty acids
GLUT	Glucose transporter
Grb-2	Growth factor receptor-bound protein 2
GSK	glycogen synthase kinase
HBSS	Hank's Balanced Salts Solution
HDL	High density lipoprotein
HGP	Hepatic glucose production
HIV	Human immunodeficiency virus
HMG	3-hydroxy-3-methylglutaric acid
HPA	Hypothalamic-pituitary-adrenal axis
icv	Intracerebroventricular
IDDM	Insulin dependent diabetes mellitus
IFN	Interferon
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance
IL	Interleukin

ip	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
IRS	Insulin receptor substrate
iv	Intravenous
Jak	Janus kinase
KRB	Krebs-Ringer buffer
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
LY	LY-294,002
M1	Metabolite 1 of sibutramine
M2	Metabolite 2 of sibutramine
M3	Metabolite 3 of troglitazone
MAP	Mitogen activated protein
Mek	Map kinase or extracellular signal-related kinase kinase
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	Messenger ribonucleic acid
Nck	non catalytic region of tyrosine kinase adaptor protein
NEFA	Nonesterified fatty acid
NHC	Natural health consultants
NIDDM	Non insulin dependent diabetes mellitus
NPY	Neuropeptide Y
NSB	Non-specific binding
Ob-R	Leptin receptor
Ob-Ra	Short form of the leptin receptor
Ob-Rb	Long form of the leptin receptor
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDK-1	3-phosphoinositide-dependent protein kinase
PFK2	6-phosphofructo-2-kinase
PH	Pleckstrin homology
PI 3-Kinase	Phosphatidylinositol 3-kinase
PI	Phosphatidylinositol
PIP_3	Phosphatidylinositol trisphosphate
PKB/RAC	Protein kinase B/Related to A and C kinases
po	Oral gavage
POMC	proopiomelanocortin
PPARγ	Peroxisome proliferator-activated receptor gamma
PRL	Prolactin
PTK	Protein tyrosine kinase
PTPases	Phosphotyrosine phosphatases
Raf-1	v-raf-1murine leukemia viral oncogene homolog
RFLP	Restriction fragment length polymorphisms
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RXR	Retinoid X receptor
SA-PMP	Streptavidin-paramegnetic particles
SD	Sprague Dawley
SEM	Standard error of the mean

SGLT1	Na^+/glucose co-transporter 1
SH2	Src homology 2
SHPTP2	SH2 domain containing tyrosine phosphatase
SNRI	Serotonin and noradrenaline reuptake inhibitor
Sos	Guanine nucleotide exchange factor Son of Sevenless
SP-1	SP-1 transcription factor
STAT	Signal transducers and activators of transcription
STZ	Streptozotocin
TA	Thioctic acid
Tag	Tyrphostin AG490
TC	Total counts
TG	Triglyceride
TNFα	Tumour necrosis factor alpha
TZD	Thiazolidinedione
UCP	Uncoupling protein
UKPDS	United Kingdom Prospective Diabetes Study
VLDL	Very low density lipoprotein
VMH	Ventromedial nucleus of the hypothalamus
WM	Wortmannin

Chapter One:
Introduction

Chapter One: Introduction

1.1 Glucose Homeostasis

Glucose metabolism is controlled by complex homeostatic mechanisms, which regulate the production, storage and utilisation of glucose. This process depends upon a balance between glucose output from the liver through glycogenolysis and gluconeogenesis, and glucose utilisation by insulin dependent tissues, such as fat and muscle, and insulin independent tissues such as brain and kidney (Bergman, 1989). Maintaining this balance requires rapid and accurate adjustments in the control of glucose metabolism to accommodate normal daily fluctuations in the supply and demands for this vital nutrient fuel. (The main metabolic processes are shown in table 1a below).

Table 1a – Summary of reactions in intermediary metabolism

Metabolic process	Reaction	Consequence
Glycogenesis	Glucose →Glycogen	↓ Blood glucose
Glycogenolysis	Glycogen →Glucose	↑ Blood glucose
Gluconeogenesis	Amino acids → Glucose	↑ Blood glucose
Protein synthesis	Amino acids → Protein	↓ Blood amino acids
Protein degradation	Protein → Amino acids	↑ Blood amino acids
Lipogenesis	Fatty acids & → Triglycerides Glycerol	↓ Blood fatty acids
Lipolysis	Fatty acids Triglycerides → & Glycerol	↑ Blood fatty acids

Table 1b – Key actions of insulin in nutrient fuel selection

Action	Tissue	Mediator	Sensitivity (mmol/l)*	Time of Onset (min)
↑ Glucose Uptake	Muscle, Fat	↑ Glucose Transporters[a]	~5x10^{-11}	<1
↑ Glycogenesis	Liver, Muscle, Fat[b]	↑ Glycogen Synthase[c] ↓ Glycogen phosphorylase[d]	~2x10^{-10} ~2x10^{-10}	<5 <5
↑ Glycolysis	Liver, Muscle, Fat	↑ Glucokinase (liver)[f] ↑ Pyruvate kinase[c]	- -	>10 <5
↓ Gluconeogenesis	Liver	↓ Phosphoenolpyruvate carboxykinase	~2x10^{-10}	>10
↑ Lipogenesis	Liver, Fat	↑Acetyl CoA carboxylase[e]	~2x10^{-10}	<5
↓ Lipolysis	Fat	↓ Triacylglycerol lipase[d]	~10^{-11}	~1
↑ Protein synthesis	Liver, Muscle	↑ RNA synthesis	~10^{-9}	>10

↑ Increase; ↓ Decrease.

*Approximate concentration of insulin at which action can be detected. Normal circulating insulin concentrations are basally <10μU/ml, 0.4ng/ml, 7x10^{-11}mol/l (70pM). Postprandial insulin concentrations rarely exceed 150μ/ml, 6ng/ml, 10^{-9}mol/l (1nM).

[a]Predominantly isoform GLUT4; [b]Glycogenesis is not a major fate of glucose in fat; [c]activated by dephosphorylation; [d]suppressed by dephosphorylation; [e]activated by phosphorylation; [f]induced [g]de-induced

The liver plays a primary role in glucose homeostasis. It is the principal site for metabolic interconversions such as gluconeogenesis, it releases glucose into the blood when it is needed and stores glycogen when there is excess glucose available. Muscle utilises the majority of energy and is the main site of amino acid storage, whereas adipose tissue is important in the regulation of fatty acid storage and supply. In the fasting state, glucose homeostasis is mainly balanced against the availability of free fatty acids (FFA). Lipolysis is initiated in adipose tissue, and triglycerides (TG) are hydrolysed to FFAs and glycerol. FFAs cause a decrease in glucose uptake and metabolism and hence a decrease in glucose utilisation. During this process ketogenesis is increased. Ketones are synthesised in the liver from FFAs and provide an energy source during prolonged starvation.

Glucose homeostasis and the pattern of glucose utilisation is highly regulated, particularly by hormones secreted by the pancreatic islets; insulin and glucagon.

1.2 Endocrine Control of Glucose Homeostasis

The islets of Langerhans are cells of the endocrine pancreas, which produce and secrete the hormones that regulate glucose homeostasis. Insulin is synthesised and secreted by the β cells of the islets, and acts in an anabolic manner to promote the cellular uptake of glucose, fatty acids and amino acids. Insulin promotes the storage of glucose as glycogen, fatty acids as triglycerides and the incorporation of amino acids into protein. In order to do this, insulin has multiple actions; it facilitates glucose transport, stimulates glycogenesis, inhibits glycogenolysis and gluconeogenesis, enhances the conversion of glucose to fatty acids, inhibits lipolysis, increases protein synthesis, inhibits protein degradation, and contributes to cellular growth, differentiation and division (Kruszynska, 1997). (Table 1b key actions of insulin in nutrient fuel selection).

A direct negative feedback loop between the β cells and the blood glucose concentration is the primary control of insulin secretion.

Glucagon is secreted by the α cells of the islets of Langerhans, and counterbalances many of the actions of insulin. The main effects of glucagon increase blood glucose levels by increasing glycogenolysis and gluconeogenesis. Glucagon, also promotes lipolysis, inhibits triglyceride synthesis, increases ketogenesis, inhibits hepatic protein synthesis, and increases amino acid metabolism as a carbon source for gluconeogenesis, although glucagon's effects on peripheral protein catabolism and blood amino acid levels are minimal.

Thus insulin and glucagon act to serve as key hormones in the acute regulation of glucose homeostasis. (The involvement of other factors is summarised in table 1c).

Table 1c – Endogenous agents which regulate glucose homeostasis

Agents that decrease blood glucose	Agents that increase blood glucose
Insulin	Glucagon
Pro insulin	Adrenaline
Growth hormone fragments	Glucocorticoids
	Growth hormone
	Sympathetic nerves controlling liver
	Increased fatty acids and amino acids

1.3 Diabetes Mellitus

Diabetes mellitus is a disturbance in the endocrine control of glucose homeostasis causing hyperglycaemia. There are three main types of diabetes mellitus; Insulin dependent (IDDM) or Type 1, Non-insulin dependent (NIDDM) or Type 2 and diabetes mellitus secondary to certain medical conditions or associated with genetic syndromes.

Type 1 diabetes results from the progressive autoimmune destruction of β cells in the islets of Langerhans, which leads to insulin deficiency. This results in hyperglycaemia due to decreased glucose utilisation despite increased hepatic glucose output, from glycogenolysis and gluconeogenesis. Blood glucose exceeds the renal threshold creating an osmotic diuresis and causing dehydration. Insulin deficiency also increases the hydrolysis of triglycerides and increases ketonogenesis from hepatic oxidation of FFAs. FFAs and ketones further inhibit glucose uptake, hence contributing to hyperglycaemia. During insulin deficiency, muscle anabolism is decreased and proteolysis is increased fuelling gluconeogenesis while causing muscle wasting. Insulin treatment is mandatory with this type of diabetes (reviewed by Campbell and Lebovitz, 1996).

Type 2 diabetes is caused by insulin resistance and abnormal β cell function, which together contribute to a series of metabolic disturbances, the pathophysiology of which remains incompletely understood. It is thought that Type 2 diabetes may be initiated by a defect in insulin action, reflected by a reduction in glucose clearance. A slight but persistent rise in hyperglycaemia may then initiate hyperinsulinaemia, which initially compensates for the insulin resistance. However in patients who are unable to sustain hyperinsulinaemia the disease progresses to a phase of β cell "exhaustion". Insulin resistance occurs in different insulin sensitive tissues and is dependent upon insulin and glucose levels. When both insulin and glucose levels are high, hepatic resistance to insulin is evident and hepatic glucose output is inadequately suppressed. Glucose uptake is significantly reduced, and it is this defect in muscle glucose uptake that is thought to be the major contributor to the cause of hyperglycaemia (reviewed by DeFronzo, 1992).

The prevalence of Type 2 diabetes is increased in populations where high energy intake and obesity are common, indeed there is a definite correlation between obesity and Type 2 diabetes, especially in individuals with an increased abdominal fat mass. The extra adiposity coupled with enhanced lipolysis in insulin resistance, leads to elevated plasma free fatty acids and stimulates lipid oxidation by peripheral tissues, thereby restricting glucose oxidation and causing defects in muscle glucose storage. The hyperinsulinaemia, and release of certain cytokines such as TNF-α are further factors contributing to insulin resistance. Therefore, obesity is a causative pathogenic factor in insulin resistance. Treatment of obese Type 2 diabetes patients is usually aimed at reducing insulin resistance by weight reduction thereby reducing hyperglycaemia, hyperinsulinaemia and hyperlipidaemia.

1.4 Type 2 diabetes

The link between insulin resistance, Type 2 diabetes, obesity and a multitude of related disorders is considered here from a clinical viewpoint, in keeping with the aims of this report, Type 2 diabetes and obesity will be the main focus.

As discussed earlier diabetes mellitus is a disease of metabolic dysregulation due to abnormal glucose metabolism and is a major worldwide health problem. It is frequently associated with obesity and approximately 60-90% of Type 2 diabetics are or have been obese (National Diabetes Data Group, 1979). Early studies demonstrated the importance of obesity as a precursor of glucose intolerance and Type 2 diabetes (Kadowaki et al., 1984; Medalie et al., 1975). Particularly notable is the high incidence of insulin resistance in obesity, and also that glucose intolerant obese subjects have an increased risk of becoming diabetic (Wilson et al., 1981).

Obesity was initially shown to be a progressive condition that comprises two main phases: an early period without insulin resistance and a later period in which insulin resistance is apparent (Jeanrenaud, 1979). However, it has recently been suggested that the evolution from obesity to Type 2 diabetes should be divided into four phases; obese glucose tolerant, obese glucose intolerant, obese diabetic with a hyperinsulinaemic response to a glucose load, and obese diabetic with a hypoinsulinaemic response (Golay et al., 1997; DeFronzo, 1992).

The increase in fat mass in obesity consequently causes an increase in lipolysis, lipogenesis and lipid oxidation (Golay et al., 1988; Schutz et al., 1992). The high plasma free fatty acid levels (FFA) and lipid oxidation rates are accompanied by a proportional decrease in glucose oxidation and an inhibition of glucose uptake, storage and glycogen synthesis (Ferrannini et al., 1983; Boden and Chen, 1995). Consequently, this leads to insulin resistance. This occurs over a long period of time, and studies have demonstrated that diabetes is preceded by years of glucose intolerance, which leads to diabetes when glycaemia does not return to normal basal levels (Felber et al., 1993; Jallut et al., 1990).

1.4.1 Epidemiology

Presently, approximately 100 million people have diabetes and this figure is rising. Type 2 diabetes accounts for 75-90% of these cases, however this is dependent on ethnic background (Campbell and Lebovitz, 1996). Hence, it is a common metabolic disorder, affecting 2-5% of the adult population of most western countries (King et al., 1993). It accounts for over 80% of diabetes in Europe and North America, and affects 10% of individuals aged 70 or over (Williams, 1994), however, despite its rising prevalence, it is still poorly diagnosed in many countries (reviewed by Scheen and Lefebvre, 1996). Indeed, for every diagnosed Type 2 diabetic, it is thought that there is an undiagnosed patient.

The onset of Type 2 diabetes occurs mainly in patients over the age of 30 and is often insidious, indeed it is thought to be a leading cause of disability and death in developed and developing countries (Songer, 1992).

Obesity is the most prevalent nutrition related problem in industrialised societies (reviewed by Chua and Leibel, 1997), for example, approximately one third of adult Americans, and 20% of American children are obese, and it is thought that, like diabetes this figure is rising (Kuczmarski et al., 1994). Obesity in adults is associated with over 70% of Type 2 diabetics in America, as well as increasing the risk of hypertension, dyslipidaemia and cancers (Troiano et al., 1995).

1.4.2 Pathophysiology

Type 2 diabetes results from an imbalance between insulin sensitivity and insulin secretion. It is an impairment in the body's ability to respond to insulin due to insulin resistance, causing a decrease in the biological effects of insulin (Reaven, 1988). However, in the earlier stages of Type 2 diabetes, the pancreas is able to compensate for this by producing elevated quantities of insulin, hence controlling blood glucose levels.

The β cells of the islets of Langerhans of the pancreas must maintain an elevated rate of insulin secretion in order to maintain normoglycaemia. With time, the β cells fail to do this, causing a relative decrease in insulin secretion, which may still be at a higher rate than that in non-diabetics, but which causes hyperglycaemia (Orci et al., 1990).

Therefore there are two main pathological defects in Type 2 diabetes; a) insulin resistance and b) relative and absolute insulin deficiency.

1.4.2a Insulin Resistance

Insulin resistance was first demonstrated over 60 years ago (Himsworth, 1936) and plays a central role in the pathogenesis and clinical course of several important diseases. It is characteristic of patients with Type 2 diabetes. Insulin resistance is discussed in detail below; section 1.5

1.4.2b Insulin Deficiency

During the onset of Type 2 diabetes, normoglycaemia is achieved due to an elevated rate of insulin secretion. However, the higher rate of insulin secretion cannot be maintained. Hence a relative insulin deficiency occurs and hyperglycaemia ensues. In Type 2 diabetes, fasting insulin levels correlate with fasting blood glucose concentrations, therefore as blood glucose levels increase, so levels of insulin increase until a point at which any further increase in the blood glucose level results in a decrease in insulin levels.

Insulin deficiency is defined as a "pathological condition in which there is an inappropriate decrease in the rate at which the β cell secretes insulin" (Taylor et al., 1994). It is caused by defective β cell function, the actual cause of which is largely unknown (Leahy, 1990). However, the following have been considered as possible sites of β cell dysfunction; cell number, cell morphology, genetic defects of the insulin gene, the presence of amylin, galanin, calcitonin gene related pepide (CGRP), glucose toxicity and lipotoxicity.

The amount of insulin secreted by the pancreas may be directly determined by the number of β cells (Gepts and Lecompte, 1981) and hence indicates that this may be a factor in insulin deficiency in Type 2 diabetes. Indeed, many studies have suggested that initially there are often moderate increases in β cell mass in patients with Type 2 diabetes (Maclean and Ogilvie, 1955; Clark et al., 1988). However, the subsequent decrease in β-cell mass has not been demonstrated to be significant enough to cause Type 2 diabetes on its own. Therefore it appears that there are other factors in addition to β-cell loss which must be responsible for the impairment of insulin secretion (DeFronzo et al., 1992).

It has been suggested that a decrease in the rate of insulin secretion in Type 2 diabetes may be due to a genetic defect in the insulin gene. Since this time, Bell et al., (1980) have sequenced the human insulin gene and it has been mapped for restriction fragment length polymorphisms (RFLP) showing no mutations on abnormalities (Permutt and Elbein, 1990).

Amylin, galanin and CGRP, have been implicated in contributing to β cell dysfunction. Amylin the precurser of amyloid deposits found in Type 2 diabetes, is produced by the β cell (Nishi et al., 1990). *In vitro* (but not *in vivo*) amylin has been found to inhibit insulin secretion (Ohsawa et al., 1989, Ghatei et al., 1990). Similarly there is no evidence to suggest that either galanin or CGRP play a major role in insulin deficiency. In contrast, there are data to suggest that glucose toxicity and lipotoxicity adversely affect insulin secretion (Rossetti et al., 1990). Indeed, good glycaemic control in Type 2 diabetes does improve insulin deficiency (Hidaka et al., 1982). Both *in vitro* and *in vivo* studies have demonstrated that an increase in plasma glucose causes inhibition of insulin secretion. (Leahy et al., 1987; Bonner-Weir et al., 1983) and hence it has been hypothesised that chronic hyperglycaemia is responsible at least in part for β cells inability to respond to an acute hyperglycaemic challenge (DeFronzo et al., 1992). Evidence that β cell function is inhibited by an excessive accumulation of fat has been recently demonstrated as experimental lowering of islet fat restored β cell function of Zucker diabetic fatty rats (Shimabukuro et al., 1998).

1.4.3 Pathogenesis

For many years, it has been recognised that hypertension is very common in obese and diabetic patients (Modan et al., 1985; Turner, 1985), and that the fasting plasma insulin concentration is closely related to the elevation in blood pressure (Christlieb et al., 1985). Other cardiovascular risk factors such as hyperlipidaemia have also been linked to diabetes and obesity, and these metabolic disorders have been established and described as syndrome X or metabolic syndrome (Reaven, 1988). The principal features of syndrome X are hypertension, abnormal glucose tolerance, increased very low density lipoprotein (VLDL) triglyceride levels, decreased high density lipoproteins (HDL) cholesterol levels, obesity and hyperinsulinaemia or with decreased insulin sensitivity (Walker and Alberti, 1993). These have been shown to cluster within an individual and are associated with an increased risk of coronary artery disease (CAD).

Epidemiological studies have suggested that hyperinsulinaemia is probably the common factor linking each of the cardiovascular risk factors of syndrome X (Ferrannini et al., 1991), although the mechanism or mechanisms underlying the development of syndrome X have yet to be exactly identified (Reaven, 1988).

1.4.4 Treatment

It is important to control the hyperglycaemia in order to reduce long-term complications (UKPDS, 1998). Treatment programmes are established with dietary management and exercise, particularly to facilitate weight loss in obese patients. When these measures are inadequate, oral hypoglycaemic drugs or insulin are required. There are presently several oral hypoglycaemic agents available to treat hyperglycaemia, with different mechanisms of action.

1.4.4a Sulphonylureas

Sulphonylureas (appendix II) are widely used to treat hyperglycaemia by stimulating insulin secretion. Thus the efficacy of these agents depends on the presence of enough β-cells with sufficient functional reserve. They have been reported to produce an acceptable degree of glycaemic control in more than two-thirds of Type 2 diabetic patients who are not successfully treated by dietary management alone (Gerich, 1989; Groop, 1992). The UKPDS however, demonstrated a progressive failure of sulphonylureas and after 10 years few patients were adequately controlled with these agents. There are several sulphonylureas available, with varying pharmacokinetic properties and different durations of insulin release. Short acting preparations are usually taken twice daily (e.g. glipizide). They may reduce the post-prandial peaks of blood glucose and help to reduce basal hyperglycaemia. Long acting preparations (e.g. glibenclamide) need to be taken only once each day. There is an increased risk of hypoglycaemia if a meal is missed and they may incur a greater increase in weight gain than shorter acting preparations. Sulphonylureas are discussed in detail in chapter 7. Very short acting insulin releasing agents, which are not sulphonylureas but act in a similar manner (e.g. repaglinide), are taken with meals to stimulated postprandial insulin release and thereby reduce postprandial hyperglycaemia.

1.4.4b Metformin

Metformin is a biguanide (appendix II), commonly used to treat Type 2 Diabetes. In contrast to the sulphonylureas, metformin acts at the sites of insulin resistance. Its hypoglycaemic properties are a consequence of increased insulin-stimulated glucose uptake and glycogenesis in skeletal muscle and reduced hepatic glucose output (Bailey and Turner, 1996). Thus metformin improves insulin sensitivity without increasing insulin concentrations (UKPDS, 1998). The mechanism of metformin action is discussed in detail in chapter 7.

1.4.4c Thiazolidinediones

Thiazolidinediones (appendix II) are a new class of oral anti-diabetic agents, which act via the nuclear peroxisome proliferator-activated receptor-gamma (PPARγ) to increase the transcription of certain insulin-sensitive genes. Troglitazone was the first thiazolidinedione to be approved for use (launched in 1997), however rosiglitazone and pioglitazone have also recently been approved for use in the USA. *In vitro, in vivo* and clinical studies for thiazolidinediones are discussed in detail in chapter 7.

1.4.4d Acarbose

α-glucosidase inhibitors slow the rate of carbohydrate digestion, thereby decreasing the rate of glucose absorption and decreasing the likelihood of postprandial hyperglycaemia. The mode of action of these is via the inhibition of α-glucosidase enzymes, which act at the brush border of the small intestine enterocytes to cleave glucose (Bailey and Flatt, 1996).

The first α glucosidase inhibitor which became available for the treatment of Type 2 diabetes was acarbose, an inhibitor of glucoamylase, sucrase, isomaltase and maltase (Hanefold, 1993). *In vivo* studies have shown that acarbose decreases postprandial hyperglycaemia and consequently lowers the insulin response (Clissold and Edwards, 1988), and similar findings have been reported in human studies (Dimitriadis et al., 1986). Side effects of this treatment include abdominal distention, diarrhoea and flatulence.

1.4.4e Agents used in the Treatment of Obesity (Anorexigenic and Satiety-Inducing Compounds)

The majority of patients with Type 2 diabetes are obese, and excess body adiposity causes insulin resistance. In obese Type 2 diabetic patients, weight loss improves insulin sensitivity (DeFronzo and Ferrannini, 1991) thus the use of anorexigenic and satiety-inducing agents for the treatment of obesity and obese Type 2 diabetes has recently gained wider acceptance.

Drugs that continue to be approved for clinical use in the treatment of obesity include noradrenergic agents, and serotoninergic agents. Noradrenergic agents include benzphetamine, phendimetrazine, diethylpropion/amfepramone, mazindol, phentermine hydrochloride, phentermine resin, phenylpropanolamine. These agents act as appetite suppressants by binding to hypothalamic receptors that are part of the central satiety signalling pathways. They carry the potential for addiction and can also exert centrally mediated on the cardiovascular system (CVS) and peripheral metabolism (Hauger et al., 1986). Hence, some side effects of these agents include insomnia, nervousness, dry mouth and increases in heart rate and blood pressure (Bray, 1985).

Fenfluramine, dexfenfluramine (both recently withdrawn) and fluoxetine are serotonin-releasing agents (appendix II) active in suppressing food intake, however have been shown to produce side-effects (Bray and Ryan, 1997). These have been related to dependency and abnormalities of heart function have been noted in approximately 20% of patients on dexfenfluramine.

Sibutramine is a serotonin and noradrenaline reuptake inhibitor (SNRI, appendix II), currently available in the USA as a treatment for obesity. Its novel mechanism of action combines the beneficial effects of both serotoninergic and adrenergic agents, in the regulation of appetite and satiety. Sibutramine has been shown to increase satiety and increase thermogenesis thus significantly increasing weight loss. Sibutramine is discussed in detail in chapter 5.

Orlistat is the first of a new class of antiobesity agents, the lipase inhibitors. It has been developed for the long-term management of obesity and has recently been launched in the UK. Orlistat acts by selectively inhibiting the absorption of dietary fat (Hauptman et al., 1992), and has been demonstrated to be more effective in reducing weight compared with diet alone (Tonstad et al., 1994; Zhi et al., 1996). The role of orlistat in the treatment of obese patients with Type 2 diabetes has recently been investigated during a 1-year randomised double-blind study (Hollander et al., 1998). After 1 year of treatment the orlistat group lost significantly more weight than the placebo group, fasting plasma glucose and HbA1c was significantly decreased. Total cholesterol, LDL cholesterol, triglycerides, apolipoprotein B, and the LDL to HDL cholesterol ratio were significantly reduced. Side effects of this treatment include mild to moderate and transient gastointestinal events.

1.5 Insulin Resistance

One of the main roles of the anabolic hormone insulin, is that of controlling blood glucose. Insulin acts mainly by increasing glucose uptake in muscle and adipose tissue, and inhibition of gluconeogenesis by the liver. Insulin has a multitude of actions which are mediated via the actions of intracellular enzymes and transcription factors for selected genes, including alterations in the synthesis and activation of various intracellular enzymes and membrane transport proteins. Hence the effects of insulin are pleiotropic (table 1b).

The term 'insulin resistance' is typically used to refer to a state of defective insulin action on glucose homeostasis. Insulin resistance has been more specifically defined as a subnormic response to a given concentration of insulin (Moller and Flier, 1991), involving a shift in the dose response curve, such that the magnitude of the biological response to insulin is decreased (Kahn, 1978).

Insulin resistance incurs a broad spectrum of clinical consequences since the impaired response to insulin may be observed over the entire range of insulin concentrations, found under physiological and therapeutic circumstances. Clinically it includes patients with diabetes mellitus who require insulin but who continue to be hyperglycaemic despite large insulin doses. It also includes those insulin resistant patients who maintain normoglycaemia although endogenous insulin secretion is highly elevated and patients who are obese and consequently diabetic due to the role excess body fat plays in the insulin resistance of Type 2 diabetes.

Evidence from studies completed in the late 1980's have shown that all major target tissues are resistant to insulin in clinically defined insulin resistance but to different extents (Reaven, 1988). Methods are now well established and provide a quantitative measurement of insulin action *in vivo*, such as the euglycaemic hyperinsulinaemic clamp technique and also computer modelled analysis of glucose tolerance testing (DeFronzo, 1988; Bergman, 1989).

Hyperinsulinaemia is a common feature of insulin resistance which correlates with levels found in precise measurements of insulin sensitivity (Bergman, 1989), and may represent a compensatory measure of insulin secretion to counter the decrease in insulin action. Studies have also confirmed that in the insulin resistant state, hyperinsulinaemia does not induce hypoglycaemia (DeFronzo, 1992). This results not only from increased secretion of insulin by β cells of the endocrine pancreas but also from impairment of receptor-mediated clearance of insulin by resistant peripheral target cells (Flier et al., 1982). However, this in turn further increases insulin resistance and unmasks or hastens the emergence of defects in insulin secretion which can eventually cause β-cell failure in susceptible individuals.

Therefore, there are two defined pathological defects in Type 2 diabetes. Firstly, the poor biological response of target tissues upon which insulin action is directed, such as impaired glucose uptake and utilisation by muscle and fat and inhibition of hepatic glucose output, i.e. insulin resistance. Secondly, the inability of β-cells to compensate for insulin resistance, consequently causing relative insulin deficiency found in Type 2 diabetes (Porte, 1991; Leahy et al., 1992; DeFronzo, 1992).

1.6 The Molecular Basis of Insulin Action

Since current evidence favours the explanation that insulin resistance is the primary, underlying cause of most cases of Type 2 diabetes, it is important to identify the reasons for insulin resistance. It is necessary to understand the signalling pathways that link the insulin receptor to its biological effectors. Hence, an understanding of the mechanisms through which insulin controls metabolic processes may eventually lead to the identification of the specific intracellular signalling lesion or lesions that cause(s) insulin resistance.

Recently, Kahn documented the 3 levels of insulin action; level one is the initial events related to receptor tyrosine kinase activity, level two comprises the serine phosphorylation/dephosphorylation cascade and level three; the final biological effectors (Kahn, 1994). Since this time there has been much research in this area and components of the insulin signalling pathways are constantly being elucidated. However the pathways identified to date are primarily based upon the kinase-phosphatase cascades documented by Kahn, White and colleagues at the Joslin Diabetes Center, Boston.

1971 saw the discovery that insulin binds to a cellular receptor (Freychet et al., 1971). When insulin binds to the receptor, its conformation changes and the kinase activity of the receptor is stimulated (Herrera and Rosen, 1986; Roth and Cassell, 1983; Baron et al., 1992). This consequently causes receptor autophosphorylation of tyrosine residues, thought to be essential for insulin signalling (White et al., 1988).

The insulin receptor acts as an allosteric enzyme and when insulin binds extracellularly, the intracellular portion of the β subunit changes conformation to expose ATP binding sites which enable the receptor to autophosphorylate at several tyrosine residues. This in turn causes phosphorylation of a number of intracellular substrate proteins (Rosen, 1987). Hence insulin receptors phosphorylate multiple proteins, of which IRS-1 is the best characterised. However, several other cellular substrates have been described for the insulin receptor, some of which have been heavily investigated, and others, which play an undetermined role in insulin action.

Pp120, a membrane glycoprotein specific to the liver, and pp15, an adipose specific substrate are 2 such substrates of unknown function which are tyrosine phosphorylated during insulin stimulation (Perroti et al., 1987; Bernier et al., 1988).

Shc is another substrate that is tyrosine phosphorylated during insulin stimulation and is thought to play an important role in insulin action (Skolnik et al., 1993). Upon insulin stimulation Shc binds to a SH2 domain in Grb-2 and mediates p21ras GTP-loading, hence it is thought to play a role in the activation of the mitogen activated protein (MAP) kinase cascade (Reviewed by White, 1997; Myers et al., 1994).

The insulin signalling pathway is a complex branching cascade of kinase and phosphatase reactions with many participating substrates (figure 1.1, Schematic diagram of the insulin receptor signalling mechanism). However, the aims of this research focus primarily on the IRS-1 → PI 3-kinase → PKB → GLUT pathway, which is particularly important for the acute metabolic effects of insulin. Although other pathways are important for other actions of insulin, they will not be further considered here, for a detailed review, see White (1997).

The first substrate protein involved in insulin signalling was identified and subsequently named insulin receptor substrate 1 (IRS-1) (Rothenberg et al., 1991). It has been hypothesised that it is a specific substrate for the insulin receptor and activation results in its phosphorylation on multiple tyrosine sites (Sun et al., 1993). This causes binding of a number of target intracellular proteins, which all possess SH2 domains. There are at least 20 of these proteins known, although few are thought to be involved in the insulin signalling pathway. One of these proteins appears to serve a key role in the signalling process; phosphatidylinositol 3-kinase.

Phosphatidylinositol 3-kinase (PI 3-Kinase), was the first SH2 protein found to associate with IRS-1 (White and Kahn, 1994). It has been heavily implicated as carrying great importance in the pathway activating glucose uptake via translocation of GLUT4 glucose transporters from the intracellular pool to the plasma membrane. This enzyme phosphorylates the D-3 position of the inositol ring of phosphatidylinositol to produce PI-3-P, PI-3,4-P_2 and PI-3,4,5-P_3. It is these lipids which are thought to constitute the first signalling intermediates of this routing of the insulin signalling pathway to control glucose transport. PI 3,4,5-P_3 has recently been documented to be the main messenger in this pathway, which is formed in the inner leaflet of the plasma membrane which is then converted to PI 3,4-P_2 by a specific phosphatase (Cohen et al., 1997; figure 1.2).

It is only within the last eighteen months that research has identified some of the possible missing links in signal transduction, much of which has been completed by Cohen, and colleagues at the MRC protein phosphorylation unit, Dundee.

It is thought that the next messenger in the insulin signalling pathway is an enzyme termed 3-phosphoinositide-dependent protein kinase (PDK-1), because it is only active in the presence of PI 3,4,5–P_3 or PI 3,4-P_2 (Cohen et al., 1997). Little is known

Figure 1.1 – Schematic diagram outlining the components of insulin signalling from insulin receptor to the biological effectors that have been identified to date.

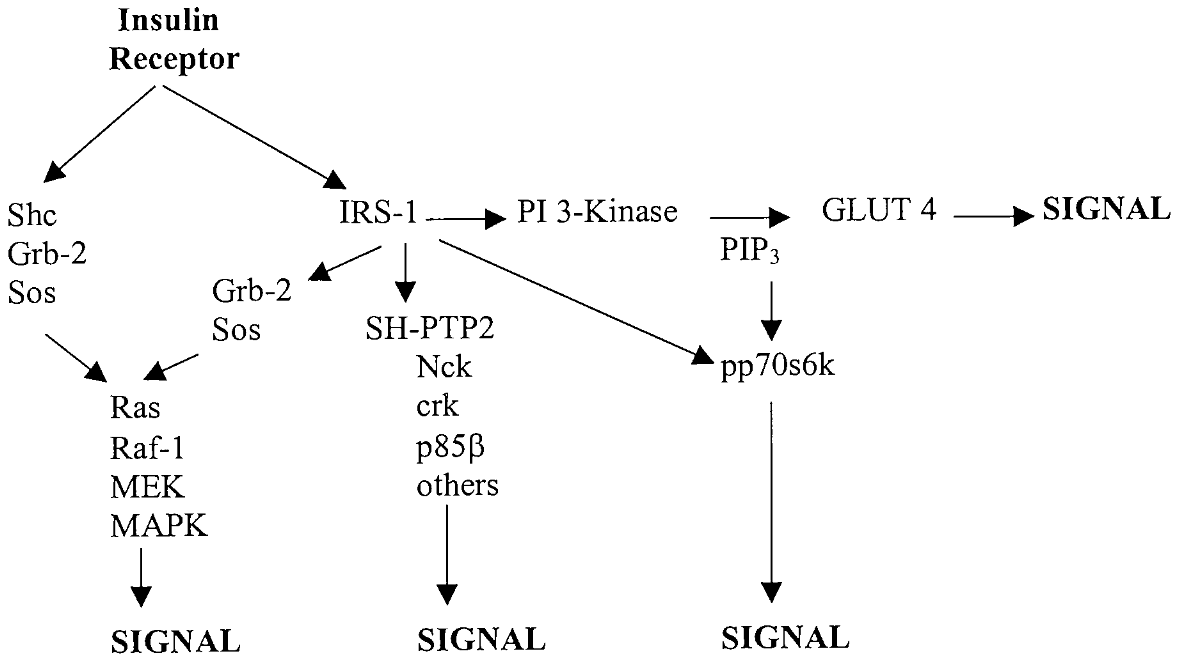
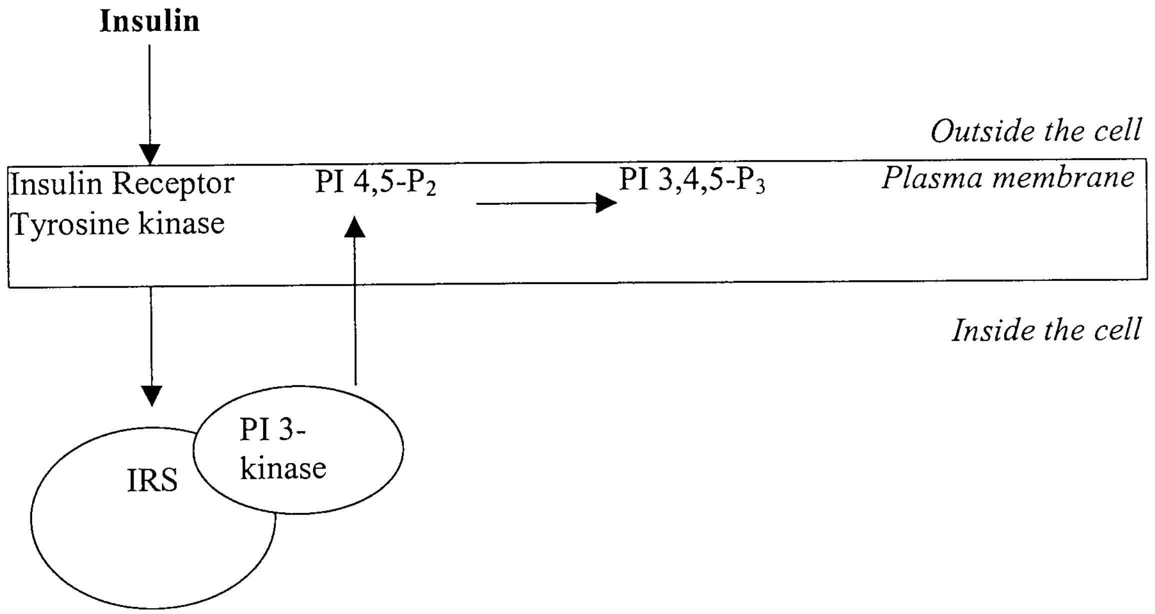


Figure 1.2 Mechanism by which insulin stimulates the formation of PI 3,4,5-P$_3$. Insulin binding activates the protein tyrosine kinase associated with the insulin receptor. Autophosphorylation of the insulin receptor triggers IRS phosphorylation which interacts with PI 3-kinase. The p110 catalytic subunit of is recruited to the plasma membrane where it converts PI 3,4-P$_2$ to PI 3,4,5-P$_3$. (Adapted from Cohen et al., 1997).



about this protein, except that it is not inhibited by wortmannin (WM), and it appears to be similar to the subfamily of protein kinases that includes protein kinase B (PKB) (Alessi et al., 1997).

Protein kinase B (PKB) has been identified as a protein messenger which regulates further downstream from PI 3-kinase, and may be the next step in the signalling pathway. Recent research has reported that PDK-1 activates PKB, disputing earlier reports that PI 3-kinase directly activates PKB (Cohen et al., 1997). PKB may be a protein of major importance in this pathway, as presently it appears that it not only activates glucose transporters to stimulate glucose uptake, but also inhibits GSK3 which is implicated in protein synthesis via eIF2B and glycogen synthesis via glycogen synthase. PKB also activates cardiac PFK2 to stimulate glycolysis (figure 1.3, the signal transduction pathway through which PKB and GSK may mediate the metabolic effects of insulin).

It is thought that possibly one of the final stages in the insulin signalling pathway is the stimulation of glucose transporters, and this may occur indirectly through the stimulation of PKB. Upon activation there is movement of glucose transporter vesicles to the plasma membrane, and glucose transport is then facilitated across the membrane and into the cell.

1.6.1 The Insulin Receptor

The insulin receptor is a large transmembrane glycoprotein complex with a molecular weight of approximately 400KDa (Collier and Gorden, 1991). The complex consists of 2 135KDa α subunits and 2 95KDa β subunits, which are linked by disulphide

Figure 1.3 Schematic cascade to demonstrate the signal transduction pathway through which PKB and GSK may mediate the metabolic effects of insulin as hypothesised by Cohen et al., 1997.

bonds to form a heterodimer.The α subunit consists of 719 or 731 amino acids (as there are 2 isoforms of the receptor due to alternative splicing of the receptor transcript), and contains the insulin binding site as it is entirely extracellular (Kasuga et al., 1982). The β subunit is composed of a 23 amino acid transmembrane domain, a 403 amino acid cytoplasmic sequence and a 194 amino acid extracellular domain (Massague et al., 1982).

Insulin binds with high affinity to the α subunit of its receptor which leads to the phosphorylation of the β subunit (reviewed by White and Kahn, 1994). This occurs via conformational changes in the extracellular domain which is transmitted to the β subunit down to its C-terminus, leading to a receptor which is then able to bind ATP (Pilch and Czech, 1980). This leads to autophosphorylation of the receptor, which induces a second conformational change allowing enzyme substrate interactions (Baron et al., 1992).

The discovery that the insulin receptor is a tyrosine kinase was a major finding that lead to the realisation that because several proteins are modified on serine/threonine residues, there must a switch kinase in the signalling pathway (reviewed by Van Obberghen, 1994). However, it was evident that the first receptor substrate in the signalling pathway must be phosphorylated on tyrosine residues, and so the search began to identify the components of this pathway (Gazzano et al., 1983).

The insulin receptor is structurally normal in Type 2 diabetes, and there is 'pool' of 'spare' receptors which ensure that a reduced population of insulin receptors does not make a major contribution to insulin resistance in most patients (Kahn, 1997).

However the variety of biological effects of insulin that are impaired in insulin resistance suggests that important rate-limiting lesions are located early in the signal transduction process, prior to the full separation of the different pathways. Indeed, decreased phosphorylation and tyrosine kinase activity of the insulin receptor β-subunit have been observed in Type 2 diabetic patients (Kahn, 1997; Khan, 1994).

1.6.2 Insulin Receptor Substrate

IRS-1 is the best characterised insulin receptor substrate (Myers and White, 1995) and was initially detected in 1985 in insulin stimulated Fao hepatoma cells by immunoprecipitation with high affinity antiphosphotyrosine antibodies (White et al., 1985). Initially named pp185, IRS-1 is a cytosolic protein with at least 30 potential serine/threonine and 22 potential tyrosine phosphorylation sites (Sun et al., 1991) and has a molecular weight of 131KDa (reviewed by Kahn, 1994).

IRS-1 is highly phosphorylated on serine residues in the basal state, however an increase in serine phosphorylation and additional tyrosine phosphorylation occurs upon insulin stimulation (Sun et al., 1991; 1993). This multisite phosphorylation results in a non-covalent binding of IRS-1 to specific intracellular proteins (Backer et al., 1992; Myers and White, 1993; Lavan et al., 1992). Hence, IRS-1 is regarded as an intracellular docking protein, due to its ability to bind other intracellular proteins following tyrosine phosphorylation and to transmit signals through non covalent binding (Fantl et al., 1993). This occurs via phosphorylation motifs in IRS-1 and specific domains on the target proteins denoted SH2 (Src homology 2) domains, of which there are at least 20 proteins which contain them (Kahn, 1994) as noted earlier.

The binding of SH2 proteins to IRS-1 serves several purposes, enzymes associate with IRS-1 through SH2 domains may be activated e.g. PI 3-kinase, and the IRS-1/SH2 protein complex is free to move independently of the internalised receptor, as it is mobile (reviewed by White, 1997).

Several proteins closely related to IRS-1 have recently been identified, particularly IRS-2, and possibly IRS-3 and IRS-4, which may undertake a similar role to IRS-1 but exist in much smaller quantities (White, 1997).

Decreased phosphorylation of IRS-1 has been observed in Type 2 diabetic patients (Kahn, 1994). Cross-talk between the insulin receptor, IGF1 receptor and IRS proteins could imbalance IRS activation (White, 1997). Other receptors also appear to communicate (directly or indirectly) with IRS proteins or affect receptor phosphorylation (e.g. cytokines). Increased phosphatase-induced dephosphorylation of the insulin receptor or IRS proteins may be implicated in insulin resistance, since phosphatase inhibitors such as vanadium compounds can improve insulin action (vanadium compounds are discussed further in chapter 7).

1.6.3 Phosphatidylinositol 3-kinase

1.6.3a Phosphoinositides

The phosphoinositide metabolic pathway has been well documented, in which phosphatidylinositol (PI) is the initial substrate. Several years ago the pathway was thought to be very simple, beginning with the phosphorylation of PI by phosphatidylinositol 4-kinase, which in turn is phosphorylated by phosphatidylinositol 4-phosphate 5-kinase to produce phosphatidylinositol 4,5-bisphosphate (PI 4,5-P_2). Diacylglycerol and inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3) are produced by the hydrolysis of PI 4,5-P_2 due to the action of phospholipase C (reviewed by Carpenter and Cantley, 1990).

However, more than one species of PI-kinase exists, and in 1988 a type of PI-kinase was discovered that phosphorylates the D3 position of PI. Hence PI 3-kinase and its products, potential intracellular signals, were discovered (Whitman et al., 1988).

1.6.3b Phosphatidylinositol 3-kinase

The identification of PI 3-kinase resulted in the discovery of new D3 phosphorylated phosphoinositides, namely PI 3-P and PI 3,4-P_2 due to the action of PI 3-kinase upon substrates PI, and PI 4-P, respectively. The product PIP_3 has also been identified due to the action of PI 3-kinase upon PI 4,5-P_2 and is thought to be PI 3,4,5-P_3. This has very recently been described an important messenger in the insulin signalling pathways (Cohen et al., 1997).

PI 3-kinase was initially identified in fibroblasts (Whitman et al., 1988) and subsequent investigations including purification and elucidation of the structure have revealed that PI 3-kinase is a 190kDa and is comprised of two peptides of 110kDa and 85kDa to form a structural dimer (Carpenter and Cantley, 1990).

1.6.3c The Physiological Role of Phosphatidylinositol 3-kinase

As noted above, the action of PI 3-kinase upon PI and polyphosphoinositides produces a group of phosphoinositides that are phosphorylated at the D3 position of the inositol ring. It is possible that these PI 3-kinase products are themselves cellular second messengers, in particular, PI 3,4,5-P_3, which is thought to be converted downstream by a specific phosphatase to PI 3,4-P_2 (Cohen et al., 1997).

The molecular mechanism by which insulin stimulates the formation of PI 3,4,5-P_3 is described below. Insulin binds to the α-subunit of the insulin receptor, activating the protein tyrosine kinase associated with the β-subunit. The β-subunit then phosphorylates itself, as described above, which triggers an interaction between the receptor and IRS, allowing the receptor to phosphorylate IRS-1, IRS-2 and possibly other proteins at multiple tyrosine residues. Particular phosphotyrosine residues in IRS-1/ IRS-2 then interact with the p85 subunit of PI 3-kinase, thereby recruiting the p110 catalytic subunit to the plasma membrane where it converts PI 3,4-P_2 to PI 3,4,5-P_3. PI 3,4,5-P_3 is subsequently converted to PI 3,4-P_2 by a specific phosphatase as described by Cohen et al., (1997).

Initial investigation into the role of PI 3-kinase has shown that it is associated with a number of cell receptors upon stimulation with the appropriate agonist and also with different oncoproteins (Carpenter et al., 1993). In particular, tyrosine and serine residues are phosphorylated in response to PDGF receptor stimulation by PDGF (Auger et al., 1989), and insulin receptor stimulation with insulin and insulinomimetic agents (Hadari et al., 1992; Jullien et al., 1996).

1.6.3d Phosphatidylinositol 3-kinase and Insulin Signalling

The activation of PI 3-kinase by insulin was demonstrated in 1990, utilising Chinese hamster ovary (CHO) cells transfected with the human insulin receptor (Ruderman et al., 1990). The subcellular distribution of PI 3-kinase was subsequently investigated, by observing the effect of insulin on PI 3-kinase activity in rat adipocytes, obtained from a collagenase digest of rat epididymal fat pads. The subcellular distribution was identified by measuring PI 3-kinase activity in plasma membranes, intracellular membranes and the cytosol, of adipocytes incubated with and without insulin, and also in anti-tyr (P) immunoprecipitates prepared from these fractions and whole cell lysates. (Kelly et al., 1992). It was found that insulin increased the activity of PI 3-kinase in adipocytes, especially in the low density intracellular membranes and (to a lesser extent) in the plasma membranes.

By 1993, it was established that PI 3-kinase activity could be stimulated by insulin (Kelly and Ruderman, 1993; Ruderman et al., 1990; Kelly et al., 1992; Giorgetti et al., 1992). However the exact mechanism of action of this effect remained unknown. To try to elucidate the intracellular pathway of the insulin signalling system, other potential regulatory molecules were investigated. The association of PI 3-kinase with IRS-1 was an important discovery in this field. It was realised that insulin has the ability to stimulate the formation of signalling complexes between PI 3-kinase, IRS-1 and the insulin receptor, (Backer et al., 1992; Yonezawa et al., 1992; Folli et al., 1992; Giorgetti et al., 1993).

Research within this field has recently focused upon the physiological role of PI 3-kinase specifically in insulin induced glucose transport and inhibition of lipolysis in rat adipocytes. Okada et al., (1994), demonstrated that PI 3-kinase activity was detectable in both adipocyte membrane fractions and anti phosphotyrosine immunoprecipitates incubated with insulin, and was inhibited by the fungal metabolite, wortmannin (WM). PI 3-kinase has also been investigated in the role of regulation of glucose transport, amino acid transport and glucose transporters (Tsakiridis et al., 1995). This suggests that PI 3-kinase is WM sensitive, and plays an important role within the insulin signalling pathway(s).

The specific role of PI 3-kinase in insulin signalling is an area of recent investigation, and little is known of its actual function and possible implication in Type 2 diabetes. However, studies using animal models have demonstrated that there may be a defect in skeletal muscle PI 3-kinase in goldthioglucose induced obese insulin resistant mice (Heydrick et al., 1993), and in *ob/ob* mice there is thought to be decreased PI 3-kinase activity (Folli et al., 1993).

Further studies into the physiological role of PI 3-kinase investigated its activity in the soleus muscle of insulin resistant gold thioglucose treated mice (Heydrick et al., 1995). PI 3-kinase activity was diminished in response to insulin stimulation compared to lean mice. Also, IRS-1 tyrosine phosphorylation was diminished in these mice, suggesting that altered PI 3-kinase stimulation in muscle is characterised by a reduced association of PI 3-kinase activity with IRS-1 tyrosine phosphorylation. Hence suggesting that a defect in PI 3-kinase activation could participate in the establishment of insulin resistance in both muscle and adipose tissue and that changes in the phosphorylation levels of IRS-1 could play an important role in insulin resistance.

Recently, PI 3-kinase has been implicated in the control of insulin secretion. PIP_3 has been found in most cell types, including the cytosolic and membrane fractions of insulin secreting cells. It has been demonstrated that PI 3-kinase is present in both fractions of rat islets and cultured β-TC3 cells (Gao et al., 1996). WM inhibited PI 3-kinase activity directly, although basal and glucose stimulated insulin release was unaffected at the concentration used. Indeed, a much higher concentration of WM was required in order to modify insulin release, suggesting that PI 3-kinase is unlikely to play a major role in glucose induced insulin secretion.

However, it appears that PI 3-kinase plays an important role within the insulin signalling system, and that this has important implications for the pathogenesis of insulin resistance and consequently Type 2 diabetes. Nevertheless, much remains to be learned about PI 3-kinase and the role that it plays. It is anticipated that this area of research may reveal answers to questions surrounding the cause of insulin resistance and may open new approaches to the treatment of Type 2 diabetes.

1.6.4 Protein Kinase B

Protein Kinase B (PKB), has recently emerged as a key intermediate in the PI 3-kinase stimulated signalling pathway, based on its stimulation by insulin and the inhibition of its activation by wortmannin (reviewed by Cohen et al., 1997).

PKB is a member of the subfamily of the second messenger-regulated serine/threonine kinases which share sequence homology to both protein kinase A and C. PKB is now recognised to be identical to the kinase Rac (Burgering and Coffer, 1995), also referred to as Rac or Akt. Three isoforms of PKB have been identified; α, β and γ, each consisting of an amino terminal pleckstrin homology (PH) domain, a central kinase domain and a serine/threonine rich carboxyl-terminal region (reviewed by Frech et al., 1997).

The exact mechanism of signal transduction from PDK-1 to PKB is unknown, however, the amino terminal PH domain has been suggested to be of importance because if it is blocked it cannot be activated by stimulatory growth factors (Andjelkovic et al., 1996).

The role that PKB plays in insulin signalling remains to be determined. So far it has been implicated in adipocyte differentiation, cell growth and regulation of glycogen metabolism by insulin (Magun et al., 1996; Bellacosta et al., 1991; Cross et al., 1995). It has been suggested that PKB may play a role in glucose transporter translocation by Tanti et al. (1997). In this study PKB was activated by insulin in isolated adipocytes, and GLUT4 translocation was demonstrated in parallel to PKB activation. Adipocytes transfected with active PKB and an epitope tagged transporter was used to demonstrate GLUT4 subcellular distribution. It was observed that expression of PKB led to an increase in the amount of GLUT4 at the cell surface, suggesting that PKB could play a role in promoting GLUT4 appearance at the cell surface following insulin stimulation (Tanti et al., 1997).

1.6.5 Glucose Transporters

There are two processes that enable the transport of glucose across the plasma membrane in mammalian cells. The dietary uptake of glucose from the lumen of the small intestine is mediated by the intestinal Na+/glucose cotransporter SGLT1, which uses the electrochemical sodium gradient to transport glucose against the concentration gradient. Energy-independent transport of glucose down its concentration gradient is facilitated by glucose transporters known as the GLUT family of carriers present on the surface of all cells (reviewed by Bell et al., 1993).

The GLUT family is characterised by a high degree of stereoselectivity, providing for the bidirectional transport of glucose. Seven members of this supergene family have been described to date, however only five of these are thought to be the main facilitators of glucose transport, named GLUT1 to GLUT4, (and also possibly GLUT-5, although this is thought to transport mainly fructose), based upon the chronological order of their identification (Mueckler, 1994). The transporters show tissue specific distribution which adapt to tissue specific demands for glucose utilisation (Mueckler et al., 1985), and although there is a degree of overlap in tissue expression of isoforms, there are several trends their in distribution (Birnbaum et al., 1986; Birnbaum et al., 1989; Charron et al., 1989; James et al., 1989; Kaestner et al., 1989; Kayano et al., 1988; Thorens et al., 1988; table 1d below).

Table 1d – Distribution and properties of glucose transporters

Glucose Transporter	**Tissue**	**N° amino acids**	**Homology to Glut 4 (%)**
GLUT1	Erythrocyte, placenta, brain	492	76
GLUT2	Liver, kidney	524	67
GLUT3	Brain, placenta, kidney	496	69
GLUT4	Muscle, fat	509	100
GLUT5	Small intestine	501	41

Mueckler et al., (1985), proposed a model for the structure of the glucose transporters in the plasma membrane consisting of 12 membrane spanning α helical segments with intracellularly located NH2 and COOH termini. The molecular structure reveals two large loops, an intracellular loop, connecting transmembrane segments 6 and 7 and which divides the structure into two halves, and an extracellular loop, connecting segments 1 and 2. The remaining loops between the helices at the cytoplasmic surfaces are very short, resulting in close packing of the helices at the inner surface of the membrane. A comparison of amino acid sequences of GLUT isoforms have revealed that the proteins are of a similar size, approximately 500 amino acids (Bell et al., 1990), however, there is only 38% homology between isoforms GLUT 1-4. The areas of highest homology were found between the membrane spanning segments.

Studies on glucose transporter biology have focused predominantly on GLUT4 and GLUT1 as GLUT4 (and GLUT1 to a lesser extent) are insulin-sensitive glucose transporters, and are expressed abundantly in skeletal muscle and adipose tissue (James et al., 1988). These tissues are the major insulin target tissues and hence the insulin-sensitive glucose transporter system is involved with the regulation of whole body glucose homeostasis. Therefore GLUT1 and GLUT4 will be considered here because when dysregulated they may have implications in the pathogenesis of diabetes. [For a detailed review of glucose transporter systems in the brain, heart, pancreatic beta cells, liver, intestines and kidney see Olsen and Pessin (1996)].

In skeletal muscle, which accounts for approximately 85% of postprandial glucose uptake, GLUT1 is found predominantly in the plasma membrane and GLUT4 in intracellular vesicles, in basal conditions. During insulin stimulation, the amount of GLUT1 and GLUT4 in the intracellular membrane increases, hence it has been hypothesised that GLUT1 is involved primarily in basal glucose uptake and GLUT4 in insulin stimulated glucose uptake (James et al., 1988). Indeed, more recently, studies using photolabelling techniques have demonstrated that an increase in glucose transport activity directly correlates with the appearance of GLUT4 at the cell surface (Wilson and Cushman, 1994), and this has been shown in both skeletal muscle and adipocytes (Cushman and Wardzala 1980).

It is not known whether an abnormality of the glucose transporters contributes to insulin resistance. Indeed, there are conflicting reports on the levels of GLUT4 in different insulin resistant states and in different tissues and cells. It appears that the majority of data suggests that the expression of GLUT4 in skeletal muscle is not significantly decreased. This is possibly due to compensatory mechanisms, although is not the case in studies using adipocytes (Karnieli et al., 1981; Berger et al., 1989) or in *in vivo* studies (Garvey et al., 1989). Therefore, until there is conclusive evidence that impaired glucose uptake is primarily due to altered GLUT4 levels, then it can be assumed that insulin resistance may be due to an unknown defect or defects in the insulin signalling cascades, which consequently modulates the action of the glucose transporter system.

1.7 Aims and Objectives

Non insulin dependent diabetes mellitus is caused by insulin resistance and abnormal β-cell function, which together contribute to a series of metabolic disturbances, the pathophysiology of which remains incompletely understood.

The aim of this work is to explore the use of a number of pharmacological agents and exogenous peptides to modify insulin action in cultured skeletal muscle cells and in animal models of diabetes, insulin resistance and obesity. Potential mechanisms of action and sites of signal transduction of these compounds will be investigated using known inhibitors of proteins known to mediate intracellular signalling. The overall aim of this work is to find new and better ways to treat Type 2 diabetes and insulin resistance and to search for other therapeutic agents that could be of use in diabetic patients who fail to respond to conventional therapy. Investigations undertaken in this thesis were completed specifically with this view in mind. The specific objectives are defined below.

As discussed previously, skeletal muscle is quantitatively the major site responsible for insulin-stimulated glucose transport (DeFronzo et al., 1988). Thus, the rat L6 skeletal muscle cell line will be characterised (chapter 3) and utilised in this study to determine the direct effects of the said agents under investigation. Other models to be investigated as potential models include isolated soleus muscles and hemidiaphragms. *In vivo* studies will be undertaken to investigate effects of compounds on glycaemia, insulinaemia, satiety and body weight.

Chapter four considers leptin, a hormone secreted by adipocytes, which is primarily involved in the regulation of body weight. There have been discrepancies in the literature pertaining to a direct action of leptin in different model systems. The objectives are two fold, to determine the effect of leptin on;

a) mammalian physiology and glucose transport by soleus muscles and

b) glucose transport in L6 skeletal muscle cells and to investigate the mechanism of direct action.

Sibutramine, an antiobesity agent is investigated in chapter 5, to identify any antidiabetic properties of the compound independent of the weight reducing effects demonstrated *in vivo*. The objectives are to explore potential antidiabetic effects of this compound and its active metabolites both *in vivo* and *in vitro*, and to compare the effects with other known antiobesity agents.

Many plants have been used for centuries for their hypoglycaemic properties (Bailey and Day, 1989). Two compounds are investigated in chapter 6 for their action on skeletal muscle *in vitro* and hypoglycaemic properties *in vivo.*

Other compounds investigated, including those already known to affect insulin sensitivity are investigated in chapter 7 e.g. metformin, tolbutamide, thiazolidinediones, vanadyl sulphate and thioctic acid. The ability of these compounds to modify glucose transport in skeletal muscle is investigated *in vitro*.

These investigations were designed to fulfil the overall aims as outlined above and to pave the way for future research to identify intervention targets to improve insulin action, which will ultimately provide the answers needed to prevent the insulin resistance syndrome and reverse Type 2 diabetes.

Chapter Two:
Materials and Methods

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2.1 Suppliers of Materials

Chemicals used were of analytical grade and supplied by BDH, Ltd, U.K., Fisons, U.K. and Sigma Chemical Company, Poole, Dorset, U.K. unless otherwise stated. Radionuclide chemicals were obtained from Amersham, Amersham International Plc., Buckinghamshire, U.K.

Cell culture media and supplements were purchased from GibcoBRL/Life technologies, Paisley, Scotland, unless stated otherwise. Plasticware was from Sarstedt. Rat L6 skeletal muscle cell line was obtained from the European Culture Collection, Porton Down.

Recipes for solutions used in this chapter are given in appendix I.

2.2 Cell Culture

2.2.1 Cell Culture Media and Supplements

DMEM (Dulbecco's Modification of Eagles Medium) was obtained as 500ml sterile 1x solutions containing 0.11g/l sodium pyruvate. DMEM was supplemented with the following additions; antibiotic/antimycotic solution (100x, containing; 100 units/ml penicillin G sodium, 10mg/ml streptomycin sulphate, 25μg/ml amphotericin B as fungizone in 0.85% saline) and L-glutamine (1mM). Foetal calf serum (FCS) was added at varying concentrations as described in section 2.2.2.

The supplements were sterilised by filtration and added to 500ml basic medium. Supplemented medium was stored at 5°C for a maximum of four weeks. FCS was stored in 25ml aliquots at –20°C. L-glutamine was made up as a 200mM stock and stored in 5ml aliquots at –20°C. Antibiotic solution was stored as 5ml aliquots at –20°C

2.2.2 Maintenance and Propagation of Cell Lines

The L6 cell line was routinely cultured in 75cm^3 flasks with 20ml DMEM supplemented with 5% (v/v) FCS and 1mM glutamine. Cells were maintained as a monolayer in a humidified atmosphere of 5% carbon dioxide, 95% air at 37°C. Cells were passaged when approximately 90% confluent to prevent contact inhibition.

DMEM was aspirated and replaced with 5ml trypsin solution (trypsin-EDTA, contains 0.5g porcine trypsin, 0.2g EDTA, 4Na/l of HBSS, Sigma), shaken and incubated at 37°C for up to 10 minutes, until all cells were detached. The trypsin-cell suspension was centrifuged at 800rpm (MSE Mistral 2,000, Fisher Scientific Loughborough, UK) for 5min to pellet the cells. Cells were disaggregated and re-suspended in 10ml DMEM supplemented with 5% FCS (complete medium) of which 1ml was transferred to a fresh flask with 20ml pre-warmed medium. The remaining cell suspension was diluted and used for experimentation.

Cells were used at passages 15-35. At P35 and higher passages, a significant decline in insulin stimulated glucose transport was observed.

Cells were seeded in 24 well plates (16mm wells containing 5×10^5 cells in 1ml medium) for all 2DG-uptake experiments. Cells were grown to confluence and serum starved with 0.5% foetal bovine serum supplemented DMEM 24 hours prior to day 1 of the experiment, in order to decrease basal glucose uptake. This also facilitated differentiation and fusion of myotubes, see chapter 3.2.

For long-term storage the cells were frozen and stored in liquid nitrogen. The cells were trypsinised and the cells recovered by centrifugation as described above. The pellet of cells was then re-suspended at a concentration of 10^7 cells/ml of 90% FCS, 10%DMSO. The resulting suspension was aliquoted into a cryotube, which was placed into an insulating container and stored at –70°C overnight. The cryotubes were then transferred to tanks of liquid nitrogen for long term storage. When required, the cells were rapidly thawed to prevent crystalline damage, and added to 20mls pre-warmed complete medium in a $75cm^3$ flask for routine culture.

2.2.3 Growth Curves

The daily rate of cell growth was assessed by daily trypsinisation of individual flasks of cells and counting the number of cells using a haemocytometer.

2.3 *In Vivo* Studies

2.3.1 Animal Care

The animals were maintained in an air-conditioned room at 22±2°C, with a light cycle of 12 hours light (0800-2000) and 12 hours dark. A standard rodent pellet diet (SDS Economy Rodent Breeder, Special Diet Services (SDS, Witham, Essex, U.K.) and tap water were provided *ad libitum* throughout the study unless decreed otherwise by experimental procedure.

2.3.2 Blood Sampling

Blood samples of approximately 60μl for determination of plasma glucose and insulin were obtained from the cut tail tip of conscious mice into microfuge tubes pre-treated with 500U/ml heparin. Blood samples were stored on ice throughout the procedure. The plasma was separated by microcentrifugation for 30 seconds. 20μl was stored at –20°C for insulin assay, and 5-10μl used immediately for glucose analysis.

For assays that require sacrificing mice (e.g. for the measurement of glucose transport into isolated tissues), mice were killed by cervical dislocation and decapitated. Blood was withdrawn by exsanguination through the aorta into a heparinised microfuge tube on ice so as to minimise the number of animals used.

2.3.3 Induction of Diabetes

Diabetes was induced in overnight fasted, lean, 6 week old, non diabetic mice by intraperitoneal injection of streptozotocin (STZ) (160mg/kg, ip, from 50mg/ml stock solution). STZ was dissolved in citrate buffer at pH4.8 immediately prior to use and kept on ice between solubilisation and injection to prevent inactivation of STZ at neutral pH. Food was replaced >4 hours after treatment.

Animals were monitored by weight loss, urine excretion, plasma glucose concentration and general appearance, to determine the extent of hyperglycaemia, 2-4 days prior to the experiment. Animals were selected only if plasma glucose was greater than 12mmol/l on the morning of the experiment.

2.3.4 Compound Administration

All compounds were administered to conscious animals by oral gavage (po) or by intraperitoneal injection (ip) unless specified otherwise.

2.3.5 Glucose Tolerance Tests

Intraperitoneal glucose tolerance tests (IPGTT) and oral glucose tolerance tests (OGTT) were performed on fasted animals. Glucose (2g/kg body weight) was administered as a 40% w/v solution by either oral gavage or by intraperitoneal injection. Blood samples were always taken immediately before (i.e. time zero) and at a number of different time points after administration of the glucose load as determined by protocol. The plasma was separated for glucose assay.

2.3.6 Insulin Hypoglycaemia Tests

Insulin induced glucose disappearance tests were performed on fasted animals. Human actrapid (1U/kg ip) was administered and blood samples taken as with glucose tolerance tests, section 2.3.5

2.3.7 Removal of Soleus Muscles and Diaphragms

Mice were humanely killed by cervical dislocation, and the soleus muscles and diaphragms immediately dissected and transferred to glass petri dishes containing unsupplemented KRB at room temperature. Diaphragms were trimmed and dissected to produce hemi-diaphragms and rinsed in KRB. Both soleus muscles and hemi-diaphragms were carefully blotted and weighed before use (appendix IV).

2.4 Analyses

2.4.1 2-Deoxy-[^{3}H]-Glucose Uptake into Cells

Glucose uptake was assessed using the tritiated glucose analogue 2-deoxy-D-glucose (2DG) (Amersham, specific activity ranging from 326Gbq/mmol to 570Gbq/mmol dependent on batch), which is taken up by the cell in an identical manner to glucose, phosphorylated but not metabolised further (Walker et al., 1989). This allows measurement of ^{3}H-2DG phosphate.

Medium was removed from the cells and washed with glucose-free Krebs Ringer Bicarbonate (KRB) buffer at room temperature (22°C). Cells were incubated with 1ml of KRB with the addition of radionuclide label 2-deoxy-D-[1-^{3}H]glucose at 7.4Kbq/ml (0.2μCi/ml), and unlabelled 2DG (Sigma) at 0.0162mg/ml (0.1mM) for 10 minutes at room temperature. Washing cells twice with ice cold, unsupplemented KRB buffer terminated glucose uptake, and cells were lysed with 0.5ml/well 1M sodium hydroxide at room temperature for 1 hour. The cells were transferred to scintillation vials, to which 5ml scintillant (Optiphase 'Hisafe' III, Fisons) was added and ^{3}H was counted for 5 minutes using a Packard 1900TR liquid scintillation counter.

2.4.2 2-Deoxy-[^{3}H]-Glucose Uptake into Whole Muscles

Glucose uptake was assessed using 2-deoxy-D-[1-^{3}H] glucose measurement. (Henriksen et al., 1990; Haber and Weinstein, 1992).

Soleus muscles or hemi-diaphragms were transferred to incubation vials containing

1ml KRB buffer containing 1mM pyruvic acid, 0.5% BSA, 1mM 2-deoxy-D-glucose, and 0.2 μCi 2-deoxy-D-[1-^{3}H] glucose, pre-gassed with 5%CO_2:95%O_2. 10μl test compound and/or insulin was added to the incubation buffer, incubation vials were sealed with suba-seal rubber stoppers, and maintained at 30°C with gentle shaking for 30 minutes.

Extracellular 2DG was removed from the muscles by thorough blotting on tissue paper. Muscles were transferred to scintillation vials and digested by the addition of 0.5ml 1M Sodium hydroxide. Digestion was aided by heating in a water bath to 90°C. Once cooled, 5ml scintillant was added to each vial and counted.

2.4.3 Insulin Radioimmunoasay

Plasma was assayed for insulin using the BIOTRAK™ rat insulin-[^{125}I] radioimmunoassay system with magnetic separation (Amersham). The general principle of radioimmunassay (RIA) is based on the immunogenic properties of polypeptide hormones. Specific antibodies can be raised when the hormone is administered to a species in which the hormone bears sufficient structural difference from the endogenous hormone to produce an immune response. This assay is based on the competition between unlabelled insulin and a fixed quantity of ^{125}I-labelled human insulin for a limited number of binding sites on an insulin antibody. Fixed amounts of antibody and radioactive ligand were added to the assay and hence the amount of radioactive ligand bound by the antibody was inversely proportional to the concentration of added non-radioactive ligand. The antibody bound insulin was reacted with a second antibody bound to magnetizable polymer particles. Separation of the antibody bound fraction was achieved by magnetic separation, and the radioactivity measured.

The antibody was a non-specific anti-insulin antibody raised against human insulin in guinea pig. The antibody showed full cross reactivity with human and rat insulin and separated A and B chains. The second antibody was a sheep anti-guinea pig serum coated onto magnetised polymer particles.

9 standard concentrations of rat insulin were prepared ranging from 0.01 to 2.5ng/tube, using the doubling dilution method from a 50ng/ml stock standard of rat insulin, with assay buffer consisting of 0.025M phosphate buffer, pH7.5, containing 0.1%(w/v) sodium azide.

Total counts (TC) tubes were prepared with 100µl of the assay tracer, containing, [^{125}I] human insulin in assay buffer as above. Non-specific binding (NSB) tubes were prepared with 200µl assay buffer and the zero standard (Bo) tubes containing 100µl assay buffer. Unknown sample tubes contained 100µl plasma appropriately diluted with assay buffer, where high concentrations were suspected, so as to provide concentrations within the middle of the standard curve.

This procedure was as follows: 100µl of antiserum in assay buffer was added to all tubes except NSB and TC. 100µl tracer was added to all tubes, which were vortexed thoroughly, covered and incubated for 4 hours at room temperature. 250µl of undiluted Amerlex-M second antibody reagent containing the second antibody (sheep anti-guinea pig serum) coated onto magnetizable polymer particles was added to all tubes except the TC. Solutions were vortex mixed and incubated at room temperature for 10 minutes. Placing the tubes in an Amerlex-M separator rack for 15 minutes separated the antibody-bound fraction. The supernatant was discarded and allowed to drain for 5 minutes. The radioactivity present in each tube was determined by counting for 1min in a gamma scintillation counter.

Each set of tubes was analysed in duplicate, data are therefore the average counts per minute for each set of replicate tubes.

The percentage NSB/TC was calculated using equation 1, the percent Bo/TC using equation 2, and the percentage bound/Bo for each standard and sample was calculated using equation 3.

Equation 1

$$\%NSB/TC = \frac{NSB\ cpm}{TC\ cpm} \times 100$$

Equation 2

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

Equation 3

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

A standard curve was generated by plotting the percent B/Bo as a function of the log of the standard rat insulin concentration. B/Bo was plotted against ng standard per tube in order to read ng per tube directly from the graph.

2.4.4 Plasma Glucose Assay

Glucose was assayed using a Beckman Glucose Analyser (Beckman Instruments, High Wycombe, Bucks, U.K) which automates the glucose oxidase procedure (Stevens, 1971), and uses the oxygen consumption rate method to assay for glucose. An oxygen electrode within the reaction well compares oxygen utilisation by the sample with oxygen utilisation of a standard glucose solution (a standard of 8.3mmol/l glucose was used). The rate of oxygen utilisation is directly proportional to the concentration of glucose in the solution. The test sample is pipetted into the glucose oxidase reagent and β-D-glucose from the sample combines with the dissolved oxygen from the solution:

$$\beta\text{-}D\text{-}glucose + O_2 \xrightarrow[H_2O]{\text{Glucose oxidase}} \text{Glucuronic acid} + H_2O_2$$

The H_2O_2 is removed by two further reactions to ensure it cannot yield back any oxygen to the solution:

$$H_2O_2 + C_2H_5OH \xrightarrow{\text{Catalase}} CH_3CHO + 2H_2O$$

$$H_2O_2 + 2H^+ + 2I \xrightarrow{\text{Molybdate}} I_2 + 2H_2O$$

The addition of ethanol to the reagent removes the peroxide by catalase without yielding oxygen. Iodide and molybdate are added as catalase activity is diminished during storage.

2.4.5 Cell Viability Assessment

Cell counts and viable cell numbers were obtained using trypan blue and the haemocytometer.

Trypan blue is one of several stains used for dye exclusion procedures for viable cells counting. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Staining facilitates the visualisation of cell morphology.

Cells were grown to confluence in 24 well plates as described in section 2.2.2. Cells were incubated with compounds tested as described in the experimental design, and were removed from the plates using Cell Dissociation Fluid (Sigma). A cell suspension was prepared by dispersing cells in 1ml PBS. 500μl 0.4% (w/v) trypan blue solution (Sigma) was transferred to a test tube and 300μl PBS and 200μl cell suspension added, and mixed thoroughly. This mixture was left to stand at room temperature for 10min. With the cover slip in place, a Pasteur pipette was used to transfer a small amount of trypan blue-cell suspension mixture to both chambers of the haemocytometer using capillary action. Cells were counted (viable and non-viable) in the centre square and four corner squares.

When cells are incubated with particularly toxic compounds this method of assessing cell viability in an adherent cell line becomes inaccurate. As adherent cells undergo apoptosis, they no longer adhere. Therefore when washing the cells prior to staining with trypan blue, the cells are lost in the washing procedure and counting becomes inaccurate. Thus to overcome this and to demonstrate decreased cell viability for potentially toxic compounds in adherent cell lines, the cell monolayer was stained. Cells were grown to confluence in 24 well plates and compounds added as determined by experimental design. 150μl 0.4% (w/v) trypan blue solution and 150μl PBS was added to the cell monolayer. Cells were incubated at room temperature for 5min and cells were photographed using an inverted microscope with attached camera (both Nikon). Although this method does not provide a quantitative assessment of cell viability it sufficiently demonstrates the effect of compounds used on cell viability in an adherent cell line.

2.5 Molecular Analyses

2.5.1 RNA Preparation

2.5.1.1 Tissue Homogenisation

2.5.1.1a Large Tissue

Large tissues were prepared by breaking up in liquid nitrogen using a pestle and mortar to obtain reasonable size fragments. Tissue was weighed and ground in liquid nitrogen until the tissue fragments became the size of sugar grains. Tissue was transferred to a glass homogeniser and 1ml solution D was added for every 100mg tissue. Tissue was homogenised as rapidly as possible.

2.5.1.1b Small Tissue

Small tissues (e.g. hypothalami) were not ground in liquid nitrogen but were transferred directly to the homogeniser, and were homogenised as 2.5.1.1a.

2.5.1.1c Cell Cultures

Homogenisation of cell cultures was found to be unnecessary, lysis was achieved by the addition of solution D. RNA was prepared from three confluent 75cm^3 flasks of cultured cells. Culture medium was aspirated and the cells were incubated for 1h at room temperature in 7ml solution D. The resulting lysate was combined and 10ml transferred to two polypropylene tubes.

2.5.1.2 RNA Extraction

2ml of 2M sodium acetate pH4 was added to the lysate and the solutions thoroughly mixed. Phenol extraction was performed by the addition of 10ml water saturated phenol and by mixing the solutions followed by the addition of 3ml chloroform:isoamyl alcohol to form two distinct phases. The phases were mixed to form an emulsion and transferred to glass tubes pre-baked to 200°C. The emulsion was cooled on ice for 15 min prior to centrifugation for 20min at 4°C at 8,000rpm (Sorvall RC-5B Superspeed Centrifuge, DuPont Instruments). The aqueous upper phase was carefully transferred to a fresh tube and precipitated for 1 hour in 10ml isopropanol at –20°C. The precipitate was collected by centrifugation at 4°C for 20min at 12,000rpm (Sorvall, as above), and the supernatant removed. The tube was allowed to drain completely, and the pellet was resuspended in 2ml solution D. A further precipitation in 10ml isopropanol was performed at –20°C overnight. The precipitate was collected as before, washed with 3ml cold 75% (w/v) ethanol, and centrifuged for 20min at 4°C, 12,000rpm. The pellet was resuspended in 1ml purified, autoclaved water and the concentration determined spectrophotometrically.

2.5.1.3 RNA Determination

2.5.1.3a Spectrophotometrically

The concentration of recovered RNA was identified spectrophotometrically. 10µl RNA solution was transferred to eppendorfs containing 1ml water, to give a 1:100 dilution. The solutions were transferred to quartz cuvettes and the absorbance determined at 240, 260 and 280nm using water as a blank. Clean RNA has a ratio of 1:2:1.5 and the concentration was calculated using the equation:

Concentration (mg/ml) = (A_{260} x dilution factor)/20

The RNA was precipitated by the addition of 100µl 2M sodium acetate pH 5.2 and 2.5ml absolute ethanol, for 1h at –20°C. RNA was recovered as before, the supernatant was drained and the pellet dried. The pellet was resuspended in the appropriate amount of water to give a final concentration of 5mg/ml.

2.5.1.3b Agarose Gel Electrophoresis

The quality of the prepared RNA was determined using agarose gel electrophoresis. The gel was prepared by boiling 1g agarose in 88ml water and when this had cooled to 60°C adding 5ml 20xMOPS buffer and 7.5ml formaldehyde (about 40%w/v) to denature the RNA. The RNA samples were prepared by transferring 12µl denaturing buffer (100µl formamide, 30µl formaldehyde, 10µl MOPS) to clean eppendorfs and adding 1µl RNA sample and heating to 65°C for 5 min. The remaining RNA solution was stored at –20°C. The gel was poured onto a glass plate and allowed to set for 10 min. 3µl of loading buffer was added to each RNA sample and these were loaded onto the gel. The gel was electrophoresed in reservoir buffer (900ml water, 50ml MOPS 75ml formaldehyde) using an Anachem gel tank, at 120 volts (Consort E443 Electrophoresis Power Supply), for 45 min. The gel was then shaken for 30 min in 100ml water with 1ml 100xTE and 10µl ethidium bromide. The gel was then washed overnight with 400ml water and 4ml 100xTE.

2.5.1.4 mRNA Isolation

mRNA was purified from the total RNA extract using the PolyATtract® mRNA Isolation Systems Kit IV (Promega Corporation, Madison). This kit uses a biotinylated oligo(dT) primer to hybridise to the 3'poly(A) region present in most mature eukaryotic mRNA species. The hybrids were captured and washed using streptavidin coupled to paramagnetic particles and a magnetic separation stand and the mRNA was eluted by the addition of ribonuclease (RNase)-free deionised water

2.5.1.4a Annealing of Probe

1mg total RNA was added to RNase-free water to give a final volume of 500µl and was incubated at 65°C for 10min. 3µl of biotinylated-Oligo(dT) Probe and 13µl 20X SSC was added to the RNA. This was gently mixed and incubated at room temperature until completely cooled.

2.5.1.4b Streptavidin-Paramagnetic Particles (SA-PMPs)

SA-PMPs were resuspended and captured by placing in a magnetic stand for 30 secs and the supernatant was removed. The SA-PMPs were washed three times with 0.3ml 0.5X SSC, and were resuspended in 0.1ml 0.5X SSC.

2.5.1.4c Capture and Washing of Annealed Oligo(dT)-mRNA Hybrids

The products of the annealing reaction (2.5.1.4a) were added to the washed SA-PMPs and were incubated at room temperature for 10mins with gentle mixing by inversion every 2 mins. The SA-PMPs were captured using the magnetic stand and the supernatant was removed. The particles were washed 4 times with 0.3ml 0.1X SSC and after the final wash the aqueous phase was removed.

2.5.1.4d Elution of mRNA

The SA-PMP pellet was resuspended in 0.1ml RNase-free water, and captured magnetically. The eluted mRNA aqueous phase was transferred to a sterile, RNase-free eppendorf. The SA-PMP pellet was resuspended in 0.15ml RNase-free water, the pellets were recaptured and the supernatant pooled with the previous eluate to give a final volume of 0.25ml.

2.5.1.4e Determination of mRNA Concentration and Purity

The concentration and purity of the eluted mRNA was determined by spectrophotometry as described in section 2.5.1.3a. Pure mRNA has an A_{260}/A_{280} absorbance ratio of >=2.0. mRNA constitutes approximately 1-2% total RNA.

2.5.1.4f Precipitation and Storage of mRNA

The mRNA was precipitated by the addition of 100μl 2M sodium acetate pH 5.2 and 2.5ml absolute ethanol overnight at –20°C. RNA was stored in sodium acetate/isopropanol solution at –70°C and centrifuged immediately prior to use.

2.5.2 Northern Blotting

The gel and RNA samples were prepared as described in section 2.5.1.3b. The gel was electrophoresed in reservoir buffer (900ml water, 50ml MOPS 75ml formaldehyde) using an Anachem gel tank, at 120 volts (Consort E443 Electrophoresis Power Supply), for 2h. The gel was then inverted onto a wick soaked in 20xSSC over a reservoir and a nylon membrane (Hybond-N, Amersham) was cut to size and placed over the gel avoiding bubbles. The nylon membrane was covered with layers of filter paper (3MM) and dry paper towels, a weight was placed on top of the pile and the RNA was left to transfer overnight. Filters were fixed by baking for 2h at 80°C and were prehybridised to block non-specific binding of the probe by incubating the filter in prehybridisation buffer for 2h at 55°C. Radionuclide labelled cDNA probes were prepared using the Pharmacia Oligolabelling Kit and α^{32}-P dATP. Oligos were diluted to 10μg/μl with riboprobe buffer and labelled with 70μCi radionuclide:1μl terminal transferase. The purity of the labelling reaction was checked by running on a small G50 column with 180μl aliquots 1xTE. The eluted fractions were checked using a bench top scintillation counter. The two 'hottest' fractions were used to probe the hybridising filter. Hybridisation was carried out at 55°C for 16h. Filters were washed twice at room temperature in 2XSSC, 0.1% SDS for 15min followed by one wash at 50°C in 0.1XSSC, 0.1%SDS and analysed using a PhosphorImager (PhosphorImager SF, Molecular Dynamics), and Image Quant Software Version 3.3 Filters were exposed to X-ray film (Fuji) at –70°C overnight.

2.6 Statistical Analyses

2.6.1 Student's 't' Test

Student's 't' test for unpaired data was used to determine if there was a difference between two separate sets of data (data was expressed as mean ± SEM, n is indicated for each experiment). The paired 't' test was used when the results were paired (e.g. where both control and treatment were applied to the same animal).

2.6.2 The Bonferroni Adjustment

If an experiment has more than two treatments there are statistical objections to comparing all of the means by paired or unpaired student's 't' tests. In these experiments data were analysed using the Bonferroni adjustment procedure. The procedure is used to adjust the alpha level (0.05) downward to consider chance capitalisation, as in the case of repeated testing (type 2 error) the chance to incorrectly declare a difference, effect or relationship as significant, might be increased. The appropriate 't' test with the Bonferroni adjustment was employed unless otherwise indicated.

2.6.3 Analysis of Variance

An ANOVA test (analysis of variance) is also used to find out if there is a significant difference between groups of mean values. It relies on the assumption that the total variation in a set of data can be calculated and that the variation of can be partitioned into separate components and that these components can be compared. Where appropriate ANOVA was employed and this is indicated in the experimental design.

2.6.4 Post hoc Analysis

Post hoc is Latin for 'after the fact' i.e. the outcome of the experiment is known prior to deciding which comparisons to make. (As opposed to planned tests, which are those tests intended to apply prior to conducting the experiment).

ANOVA analysis simply indicates that there is a significant difference between two or more group means but it does not tell you between which means there are significant differences. In order to determine this, a post hoc test was utilised. The Tukey Test is a post hoc test designed to perform a pair wise comparion of the means, this was employed to determine where the significant difference was. The use of this test is indicated in the experimental design text.

Chapter Three:
Characterisation of The Rat L6 Skeletal Muscle Cell Line

Chapter Three: Characterisation of the Rat L6 Skeletal Muscle Cell Line

3.1 Introduction

The primary L6 culture line was originally prepared from thigh skeletal muscle of newborn rats by Yaffe (1968). The first cultured cells of this line were described as a heterogeneous population of mononucleated cells, the majority of which were spindle shaped myoblasts, the mononuclear precurser cells of muscle fibres. These cells were observed to divide in culture two to three times, at which point they began to aggregate and fuse to form postmitotic multinucleated muscle fibres. Further differentiation was observed with the appearance of cross-striation. (Yaffe, 1968).

Characterisation studies of the L6 cell line have determined that if these cells are maintained in exponential growth in tissue culture, they pass on the capacity to fuse and differentiate into muscle fibres to their progeny, hence, the cells have over time become an established line. However, the cells are not capable of replicating indefinitely in culture, although they withstand freeze storage at -80°C for extended periods of time without loss of viability (Yaffe, 1968).

During the process of L6 muscle cell differentiation *in vitro*, the myoblasts undergo membrane fusion to form multinucleated myotubes. This process mimics the myoblast to myotube maturation process that occurs during muscle ontogenesis (Klip and Leiter, 1990). The cells have also been observed to undergo biochemical differentiation, mediated by the reorganisation of the cytoskeletal structure, synthesis of new and destruction of old muscle-specific proteins (Ebisui et al., 1994).

The rat L6 cell line has been extensively used as a model for the study of glucose uptake into skeletal muscle (Klip et al., 1984) and the L6 line is used in this study to assess insulin-mimetic properties of a number of pharmacological agents. To this end, preliminary studies are included here in order to characterise the cultured L6 skeletal muscle cell line as a model of skeletal muscle.

3.2 L6 Cell Growth and Fusion

3.2.1 Experimental Design

L6 skeletal muscle cells (European Type Culture Collection) were cultured as described in 2.2.2, and cell growth was determined over a 4-day period (section 2.2.3). Four separate 75cm^3 tissue culture flasks were seeded with an identical number of L6 cells (10^9), and were allowed to settle to form a monolayer overnight. After 24 hours, the cells of flask 1 were removed by trypsinisation and were resuspended in 5ml of fresh media. Cells were subsequently counted using a haemocytometer, adjusted for dilution factors and expressed as the number of cells per ml of culture media. This procedure was repeated at the same time each day for 4 days using the cells that had been seeded on day one of the study. To obtain accurate cell counts, this experiment was repeated 3 times, therefore data are expressed as the mean ± SEM, n=3. Photographs of L6 cells were taken 48h, 72h after an initial inoculation of 1×10^9 cells. Cells were serum starved by removing complete media and replacing with media supplemented with 0.5% foetal calf serum (FCS) as described in section 2.2.2

and photographs taken after 6h and 24h after serum starvation (figure 3.1a, b, c and d).

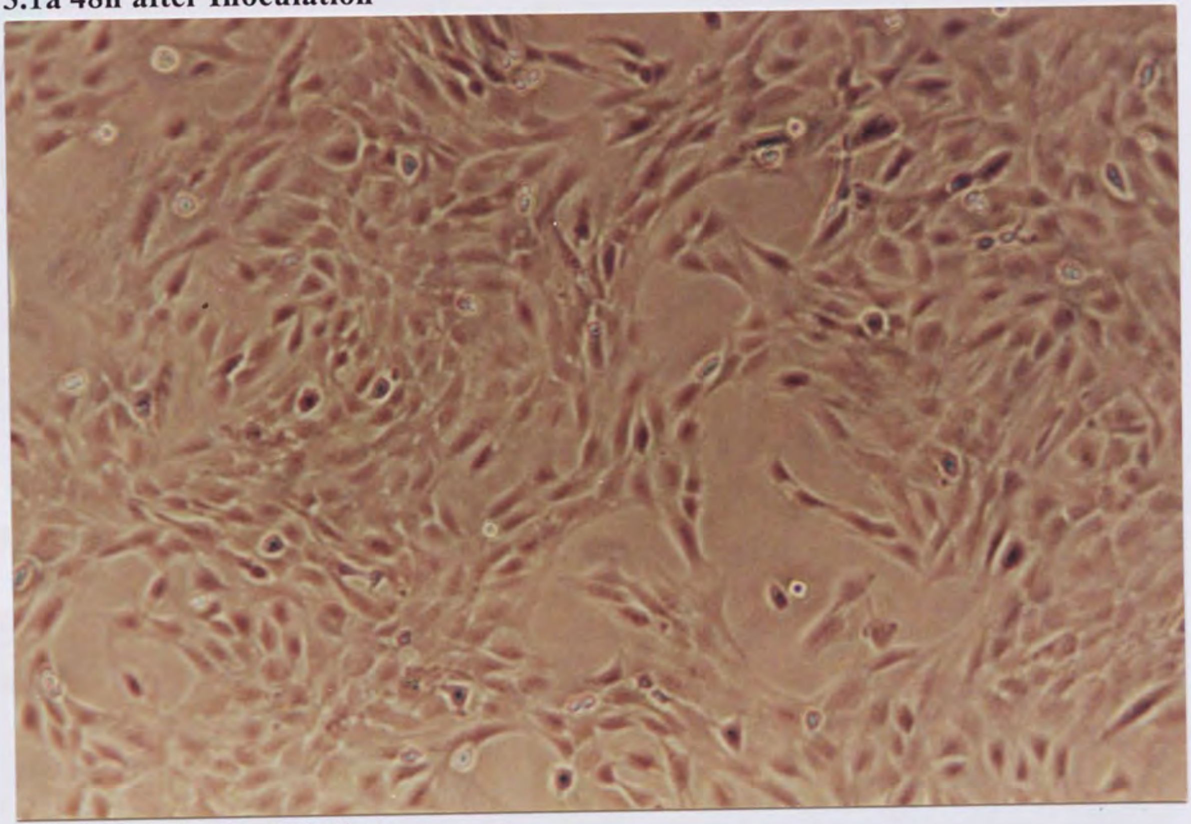
3.2.2 Results

The L6 muscle cell growth curve (figure 3.2) demonstrates that over the first 48h after cell seeding, the cells grow at a very slow rate. They adhered to the bottom of the flask evenly but were growing in a random manner. However between 48 and 72h, there was a significant increase in cell growth ($2x10^9$ to $4.5x10^9$ cells/ml, $p<0.01$, 48h versus 72h), leading to confluence at 96h ($p<0.02$, 96h versus time zero). After 72h of growth in unchanged media, the cells began to align into muscle fibres, and after 96h the cells began to differentiate and fuse to form myotubes. After this time point it became impossible to count the cells as approximately 90% of the cells had undergone the fusion process.

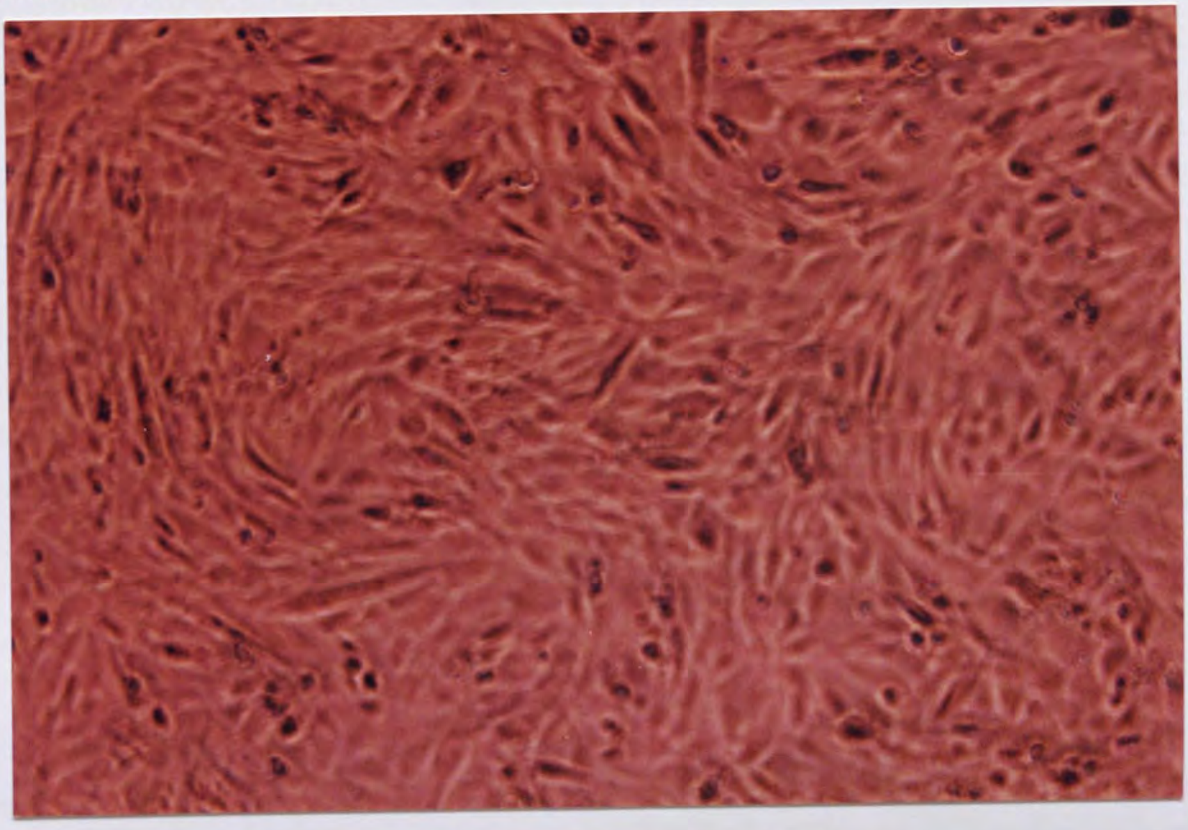
As the growth rate of the L6 cells in culture increased after 48h, it can be postulated that the cells release one or more factors that enhance cell division, such as growth factors. If culture media is removed and replaced with fresh medium daily, this hinders the rate of cell growth.

Figure 3.1 L6 muscle cell differentiation *in vitro*. Photographs were taken as described in Chapter Two using a x10 objective. A single 75cm^3 tissue culture flask was seeded with 1x10^6 L6 cells suspended in DMEM supplemented with 5% foetal calf serum (FCS) at time zero, and photographs taken to demonstrate growth and fusion over time. **3.1a.** 48h after the initial inoculation, the cell suspension had formed a preconfluent monolayer. **3.1b.** 72 hours incubation, cells had grown to form a confluent monolayer, which completely covered the bottom of the flask. **3.1c.** Medium was removed and replaced with DMEM supplemented with 0.5% FCS (serum deprivation) in order to induce fusion to myotubes (photograph taken 6h after serum deprivation). **3.1d.** The cells were maintained in DMEM with reduced serum for 24h to form multinucleated myotubes.

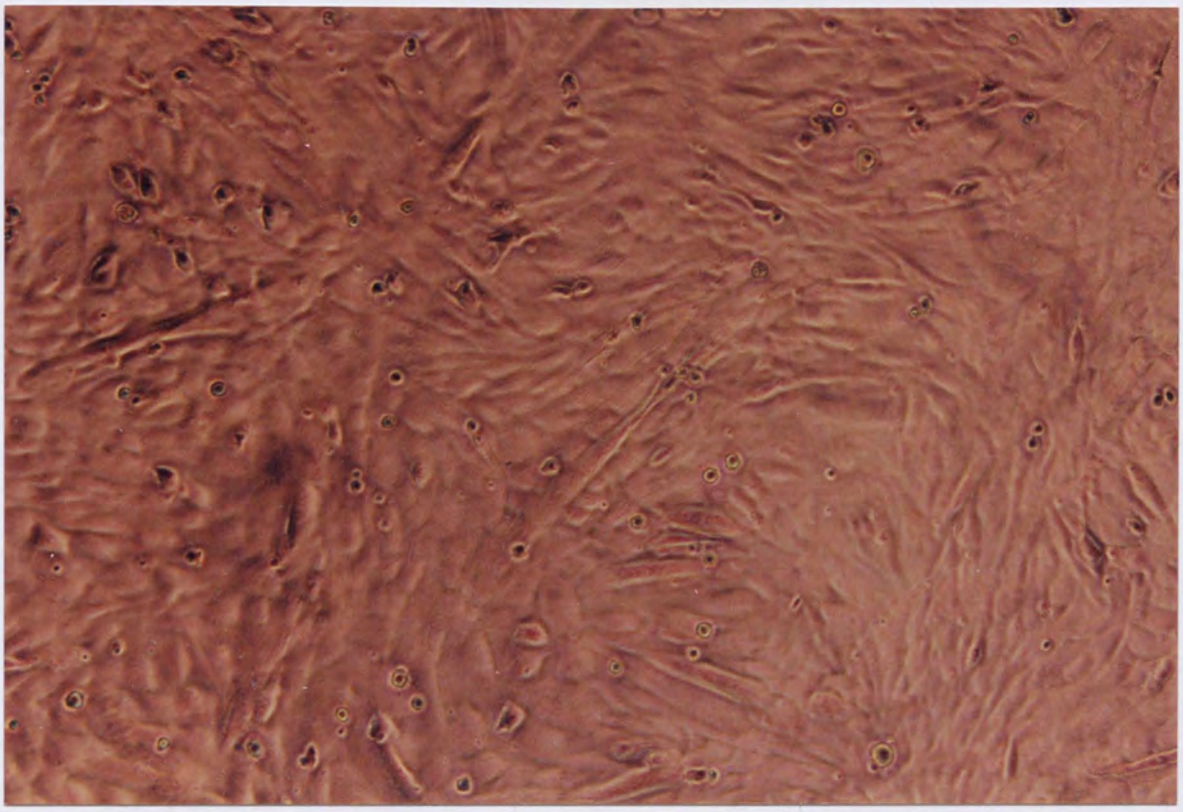
3.1a 48h after Inoculation



3.1b 72h after Inoculation



3.1c 6h after Serum Deprivation



3.1d 24h after Serum Deprivation

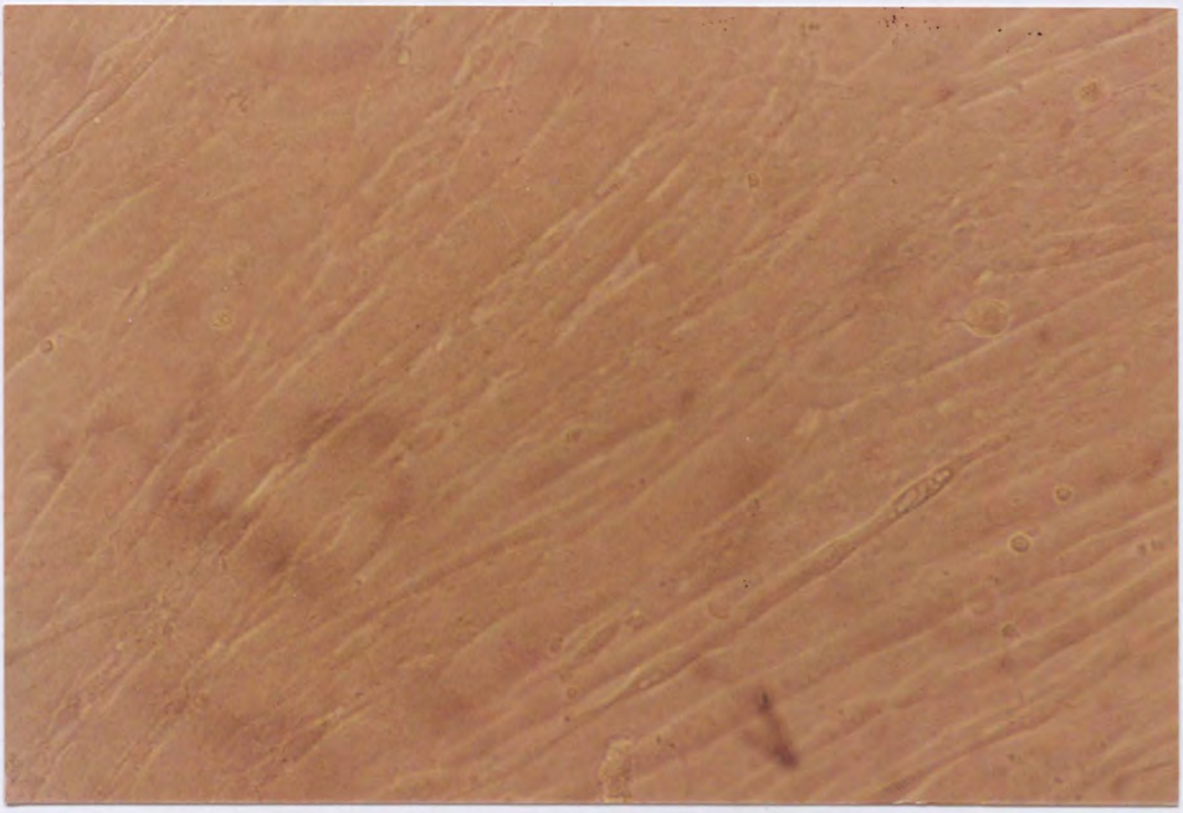
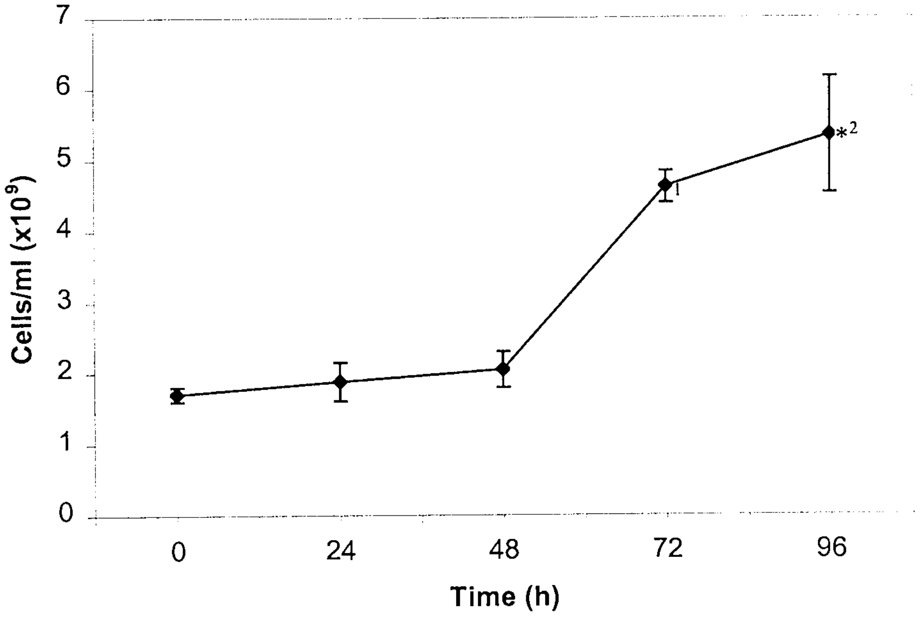


Figure 3.2 L6 muscle cell growth and fusion. Cultured L6 cells were trypsinised daily and growth was determined by counting using a haemocytometer. After the initial inoculation cells grew at a steady rate to form a monolayer of spindle shaped myoblasts. After 48h, the rate of growth significantly increased ($p<0.01$), until contact inhibition prevented further multiplication. The differentiation process began after 72h, and at 96h approximately 90% of the cells had fused to form myotubes*. Data are mean ± SEM ($n=3$) $^{1}p<0.01$, $^{2}p<0.02$



3.3 Acute and Sub-chronic Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

3.3.1 Introduction

2-Deoxyglucose (2DG) uptake has been validated as a widely used technique for assessing cellular or whole tissue insulin resistance and is used here to determine glucose uptake into cultured muscle cells.

2DG is a glucose analogue which is taken up into the cell by glucose transporters (GLUTS, section 1.6.5) in a manner identical to glucose, which is then phosphorylated by hexokinase to form glucose 6-phosphate. The next step in the glycolytic pathway is the isomerisation of glucose 6-phosphate to fructose 6-phosphate by phosphoglucoisomerase, hence the six membered pyranose ring of glucose 6-phosphate is converted to the five-membered furanose ring. It is the keto group on C-2 that reacts, with the C-5 hydroxyl group to form the furanose ring of fructose 6-phosphate. 2DG however lacks the keto group on C-2 thereby preventing the isomerisation reaction. Therefore 2DG is taken up into the cell and phosphorylated but is not metabolised further and hence it is a useful tool in determining intracellular concentrations. This method relies on detection using a radionuclide-label, in this case ^{3}H, which enables detection of the internal glucose concentration. Labelled 2DG has also been used as a tracer to quantitatively assess rates of local cerebral glucose utilisation using autoradiography and positron emission tomography (Sokoloff et al., 1977; Reivich et al., 1985; Virkamaki et al., 1997).

An alternative quantitative measurement of glucose uptake can be achieved using 3-O-methyl glucose which, like 2DG, is taken up into the cell but is not phosphorylated. Similar data have been obtained using these two methods, and 2DG is the method of choice for these experiments. The kinetics of 2DG transport in skeletal muscle have been well documented and the effects of insulin and contraction have been previously determined (Hansen et al., 1995).

3.3.2 Experimental Design

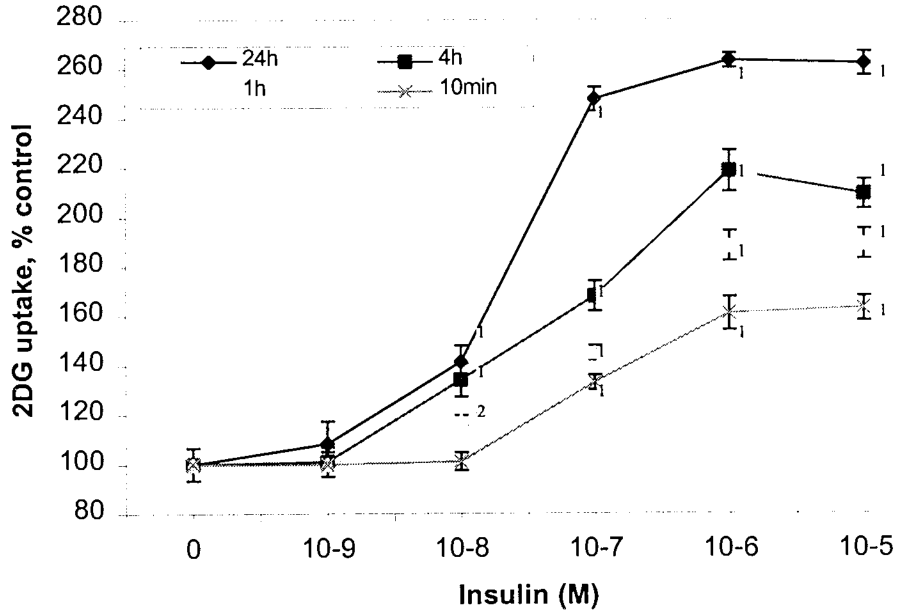
Bovine insulin (Sigma) was dissolved in PBS and sterilised by filtration. L6 cells were grown to confluence in 24 well plates and serum starved for 24h. Cells were incubated with insulin (10^{-9}M – 10^{-5}M), for 10mins, 1h, 4h or 24h. Experiments were undertaken in multiples of six wells on at least three occasions. Six control wells were included in each 24 well plate. Glucose uptake was measured using the 2DG-uptake method as described in chapter 2 Section 2.4.1 Uptake of 2DG was expressed as the percentage change compared with control (100%). Data are presented as mean ± SEM and compared using Student's 't' test. Probability values of $p<0.05$ were considered to be significant.

3.3.3 Results

Insulin increases 2DG uptake in L6 cells both in a concentration dependent manner and a time-dependent manner (figure 3.3).

After 24h incubation, insulin had a significant effect on 2DG uptake at concentrations 10^{-5}M-10^{-8}M ($p<0.01$ versus control). 10^{-8}M insulin increased 2DG uptake by 41% and a maximal effect of 2DG uptake in L6 cells was observed after incubating cells

Figure 3.3 Insulin-stimulated 2DG uptake in L6 skeletal muscle cells. 2DG uptake was stimulated with insulin (10^{-9}M - 10^{-5}M) for 10min, 1h, 4h and 24h. Insulin stimulated 2DG uptake in a time and concentration dependent manner. Maximal stimulation of 2DG uptake by L6 cells was after 24h exposure to 10^{-6}M insulin, (163%, $p<0.01$ versus control). Data are expressed as % control, values are mean ± SEM, (n=18). $^{1}p<0.01$ versus control, $^{2}p<0.02$ versus control, $^{3}p<0.05$ versus control.



for 24h with 10^{-6}M insulin (163% increase). 10^{-9}M insulin increased glucose uptake by 8%, this was not statistically significant. The ED_{50} of this concentration-response curve was 2.19 x 10^{-8}M

2DG uptake was also significantly increased by insulin of the range 10^{-8}M-10^{-5}M after 4h cell exposure, by 34% - 109% ($p<0.01$ versus control). Maximal stimulation was produced by 10^{-6}M insulin, which significantly increased glucose uptake by 118%.

This concentration-dependent curve was also observed after cell exposure to insulin for 1h. Insulin significantly increased glucose uptake by 45% - 89% (10^{-7}M – 10^{-5}M, $p<0.01$ versus control) and 18% by 10^{-8}M insulin ($p<0.02$ versus control). 10^{-9}M insulin had no effect.

After 10min cell exposure to insulin, 2DG uptake was greatly diminished as compared to the 24h time point. However glucose uptake was significantly increased at high insulin concentrations. 10^{-7}M - 10^{-5}M insulin significantly increased glucose uptake by 33% - 63% ($p<0.01$ versus control). 10^{-9}M and 10^{-8}M insulin did not exert a significant effect after 10min exposure.

This experiment was repeated at the beginning of each set of studies and after freeze/thawing cells to ensure that the experimental protocol was working. The data in figure 3.3 are typical of those generally observed as the positive control experiment of each subsequent study.

3.4 Mechanism of Insulin Action in L6 Cells

3.4.1 Effect of Wortmannin on Basal and Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

3.4.1.1 Introduction

Wortmannin (appendix II) is a microbial metabolite, isolated from the culture filtrates of *Penicillium Wortmanni.* It was initially identified as a potent anti-inflammatory agent, but the toxicity rendered it unsuitable for clinical development (Hauser et al., 1973; Wiesinger et al., 1974). It has recently been shown to be a potent and selective inhibitor of PI 3-kinase (Evans et al., 1995; Gao et al., 1996; Rahn et al., 1994), and has been demonstrated to inhibit serine and lipid kinase activities of PI 3-kinase in adipocytes (Lam et al., 1994). It reacts with p110alpha sub-unit of PI 3-kinase (Wymann et al., 1996; Thelen et al., 1994) and the site of this modification has recently been located at Lys-802 (Wymann et al., 1996). Site-directed mutagenesis studies have demonstrated that Lys-802 is essential for catalytic activity. The electrophilic 21-site makes a covalent linkage with the enzyme and the inhibition of PI 3-kinase is irreversible.

The use of wortmannin has been focused as a probe to understand the structural requirements necessary for PI 3-kinase inhibition, and has proven to be a valuable tool to investigate the PI 3-kinase pathway in signal transduction. Effects of wortmannin have been documented on insulin stimulated glucose transport, Glut 4 translocation, antilipolysis and DNA synthesis in adipocytes (Evans et al., 1995; Okada et al., 1994). It has also been used to determine the role of PI 3-kinase in glucose induced insulin secretion in intact pancreatic islets (Gao et al., 1996). Other applications have demonstrated that wortmannin is capable of blocking contraction of gastro-intestinal, vascular smooth muscle and skeletal muscle possibly by inhibition of myosin light chain kinase (Burke et al., 1996; Takayama et al., 1996; Hong and Chang, 1998). Wortmannin has also been reported to have interesting anti-cancer activity, however it is too unstable for clinical use.

Wortmannin was used in this study to determine basal and insulin-stimulated PI 3-kinase mediated glucose uptake and as a prelude to further studies to analyse PI 3-kinase mediated glucose uptake by other pharmacological agents in L6 cells. The use of wortmannin as a PI 3-kinase inhibitor is well documented but, there are very few who have considered the toxicity of this metabolite. Wortmannin has previously been used as an inhibitor of PI 3-kinase at concentrations as high as 10^{-4}M and cells have been exposed *in vitro* to wortmannin for as long as 20h (Evans et al., 1995; Okada et al., 1994). It is necessary to determine the toxicity of this metabolite to ensure that the decrease in glucose uptake observed is due to an inhibition in PI 3-kinase and not due to cell lysis. This is true of all studies in which a compound is being used to inhibit a physiological mechanism. Thus the effect of wortmannin on cell viability is also considered here.

3.4.1.2 Experimental Design

Wortmannin (Sigma) was dissolved in dimethyl sulphoxide (DMSO) at 10mM, stored at –20°C in the dark in 100μl aliquots, and diluted with PBS immediately before use. L6 cells were grown to confluence in 24 well plates and serum starved for 24h as described in chapter 2 Section 2.2.2.

Wortmannin was diluted and added to the cell incubation medium to give a final concentration of 10^{-9}M to 10^{-4}M. Insulin was diluted as described in 3.3.2 above and added to the incubation medium at final concentrations of 10^{-8}M to 10^{-6}M. Cells were incubated with insulin for 40min and with wortmannin for 50min, to allow cell pre-exposure to wortmannin for a 10min time period.

Experiments were undertaken in multiples of three wells on at least three separate occasions. In order to determine the effect of wortmannin on insulin action, 24 well plates were divided in half. One set of twelve 1ml wells received insulin only at concentrations 10^{-8}M to 10^{-6}M, and the second set received insulin and one concentration of wortmannin. This was to determine the effect of wortmannin on the concentration dependent insulin-stimulated 2DG uptake in L6 cells as identified in 3.3. Three control wells were included in each 24 well plate and three wells were used to determine the effect of wortmannin on basal glucose transport in these cells. Glucose uptake was measured using the 2DG-uptake method as described in chapter 2, Section 2.4.1 Uptake of 2DG was expressed as described above in Section 3.3.2.

Wortmannin was assessed for toxicity using the trypan blue exclusion method as described in 2.4.5 (chapter 2). L6 skeletal muscle cells were grown to confluence in 24 well plates (2.2.2) and incubated for 50min with wortmannin (the time period used to assess inhibition of glucose uptake – as described above) at concentrations 10^{-8}M – 10^{-4}M. Media was removed, trypan blue solution (Sigma) was added to stain the monolayer and removed before photography.

3.4.1.3 Results

Incubation of L6 muscle cells for 50 min with wortmannin decreased basal glucose uptake in a concentration dependent manner (figure 3.4). 10^{-4}M wortmannin decreased basal glucose uptake by 81%, which was the most potent effect observed, and was highly significant ($p<0.01$). Wortmannin at concentrations 10^{-5} – 10^{-8}M decreased basal glucose uptake in a concentration dependent manner (21%-8%), and 10^{-9}M wortmannin had no effect on basal glucose uptake in L6 cells.

Cell exposure to insulin (10^{-8}M-10^{-6}M) for 40 min increased glucose uptake in a concentration dependent manner (9%-26% respectively, 10^{-6}M insulin produced a statistically significant increase; $p<0.01$ versus control). This effect was decreased by wortmannin (50 min). 10^{-4}M wortmannin, decreased insulin stimulated glucose uptake by 87% - 107% (10^{-8}M – 10^{-6}M insulin), which was highly significant ($p<0.01$ versus insulin alone). 10^{-5}M wortmannin decreased glucose uptake in these cells by 31% - 45% ($p<0.01$ versus 10^{-8}M – 10^{-6}M insulin alone) and 10^{-6}M wortmannin by 23% ($p<0.05$ versus 10^{-8}M insulin) - 35% ($p<0.01$ versus 10^{-6}M insulin), thereby decreasing insulin-stimulated glucose uptake below basal levels. 10^{-7}M wortmannin, decreased insulin stimulated glucose uptake by 5% (not significant) - 20% ($p<0.02$ versus 10^{-6}M insulin), at this concentration wortmannin appeared to inhibit the effects of insulin without decreasing glucose uptake below control values. Incubation of L6 cells with wortmannin at concentrations 10^{-8}M and 10^{-9}M decreased glucose uptake by 4% - 15% (not statistically significant), glucose uptake was not inhibited below basal values. This effect was dependent on the concentration of insulin used to stimulate glucose uptake e.g. low wortmannin concentrations (10^{-8}M and 10^{-9}M) did

Figure 3.4 Inhibition of basal and insulin-stimulated 2DG uptake by the fungal metabolite wortmannin in L6 cells. Cells were pre-stimulated with wortmannin (10^{-9}M – 10^{-4}M) for 10min. 2DG uptake was determined after cells were cultured with added insulin (10^{-8}M – 10^{-6}M) for a further 40min. Insulin stimulated 2DG uptake in these cells in a concentration dependent manner. Both basal and insulin stimulated 2DG uptake were inhibited by wortmannin in a concentration dependent manner. Data are expressed as % control, values are mean ± SEM, n=9, *significance versus control, †significance versus insulin. $^{1}p<0.01$, $^{2}p<0.02$, $^{3}p<0.05$

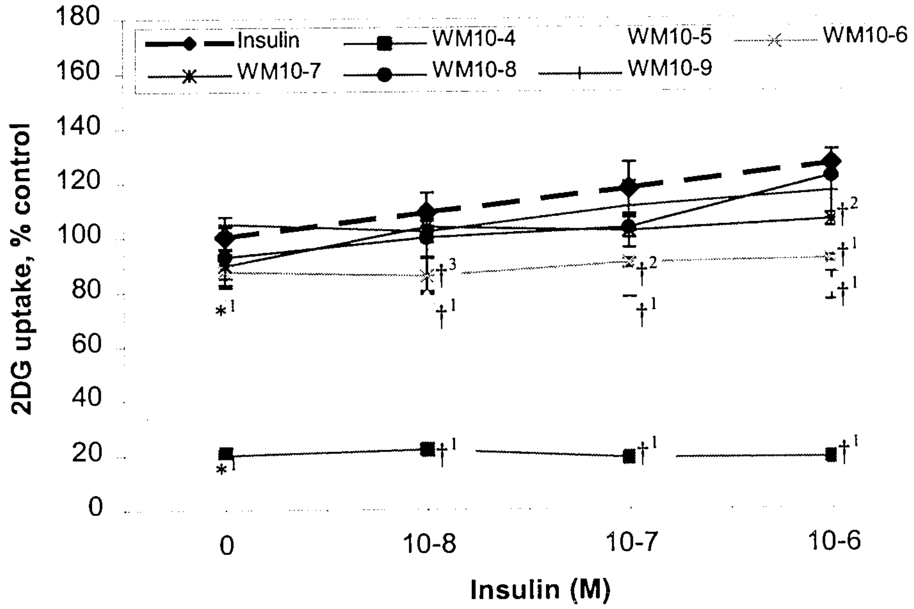
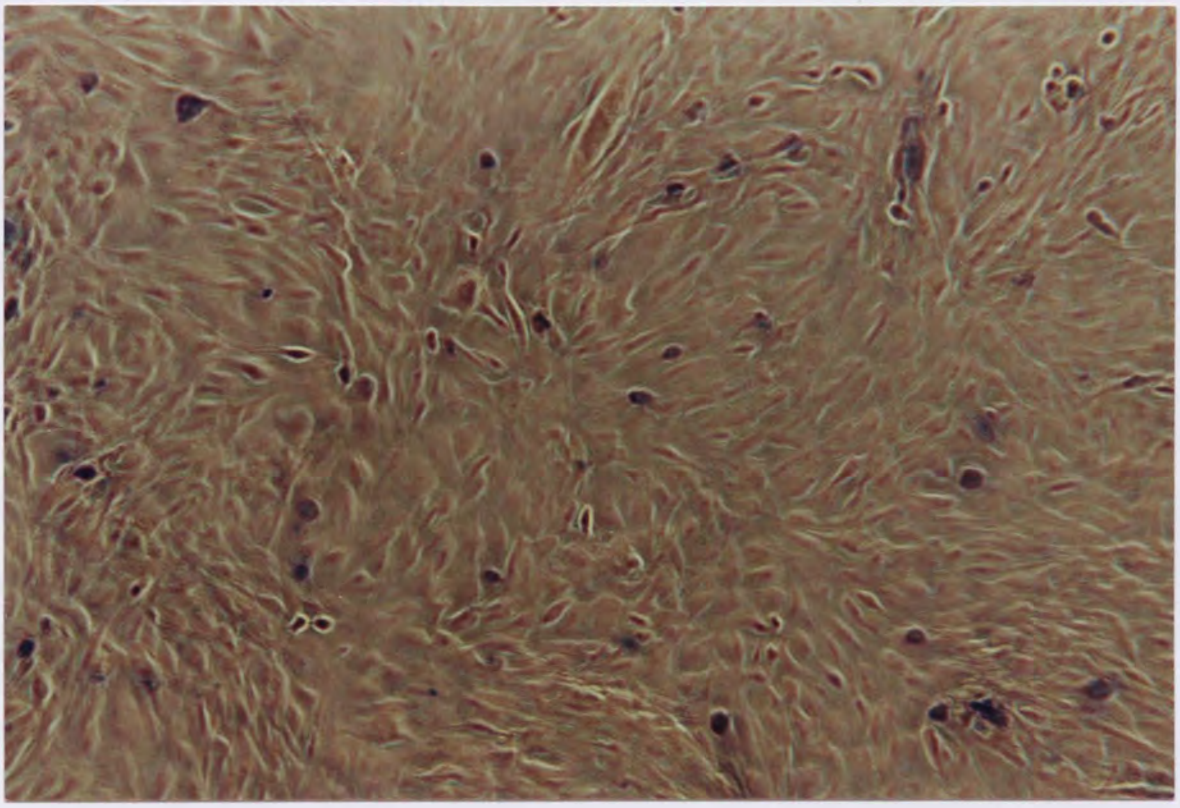
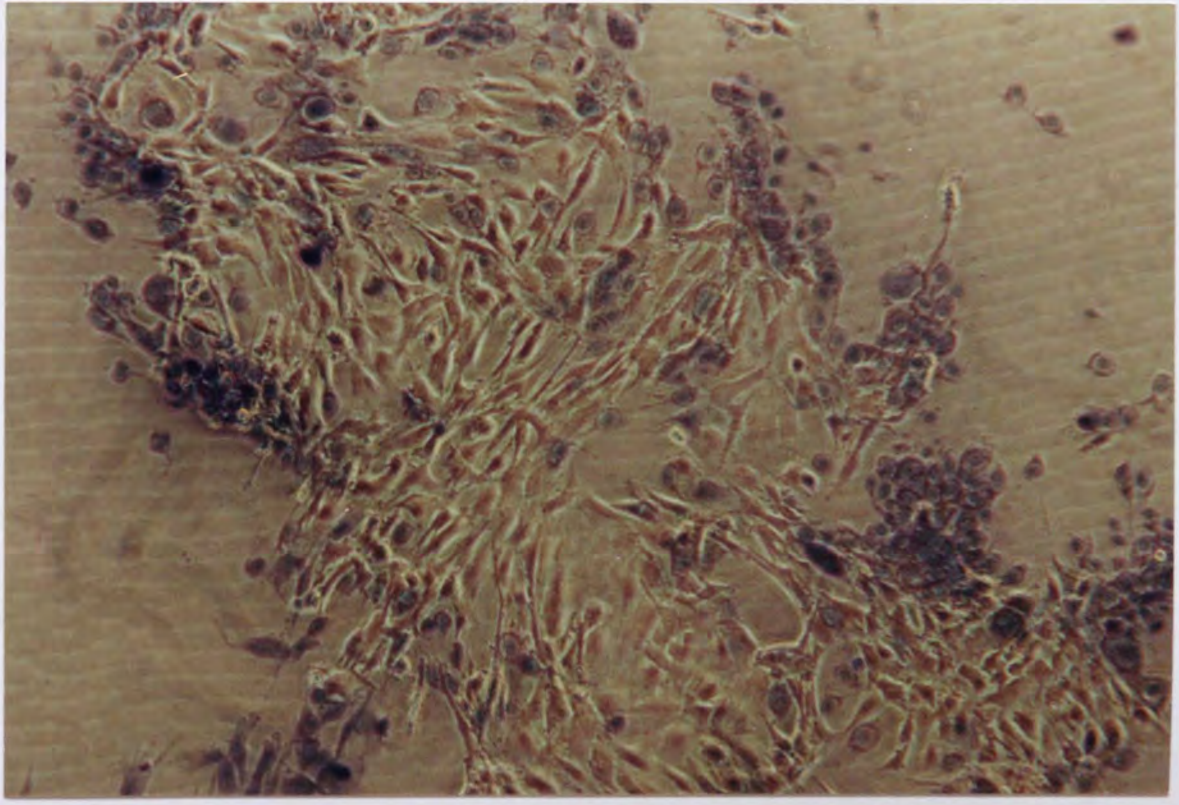


Figure 3.5 Photographs to demonstrate the effect of wortmannin on viability of L6 skeletal muscle cells. **3.5a.** Healthy cells stained with trypan blue. **3.5b** Cells were incubated with 10^{-4}M wortmannin for 50min (as for glucose uptake studies) and stained with trypan blue. Photograph **3.5b** demonstrates that incubation of cells for 50min with 10^{-4}M wortmannin caused approximately 50% of the cells to become detached from the surface of the culture plate and approximately 30% of the cells stained blue, indicating that wortmannin at this concentration is toxic to the cells. 10^{-6}M and 10^{-8}M wortmannin did not increase the number of cells that stained blue upon incubation with trypan blue (photographs can be found in appendix III).

3.5a Healthy Control Cells



3.5b Cells Exposed to Wortmannin (10^{-4}M for 50 minutes)



not fully inhibit glucose uptake stimulated by 10^{-6}M insulin (10^{-8}M=22% and 10^{-9}M=16% versus insulin =27%).

Healthy cells stained with trypan blue are shown in figure 3.5a. A small number of control cells had stained blue (approximately 3% of total cells). Addition of 10^{-4}M wortmannin for 50min caused an increase in the number of cells that stained blue and caused a number of cells to become detached from the plate surface which were removed with the excess trypan blue (figure 3.5b). Approximately 50% of cells had become detached and of the adhered cells, at least 30% were stained blue. Incubation of the cells with 10^{-6}M or 10^{-8}M wortmannin did not increase the number of cells that stained blue (photographs included in appendix III) in comparison with control cells (figure 3.5a). It is unlikely that toxicity impairs glucose transport without decreasing cell viability. Incubation of cells with wortmannin for time periods longer than 1h decreased cell viability even at low concentrations, therefore in subsequent investigations, cells were only exposed to wortmannin for a maximum of 50min.

3.4.2 Effect of LY-294,002 on Basal and Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

3.4.2.1 Introduction

2-(4-morpholinyl)-8-phenyl-[4H]-1-benzopyran-4-one also known as 2-(4-morpholinyl)-8-phenylchromone (LY-294,002, appendix II) was developed at the Lilly Research Laboratories as a potential treatment of proliferative diseases (Vlahos et al., 1994). Previous to the development of LY-294,002, quercetin, a naturally occurring bioflavonoid was found to inhibit PI 3-kinase and inhibition appeared to be directed at the ATP-binding site of the kinase (Matter et al., 1992). It has since been established that quercetin inhibits other kinases including PI 4-kinase and several tyrosine and serine/threonine kinases (Nishioka et al., 1989; Levy, et al., 1984; Graziani et al., 1983; Cochet et al., 1982; Srivastava, 1985). Quercetin was however used as a model compound for the development of LY-294,002 which was demonstrated to be a selective inhibitor of PI 3-kinase, by completely inhibiting PI 3-kinase activity against purified PI 3-kinase (Vlahos et al., 1994).

LY-294,002 has since been used in many studies in the determination of signal transduction via PI-3kinase. In order to study the role of PI 3-kinase in insulin action Sanchezmargalet et al., (1994) analysed the effects of LY-294,002, and measured five biological functions of insulin in mouse 3T3 fibroblasts over expressing human insulin receptors. LY-294,002 had no effect on tyrosine phosphorylation of the insulin receptor beta-subunit or insulin receptor substrate 1 (IRS-1) and it was found not to influence the association of the p85 subunit of PI3-kinase with IRS-1. However, LY-294,002 partially inhibited insulin-stimulated glucose uptake, amino acid uptake and protein synthesis, while it completely inhibited insulin stimulation of DNA synthesis and p70 S6 kinase activation. LY-294,002 has also been used to determine the role of IRS-1 in insulin signalling. It was determined that chronic exposure to insulin down regulates IRS-1 in 3T3-L1 adipocytes by stimulating its degradation and that this insulin response was completely inhibited by wortmannin and LY-294,002. Neither wortmannin nor LY-294,002 was shown to have any effect on the calcium-dependent degradation of IRS-1 *in vitro* nor did they inhibit the tyrosine phosphorylation of IRS-1 in response to insulin in intact cells (Smith et al., 1995).

Wortmannin is now the compound of choice to inhibit PI 3-kinase-sensitive signalling pathways. However due to the high toxicity of wortmannin, it cannot be used in cultured cell lines for extended periods of time. Therefore there are limitations to the use of wortmannin in the study of compounds that exert their effects over a longer time period. To this end, LY-294,002 is studied here as an alternative to wortmannin as a structurally distinct inhibitor of PI 3-kinase in cultured cells. However due to the inhibitory effect of LY-294,002, toxicity was also assessed by cell viability.

3.4.2.2 Experimental Design

LY-294,002 (Alexis Corporation (UK) Ltd. Nottingham) was dissolved in dimethyl sulphoxide (DMSO) at 10mM, stored at –20°C in 100μl aliquots, and diluted with PBS immediately before use. L6 cells were grown to confluence in 24 well plates and serum starved for 24h (2.2.2). LY-294,002 was diluted and added to the cell incubation medium to give a final concentration of 10^{-9}M to 10^{-4}M. Insulin was added to the incubation medium at final concentrations of 10^{-8}M to 10^{-6}M. As with wortmannin, experiments were undertaken in multiples of three wells on at least three separate occasions. In order to determine the effect of LY-294,002 on insulin action, 24 well plates were divided in half. One set of twelve 1ml wells received insulin only at concentrations 10^{-8}M to 10^{-6}M, and the second set received insulin and one concentration of LY-294,002. This was to determine the effect of LY-294,002 on the concentration dependent insulin-stimulated 2DG uptake in L6 cells (3.3). Three control wells were included in each 24 well plate and three wells were used to determine the effect of LY-294,002 on basal glucose transport in these cells.

Cells were exposed to LY-294,002 and/or insulin for 4h or 24h to determine any long term effects. Alternatively for acute effects cells were incubated with insulin for 40min and with LY-294,002 for 50min, to allow cell pre-stimulation with LY-294,002 for a 10min time period

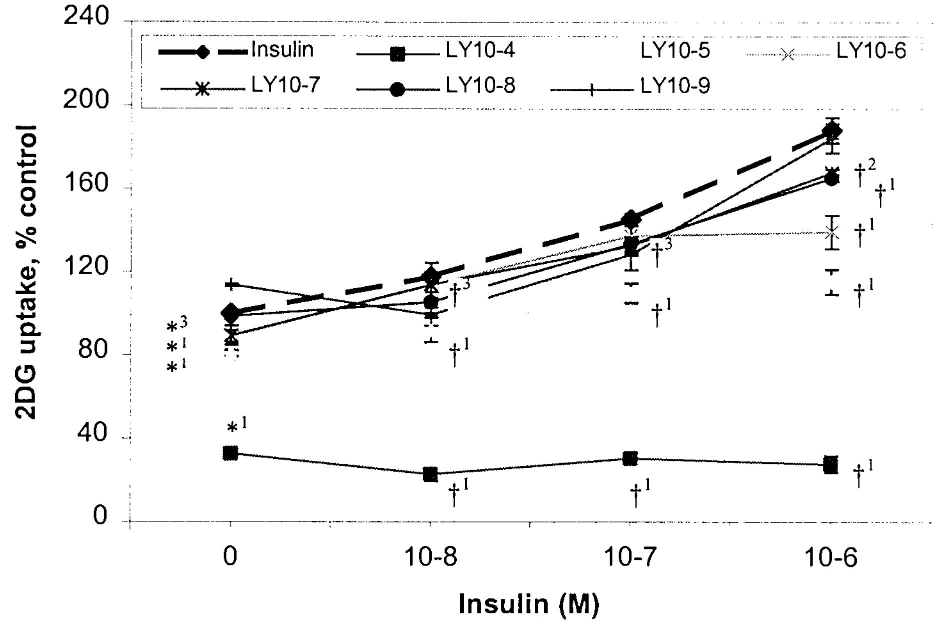
Glucose uptake was measured using the 2DG-uptake method as described in 2.4.1 (chapter 2). Uptake of 2DG was expressed as described above in Section 3.3.2. Viability studies were completed as outlined in section 2.4.5 (chapter 2) to determine possible toxicological effects of LY-294,002. The effects of LY-294,002 on L6 skeletal muscle cell viability was determined using the same concentration range as used to determine metabolic effects in section 3.4.2 (10^{-9}M – 10^{-4}M) after cell exposure for 50min, 4h and 24h.

3.4.2.3 Results

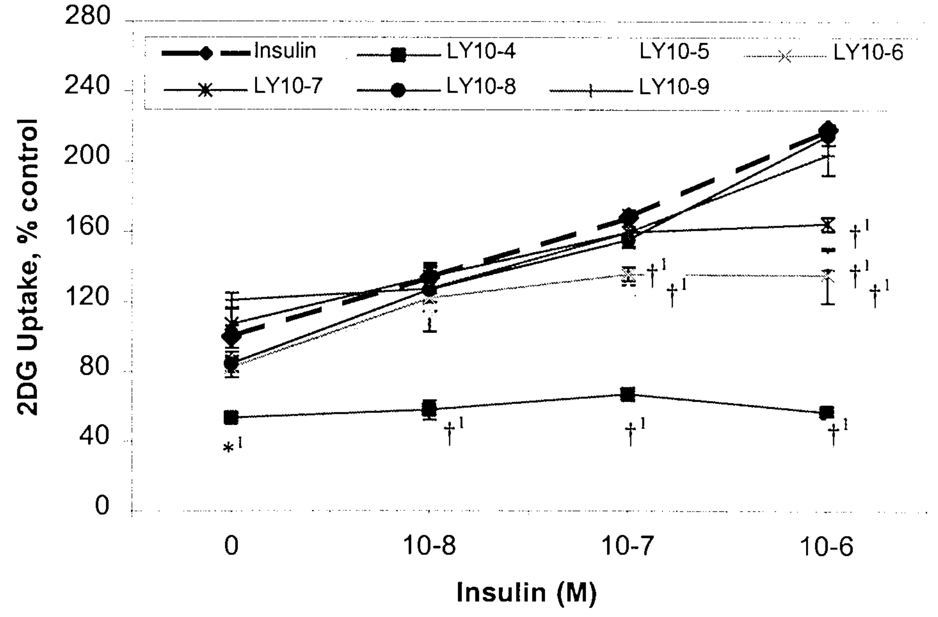
Incubation of L6 muscle cells with LY-294,002 decreased basal glucose transport in a concentration dependent manner. 10^{-4}M LY-294,002 was the highest concentration used and this produced a significant decrease in basal glucose uptake at each incubation period investigated ($p<0.01$ versus control). The shortest time point investigated was 50 mins and 10^{-4}M LY-294,002 decreased basal glucose uptake by 67% (figure 3.6a). After 4h cell exposure to LY-294,002, glucose uptake was inhibited by 47% (3.6b) and after 24h by 66% (3.6c). The effect of LY-294,002 on basal glucose transport was also determined at lower concentrations 10^{-9}M – 10^{-5}M. After a 50 minute incubation, LY-294,002 decreased basal glucose transport by 11% - 19% (10^{-7}M, $p<0.05$ versus control – 10^{-5}M, $p<0.01$ versus control), but little effect was observed with 10^{-9}M and 10^{-8}M (3.6a). After 4h incubation, glucose transport

Figure 3.6 Inhibition of basal and insulin-stimulated 2DG uptake by the structurally distinct inhibitor of PI 3-kinase LY-294,002 in L6 cells. In acute studies, cells were pre-stimulated with LY-294,002 (10^{-9}M – 10^{-4}M) for 10min. 2DG uptake was determined after cells were cultured with added insulin (10^{-8}M – 10^{-6}M) for a further 40min **3.6a**. In longer studies, cells were cultured with LY-294,002 and/or insulin for 4h **3.6b** or 24h **3.6c**. Insulin stimulated 2DG uptake in these cells in a concentration dependent manner. Both basal and insulin stimulated 2DG uptake was inhibited by LY294,002 in a concentration dependent manner at all time points investigated. 24h incubation of LY-294,002 produced the most potent inhibitory effect. Data are expressed as % control, values are mean ± SEM, n=9, *significance versus control, †significance versus insulin. $^1p<0.01$, $^2p<0.02$, $^3p<0.05$

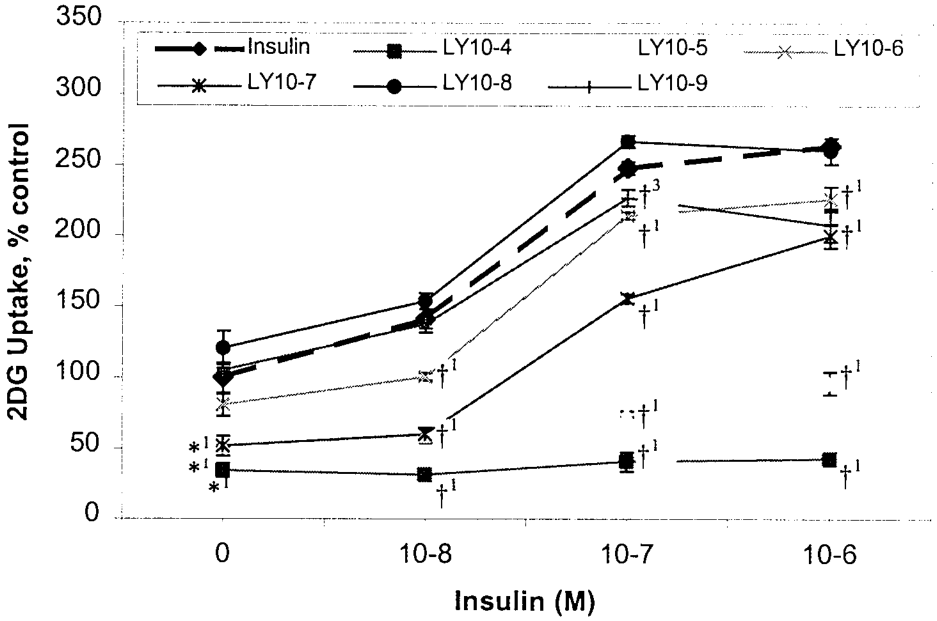
3.6a 50 minutes



3.6b 4 hours



3.6c 24 hours


Insulin
LY10-4
LY10-5
LY10-6
LY10-7
LY10-8
LY10-9
2DG Uptake, % control
350
300
250
200
150
100
50
0
0
10-8
10-7
10-6
Insulin (M)
*1
*1
*1
†1
†3
†1
†1
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†1
†1
†1
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†1
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was depressed by 14% - 18% (10^{-5}M – 10^{-9}M) however this was found not to be statistically significant (3.6b). Incubation for 24h with LY-294,002 decreased glucose transport by 48% (10^{-5}M and 10^{-7}M, $p<0.01$ versus control), concentrations 10^{-8}M and lower produced no inhibitory effect (3.6c).

Cell exposure to insulin (10^{-8}M – 10^{-6}M) increased glucose uptake in a concentration and time dependent manner as described in section 3.3 above. 10^{-4}M LY-294,002 decreased insulin-stimulated glucose uptake in a manner similar to that observed with basal glucose uptake i.e. by approximately 70% (50min exposure), 42% (4h) and 65% (24h) below control, regardless of insulin concentration used to stimulate glucose transport. The action of lower concentrations of LY-294,002 were effected by insulin concentration and inhibition was rarely decreased below that of basal glucose uptake. 10^{-8}M – 10^{-6}M insulin-stimulated glucose uptake was decreased after 50min incubation by 25% - 73% ($p<0.01$, 10^{-5}M LY-294,002 versus insulin), 4% - 49% (10^{-6}M), 4% - 21% (10^{-7}M), 12% - 23% (10^{-8}M) and 18% - 4% (10^{-9}M), figure 3.6a. LY-294,002 affected insulin-stimulated glucose transport after 4h in a manner similar to, but less potent than 50min incubation (3.6b). It decreased insulin-stimulated glucose uptake by 19% - 74% (10^{-5}M), 12% - 83% (10^{-6}M), 0% - 53% (10^{-7}M), 7% - 12% (10^{-8}M) and 7% - 14% (10^{-9}M). 24h incubation of LY294,002 on insulin-stimulated glucose transport also had an inhibitory effect (3.6c). Glucose transport was inhibited by 82% - 167% (10^{-5}M), 40% - 37% (10^{-6}M), 81% - 63% (10^{-7}M), 0% - 3% (10^{-8}M) and 4% - 56% (10^{-9}M), see figures for levels of significance.

LY-294,002 did not appear to affect cell viability by method of trypan blue exclusion at concentrations 10^{-9}M – 10^{-5}M at each time point tested. Cells incubated with LY-

294,002 at these concentrations did not appear to be significantly different from control cells either in morphology, ability to exclude trypan blue or ability to adhere. However 10^{-4}M LY-294,002 did decrease cell viability determined by an increase in intracellular trypan blue (data/photographs not shown). LY-294,002 will only be utilised at concentrations 10-9M – 10-5M in subsequent investigations. Unlike wortmannin, the length of time the cells were exposed to LY-294,002 did not affect cell viability at lower concentrations.

3.4.4 Effect of Tyrphostin AG490 on Basal and Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

3.4.4.1 Introduction

3.4.4.1.1 Protein Tyrosine Kinases

The protein-tyrosine kinases (PTKs) are a large family of proteins that possess a conserved domain of 250 to 300 amino acids capable of phosphorylating substrate proteins on tyrosine residues.

Approximately 100 tyrosine kinases have been identified to date and although the biological role of PTKs has been characterised for only very few, many of them are thought to signal cell proliferation (Levitzki, 1990).

3.4.4.1.2 Janus Kinase

Numerous biological functions are controlled by a group of ligands termed cytokines, all of which signal through a related set of receptors. All known receptors responding to these ligands are associated with one or more members of the Jak family. The Janus

Kinase (Jak) family is a PTK family involved in cytokine signalling, activated by type I and type II cytokine receptors, and plays a pivotal role in the signal transduction process mediated by cytokines. The activation of Jak family is associated with rapid tyrosine phosphorylation of Signal Transducers and Activators of Transcription (STAT) proteins.

At present Jak family members include Jak1, Jak2, Jak3 and Tyk2.

3.4.4.1.3 Jak1 Tyrosine Kinase

Jak1 is a large, widely expressed membrane-associated phosphoprotein of approximately 130,000 Da, and is characterised by a second phosphotransferase-related domain immediately N-terminal to the protein tyrosine kinase (PTK) domain.

The second phosphotransferase domain bears all the characteristics of a protein kinase, although its structure differs significantly from that of the PTK and threonine/serine kinase family members. The PTK activity of Jak1 has been located in the C-terminal PTK-like domain.

By site-directed mutagenesis and selection for resistance to interferon, Muller et al. (1993) produced a cell line that lacked Jak1 and was completely defective in an interferon response. Addition of Jak1 to the culture system restored the response, establishing the requirement for Jak1 in the interferon-α, β and γ signal transduction pathways.

3.4.4.1.4 Jak2 Tyrosine Kinase

A second member of the family, Jak2, has been partially characterized and exhibited a similar array of kinase-related domains.

Watling et al. (1993) isolated a cell line, selected for its inability to express interferon (IFN)-gamma-inducible cell-surface markers. The cell line was deficient in all aspects of IFN-gamma response tested but responded normally to alpha and beta IFNs. The mutant cells could be complemented by the expression of Jak2. Unlike IFNs alpha and beta, IFN-gamma induced rapid tyrosine phosphorylation of Jak2 in wildtype cells, and Jak2 immunoprecipitates from these cells showed tyrosine kinase activity. These responses were absent in the mutant cell line. Jak2 is, therefore, required for the response to interferon-gamma but not to IFNs alpha and beta.

Campbell et al. (1994) presented evidence that Jak2 is constitutively associated with the prolactin receptor and that it is activated and tyrosine phosphorylated upon PRL binding to the PRL receptor. These results are consistent with Jak2 serving as an early, perhaps initial, signaling molecule for prolactin.

To assess the role of Jak2, Parganas et al. (1998) derived Jak2-deficient mice by targeted disruption of the mouse gene in embryonic stem cells. The mutation caused an embryonic lethality due to the absence of definitive erythropoiesis. Fetal liver myeloid progenitors, failed to respond to erythropoietin, thrombopoietin, interleukin-3 (IL3), or granulocyte/macrophage colony-stimulating factor. In contrast, the response to granulocyte-specific colony-stimulating factor was unaffected. Jak2-deficient fibroblasts failed to respond to IFN-gamma, although the responses to IFN-alpha/beta and IL6 were unaffected. Reconstitution experiments demonstrated that Jak2 was not required for the generation of lymphoid progenitors, their amplification, or their functional differentiation. Parganas et al. (1998) concluded that Jak2 plays a critical role in the function of a specific group of cytokine receptors.

Neubauer et al. (1998) also performed a targeted inactivation of Jak2 in mice. Jak2-/- embryos were anaemic and died around day 12.5 postcoitus. Primitive erythrocytes were found, but definitive erythropoiesis was absent. Compared to erythropoietin receptor-deficient mice, the phenotype of Jak2 deficiency was more severe. However, multilineage hematopoietic stem cells were found, and B lymphopoiesis appeared intact. In contrast to IFN-alpha stimulation, Jak2-/- cells did not respond to IFN-gamma. These data also demonstrated that Jak2 has pivotal functions for signal transduction of a set of cytokine receptors required in definitive erythropoiesis.

The leptin receptor is found in many tissues in several alternatively spliced forms, raising the possibility that leptin exerts effects on many tissues including the hypothalamus. The leptin receptor is a member of the gp130 family of cytokine receptors that are known to stimulate gene transcription via activation of cytosolic STAT proteins. In order to identify the sites of leptin action *in vivo*, Vaisse et al. (1996) assayed for activation of STAT proteins in mice treated with leptin. The STAT proteins bind to phosphotyrosine residues in the cytoplasmic domain of the ligand-activated receptor, where they are subsequently phosphorylated. The activated STAT proteins dimerize and translocate to the nucleus, where they bind DNA and activate transcription. The investigators assayed the activation of STAT proteins in response to leptin in a variety of mouse tissues known to express the leptin receptor, Ob-R. Leptin injection activated STAT3 but no other STAT protein in the hypothalamus of *ob/ob* and wildtype mice but not *db/db* mice, mutants that possess a truncated form of the leptin receptor. Leptin did not induce STAT activation in any of the other tissues tested. The dose-dependent activation of STAT3 by leptin was first observed after 15 minutes and later at 30 minutes. The data indicated to Vaisse et al. (1996) that the hypothalamus is a direct target of leptin action and this activation is critically dependent on the gp130-like leptin receptor isoform missing in *db/db* mice.

Pfeffer et al. (1997) found that STAT3, a transcription factor for acute phase response genes, acts as an adapter molecule in signal transduction from the type I interferon receptor. They found that it binds to a conserved sequence in the cytoplasmic tail of the receptor and undergoes interferon-dependent tyrosine phosphorylation. The p85 regulatory subunit of phosphatidylinositol 3-kinase, which activates a series of serine kinases, was found to bind to phosphorylated STAT3 and subsequently to undergo tyrosine phosphorylation. The authors concluded that STAT3 acts as an adaptor to couple to another signalling pathway.

3.4.4.1.5 Tyrosine Kinase Inhibitors

Protein tyrosine kinases have been identified as a potential target for drug targeting (Levitzki, 1990), and potential blockers for the substrate domain of the protein tyrosine kinase has been proposed as a putative site of action for an antiproliferative agent.

Quercetin and its related compound genistein (Akiyama et al., 1987) inhibit a variety of protein tyrosine kinases and other protein kinases such as cAMP-dependent protein kinase and protein kinase C, rendering them highly toxic.

Tyrphostins are compounds that were designed as selective inhibitors of protein tyrosine kinases, which target the particular substrate sites of different protein tyrosine kinases (Yaish et al., 1988). These compounds were developed as antiproliferative agents and have been demonstrated to be highly effective. Meydan et al., (1996) demonstrated that leukaemic cells from patients in relapse have constitutively activated Jak2 protein tyrosine kinase. Inhibition of Jak2 activity by a specific tyrosine kinase blocker, tyrphostin AG490, selectively inhibits leukaemic cell growth *in vitro* and *in vivo* by inducing programmed cell death, with no deleterious effect on normal haematopoiesis. More recently tyrphostins have been used as molecular tools to investigate signal transduction pathways of protein tyrosine kinases.

Tyrphostin AG490 (N-Benzyl-3,4-dihydroxy-benzylidinenecyanoacetamide α-cyano-(3,4-dihydroxy)-]N-benzylcinnamamide (appendix II) is a potent and specific inhibitor of Jak2 tyrosine kinase and has been used in a number of studies to investigate the Jak/STAT (Janus kinase/signal transducer and activator of transcription) signalling pathway. Tyrphostin AG490 is used here to determine whether Jak2 tyrosine kinase plays a role in signal transduction in the insulin receptor/glucose uptake pathway. It is also investigated as a prelude to further experimentation of compounds thought to stimulate Jak2 tyrosine kinase activity.

3.4.4.2 Experimental Design

Tyrphostin AG490 (Alexis Corporation (UK) Ltd. Nottingham) was dissolved in dimethyl sulphoxide (DMSO) at 10mM, stored at –20°C in the dark in 100μl aliquots, and diluted with PBS immediately before use. L6 cells were grown to confluence in 24 well plates and serum starved for 24h as described in 2.2.2.

Tyrphostin AG490 was diluted and added to the cell incubation medium to give a final concentration of 10^{-9}M to 10^{-4}M. Insulin was diluted and added to the incubation medium at final concentrations of 10^{-8}M to 10^{-6}M. As described previously, experiments were undertaken in multiples of three. In order to determine the effect of tyrphostin AG490 on insulin action, 24 well plates were divided in half. One set of twelve 1ml wells received insulin only at concentrations 10^{-8}M to 10^{-6}M, and the second set received insulin and one concentration of tyrphostin AG490. This was to determine the effect of tyrphostin AG490 on the concentration dependent insulin-stimulated 2DG uptake in L6 cells as identified in section 3.3 above. Three control wells were included in each 24 well plate and three wells were used to determine the effect of tyrphostin AG490 on basal glucose transport in these cells. Glucose uptake was measured using the 2DG-uptake method as described in 2.4.1 Uptake of 2DG was expressed as described above (3.3.2).

To determine the full range of effects of tyrphostin AG490 on insulin-stimulated and basal glucose transport this experiment was completed after incubating the cells for either 24h or 4h with typhostin and/or insulin. To determine the acute effects of tyrphostin AG490, cells were prestimulated for 10mins with the inhibitor, and insulin was added for a further 40mins.

3.4.4.3 Results

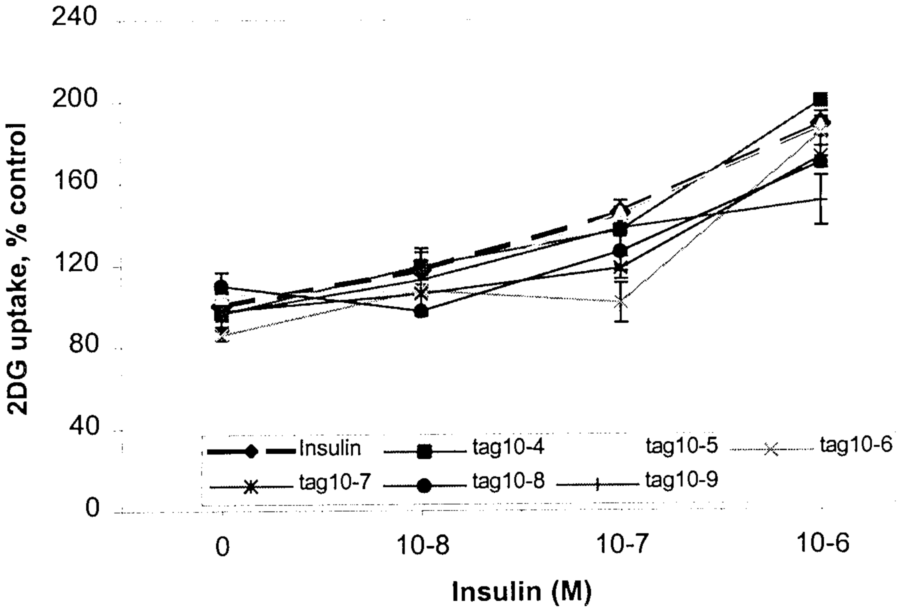
L6 cell exposure to tyrphostin AG490 for 50 min produced little effect on both basal and insulin stimulated glucose transport (figure 3.7a). Basal glucose uptake was unaffected by incubations of 10^{-9}M – 10^{-4}M tyrphostin AG490. As described previously, insulin increased glucose uptake in a concentration-dependent manner section 3.3, using concentrations 10^{-8}M – 10^{-6}M. Preincubation with tyrphostin AG490 (10^{-9}M – 10^{-4}M) did not significantly alter this response.

4h exposure to tyrphostin AG490 (10^{-9}M – 10^{-4}M) did not affect basal glucose transport, however high concentrations appeared to increase insulin-stimulated glucose transport (figure 3.7b). 10^{-4}M tyrphostin AG490 increased the insulin dose response curve by 47% - 67% (10^{-8}M – 10^{-6}M insulin, $p<0.01$). 10^{-5}M tyrphostin AG490 increased the response by 41% (10^{-7}M insulin only, $p<0.01$) and 10^{-9}M – 10^{-6}M did not significantly alter the insulin-stimulated glucose uptake response.

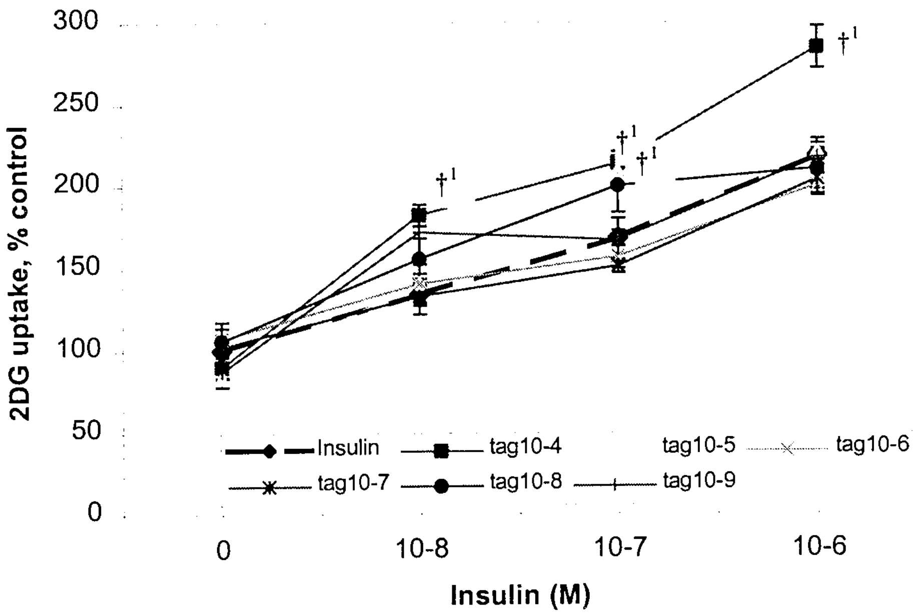
24h exposure of the cells to 10^{-4}M tyrphostin AG490 increased basal glucose transport by 113% ($p<0.01$ versus control) however; lower concentrations (10^{-5}M-10^{-9}M) had no effect (figure 3.7c). Insulin stimulated glucose transport after 24h incubation by 41% - 163% (10^{-8}M – 10^{-6}M) as described in section 3.3. 10^{-4}M tyrphostin increased insulin-stimulated (10^{-8}M) glucose uptake by 113% ($p<0.01$ versus 10^{-8}M insulin) but did not affect glucose uptake stimulated by higher concentrations of insulin (10^{-7}M and 10^{-6}M). Lower concentrations of tyrphostin AG490 (10^{-9}M – 10^{-5}M) had no significant effect on insulin-stimulated glucose uptake in these cells.

Figure 3.7 Effect of the janus kinase 2 inhibitor tyrphostin AG490 (tag) on basal and insulin-stimulated 2DG uptake In acute studies, cells were pre-stimulated with tyrphostin AG490 (10^{-9}M – 10^{-4}M) for 10min. 2DG uptake was determined after cells were cultured with added insulin (10^{-8}M – 10^{-6}M) for a further 40min **3.7a**. In longer studies, cells were cultured with tyrphostin AG490 and/or insulin for 4h **3.7b** or 24h **3.7c**. Insulin stimulated 2DG uptake in these cells in a concentration dependent manner. Tyrphostin AG490 did not inhibit basal or insulin-stimulated 2DG uptake at any concentration or time-point investigated. Conversely, high concentrations (10^{-4}M) of tyrphostin AG490 significantly increased insulin-stimulated glucose uptake after 4h and 24h incubations and increased basal glucose transport after 24h. Data are expressed as % control, values are mean ± SEM, n=6, *significance versus control, †significance versus insulin.^{1}p<0.01, ^{2}p<0.02, ^{3}p<0.05

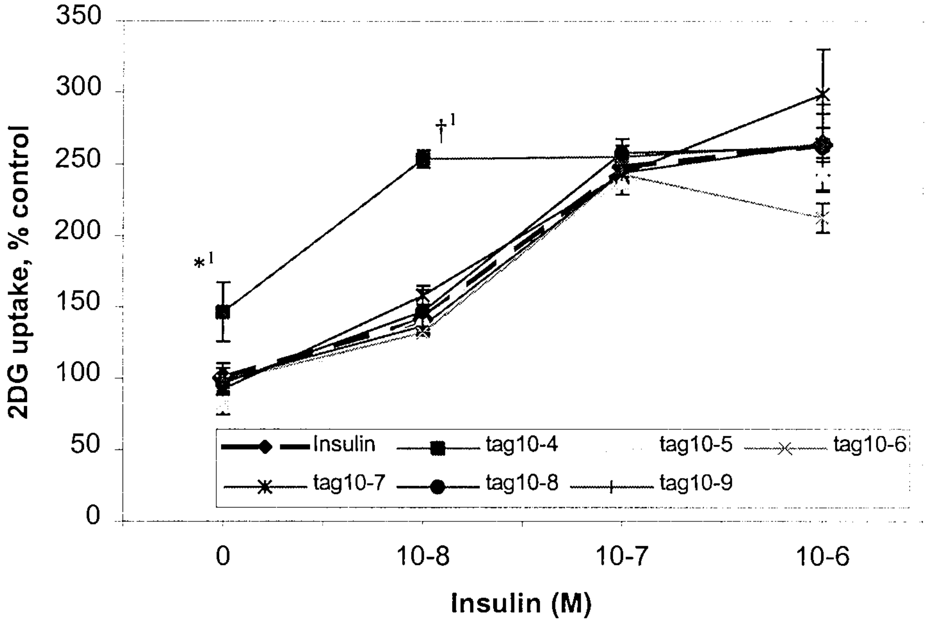
3.7a 50 minutes



3.7b 4 hours



3.7c 24 hours


350
300
250
200
150
100
50
0
2DG uptake, % control
*1
†1
Insulin
tag10-4
tag10-5
tag10-6
tag10-7
tag10-8
tag10-9
0
10-8
10-7
10-6
Insulin (M)

Tyrphostin AG490 did not decrease either basal or insulin-stimulated glucose transport and was observed microscopically to have no effect on cell morphology at either high concentration or after chronic cell exposure. The data obtained above, were thought not to be confounded by any toxicological implications of this agent.

3.4.5 Effect of Cytochalasin B on Basal and Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

3.4.5.1 Introduction

Skeletal muscle is quantitatively the major site responsible for insulin stimulated glucose uptake (DeFronzo et al., 1988) facilitated by glucose transporters (GLUTS, section 1.6.5).

Cytochalasin B, (appendix II) a fungi alkaloid, acts on microfilaments and interferes with the assembly of actin filaments by capping one of their ends. It is a high affinity inhibitor of glucose transporters, hence glucose competes with cytochalasin B for reversible binding to the transporter. Early studies utilised [^{3}H]-photoaffinity-labelled cytochalasin B to identify the glucose transporter in rat skeletal muscle (Klip et al., 1983), and has been used to characterise the kinetic and pharmacological properties of glucose uptake in muscular cells (Klip et al., 1982).

More recently, glucose transporter subtype and quantity in numerous tissues has been identified by the preparation of cell membranes and identification of glucose transporters using anti-GLUT antibodies raised to the C-terminal end of their amino acid sequences (James et al., 1989; Birnbaum et al., 1989) and detection by western blotting. L6 skeletal muscle cells were found to contain multiple glucose transporter isoforms, which include GLUT1, GLUT3 and GLUT4. Subcellular fractionation demonstrated that levels of glucose transporter proteins increased in the plasma membrane in response to insulin stimulation. Acute stimulation of the cells with insulin did not alter the total protein levels of GLUT1, 3 or 4 or the mRNA of GLUT1 or GLUT4, indicating redistribution of transporters (Bilan et al., 1992; Wilson et al., 1995).

The subcellular fractionation technique is now considered to possess a number of drawbacks including a poor recovery of plasma membranes and a high degree of cross-contamination among membrane subfractions. Subsequently, this technique has now been superseded by the use of the more sensitive method of exofacial bis-mannose photolabelling (ATB-BMPA), (Lund et al., 1997). Photolabelling has demonstrated that there are similar amounts of GLUT1, 3 and 4 at the surface of L6 cells in the basal state and that similar quantities were found at the cell surface of insulin-stimulated cells (Wilson et al., 1995)

Due to financial implications this method was not employed here to determine glucose transporter quantity in the L6 muscle cells as these data are already widely published (Wilson et al., 1995; Klip et al., 1996). However, cytochalasin B was used here as an inhibitor of carrier-mediated glucose uptake. It does not inhibit diffusion of the sugars across the cell membrane, and therefore an inhibitory effect of cytochalasin B demonstrates the presence of a facilitated transport system in these cells.

3.4.5.2 Experimental Design

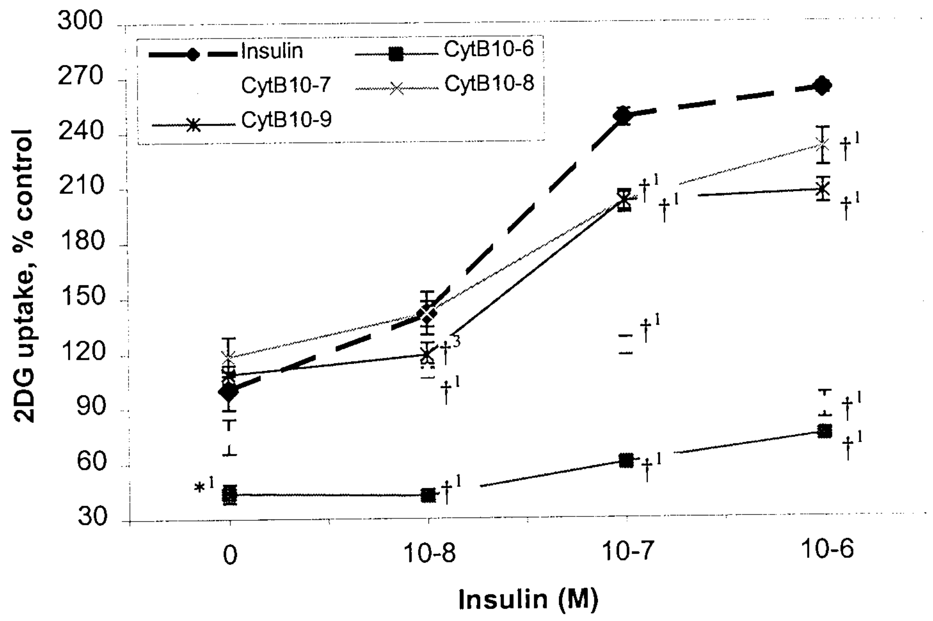
Cytochalasin B (Sigma) was dissolved in dimethyl sulphoxide (DMSO) at 10mM, stored at –20°C in the dark in 100μl aliquots, and diluted with PBS immediately before use. L6 cells were grown to confluence in 24 well plates and serum starved for 24h as described in 2.4.1.

Cytochalasin B (Cyt B) was diluted and added to the cell incubation medium to give a final concentration of 10^{-9}M to 10^{-6}M. Insulin was added to the incubation medium at final concentrations of 10^{-8}M to 10^{-6}M. As described previously, experiments were undertaken in multiples of three wells on at least three separate occasions. In order to determine the effect of Cyt B on insulin action, 24 well plates were divided in half. One set of twelve 1ml wells received insulin only at concentrations 10^{-8}M to 10^{-6}M, and the second set received insulin and one concentration of Cyt B. This was to determine the effect on the concentration dependent insulin-stimulated 2DG uptake in L6 cells as identified in 3.3. Three control wells were included in each 24 well plate and three wells were used to determine the effect of Cyt B on basal glucose transport in these cells. Glucose uptake was measured using the 2DG-uptake method as described in 2.4.1 Uptake of 2DG was expressed as described above (3.3).

Studies were completed after cell incubation of insulin and/or Cyt B for 24h.

The effect of Cyt B on cell viability was assessed using the method of trypan blue exclusion as described in section 2.4.5.

Figure 3.8 Inhibition of basal and insulin-stimulated 2DG uptake by the high affinity inhibitor of glucose transporters cytochalasin B (Cyt B) in L6 cells. Cells were cultured with cytochalasin B (10^{-9}M – 10^{-6}M) and/or insulin (10^{-8}M – 10^{-6}M) for 24h. Insulin stimulated 2DG uptake in these cells in a concentration dependent manner. Both basal and insulin stimulated 2DG uptake were inhibited by cytochalasin B in a concentration dependent manner. Data are expressed as % control, values are mean ± SEM, n=9. *significance versus control, †significance versus insulin. $^{1}p<0.01$, $^{2}p<0.02$, $^{3}p<0.05$.



3.4.5.3 Results

Cell exposure to 10^{-6}M Cyt B for 24h significantly decreased basal glucose transport by 56% ($p<0.01$ versus control), figure 3.8. Lower concentrations of Cyt B did not affect basal glucose uptake (10^{-9}M, 10^{-8}M and 10^{-7}M Cyt B). Insulin stimulated glucose uptake as described in previously in this chapter, and Cyt B decreased insulin-stimulated glucose transport in a concentration dependent and insulin-concentration-dependent manner. 10^{-6}M Cyt B significantly decreased insulin-stimulated glucose uptake by 99% - 188% ($p<0.01$ versus 10^{-8}M – 10^{-6}M insulin), therefore inhibiting glucose uptake to levels below basal transport. Lower concentrations of Cyt B decreased insulin-stimulated glucose transport but inhibition did not decrease below basal levels, see figure 3.8 for levels of significance. 10^{-9}M Cyt B was the lowest concentration investigated and this decreased insulin-stimulated glucose uptake by 22% - 56% ($p<0.05$ versus 10^{-8}M insulin, $p<0.01$ versus 10^{-7}M and 10^{-6}M insulin). Cyt B had no effect on cell viability (photographs in appendix III).

3.4.6 Effect of Cycloheximide on Basal and Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

3.4.6.1 Introduction

Cycloheximide (appendix II) is an antibiotic derived from *Streptomyces griseus*. It inhibits protein synthesis in eukaryotic cells by blocking translation of messenger RNA by inhibiting the peptidyl transferase activity of the 60s ribosomal subunit.

Cycloheximide has been used previously as a molecular tool to block protein synthesis in order to determine glucose transporter action. Walker et al., (1990) demonstrated that cycloheximide prevents the stimulation of glucose uptake caused by prolonged incubation with insulin and has since been an accepted method of determining the action of specific pharmacological agents (Klip et al., 1992).

Cycloheximide is used here to determine the effects protein inhibition on basal and insulin stimulated glucose transport in L6 cells over a range of incubation periods.

3.4.6.2 Experimental Design

Cycloheximide (Sigma) was dissolved in dimethyl sulphoxide (DMSO) at 10mM, stored at –20°C in the dark in 100μl aliquots, and diluted with PBS immediately before use. L6 cells were grown to confluence in 24 well plates and serum starved for 24h as described in 2.2.2.

Cycloheximide was diluted and added to the cell incubation medium to give a final concentration of 10^{-8}M to 10^{-5}M. Insulin was added to the incubation medium at final concentrations of 10^{-8}M to 10^{-6}M. As described previously, experiments were undertaken in multiples of three wells. In order to determine the effect of cycloheximide on insulin action, 24 well plates were divided in half. One set of twelve 1ml wells received insulin only at concentrations 10^{-8}M to 10^{-6}M, and the second set received insulin and one concentration of cycloheximide. Three control wells were included in each 24 well plate and three wells were used to determine the effect of cycloheximide on basal glucose transport in these cells. Glucose uptake was measured using the 2-deoxy-[^{3}H]-glucose uptake method as described in 2.4.1 Uptake of 2DG was expressed as described above (3.3).

To determine the full range of effects of cycloheximide on insulin-stimulated and basal glucose transport this experiment was completed after incubating the cells for either 24h or 4h with cycloheximide and/or insulin. To determine the acute effects of cycloheximide, cells were prestimulated for 10mins with the inhibitor and insulin was added for a further 40mins

The effect of cycloheximide on cell viability was assessed as described previously 2.4.5.

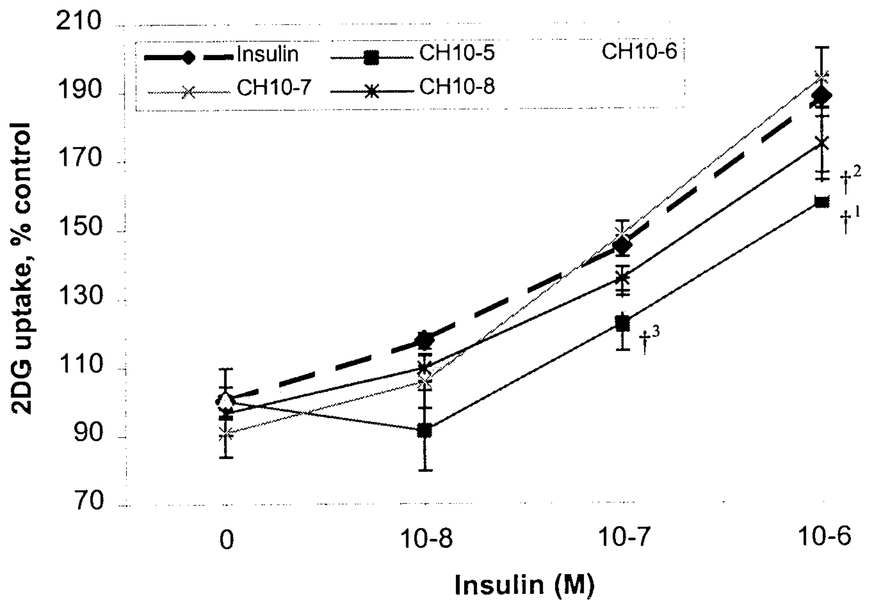
3.4.6.3 Results

50min incubation of cycloheximide had no effect on basal glucose uptake in L6 cells. When the cells were stimulated with insulin (10^{-8}M – 10^{-6}M), high concentrations of cycloheximide (10^{-6}M and 10^{-5}M) decreased glucose uptake (10^{-5}M cycloheximide = $p<0.01$ versus 10^{-6}M insulin, $p<0.05$ versus 10^{-7}M insulin, 10^{-6}M cycloheximide = $p<0.02$ versus 10^{-6}M insulin), lower concentrations had no effect, figure 3.9a.

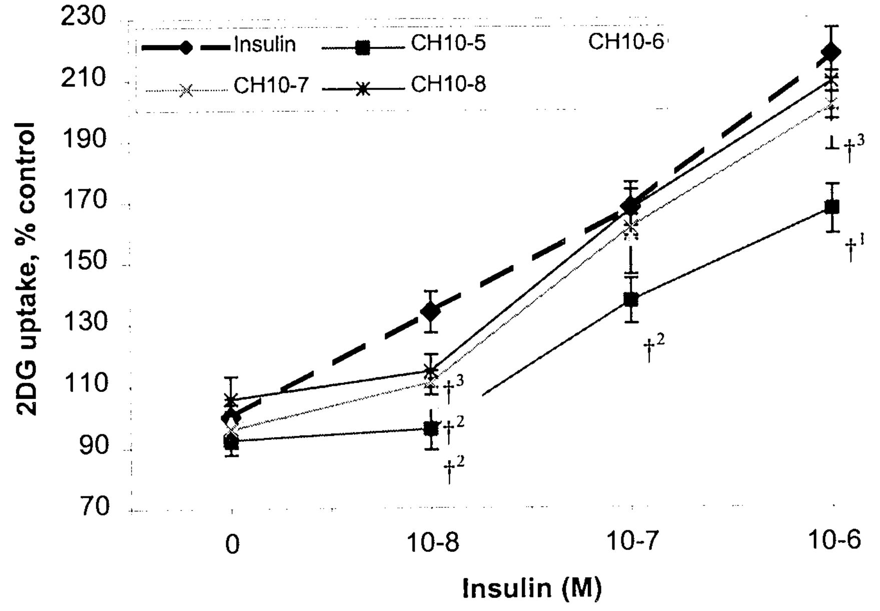
After 4h cell exposure to cycloheximide, basal glucose uptake was not significantly altered (figure 3.9b). Insulin-stimulated glucose uptake was decreased by cycloheximide at high concentrations (10^{-5}M) by 38% - 51% ($p<0.02$ versus 10^{-8}M and 10^{-7}M insulin, $p<0.01$ versus 10^{-6}M insulin). 10^{-6}M cycloheximide significantly decreased insulin-stimulated glucose uptake by approximately 25% ($p<0.02$ versus 10^{-8}M insulin and $p<0.05$ versus 10^{-6}M insulin). Lower concentrations of

Figure 3.9 Inhibition of basal and insulin-stimulated 2DG uptake by the inhibitor of protein synthesis, cycloheximide (CH), in L6 cells. In acute studies, cells were stimulated with cycloheximide (10^{-8}M – 10^{-5}M) and/or insulin (10^{-8}M – 10^{-6}M) for 50min **3.9a**. In longer studies, cells were cultured with cycloheximide and/or insulin for 4h **3.9b** or 24h **3.9c**. Insulin stimulated 2DG uptake in these cells in a concentration dependent and time dependent manner. Cycloheximide inhibited both basal and insulin-stimulated 2DG uptake in a concentration dependent and time dependent manner. Incubation of cells with cycloheximide for 24h produced the most potent inhibitory response. Data are expressed as % control, values are mean ± SEM, n=6, *significance versus control, †significance versus insulin. ^{1}p<0.01, ^{2}p<0.02, ^{3}p<0.03.

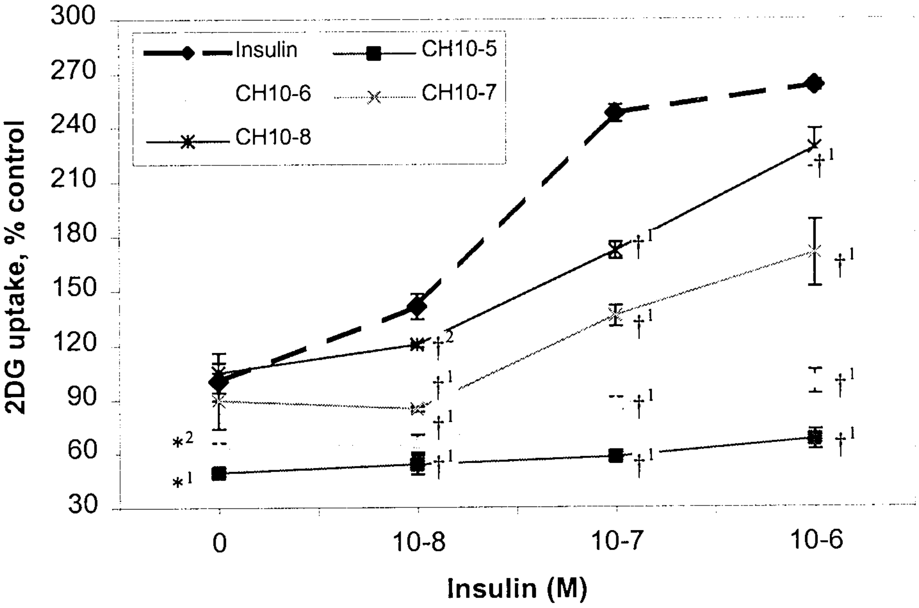
3.9a 50 minutes



3.9b 4 hours



3.9c 24 hours



300
270
240
210
180
150
120
90
60
30
2DG uptake, % control
Insulin
CH10-5
CH10-6
CH10-7
CH10-8
*2
*1
†1
†2
0
10-8
10-7
10-6
Insulin (M)

cycloheximide (10^{-8}M– 10^{-7}M) did not significantly inhibit insulin stimulated glucose transport.

Incubation of L6 cells with cycloheximide for 24h significantly decreased basal glucose transport (figure 3.9c). The inhibition was concentration dependent, 10^{-5}M cycloheximide decreased basal glucose uptake by 51% ($p<0.01$, versus control), 10^{-6}M inhibited by 37% ($p<0.02$ versus control), 10^{-7}M by 11% (not significant) and 10^{-8}M had no effect. Insulin-stimulated glucose transport was significantly decreased by cycloheximide. 10^{-5}M cycloheximide completely diminished the insulin stimulated response by decreasing glucose uptake to levels on average 42% below basal ($p<0.01$ versus insulin). 10^{-6}M cycloheximide decreased glucose uptake by 79% - 164% ($p<0.01$ versus 10^{-8}M – 10^{-6}M insulin) and 10^{-7}M inhibited by 56% - 112%, ($p<0.01$ versus insulin) and 10^{-8}M by 21% - 76% ($p<0.02$ versus 10-8M insulin, $p<0.01$ versus10^{-7}M and 10^{-6}M insulin).

The inhibitory action of cycloheximide on insulin-stimulated glucose transport was dependent on the concentrations of cycloheximide and insulin used and upon incubation time. Cycloheximide had no effect on cell viability (appendix III).

3.4.7 Discussion

There are obvious advantages for utilising a cell culture system over intact organ preparations or *in vivo* studies. Cell type homogeneity is one of the major reasons culture systems are used in many forms of experimentation. Lines of defined origin can be grown in suitable quantities and it is possible to study the maturation of the hormonal response through myogenesis. However, cell culture is particularly advantageous for this type of study as measurements are not complicated by surgical damage and the technique allows even and rapid accessibility of all cells to substrates. The geometry of skeletal muscle precludes the homogenous exposure of individual fibres to externally added substrates or ligands (Klip et al., 1984) and in the past this has been one of the limitations for acquisition of knowledge surrounding the molecular properties of glucose transport into tissue. There are added benefits of using an *in vitro* system for the study of insulin sensitivity. There are no confounding effects of weight loss to take into consideration, no sympathetic nervous system involvement, no effects due to changes in the vascular supply, tight, even control of insulin levels can be achieved and any observed effect is likely to be due to a direct action.

It is widely recognised that cell culture systems offer unique advantages over intact tissue preparations for the study of molecular mechanisms of hormone action and of the regulation of transport functions. This chapter investigates the use of the rat L6 skeletal muscle cell line as a potential model for skeletal muscle *in vitro.* L6 cells have previously been shown to possess several characteristics of skeletal muscle *in vivo* (Shainberg et al., 1971), and this is supported by the data obtained in this chapter. Cell growth and fusion of cells to form myotubes was investigated phenotypically in section 3.2, (figure 3.1 and 3.2). The data demonstrate that the cells divide in culture a finite number of times before subsequent fusion to form multinucleated muscle fibres. This was originally described by Yaffe (1968) and demonstrates that multiple sub-cloning, freeze/thaw cycling and repeated passaging have not destroyed the original phenotypical features of the cell line.

Glucose transport in these cells was investigated in section 3.3 (figure 3.3). The significant increase in 2DG uptake observed upon cell exposure to insulin establishes this cell line a suitable system in which the response of glucose transport to insulin can be studied. The data obtained in section 3.3 demonstrated that these cells increase glucose transport in response to insulin in a concentration and time dependent manner. Near-physiological concentrations increased glucose uptake after cell exposure to insulin for 1h or longer and maximal stimulation was observed after cell exposure to insulin for 24h. However, higher concentrations of insulin increased glucose transport after only 10min exposure. This stimulation of glucose transport activity by acute insulin treatment has been previously reported in L6 cells and other models of skeletal muscle (Simpson and Cushman, 1986; Klip and Ramlal, 1987; Klip et al., 1984; Bequinot et al., 1986). This effect is either due to a rapid translocation of preformed GLUTS to the cell membrane from an intracellular pool or due to intrinsic activation of pre-existing cell surface GLUTS (Simpson and Cushman, 1986; Walker et al., 1989). The chronic stimulation of L6 cells with insulin produced a highly significant glucose uptake response when stimulated with both high and physiological concentrations. This effect is thought to be due to protein transcription and the formation of new GLUTS. The acute and chronic effects of insulin on protein synthesis were investigated further in section 3.4.6 using cycloheximide. The effects of insulin concentration, time and cycloheximide concentration on GLUT transcription and translocation is discussed below.

As described previously (1.6.3), PI 3-kinase is a component of the signalling pathway of a large number of tyrosine kinase receptors and is integral to insulin signalling. Insulin activates the tyrosine kinase activity of its receptor to phosphorylate IRS and allow binding of the SH2 domains of p85 to IRS and activation of PI 3-kinase (1.6.3d). Numerous studies have determined that activation of PI 3-kinase is an important step in the activation of glucose transport by insulin in many tissues and cell lines (Kanai et al., 1993; Clarke et al., 1994; Okada et al., 1994; Cheatham et al., 1994; Shimizu and Shimizu 1994; Hara et al., 1994). The role of PI 3-kinase in basal and insulin-stimulated glucose transport was investigated in L6 cells in this chapter (3.4.1 and 3.4.2). Wortmannin has been used in many studies to investigate PI 3-kinase mediated signalling, and its inhibitory effect on insulin signalling is well documented. Nevertheless many studies have investigated this mechanism without consideration of the toxicological properties of the metabolite or the effects on basal glucose transport. The data obtained in section 3.4.1.3 demonstrated that wortmannin decreased both basal and insulin-stimulated glucose uptake in a concentration dependent manner. High concentrations of wortmannin decreased insulin-stimulated glucose transport to levels below basal. However lower concentrations of wortmannin inhibited insulin-stimulated transport when cells were stimulated with physiological concentrations of insulin. Acute stimulation of the cells with high concentrations of insulin decreased the inhibitory effect of wortmannin. Thus, in mediating this response there may be alternate mechanisms by which high concentrations of insulin can mediate glucose uptake or it is possible that inhibition of PI 3-kinase action by wortmannin may be competitive in nature. Toxicological investigations into the effect of wortmannin on cell viability (figure 3.5) demonstrated that incubation of the cells for protracted periods of time or with high concentrations of wortmannin decreased cell viability. Therefore cell exposure to wortmannin to produce an optimum effect without causing a decrease in cell viability was found to be 50 minutes. Wortmannin was found not to have an effect on cell viability if utilised at 10^{-5}M or lower. Thus wortmannin can only be used in this tissue culture system at short time points. The acute effects of insulin can be investigated using wortmannin however, in L6 cells the chronic effects of insulin on glucose uptake via protein synthesis can not be determined using this inhibitor.

In order to determine the effects of chronic stimulation of L6 cells with insulin, LY-294,002 was employed as a potential inhibitor of PI 3-kinase (3.4.2). It was investigated for toxicological properties and was found not to decrease cell viability after protracted exposure in L6 cells. Thus the compound could potentially be utilised in place of wortmannin for investigating insulin-stimulated protein synthesis. LY-294,002 was found to decrease both basal and insulin-stimulated glucose transport in a concentration-dependent manner. Extremely high concentrations resulted in a small decrease in L6 cell viability and thus concentrations of 10^{-5}M and lower (which had no effect on cell viability) were utilised in subsequent experimentation. Importantly the effect of LY-294,002 in inhibiting basal and insulin-stimulated glucose transport was not lost after 24h cell exposure and thus indicates that PI 3-kinase is pivotal in both acute and chronic responses to insulin in L6 cells. As with the acute wortmannin studies, glucose uptake in response to insulin and LY-294,002 resulted in inhibition. However glucose uptake was not decreased below basal values, indicating that in acute insulin stimulated responses that wortmannin was more potent in inhibition of PI 3-kinase. 24h incubations resulted in a more potent effect of LY-294,002 and both basal and insulin-stimulated glucose transport was decreased below basal values. To this end, in subsequent investigations in which acute (non-transcriptional via translocation or intrinsic activity) responses are explored in these cells, wortmannin was the inhibitor of choice. However because of the toxicological implications of wortmannin, LY-294,002 was used to inhibit PI 3-kinase during chronic (at least partly mediated by protein synthesis) investigations into glucose transport by L6 cells.

Acute and chronic insulin-mediated glucose uptake was further investigated in section 3.4.4 to determine if janus kinase 2 (Jak2) is involved signal transduction in L6 skeletal muscle cells. Jak2 has not previously been implicated in insulin signalling, however it is thought to play a significant role in the effect of leptin on mammalian physiology. Intracellular signalling via the long isoform of the leptin receptor is thought to act directly via Jak2 and a Jak2 binding site has recently been mapped on the leptin receptor tail (Banks et al., 1999). Thus the investigations carried out in this chapter determine whether Jak2 plays a role in insulin signalling, and also as a prelude to investigations in chapter four (leptin). Tyrphostin Ag490 is an inhibitor of Jak2, and L6 cell exposure to this compound did not inhibit basal or insulin-stimulated glucose uptake thus indicating that insulin signalling is not mediated via Jak2. However, it was observed that an extremely high concentration of tyrphostin significantly increased basal and insulin-stimulated glucose uptake in L6 cells after prolonged exposure. This effect was repeated in a number of independent experiments and therefore must be acknowledged as a 'real' effect. This has not previously been reported. Tyrphostin AG490 is purportedly a highly specific inhibitor of Jak2 and the observed effect was therefore unexpected. Tyrphostin AG490 is one of over 70 tyrphostins produced specifically as inhibitors of protein kinases. It is unlikely that that there is cross-reactivity between tyrphostins or that the tyrphostin AG490 was contaminated with other products, hence it can be assumed that tyrphostin increased glucose transport by some other mechanism. Intracellular signalling is a complex cascade of kinase and phosphatase reactions with many participating substrates. Thus it is possible that within this cascade system is a mechanism of protein kinase activation that results in downstream substrate inhibition possibly via the action of phosphatases and therefore inhibition of the kinase with tyrphostin results in a net positive effect. This effect highlights the complexity of the signalling pathways involved in this system and although a number of principal components of the insulin signalling pathway have been determined there are many substrates yet to be identified and sequenced.

The kinetics of glucose transport in L6 cells have previously been characterised (Klip et al., 1982) and much is already known about the nature of glucose transporters in L6 cells (Klip et al., 1982; Wilson et al., 1995). In this chapter glucose transporters were investigated using cytochalasin B (section 3.4.5). This study was based on the knowledge that glucose competes with cytochalasin B for reversible binding to the glucose transporter during facilitated diffusion of glucose (3.4.5.1). Basal and insulin-stimulated glucose uptake was inhibited by cytochalasin B in L6 cells, in a concentration dependent manner without affecting cell viability. This is in agreement with similar studies that have utilised [^{3}H]-cytochalasin B to identify glucose binding sites (Klip et al., 1982). However analysis of cytochalasin B inhibition of glucose transport does not distinguish between the GLUT isoforms present on the cell surface. This has previously been completed to determine the ratios of each isoform and the quantities of functional transporters on the cell surface of L6 cells (Wilson et al., 1995; discussed in 3.4.5.1). Thus it has been stated that L6 cells express saturable glucose transport kinetics of similar pharmacology as adult skeletal muscle (Klip et al., 1982).

As discussed above, insulin-stimulated glucose uptake via the regulation of surface GLUTS can either be acute or chronic, and time is a major determinant in mediating the overall metabolic effects. Facilitated transport across the cell membrane by GLUTS is rate limiting and this has previously been determined in L6 cells (Klip et al., 1982). Insulin may regulate the number of GLUTS at the cell surface via a number of different mechanisms, i.e. via GLUT protein synthesis, via GLUT translocation from an intracellular pool to the cell surface and via an increase in the intrinsic activity of GLUTS already present at the cell surface. Cycloheximide was used in this study to determine the effect of insulin on GLUT protein synthesis (section 3.4.6). The inhibitory effect of cycloheximide on basal and insulin-stimulated glucose uptake in L6 cells was found to be significantly dependent upon time. Cycloheximide had no effect on glucose transport after cell exposure for 50mins, however an inhibitory effect was observed after 4h and this was highly significant after 24h. This appears to apply to both basal and insulin-stimulated glucose transport, thus in L6 cells both GLUT1 and GLUT4 appear to be regulated by both protein synthesis and translocation/intrinsic activity (James et al., 1989).

In conclusion, L6 cells appear to retain many of the features of adult human skeletal muscle and as a consequence have been used extensively to explore glucose transport and insulin signalling. Similar cell lines have also been utilised as models of skeletal muscle, such as C_2C_{12} myotubes. These were utilised in a preliminary experiment to compare the effect of insulin on glucose transport with those data obtained in L6 cells (data not shown). Phenotypically these cells are very similar, however preliminary data showed that acute stimulation of glucose uptake by insulin was not preserved in these cells, possibly due to decreased GLUT1 expression or expression of signalling proteins upstream from GLUTS. Thus in subsequent studies, the cultured rat L6 skeletal muscle cell line was the model of choice for glucose uptake analysis. Although an appropriate model for insulin signalling studies, L6 cells are not of human origin. Due to the therapeutic approach of this thesis, the results obtained in the forthcoming chapters are extrapolated to and compared to effects previously observed in a clinical setting and thus it must be noted here that these cells are of rat origin.

Chapter Four:
Leptin

Chapter Four: Leptin

4.0 Introduction

Despite having been identified as a major risk factor for the development of Type 2 diabetes (amongst other co-morbidities such as hypertension, dyslipidaemia, cardiovascular disease, cancer, gall bladder disease) obesity is a disease for which the prevalence continues to increase. Its incidence is growing worldwide and it has become a major health issue in western countries where up to 30% of adults are overweight or obese.

The basic pathophysiological mechanisms of obesity are poorly understood and the mechanisms that link obesity to insulin resistance and islet β-cell failure in Type 2 diabetes remain unclear. Excess storage of body fat occurs when an individual's overall energy intake exceeds energy expenditure. However until recently, the study of energy balance at a detailed molecular level was impractical, as the mechanisms for regulating energy intake and expenditure were not known.

Early parabiosis experiments in which the circulatory systems of two mice were connected to allow the exchange of circulating hormones, suggested that a blood-borne factor acts on the hypothalamus to control body adiposity (Hervey, 1958; Hausberger, 1959; Coleman and Hummel, 1969). Such parabiosis experimentation with *ob/ob, db/db* and wild type mice resulted in the discovery that the circulating factor was present in the normal mice, absent in the *ob/ob* mice and that *db/db* mice were resistant (Coleman, 1978).

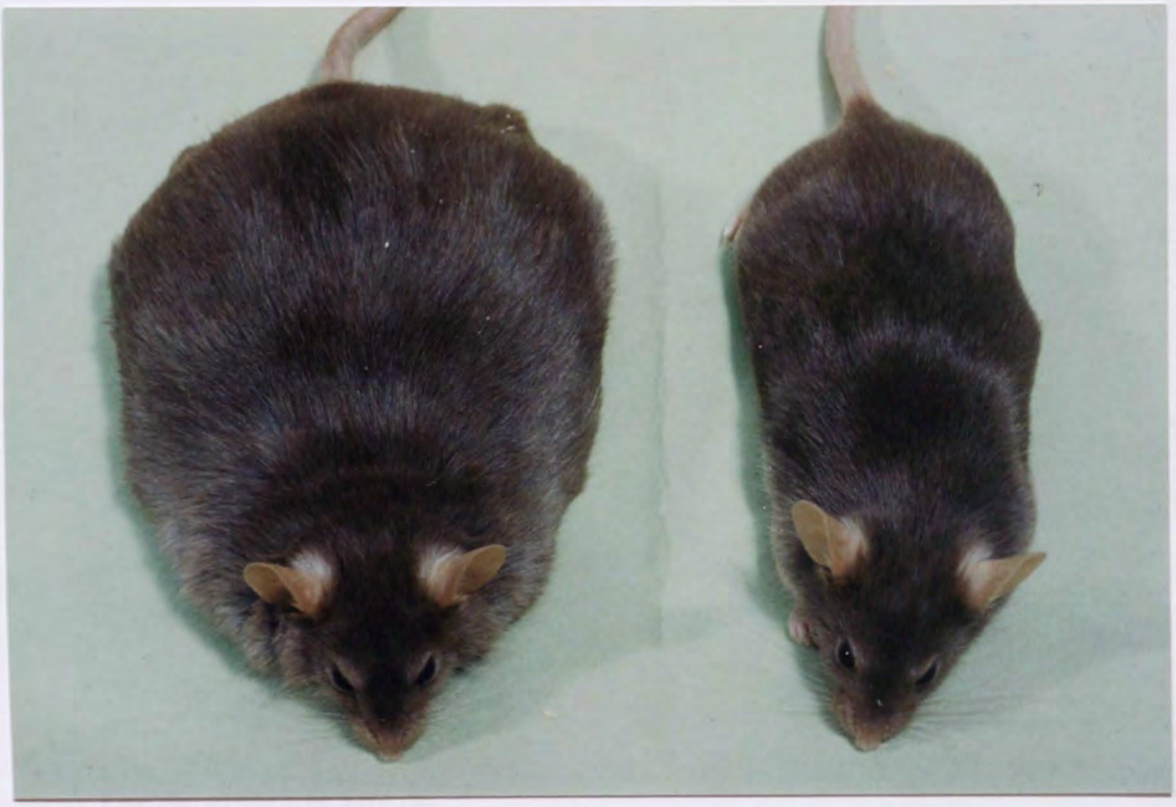
Leptin was identified in 1994 as the product of a gene that is defective in an obese strain of mice, *ob/ob* (Zhang et al., 1994). The *ob/ob* mouse (figure 4.1), which lacks leptin, and the *db/db* mouse and *fa/fa* rat, which are insensitive to leptin are profoundly obese and may additionally develop Type 2 diabetes. Treatment of the *ob/ob* mouse with exogenous leptin, reduces food intake and normalises obesity and metabolism (Bray and Ryan, 1997).

4.0.1 The *Ob* Gene

The *Ob* gene has been demonstrated to consist of 3 exons separated by 2 introns in both rodents and humans (Considine and Caro, 1996). The coding region of the *Ob* gene (501 nucleotides in length) is contained in exons 2 and 3, which are separated by an intron of approximately 2kb. The gene has been localised to chromosome 6 in mice (Zhang et al., 1994), and to chromosome 7q31.3 in humans (Isse et al., 1995). The *Ob* gene promoter region spans a region of approximately 3kb (Gong et al., 1996; He et al., 1995; Miller et al., 1996). SP-1 sites have been found in the promoter region, as well as a glucocorticoid response element and several cAMP response element-binding protein sites (Gong et al., 1996). Only the first 217 base pairs of the promoter are needed for basal adipose tissue expression of the *Ob* gene (Gura, 1997).

The obese phenotype is linked to a mutation in the *Ob* gene that results in expression of a truncated inactive protein. A nonsense mutation in codon 105 has been found in the original congenic C57BL/6J *ob/ob* mouse strain, which was shown to express a 20

Figure 4.1a Photograph of a male, genetically obese, *ob/ob* mouse and (for comparison) a lean, male, (+/+) mouse, both from the Aston Colony. Both mice were approx. 11 weeks of age when this photograph was taken. The *ob/ob* mouse weighed 85g and the lean mouse 29g.



fold increase in abnormal *Ob* mRNA. This mutation occurs in the Aston strain of *ob/ob* mice (Fraser et al., 1998). A second mutation has been found in the co-isogenic SM/Ckc-$+^{\mathrm{Dac}}$*ob*^{2j}/*ob*^{2j} mouse, in the promoter region of the *Ob* gene, which prevents the synthesis of *Ob* mRNA (Caro et al., 1996).

The nucleotide sequence of human *Ob* cDNA coding region has been found to be 83% identical to the mouse *Ob* cDNA coding region (Masuzaki et al., 1995). Identification of adipose tissue as the site of leptin secretion was identified (Zhang et al., 1994), and northern blot analysis using the cloned human *Ob* cDNA fragment as a probe identified a single mRNA species 4.5kb in size. This was found abundantly in the adipose tissues obtained from the subcutaneous, omental, retroperitoneal, perilymphatic and mesenteric fat pads. However, no significant amount of *Ob* mRNA was present in the brain, heart, lung, liver, stomach, pancreas, spleen, small intestine, kidney, prostate, testis, colon or skeletal muscle (Masazuki et al., 1995). It was also found that the *Ob* mRNA in the adipose tissue varied from region to region even in the same individual.

4.0.2 The Structure of Leptin

The mouse *Ob* gene encodes a 4.5 kb adipose tissue mRNA with a highly conserved 167 amino acid open reading frame and a 21 amino acid secretory signal sequence. The predicted amino acid sequence is 84% identical to that in humans and mice and 83% to that in rats, and has features of a secreted protein, (Masuzaki et al., 1995). Mouse and rat leptin exhibit 96% amino acid homology with each other (Ogawa et al., 1995).

Human leptin is a 16kDa, 146 amino acid residue non-glycosylated polypeptide (Cohen et al., 1996). It contains no consensus sites for N-linked glycosylation, but does contain two cysteines in the carboxyterminal region, both of which are believed to participate in an intramolecular disulphide linkage. Recent studies have shown this protein to be a member of the helical cytokine family (Bryant et al., 1995), however the exact conformational structure of leptin has been difficult to determine because it is known to aggregate and not crystallise, hence this eliminates the use of X-ray crystallography. A mutant form of leptin has been shown to crystallise, known as leptin E100, and was shown to consist of 4 antiparallel helices, each about 5-6 turns long, with the characteristic up-up-down-down arrangement of cytokines which forms two layer packing of the helices (Zhang et al., 1997).

4.0.3 The Leptin Receptor

In late 1995, mouse leptin receptor (Ob-R) cDNA was cloned by screening a cDNA expression library, prepared from mouse choroid plexus (Tartaglia et al., 1995).

The predicted extracellular domains of Ob-R was large (816 amino acids) while the predicted intracellular domain was fairly short (34 amino acids), suggesting that this protein might not have signal transducing capability. However, further screening and analysis of cDNA libraries using the original Ob-R cDNA sequence as a guide, revealed that there are multiple forms of Ob-R in both mice and humans, including a long isoform with an intracellular domain of about 303 amino acids (Tartaglia et al., 1995; Chen et al., 1996). The intracellular domain of the long receptor isoform contained sequence motifs suggestive of intracellular signal–transducing capabilities. The long receptor has also been found to be most abundantly expressed in the hypothalamus, in keeping with the notion that this part of the brain represents a major site of leptin action (Mercer et al., 1996). The *db/db* mouse possesses a mutation that prevents the expression of the long receptor form (Chen et al., 1996). Thus, the long form of the receptor (Ob-Rb) appears to be essential for leptin signalling.

The structure of the long receptor isoform appears to be a single membrane spanning receptor and includes a large extracellular domain, a short hydrophobic transmembrane domain (Lee et al., 1996) and a short intracellular domain of 34 amino acids. This long receptor isoform has also been shown to closely resemble the gp130, the common signal transducing subunit of a group of cytokine receptors, which includes interleukin-6 (Il-6), the leukaemia inhibitor factor and the ciliary neurotrophic factor (Tartaglia et al., 1995).

Ob-Rb is also expressed in some tissues outside of the hypothalamus, such as pancreatic β-cells, adrenal cortex, gonads, haempoetic cells and adipose tissue (Fei et al., 1997; Kulkarni et al., 1997; Gainsford et al., 1996; Bornstein et al., 1997; Ghilardi et al., 1996; Liu et al., 1997). The direct peripheral effects of leptin on these tissues remain controversial, although it is now well established that signals mediated by Ob-Rb in the hypothalamus are important regulators of energy homeostasis. The effects of leptin action via Ob-Rb include the regulation of appetite and metabolic rate, immune function and also endocrine function. These include growth, the hypothalamic-pituitary-adrenal (HPA) axis, thyroid function and reproduction. (Campfield et al., 1995; Friedman et al., 1998; Elmquist et al., 1998; Muzzin et al., 1996; Chehab et al., 1997; Ahima et al., 1996; Lord et al., 1998).

It is now known that mice and rats can possess up to five variants of the leptin receptor, generated through alternative splicing of a common mRNA precurser (Takaya et al., 1996; Lee et al., 1996). The extracellular domains of the short and long isoforms are identical throughout their length, it is the intracellular domains which possess differing length and sequence composition (Chen et al., 1996; Lee et al., 1996).

The short leptin receptor isoforms were originally thought to lack the signalling capabilities of the long form, the shortest of which lacks a hydrophobic transmembrane domain and probably represents a soluble form of the receptor, possibly a transport protein (Lee et al., 1996). Expression of these receptor isoforms is abundant in the choroid plexus, where they may mediate uptake of leptin across the blood-brain or blood-cerebrospinal fluid barrier (Tartaglia, 1997) although recently short receptor isoforms have been identified in peripheral tissues (Fruhbeck et al., 1999).

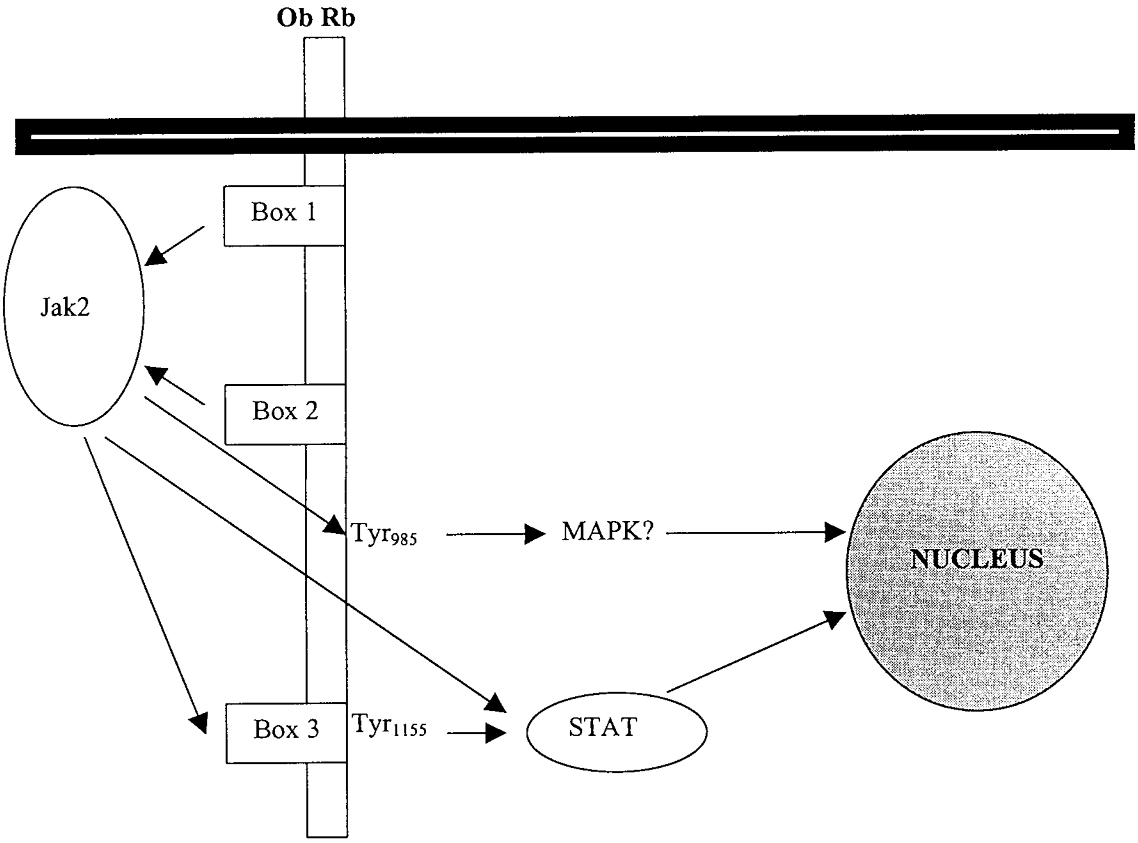
4.0.4 Leptin Intracellular Signalling

The homology of Ob-R to class 1 cytokine receptors indicates the possible mechanism of leptin intracellular signalling. Baumann et al., (1996) addressed whether cloned Ob-R isoforms are signalling receptor subunits, by introducing Ob-R into established tissue culture cell lines and compared the cell response to leptin treatment with that mediated by Il-6 type cytokine receptors. This provided evidence that the Ob-R long form (Ob-Rb) is a signal-transducing subunit and shares functional specificity with IL-6-type cytokine receptors.

The leptin receptor is now considered to be a member of the Il-6 family of class I cytokine receptors (Tartaglia et al., 1995; Tartaglia 1997). The cytoplasmic domains of these family members are quite variable but they are linked by the presence of three conserved Box motifs, required for signalling capabilities (Taga and Kishimoto, 1997). The intracellular domains of all leptin receptor isoforms contain Box 1 but only the long isoform (Ob-Rb) contains Box 2 (Tartaglia, 1995).

Class 1 cytokine receptors are known to act through Jak and STAT proteins (Heldin 1995; Kishimoto et al., 1994). These receptors lack enzymatic motifs in their cytoplasmic domains and instead associate with members of the janus kinase (Jak) family (Ihle 1995), a class of cytoplasmic tyrosine kinases. Ligand binding to the receptor results in a conformational change in the receptor/Jak kinase complex, stimulating Jak kinase autophosphorylation and activation. To date there are four known members of the Jak kinase family, Jak1, 2 and 3 and Tyk 2 (Ihle 1995). The leptin receptor, as with many members of the IL-6 receptor family preferentially associates with Jak2 which is required for cytokine and leptin function (Ihle 1995). Box 1 and Box 2 motifs mediate association with Jak kinases (Taga and Kishimoto, 1997). Hence both the long form and some short forms of the leptin receptor can activate Jak2 when artificially overexpressed with Jak2 in cultured cells. However, the efficiency of Jak2 binding by the long form of the receptor is much greater than for the short forms of the receptor (Ghilardi and Skoda, 1997; Bjorbaek et al., 1997). Indeed, when Ob-Rb is stimulated with leptin it not only activates the autophosphorylation of the associated Jak2 kinase but also the tyrosine phosphorylation of Tyr_{1155} of Box 3 and Tyr_{985} of the intracellular tail of the Ob-Rb

Figure 4.1b Summary of signalling by the leptin receptor. Leptin binding to the long form of the receptor (Ob-Rb) activates the associated Jak2 kinase. This in turn phosphorylates Tyr_{985} and Tyr_{1155} of the receptor. Tyr_{1155} binds and activates STAT 3resulting in the transcription of STAT3 controlled genes. Tyr_{985} may control transcription via MAPK.



(Ghilardi and Skoda, 1997). These tyrosine residues are present only on the long form of the receptor (Tartaglia 1997; Tartaglia et al., 1995), see figure 4.1b.

The phosphorylated receptor intracellular domain then provides a binding site for STAT proteins, a class of cytoplasmic transcription factors known as signal transducers and activators of transcription (STAT). Ob-Rb has been demonstrated to activate STAT proteins in response to ligand binding. The STAT family comprises of 6 known members to date, (Schindler et al., 1992; Fu et al., 1992; Zhong et al., 1994; Yamamoto et al., 1994; Wakao et al., 1994; Hou et al., 1994), of which three have been shown to be activated by Ob-R, STAT3, STAT5, and STAT6 (Ghilardi et al., 1996).

Tyrosine phosphorylation of the Box 3 motif in Il-6 receptor family members has been shown to recruit STAT3 (Heim et al., 1995; Haan et al., 1999). Similarly, in the hypothalamus, Ob-Rb has been shown to activate STAT3 via the Box 3 motif (Vaisse et al., 1996). In addition, it has been demonstrated that the short form of Ob-R is unable to activate STAT proteins due to the absence of a Box 3 motif and therefore reduced expression of the long form is sufficient to cause the *db/db* phenotype (Ghilardi et al., 1996).

It is known that the phosphorylation of STAT proteins induces dimerisation and translocation into the nucleus and stimulates gene transcription (Darnell et al., 1994), however the mechanism of leptin signalling downstream of STAT protein is not established.

Some class 1 cytokine receptors are able to stimulate signal transduction pathways that are distinct from the Jak/STAT pathway, such as the mitogen-activated protein, c-fos and PI 3-kinase pathways. Ob-Rb has been shown to activate MAP kinase in cultured cells (Bjorbaek et al., 1997). Also, an increase in c-fos expression by leptin stimulation has been observed in Ob-Rb-expressing hypothalamic nuclei (Elmquist et al., 1997; Elmquist, et al., 1998; Woods and Stock, 1996). It is possible that Ob-R is involved in the coordination of energy homeostasis via these pathways also.

Signalling via the short forms of the leptin receptor is controversial. As described previously, the long form of the receptor is presumed to mediate most or all of leptin's signalling, however the short forms of the receptor are widely expressed in many tissues.

4.0.5 The Function of Leptin

Leptin is produced exclusively by adipose tissue where it is secreted to circulate in the blood. The notion that adipose tissue produces a hormone that regulates body size was first introduced by Kennedy in 1953. The action of leptin in the control of energy balance is now thought to be two-fold. A greatly reduced concentration of leptin during starvation facilitates the mobilisation of alternate energy stores. However, adequate leptin levels activate central satiety signal transduction that results in decreased food intake and increase metabolic rate (Flier, 1997).

The physiological role of leptin is gradually being determined. In 1995, it was discovered that administration of leptin to *ob/ob* mice, results in a sharp decrease in weight, mediated through reduced food intake, increased energy expenditure and increased thermogenesis (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). Intracerebroventricular (icv) administration of leptin results in a more potent response suggesting that the central nervous system is a major site of leptin action (Schwartz et al., 1996). Indeed it is now established that leptin stimulates the production of anorectic neuropeptides in the hypothalamus and decreases levels of orexigenic neuropeptides (Sahu, 1998; Schwartz et al., 1996; Wilson et al., 1999).

Ob-Rb is highly expressed in the arcuate and VMH nuclei of the dorsomedial hypothalamus. The investigation of hypothalamic lesions has determined the existence of dorsomedial satiety centres, which include the arcuate and VMH nuclei (Elmquist et al., 1998; Schwartz et al., 1996).

In the arcuate nucleus there are neurons which express Ob-Rb that also express NPY (neuropeptide Y) and AgRP (agouti-related protein) which are orexigenic peptides (Stephens et al., 1995; Baskin et al., 1999; Spanswick et al., 1997). There are also neurons which express Ob-Rb, POMC (proopiomelanocortin) and CART (cocaine and amphetamine-regulated transcript) neuropeptides, that are anorectic. (Kristensen et al., 1998; Elias et al., 1998). Thus it is thought that leptin may act via Ob-Rb to inhibit NPY/AgRP and to activate POMC/CART (Baskin et al., 1999; Spanswick et al., 1997; Elmquist et al., 1998).

Leptin has been shown to decrease the release of the orexigenic neuropeptide Y (NPY) *in vitro* and *in vivo* (Stephens et al., 1995; Wang et al., 1997). Administration of NPY to rodents results in inhibition of the sympathetic nervous system, decreasing energy expenditure and improving storage of fat (Schwartz et.al. 1997). Hence, regulation of NPY expression may be involved in mediating the effects of leptin in the CNS.

Recently, leptin has also been implicated as an agent which can directly influence peripheral tissues. However there are many discrepancies in the literature over this issue.

In 1997, Berti et al., reported one of the earliest findings of the effects of leptin on cultured myotubes. They reported that preincubation of cultured C_2C_{12} myotubes with leptin (1-500ng/ml) did not significantly affect insulin-stimulated glucose transport and glycogen synthesis. However, it was observed that leptin by itself, (1ng/ml) was able to mimic approximately 80-90% of the insulin effect on glucose transport and glycogen synthesis. This effect was inhibited by wortmannin (an inhibitor of phosphatidylinositol 3-kinase), but the S6-kinase inhibitor rapamycin had no effect on the action of leptin. Therefore it was established that leptin directly increases basal glucose uptake via the insulin signalling pathway of IRS-1 and PI 3-kinase. The stimulatory effect of leptin on basal glucose uptake has since been reported to be mediated via janus kinase-2 (Jak2) and insulin receptor substrate-2 (IRS-2) dependent pathways (Kellerer et al., 1997).

In contrast, it has also been reported that leptin does not have a direct effect on glucose transport in either skeletal muscle or adipocytes. Zierath et al., (1998), reported that exposure of epitrochlearis muscles or isolated adipocytes from male SD rats to leptin (300ng/ml for 2 h) did not alter the basal, submaximal or maximal response of glucose transport to insulin in skeletal muscle or adipocytes.

In addition, the effects of leptin on rat soleus strips were investigated by Furnsinn et al., (1998), and it was demonstrated that leptin had no effect on the rate of glucose uptake or glucose incorporation into glycogen. Also, leptin did not affect rates of aerobic and anaerobic glycolysis as well as muscle glycogen content.

Leptin has also been shown to have a direct effect on metabolic actions of insulin in isolated rat adipocytes. Muller et al., (1997) have demonstrated that leptin impairs several metabolic actions of insulin, i.e. stimulation of glucose transport, glycogen synthase, lipogenesis, inhibition of isoproterenol-induced lipolysis, and protein kinase A activation and stimulation of protein synthesis.

These effects have been studied in isolated soleus muscles of obese *ob/ob* mice. It has been demonstrated that leptin causes a significant and dose-dependent inhibition of basal and insulin-stimulated glycogen synthesis in isolated soleus muscles of these mice. It was also found that there was no effect in isolated soleus muscles from diabetic *db/db* mice (Liu et al., 1997).

Furthermore, leptin modulates insulin secretion, as well as insulin-regulated responses. It has been clearly demonstrated that leptin decreases insulin secretion (Kulkarni et al., 1997). This, coupled with the evidence that insulin stimulates leptin secretion, suggests the existence of a negative feedback loop between leptin and insulin (Tritos and Mantzoros, 1997; Cusin et al., 1995). The effects of leptin in insulin secretion have been investigated in isolated rat and human islets and in cultured insulinoma cells. Leptin was found inhibit insulin secretion in all cells

(Kulkarni, et al., 1997). Furthermore, inhibition of insulin secretion has also been demonstrated in a perfused pancreas preparation from *ob/ob* mice (Emilsson et al., 1997).

4.0.6 Human Obesity

Leptin is known to act via the hypothalamus to mediate behaviour, including decreased voluntary feeding and metabolic efficiency by increasing thermogenesis. Obese individuals have higher circulating leptin levels than lean individuals, hence it has been found that leptin correlates positively with body adiposity (Matson et al., 1996). However, leptin deficiency does not mediate obesity in most humans or most animal models (Leibel et al., 1997; Friedman et al., 1998), indeed obesity caused by leptin deficiency is thought to be extremely rare in humans (Montague et al., 1997). Most obese humans are instead resistant to the effects of leptin (Ahima et al., 1996) although the mechanism for leptin resistance in obesity in unclear. Mutations of the leptin receptor gene have been investigated in obese human populations, and it is thought that most leptin resistance is not mediated by mutations in the leptin receptor gene (Leibel et al., 1997; Elmquist et al., 1998; Friedman and Halaas, 1998; Carlsson et al., 1997). Further research will determine the role of leptin in signalling mechanisms, and will consequently provide an important insight into the functional significance of leptin itself (Matson et al., 1996). Presently, clinical trials with leptin as a potential anti-obesity agent are underway (Bray and Ryan, 1997).

Part One: Effect of Leptin on Mammalian Physiology *In Vivo* and Glucose Transport by Soleus Muscles

4.1.1 Leptin Viability Test

4.1.1.1 Introduction

There have been many discrepancies in the literature pertaining to a direct action of leptin in different model systems. Indeed, it has since been established that some early batches of commercially available leptin were inactive due to production problems. Biological activity cannot be presumed, even if the amino acid sequence is correct, as the molecule can become inactive due to conformational abnormalities. Thus the biological activity of the leptin needs to be determined prior to experimentation. To ensure that the leptin used in these studies was viable, it was tested *in vivo* to determine the effects on food intake in *ob/ob* mice.

4.1.1.2 Experimental Design

12 male and female *ob/ob* mice (mean weight 100g) were caged individually for the duration of the experiment. Food (ordinary chow, see section 2.3.1) was removed 18h before the start of the experiment, drinking water was freely available. Leptin (a kind gift from Amgen, Thousand Oaks, CA, USA), (10µg/kg) was administered intraperitoneally (section 2.3.4) to 6 of the mice, the remaining mice received placebo (vehicle-only, PBS) and preweighed food was added to the cages. Food remaining in the cages was weighed every 30min for 2.5h. Data were analysed by Student's unpaired 't' test (2.6.1).

4.1.1.3 Results

Leptin rapidly decreased food intake in *ob/ob* mice (figure 4.2). Within 30min of leptin administration food intake was reduced from 0.9±0.1g to 0.4±0.2g i.e. intake was significantly reduced by approximately half ($p<0.05$ versus vehicle-only control animal food intake). 2.5h after leptin administration, leptin treated mice had consumed 1±0.3g food and control mice 2.2±0.2g ($p<0.02$). The effects of leptin in reducing food intake in *ob/ob* mice were rapid and significant.

This experiment indicated that the leptin was capable of acutely suppressing food intake in *ob/ob* mice, confirming biological potency. Thus, from the results of this preliminary investigation, it was concluded that the batch of murine recombinant leptin could be utilised in the following experiments.

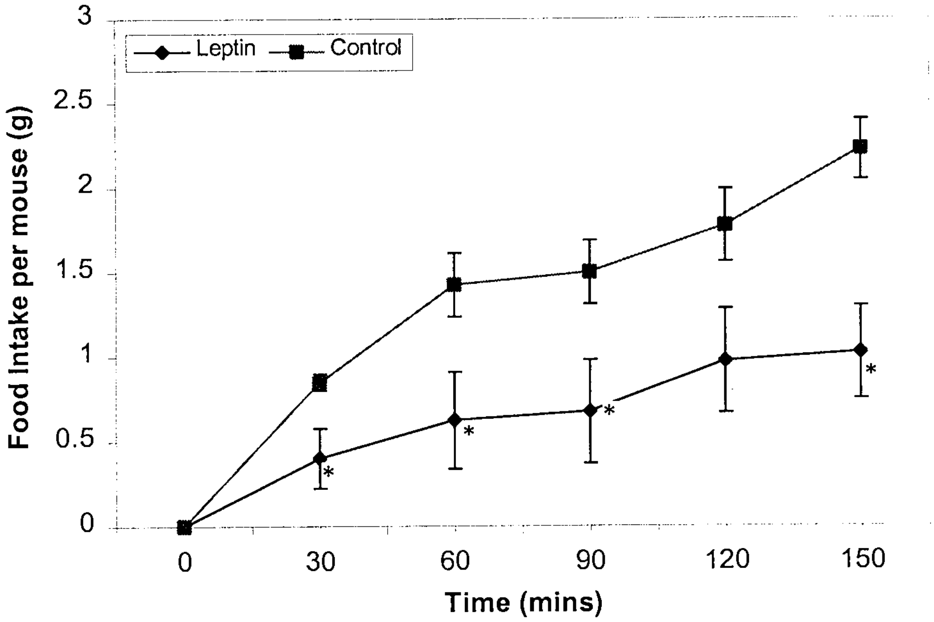
4.1.2 Chronic *In Vivo* and Acute *In Vitro* Effects of Leptin on Body Weight, Food Intake and Glucose Uptake by Soleus Muscles of *ob/ob* and Lean Mice

4.1.2.1 Introduction

In *ob/ob* mice, which do not produce functional leptin (Zhang et al., 1994), administration of leptin reverses the hyperphagia, adiposity, hyperglycaemia and hyperinsulinaemia (Pelleymounter et al., 1995; Halaas et al., 1995; Campfield et al., 1995).

These effects cannot be explained fully by the centrally mediated effect on food intake, since changes in glucose homeostasis occur more rapidly and with lower doses of leptin than required to reduce food intake (Pelleymounter et al., 1995). Also, leptin

Figure 4.2 Confirmation of biological activity of recombinant murine leptin. 12 male and female *ob/ob* mice were fasted for 18h. 6 mice received 10mg/kg leptin ip, and 6 mice received vehicle only. Mice were caged individually for the duration of the experiment and food intake was monitored half-hourly. Leptin significantly decreased food intake and this effect was observed within 30mins of administration. Data are expressed as mean food intake (g) ± SEM, n=6. *p<0.05 versus control mice.



administration will slightly reduce food intake and body weight in lean mice without a significant change in serum glucose and insulin concentrations (Pelleymounter et al., 1995). Although leptin is produced by adipocytes, the fact that excess fat correlates with high concentrations of leptin in serum and the well established linkage between obesity and insulin resistance (Felber and Golay 1995), led to this investigation of the possibility that leptin may modulate insulin-regulated responses.

Skeletal muscle is the major site of insulin-stimulated glucose disposal (DeFronzo, 1988; Baron et al., 1988), and both the short and long isoforms of the leptin receptor are expressed in this tissue (Ghilardi et al., 1996; Tartaglia, 1997; Liu et al., 1997). However, *in vitro* studies with skeletal muscles isolated from normal rodents found that leptin (1-100nmol/l) does not exert an acute or direct effect on basal or insulin-stimulated glucose uptake or glycogen synthesis (Furnsinn et al., 1998; Muoio et al., 1997; Liu et al., 1997; Zierath et al., 1998). Nevertheless, leptin (1-500ng/mg, approx. 0.06-30nmol/l) rapidly but transiently increased basal glucose uptake in C_2C_{12} muscle cells (Berti et al., 1997; Kellerer et al., 1997).

The effect of leptin on glucose metabolism by skeletal muscle in insulin resistant and diabetic states is not established. Initial *in vitro* studies in muscle of *ob/ob* mice have found no effect on glucose transport but decreased glycogen synthesis (Zierath, 1998; Liu et al., 1997).

Rodent soleus muscle has many features that make it particularly suitable for *in vitro* experimentation. It is a cylindrical muscle, terminating at each end in a well-defined tendon (appendix IV and 2.3.7). By severing these tendons the muscle can be removed from the animal without cutting its fibres and avoiding trauma, thus providing an intact preparation (reviewed by Chaudry and Gould, 1969). This study was undertaken to investigate the chronic effects of leptin on glucose uptake into soleus muscle of *ob/ob* mice.

4.1.2.2 Experimental Design

The chronic *in vivo* effects of leptin (10μg/mouse twice daily, ip) were determined after 2 and 7 days of treatment in *ob/ob* mice:

4.1.2.2a Chronic Effect of Leptin on Body Weight, Food Intake and Glucose Uptake by Soleus Muscles of *ob/ob* Mice: 7 day study

24 male and female *ob/ob* mice (mean weight 100g) were grouped in cages of between 2-6 mice per cage. Mice were divided into three groups of 8; the first group received leptin (10μg/mouse twice daily, ip, section 2.3.4) for the duration of the experiment, receiving a total of 14 injections. Group 2 mice received placebo only, and group 3 mice were pair fed to the leptin treated mice. Water was available *ad libitum* and the leptin treated and control animals had free access to food. Food intake and body weight were monitored daily throughout the study.

At the end of the 7 day period of leptin administration, mice were killed by cervical dislocation. Intact soleus muscles were dissected (section 2.3.7 and appendix IV) and glucose uptake was determined in the presence and absence of added insulin as described in section 2.4.2. Blood was collected as described in 2.3.2 and analysed for plasma insulin concentration (2.4.3) and glucose concentration (2.4.4).

Data are expressed as mean±SEM, expressed in g (body weight or food intake) or dpm/mg (glucose uptake). Body weight and food intake data were analysed by Student's unpaired 't' test (2.6.1). Glucose uptake data was analysed by Student's paired 't' test. Plasma insulin concentrations are expressed pmol/l (mean±SEM) and glucose concentrations as mmol/l (mean±SEM). Significance was determined by ANOVA followed by Tukey post-hoc analysis (2.6.3 and 2.6.4).

4.1.2.2b Chronic Effect of Leptin on Body Weight, Food Intake and Glucose Uptake by Soleus Muscles of *ob/ob* Mice: 48h study

35 male and female *ob/ob* mice (mean weight 100g) were grouped as described above. Leptin (10μg/mouse) was given intraperitoneally to the test group, twice daily for 2 days. As described previously (above 4.1.2.2a) the control group received placebo only, and the pair fed group received food as consumed by the leptin treated group. Food was freely available to both the leptin treated and control groups of mice, and water was available for all groups of animals. Food intake and body weight were monitored daily for the duration of the experiment.

After the 2 day period of investigation, mice were killed by cervical dislocation. Intact soleus muscles were dissected and glucose uptake in the presence and absence of added insulin determined, as described in 2.4.2. Data are expressed as mean±SEM, and analysed using Student's paired 't' test.

4.1.2.2c Acute *In Vitro* Effect of Leptin on Glucose Uptake by Soleus Muscles of *ob/ob* and Lean (+/+) Mice

The acute effect of leptin (10^{-9}M) on basal and insulin-stimulated (10^{-6}M) 2-deoxyglucose uptake was determined using isolated soleus muscles of non-fasted lean and *ob/ob* mice. Male and female 16 week old, lean (n=18) and *ob/ob* (n=18) mice were killed by cervical dislocation and the soleus muscles dissected (as described previously, 2.3.7 and appendix IV). Leptin was added to the muscle after dissection and was incubated immediately for 30mins at 30°C (2.4.2) for glucose uptake determination. Blood samples were taken for the measurement of plasma glucose and insulin concentrations (2.3.2, blood sampling, 2.4.3, insulin radioimmunoassay, 2.4.4, plasma glucose assay). Data (mean±SEM) were analysed by Student's paired 't' test (2.6.1).

4.1.2.3 Results

4.1.2.3a Chronic Effect of Leptin on Body Weight, Food Intake and Glucose Uptake by Soleus Muscles of *ob/ob* Mice: 7 Day Study

Leptin administration (10µg/mouse, twice daily, ip) to obese mice for 7 days reduced food intake by 62% ($p<0.01$, figure 4.4) and body weight by 18% ($p<0.01$, figure 4.3) compared with placebo control obese mice. Pair-fed mice showed a 9% ($p<0.05$) reduction in body weight which was significantly ($p<0.05$) less than with leptin (figure 4.3 and 4.4). Leptin reduced plasma insulin concentrations to within the normal range, whereas pair-feeding produced a smaller reduction in the hyperinsulinaemia (table 4a). The hyperglycaemia was similarly reduced by leptin and pair-feeding (table 4b). Basal uptake of glucose by soleus muscles was not

Figure 4.3 Chronic effect of leptin (10µg/mouse) on body weight of *ob/ob* mice. Mice received either leptin, vehicle-only (control group) or were pair fed to the leptin treated group for 7 days. Body weight was monitored on a daily basis. A lean group of animals were monitored for comparative purposes. Leptin significantly reduced body weight by 18% ($p<0.01$) compared with control mice. Pair-fed mice showed a 9% ($p<0.05$) reduction in body weight which was significantly ($p<0.05$) less than the weight reduction observed in leptin treated mice. The body weight of control and lean mice remained constant throughout the test period. Data are expressed as mean body weight (g) ± SEM, n=8. *$p<0.05$ versus control, †$p<0.05$ versus pair-feeding.

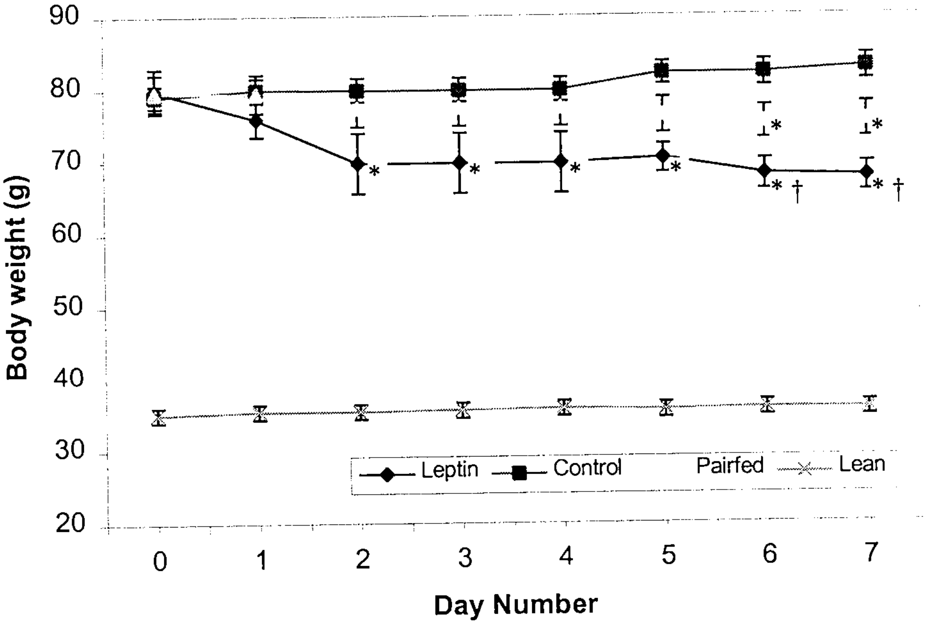


Figure 4.4 Chronic effect of leptin (10μg/mouse) on food intake of *ob/ob* mice. Mice received either leptin, vehicle-only (control group) or were pair fed to the leptin treated group for 7 days. Food intake was monitored on a daily basis. A lean group of animals was monitored for comparative purposes. Leptin administration for 7 days reduced food intake in *ob/ob* mice by 62% comparable to levels of food intake observed in lean (+/+) mice. Leptin significantly ($p<0.01$) decreased food intake within 1 day of administration and levels of food intake were significantly ($p<0.01$) decreased on each day throughout the duration of the experiment compared to control mice. Data are expressed as mean food intake (g) ± SEM, n=8.

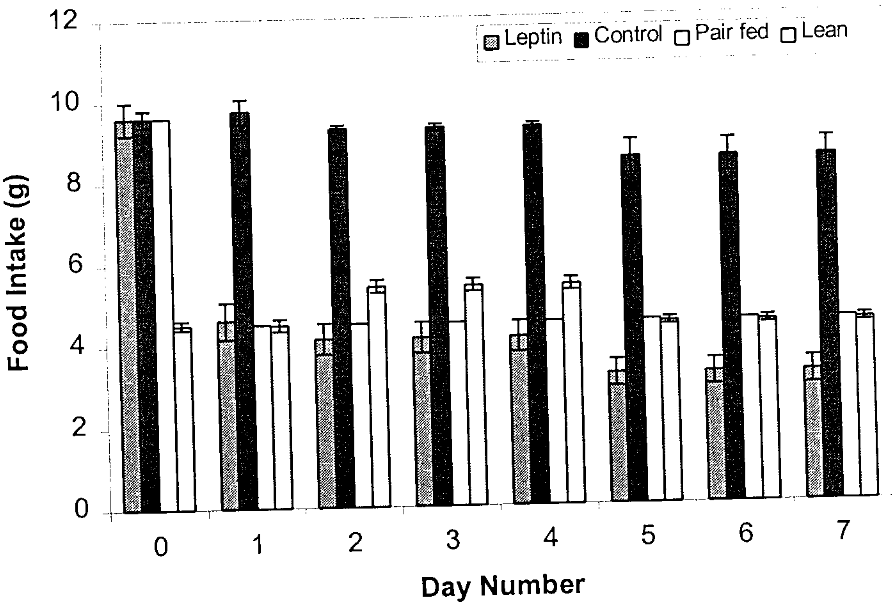


Figure 4.5 Chronic effect of leptin (10μg/mouse) on 2DG uptake in isolated soleus muscles of *ob/ob* mice. Mice received either leptin, vehicle-only (control group) or were pair fed to the leptin treated group for 7 days. A lean group of animals were included for comparative purposes. After the 7 day treatment period, soleus muscles were dissected, and 2DG uptake measured in the presence and absence of added insulin (10^{-6}M). Basal uptake of 2DG was not significantly altered after leptin treatment or pair feeding. Leptin restored an increase in 2DG uptake in response to insulin by 18% and pair feeding increased insulin-stimulated glucose uptake by 28%. Insulin increased 2DG uptake in soleus muscles of lean mice by 41%. Data are expressed as dpm/mg soleus muscle, values are mean±SEM, n=8. *p<0.05 versus basal glucose uptake.

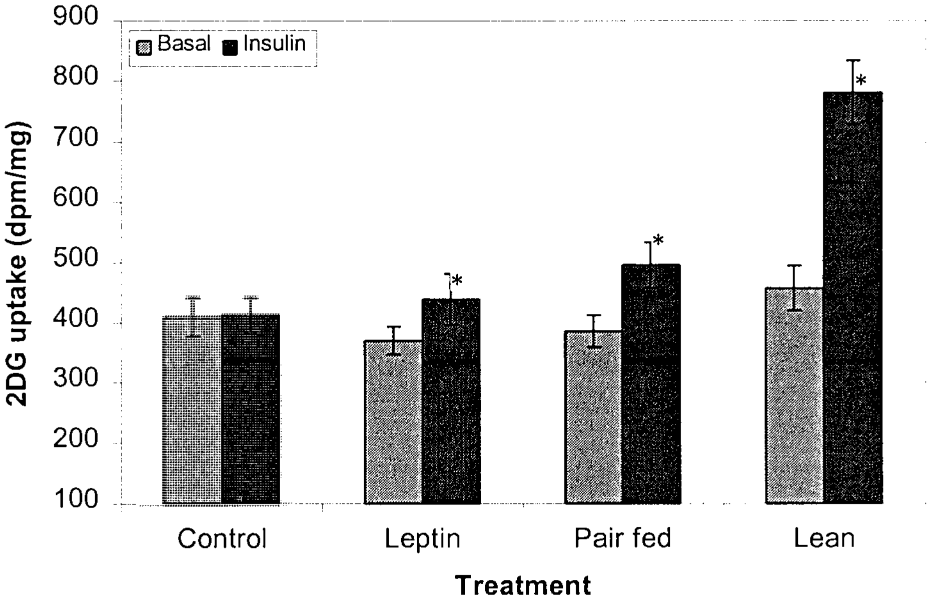


Table 4a Chronic effect of leptin (10µg/mouse) on plasma insulin concentration of *ob/ob* mice. Mice received either leptin, vehicle-only (control group) or were pair fed to the leptin treated group for 7 days. The plasma insulin concentration of a group of lean mice was measured for comparative purposes. Leptin reduced plasma insulin concentrations to within the normal range whereas pair feeding produced a smaller reduction in hyperinsulinaemia. Data are mean±SEM; n is indicated per group.

	Plasma Insulin (pmol/l)			
	Control (n=6)	**Leptin** (n=8)	**Pair fed** (n=8)	**Lean** (n=7)
Mean	5631	364	2721	19
SEM	1237	147	752	2
TUKEY v Control		0.0001	0.023	0.0001
TUKEY v Leptin	0.0001		0.046	ns
TUKEY v Lean	0.0001	ns	0.029	

Table 4b Chronic effect of leptin (10µg/mouse) on plasma glucose concentration of *ob/ob* mice. Mice received either leptin, vehicle-only (control group) or were pair fed to the leptin treated group for 7 days. The plasma glucose concentration of a group of lean mice was measured for comparative purposes. Both leptin and pair-feeding reduced plasma glucose concentrations to near-normal levels. Data are mean±SEM; n is indicated per group.

	Plasma Glucose (mmol/l)			
	Control (n=5)	**Leptin** (n=7)	**Pair fed** (n=6)	**Lean** (n=5)
Mean	18.1	9.1	10.8	3.156
SEM	1.7	1.7	2	0.5
TUKEY v Control		0.005	0.031	0.0001
TUKEY v Leptin	0.005		ns	0.09
TUKEY v Lean	0.0001	0.09	0.025	

significantly altered after 7 days of leptin treatment or pair-feeding (figure 4.5). However leptin and pair-feeding restored an increase in glucose uptake in response to 10^{-6}M insulin (by 18% and 28% respectively, $p<0.05$). Soleus muscles isolated from lean mice responded to insulin with a 41% increase in glucose uptake.

4.1.2.3b Chronic Effect of Leptin on Body Weight, Food Intake and Glucose Uptake by Soleus Muscles of *ob/ob* Mice: 48h Study

Leptin administration (10µg/mouse, twice daily, ip) to obese mice for 2 days reduced food intake more than half that of placebo control obese mice (4.1±0.4 versus 9.0±0.2g/mouse/day; $p<0.001$). This was accompanied by a small reduction in body weight (2g loss in weight versus a small increase in body weight of control mice, table 4d). The group of obese mice that were pair-fed to the leptin-treated mice also demonstrated a 2g loss in body weight.

Basal and insulin-stimulated glucose uptake by soleus muscles of the control, leptin-treated and pair-fed *ob/ob* mice were not significantly different after 2 days of treatment (figure 4.6). For comparison, insulin induced a 41% increase in glucose transport of soleus muscles of lean non-diabetic mice.

4.1.2.3c Acute *In Vitro* Effect of Leptin on Glucose Uptake by Soleus Muscles of *ob/ob* and lean (+/+) Mice

The obese (*ob/ob*) mice were characteristically heavier than the lean (+/+) mice (78.7 ± 1.7g versus 33.9 ± 1.2g, n=18, $p<0.001$), with higher plasma concentrations of glucose (12.5 ± 0.5 versus 10.2 ± 0.5mmol/l; $p<0.01$) and insulin (33.3 ± 7.2 versus 2.7 ± 0.5ng/ml; $p<0.001$). Soleus muscles of the *ob/ob* mice were smaller than lean

Figure 4.6 Effect of leptin (10μg/mouse) treatment on 2DG uptake in isolated soleus muscles of *ob/ob* mice. Mice received either leptin (bi-daily), vehicle-only (control group) or were pair fed to the leptin treated group for 2 days. A lean group of animals were included for comparative purposes. After the 48h treatment period, soleus muscles were dissected, and 2DG uptake measured in the presence and absence of added insulin (10^{-6}M). Leptin treatment or pair feeding for 48h did not significantly alter basal and insulin-stimulated 2DG uptake into soleus muscles of *ob/ob* mice. Insulin increased 2DG uptake into soleus muscles of lean mice by 41%. Data are expressed in dpm/mg soleus muscle, values are mean±SEM, n=8. *p<0.05 versus basal glucose uptake.

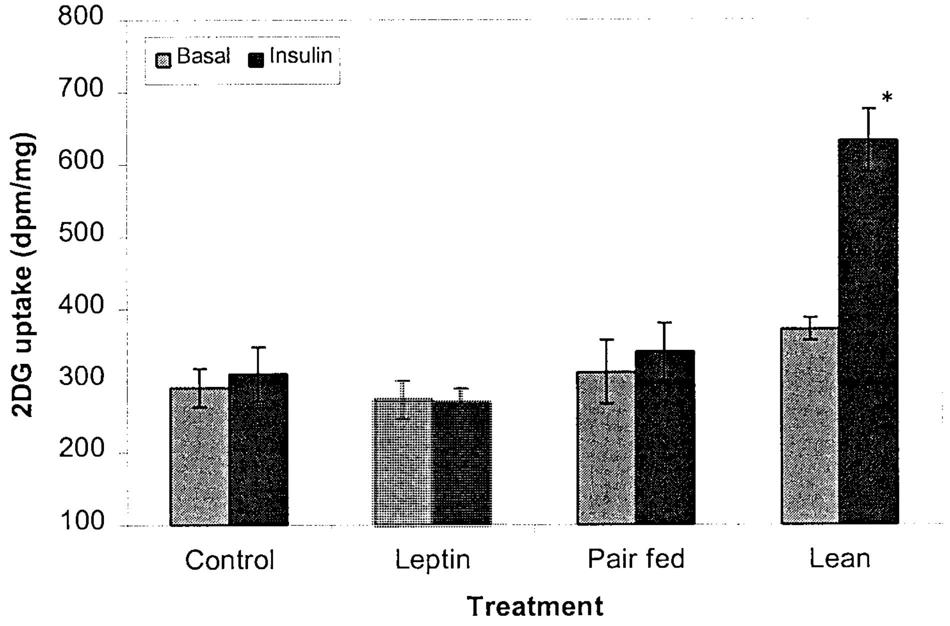


Table 4c Effect of leptin (10μg/mouse) on food intake in *ob/ob* mice. Mice received either leptin, vehicle-only (control group) or were pair fed to the leptin treated group for 48h. A lean group of mice was monitored for comparative purposes. Food intake was monitored on a daily basis. Leptin administration for 48h reduced food intake by approximately half compared to control obese mice. Data are expressed as mean food intake (g) ± SEM; n is indicated per group.

	Mean Food Intake (g)							
Day	**Control** (n=14)	SEM	**Leptin** (n=11)	SEM	**Pair fed** (n=10)	SEM	**Lean** (n=10)	SEM
0	9.6	0.2	9.6	0.2	9.6	0.1	4.5	0.1
1	8.5	0.4	4.6	0.2	4.5	0	4.47	0.15
2	8	0.2	3.9	0.3	4.5	0	5.4	0.16

Table 4d Effect of leptin (10μg/mouse) on body weight of *ob/ob* mice. Mice received either leptin, vehicle-only (control group) or were pair fed to the leptin treated group for 48h. A lean group of mice was monitored for comparative purposes. Body weight was monitored on a daily basis. Both leptin treated and pair fed mice demonstrated a small decrease in body weight compared to a 1-2g increase in body weight in control mice. Data are expressed as mean body weight (g) ± SEM; n is indicated per group.

	Mean Body Weight (g)							
Day	**Control** (n=14)	SEM	**Leptin** (n=11)	SEM	**Pair fed** (n=10)	SEM	**Lean** (n=10)	SEM
0	101	1.5	97	4	83	1	35	1
1	102.83	1.579	96.857	4.778	83	1.14	35	1.018
2	101.83	1.515	95.143	4.394	80.6	1.288	35.36	1.04

Figure 4.7 Acute effect of leptin on 2DG uptake in isolated soleus muscles of nonfasted *ob/ob* mice. Leptin (10^{-9}M) and/or insulin (10^{-6}M) was added to the isolated muscle and 2DG uptake was measured for 30min. Insulin did not affect 2DG uptake into soleus muscles of these mice demonstrating severe insulin resistance. Leptin did not significantly change 2DG uptake in the basal state or in the presence of added insulin. Data are expressed as dpm/mg soleus muscle, values are mean±SEM, n=9.

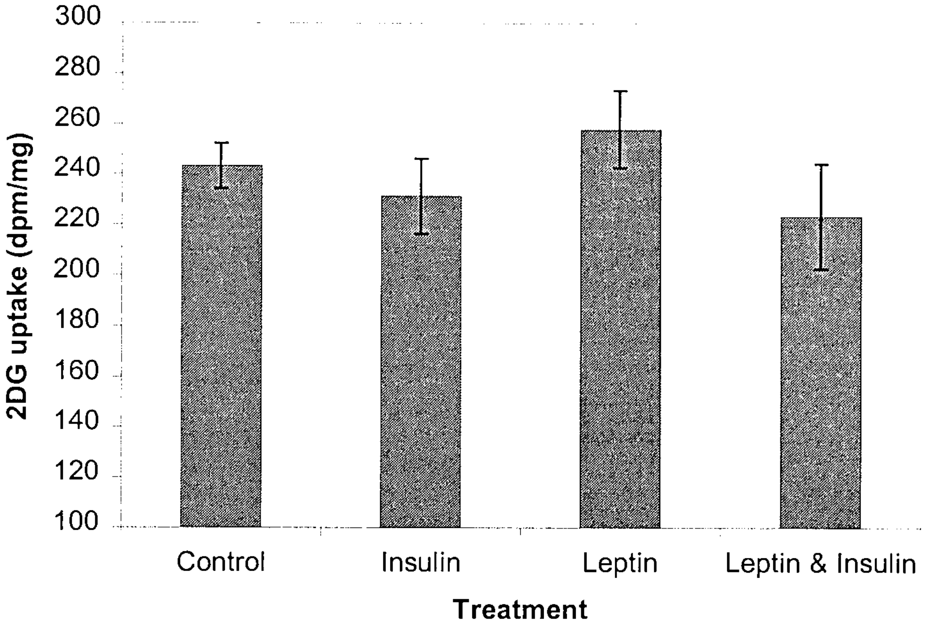
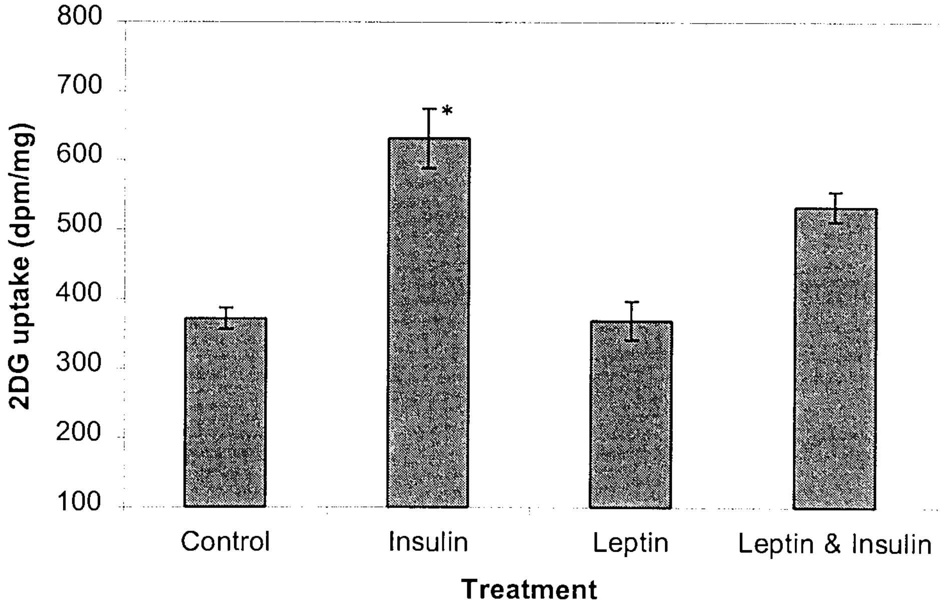


Figure 4.8 Acute effect of leptin on 2DG uptake in isolated soleus muscles of nonfasted lean mice. Leptin (10^{-9}M) and/or insulin (10^{-6}M) was added to the isolated muscle and 2DG uptake was measured for 30min. Insulin increased 2DG uptake by soleus muscles of lean mice by 41%. Leptin did not significantly change basal or insulin-stimulated 2DG uptake by soleus muscles of lean mice. Data are expressed as dpm/mg soleus muscle, values are mean±SEM, n=9. *$p<0.05$ versus control.



mice (5.07 ± 0.15mg versus 6.25 ± 0.26mg; n=36; $p<0.01$). Whereas insulin (10^{-6}M) increased glucose uptake by muscles of lean mice (by 41%, figure 4.8) insulin was ineffective in obese mice (figure 4.7), hence illustrating the severe insulin resistance of obese mice. Leptin (10^{-9}M) did not significantly change glucose uptake by soleus muscles of obese or lean mice either in the basal state or in the presence of insulin (10^{-6}M, figures 4.7 and 4.8).

4.1.2.4 Discussion

Chronic repeated treatment of *ob/ob* mice with leptin (ip) significantly decreases food intake and body weight. Leptin treatment reinstated normal circulating leptin concentrations (Kulkarni et al., 1997), which subsequently reinstated an increase in glucose uptake by soleus muscle in response to insulin. However, the long term improvement in glucose uptake by leptin was not as great as pair feeding, suggesting leptin may attenuate the improvement in muscle glucose metabolism brought about by the decrease in feeding. This is reflected in the increase in weight loss that is consistent with an increase in uncoupling proteins (UCPs) to increase glucose dissipation (Cusin et al., 1998) and increase glucose turnover (Kamohara et al., 1997).

The effects of leptin in decreasing food intake and body weight are rapid (within 48h of treatment), but after 48h treatment there was no effect on glucose uptake in soleus muscles. Thus the effect of leptin on muscle insulin sensitivity may occur via indirect (not acute) mechanisms. In addition, the acute *in vitro* study indicates that leptin does not exert a direct acute effect on glucose uptake by soleus muscles of obese (*ob/ob*) mice. This is consistent with previous observations of a lack of a direct acute effect of leptin on skeletal muscle of non-diabetic rodents (Furnsinn et al., 1998; Zierath et al., 1998; Liu et al., 1997). In an initial observation in *ob/ob* mice (Zierath et al., 1998), *in vitro* incubation with leptin did not affect glucose uptake by soleus muscles of *ob/ob* mice, but acute iv leptin increased insulin-stimulated glucose disposal in normal rats (Sivitz et al., 1997). Iv and icv administration of leptin for 5h to lean mice increased basal glucose uptake by muscle (Kamohara et al., 1997). Since the same effects were achieved by iv and icv leptin administration (Kamahara et al., 1997), this has been attributed to a neurally mediated effect of leptin. Administration of icv leptin to lean rats for 14 days increased insulin-stimulated glucose utilisation by muscle and UCP1-3 expression which may favour more energy utilisation than pair-fed animals (Cusin et al., 1998). Since icv infusion of leptin does not appear to alter plasma levels of leptin (Cusin et al., 1998), it appears that effects of icv leptin are mediated neurally.

Leptin effects on other cells involved in glucose homeostasis are controversial. Leptin did not alter hepatic glucose production (HGP) in lean mice during the 4 days of administration (Cusin et al., 1998), and it has been reported to have no acute effect on adipocytes (Zierath et al., 1998). However, leptin may alter lipid partitioning in skeletal muscle since leptin decreased fatty acid oxidation and decreased fatty acid incorporation into triglycerides (TG) (contrary to effects of insulin). Also leptin reduced the lipogenic effect of insulin *in vitro* in soleus and EDL of lean mice but leptin did not alter insulin stimulated glycogen synthesis (Muoio et al., 1997). Thus, leptin may have a direct inhibitory effect on insulin-stimulated lipid metabolism by muscle.

The controversial effects of leptin on glucose homeostasis have given rise to numerous studies that have utilised cell lines to investigate any potential effect. The data from these investigations have not provided the answers, as there is much discrepancy in the literature to date, not least because of the problems associated with biological activity of early batches of leptin. The effect of leptin on glucose transport in the rat L6 skeletal muscle cells line is investigated in Part Two.

Part Two: Effect of Leptin on Glucose Transport in the Rat L6 Skeletal Muscle Cell Line and Investigation of the Mechanism of Direct Action

4.2.1 Introduction

To determine whether there is a direct action of leptin in skeletal muscle, rat L6 skeletal muscle cells (characterised in chapter 3) were exposed to a number of different concentrations of leptin, at various time intervals. The effects of leptin were further investigated using known inhibitors of protein components that mediate intracellular signalling, such as the PI 3-kinase inhibitor, wortmannin (see chapter 3 Section 3.4.1) and the Jak 2-kinase inhibitor tyrphostin AG490 (chapter 3 Section 3.4.4). Leptin-stimulated 2DG uptake in these cells was measured. Leptin receptor subtype was assessed in these cells by determining leptin receptor mRNA.

4.2.2 Experimental Design

4.2.2a Effect of Leptin on Basal and Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake

In experiments measuring basal glucose uptake and insulin stimulated glucose uptake, leptin was used within the concentration range of 10^{-8}M to 10^{-11}M. All studies were repeated to include cell exposure times of 10 minutes, 1h, 4h and 24h to leptin. For experiments in which insulin-stimulated glucose uptake was determined, insulin was added at a concentration of 10^{-6}M, in order to produce a highly significant response to insulin at short time intervals (see section 3.3 and figure 3.3). Cells were incubated with insulin for exactly the same time periods as with leptin. Glucose uptake was determined as described in 2.4.1 Data are expressed as the mean±SEM of a combination of up to 4 separate experiments each of which contained up to 6 repetitions and analysed by Student's unpaired 't' test (2.6.1).

4.2.2b Effect of Wortmannin on Leptin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake

In order to investigate the mechanism of action of leptin on glucose uptake in L6 cells, experiments were undertaken using the PI 3-kinase inhibitor wortmannin (see section 3.4.1). Wortmannin has previously been utilised to investigate the mechanism of leptin action in cultured muscle cells (Berti et al., 1997) and this method was adopted for use in the L6 cell line. L6 muscle cells were pre-incubated with wortmannin for 10min at concentrations 10^{-9}M-10^{-4}M and the cells were exposed to leptin (10^{-10}M-10^{-8}M) for a further 40min. Experimental design was identical to that described in chapter 3 Section 3.4.1 except in place of insulin, cells were incubated with leptin. 2DG uptake was determined as described in chapter 2 Section 2.4.1.

4.2.2c Effect of Tyrphostin AG490 on Leptin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake

Tyrphostin AG490 is an inhibitor of Jak2-kinase (see section 3.4.4.1). To determine whether leptin acts via a Jak2-kinase–dependent pathway, cells were incubated with for 40min leptin and/or tyrphostin AG490 for 50min. Experimental design was identical to that described in chapter 3 section 3.4.4, (effect of Tyrphostin AG490 on insulin-stimulated 2DG uptake), except instead of insulin, cells were stimulated with leptin (10^{-10}M – 10^{-8}M). As the activity of leptin is acute i.e. an effect is observed within 1h of cell incubation with leptin, the use of tyrphostin AG490 was restricted to determining the effects on leptin-stimulated 2DG uptake within 1h of incubation. 2DG uptake was measured as described in chapter 2, 2.4.1.

4.2.2d Identification of Leptin Receptor mRNA

Three confluent 75cm^3 flasks of L6 cells were used to extract total RNA as described in chapter 2 Section 2.5.1. mRNA was subsequently isolated and probed for the long form and the short forms of the leptin receptor. Binding of the probe was detected by northern blotting (chapter 2 Sections 2.5.1.3b – 2.5.2). This work was completed in collaboration with Dr J Gardiner, Imperial College School of Medicine, London.

4.2.3 Results

4.2.3a Effect of Leptin on Basal and Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake

L6 muscle cells incubated with leptin (10^{-11}M-10^{-8}M) showed both a concentration-dependent and a time-dependent stimulatory effect on basal glucose uptake (in the absence of added insulin, figure 4.9). Cell exposure to leptin for 10min increased basal glucose uptake in this culture system. 10^{-11}M, 10^{-10}M, 10^{-9}M and 10^{-8}M leptin increased glucose uptake by 15%, 16%, 24% and 21% respectively. These increases all proved to be significant, ($p<0.02$ versus control). An increase in cell exposure to leptin to 1h produced similar results. Glucose uptake was significantly increased in response to stimulation with leptin by 23% (10^{-9}M, $p<0.01$ versus control), 17% (10^{-}

^{8}M, $p<0.02$ versus control), 16% (10^{-10}M and 10^{-11}M, $p<0.02$). A statistically significant increase in glucose uptake was not observed upon cell exposure to leptin at 4h or 24h but a minor increase in the mean value for glucose uptake was observed on each occasion (3% - 7%).

Insulin (10^{-6}M) stimulated glucose uptake in a time dependent manner up to 24h as described in chapter 3, section 3.3. Leptin had no effect on insulin-stimulated 2DG uptake after 1h, 4h or 24h incubation, but after an acute (10min) incubation, leptin significantly reduced insulin-stimulated 2DG uptake by 25%, ($p<0.05$ versus insulin alone) and 12% ($p<0.05$ versus insulin), 10^{-10}M and 10^{-9}M respectively. This effect was confirmed in repeated independent investigations (figure 4.10)

4.2.3b Effect of Wortmannin on Leptin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake

Wortmannin is a specific inhibitor of PI 3-kinase (see chapter 3 section 3.4.1) and was previously demonstrated to decrease insulin-stimulated glucose uptake in a concentration-dependent manner (chapter 3 section 3.4.1.3, figure 3.4). Preliminary studies demonstrated that wortmannin exerts a maximal inhibitory effect on insulin-stimulated glucose uptake after a 50min preincubation period without affecting cell viability.

Leptin alone increased basal glucose uptake as described in section 4.2.3a above. Wortmannin reduced basal glucose uptake as previously described in chapter 3 (figure 3.4). The inhibitory effect of wortmannin on leptin-stimulated glucose uptake was similar to the effect observed in the insulin-stimulated response (3.4.1.3). 10^{-4}M wortmannin produced a decrease in cell viability as established previously (figures

Figure 4.9 Effect of leptin on basal 2DG uptake in L6 cells. Cells were exposed to leptin (10^{-11}M – 10^{-8}M) for 10min, 1h, 4h or 24h. Leptin increased 2DG uptake in a time dependent manner. Cell exposure to leptin for 10min significantly increased 2DG uptake by 15% - 24%. Incubation of the cells for 1h with leptin significantly increased basal 2DG uptake by 16% - 23%. 4h and 24h incubation of cells with leptin did not significantly alter basal 2DG uptake. Data are expressed as % control, values are mean±SEM, n=18. *Significance versus control, $^1p<0.01$, $^2p<0.02$

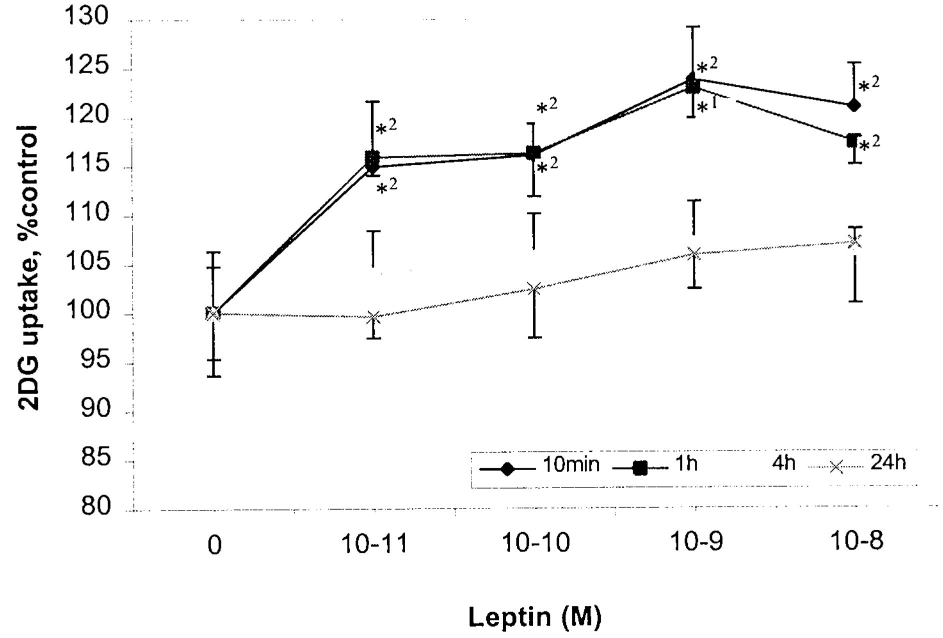


Figure 4.10 Effect of leptin on insulin-stimulated 2DG uptake in L6 cells. Cells were exposed to leptin (10^{-11}M – 10^{-8}M) and/or insulin (10^{-6}M) for 10min, 1h, 4h or 24h. Insulin alone stimulated 2DG uptake in a time-dependent manner. Leptin had no effect on insulin-stimulated 2DG uptake in L6 cells after 1h, 4h and 24h incubation, but after a 10min incubation, leptin significantly reduced insulin-stimulated 2DG uptake by 12% - 25%. Data are expressed as % control, mean±SEM, n=18. *Significance versus control, † versus insulin alone, $^1p<0.01$, $^2p<0.02$, $^3p<0.05$.

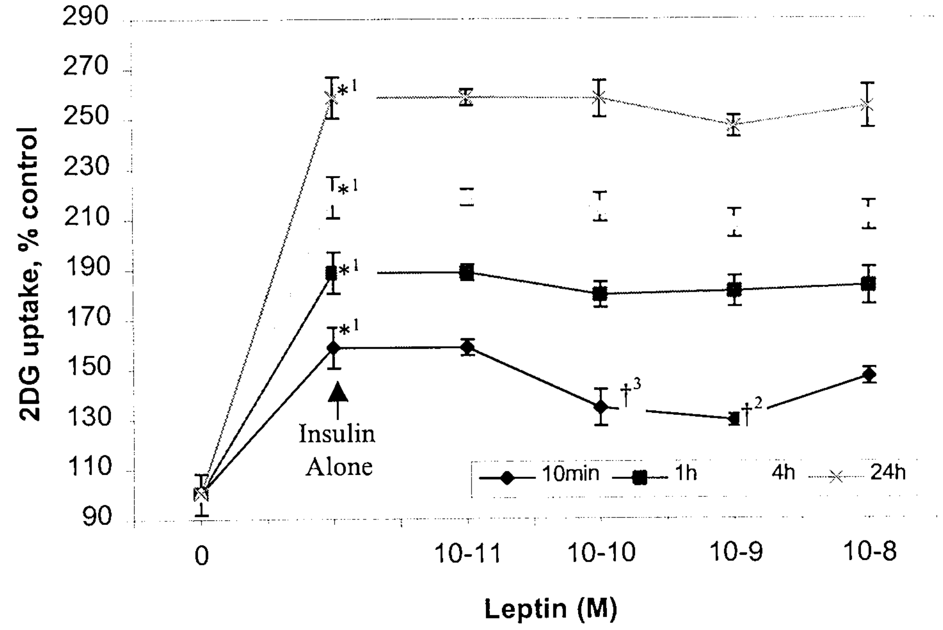
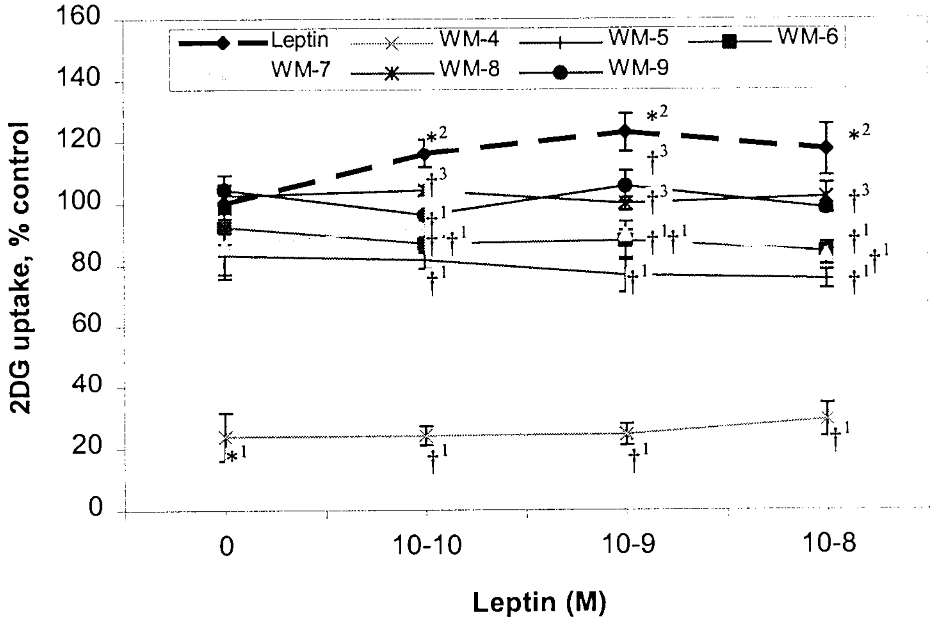


Figure 4.11 Inhibition of leptin-stimulated 2DG uptake by the fungal metabolite wortmannin in L6 cells. Cells were pre-stimulated with wortmannin (10^{-9}M – 10^{-4}M) for 10min. 2DG uptake was determined after the cells were cultured with leptin (10^{-10}M – 10^{-8}M) for a further 40min. Leptin-stimulated 2DG uptake was inhibited by wortmannin in a concentration dependent manner. Data are expressed as % control, values are mean±SEM, n=9. *Significance versus control, †significance versus leptin alone. ^{1}p<0.01, ^{2}p<0.02, ^{3}p<0.05.



3.5a and 3.5b), however lower concentrations (10^{-9}M - 10^{-5}M) decreased glucose uptake in a concentration dependent manner. 10^{-9}M wortmannin (the lowest concentration investigated) completely inhibited the leptin-stimulated response, glucose uptake values were decreased to basal levels (approximately 100%, $p<0.05$ versus leptin-stimulated glucose uptake). Higher concentrations of wortmannin inhibited leptin-stimulated glucose uptake to levels below basal (i.e. less than 100% glucose uptake) and were highly significant (figure 4.11).

4.2.3c Effect of Tyrphostin AG490 on Leptin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake

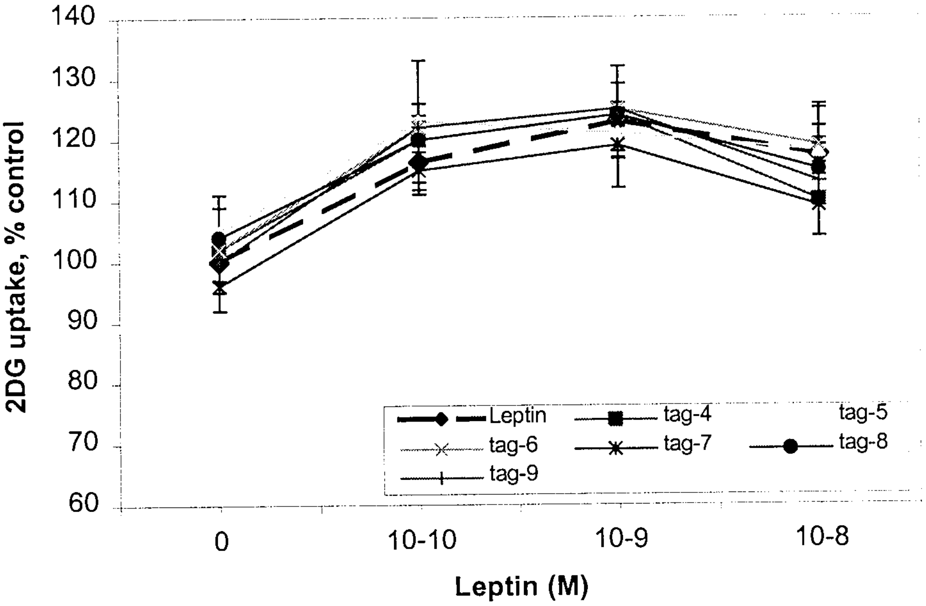
Tyrphostin AG490 (as described in section 3.4.4) is an inhibitor of the signalling component Jak2-kinase. Tyrphostin AG 490 had no inhibitory effect on basal or insulin-stimulated glucose uptake in L6 cells in chapter 3 (3.4.4, figures 3.7a, b and c).

Leptin increased basal glucose transport as described above (4.2.3.1). Tyrphostin AG490 (10^{-9}M – 10^{-4}M) had no significant effect on leptin-stimulated glucose transport in these cells (figure 4.12).

4.2.3d Identification of Leptin Receptor mRNA

L6 skeletal muscle cells were found to express the short form of the leptin receptor but not the long form of the receptor (Gardiner, personal communication).

Figure 4.12 Effect of the janus kinase 2 inhibitor tyrphostin Ag 490 on leptin-stimulated 2DG uptake. Cells were pre-stimulated with tyrphostin Ag 490 (tag) (10^{-9}M – 10^{-4}M) for 10min. 2DG uptake was determined after cells were cultured with leptin (10^{-10}M – 10^{-8}M) for a further 40min. Tyrphostin did not affect basal or leptin-stimulated 2DG uptake in L6 cells. Data are expressed as % control, values are mean±SEM, n=6.



4.2.5 Discussion

As discussed previously there is conflicting evidence concerning the effects of leptin on skeletal muscle *in vitro* (Cohen et al., 1996; Bertie et al., 1997; Liu et al., 1997; Muller et al., 1997; Furnsinn et al., 1998; Zierath et al, 1998).

This study was undertaken to assess the effects of leptin on basal and insulin-stimulated glucose uptake in L6 cells, in order to determine any possible influence on insulin sensitivity in muscle cells. The results showed evidence of considerable leptin action on glucose uptake in L6 cells. However the results do not suggest a simple mechanism of action. Basal glucose transport was significantly increased by leptin after acute incubation periods, yet in contrast with this stimulatory effect, incubation of the cells with leptin and insulin consistently demonstrated inhibition of insulin-stimulated glucose uptake.

The data suggest that *in vitro,* leptin alone can mimic the effects of insulin with regard to the stimulation of glucose uptake. This effect was observed in the absence of added insulin, therefore leptin may act to increase glucose transport by an unknown pathway. Alternatively, leptin may act to initiate glucose transporter translocation via the insulin-signalling pathway, thus acting on protein components of intracellular signalling down-stream from the insulin receptor. The effect of leptin to inhibit insulin-mediated glucose transport will be discussed later; the following discussion will focus primarily on the acute insulin mimetic effects of leptin.

The effects of leptin in this system were observed within a 1h incubation period, indicating that leptin must possess an acute paracrine action. This has previously been demonstrated *in vivo* by intravenous (iv) and intracerebroventricular (icv) infusion of leptin over a 5h period, which resulted in an increase in glucose uptake in skeletal muscle and brown adipose tissue (Kamohara et al., 1997). Although these effects were demonstrated to be acute metabolic responses to leptin administration, they were thought to be mediated by the CNS as both iv and icv administered leptin produced similar data.

The acute direct action of leptin of glucose metabolism has been investigated in other cell models thus removing the possible confounding effects of weight loss on glucose homeostasis. This stimulatory effect of leptin on basal glucose uptake in skeletal muscle has also been observed utilising the C_2C_{12} skeletal muscle cell line (Berti et al., 1997). In this study it was demonstrated that preincubation of C_2C_{12} cells with leptin did not significantly affect insulin-stimulated glucose transport and glycogen synthesis. Nevertheless leptin by itself was able to mimic approximately 80-90% of the insulin effect on glucose transport and glycogen synthesis. Furthermore it was demonstrated that the effects of leptin on basal glucose transport were inhibited by the PI 3-kinase inhibitor wortmannin. This is in keeping with data obtained in this study which clearly demonstrate that the leptin-stimulated glucose transport is potently inhibited by wortmannin with less than 1h cell exposure to both compounds. This suggests that leptin directly activates the insulin signalling pathway possibly at the level of PI 3-kinase, thus increasing GLUT translocation and subsequent glucose uptake into the cell. Furthermore leptin has been demonstrated to activate PI 3-kinase in C_2C_{12} myotubes via a Jak2 and IRS2 dependent pathway (Kellerer et al., 1997). C_2C_{12} myotubes were stimulated with insulin or leptin and PI 3-kinase activity determined in immunoprecipitates with specific antibodies against IRS 1, IRS 2 and

Jak1 and 2. Insulin stimulated PI 3-kinase activity was detected in IRS 1 and IRS 2 immunoprecipitates, however leptin stimulated PI 3-kinase activity was detected only in IRS 2 immunoprecipitates suggesting that the leptin signal to PI 3-kinase occurs via IRS 2 and not IRS 1. Leptin-stimulated (but not insulin-stimulated) PI 3-kinase activity was also detected in immunoprecipitates with antibodies against Jak2 (but not Jak1). Therefore it was postulated that leptin signals via Jak2 and IRS 2 prior to coupling to the insulin intracellular signalling pathways. However the data obtained in this chapter demonstrate that the leptin stimulated increase in glucose transport observed in L6 cells was not conducted via Jak2. Tyrphostin AG490 is a potent inhibitor of Jak2 and its effects on leptin stimulated glucose transport were investigated using a wide concentration range. Tyrphostin AG490 did not affect leptin stimulated glucose transport in L6 cells, thus it can be deduced that leptin does not stimulate glucose transport in these cells via a Jak2 dependent pathway. Furthermore, the long form of the leptin receptor isoform was not detected in C_2C_{12} myotubes indicating signalling via a short leptin receptor isoform. It is now known that Jak 2 is a major component of leptin signalling via the long form of the receptor but it is unknown whether Jak2 is involved with signalling via the short form of the receptor. The work of Kellerer et al., (1997) suggests that leptin signalling via the short form of the receptor increases glucose transport due to a Jak2/IRS2/PI 3-kinase mediated cascade. However it has recently been demonstrated that the short form of the leptin receptor is unlikely to signal via Jak2 (see section 4.0.4) as it is thought that signalling via Jak2 is mediated via the phosphorylation of tyrosine residues present only on the intracellular tail of the long receptor form (Tartaglia 1997).

The tissue expression of the leptin receptor is likely to play an important role in mediating the effects of leptin. L6 cells appear to express the short form of the receptor. Thus it can be postulated that soluble leptin receptors e.g. OB-Ra are present in the cell membranes. These may transport leptin into the cells in a similar mechanism to their postulated action in the choroid plexus.

A further direct effect of leptin in L6 cells was demonstrated in this chapter. At high leptin concentrations it was observed that leptin significantly inhibited insulin-stimulated glucose transport. This suggests that high leptin concentrations that are comparable with those present in obese individuals cause attenuation of insulin-induced responses. There are few studies that have also demonstrated this effect due to the short time periods and high concentrations of leptin needed to induce a response. Nevertheless a similar effect has been reported in isolated rat adipocytes (Muller et al., 1997). It was observed that leptin impaired several metabolic actions of insulin including stimulation of glucose transport, glycogen synthesis, lipogenesis, inhibition of isoproterenol-induced lipolysis and protein kinase A activation as well as stimulation of protein synthesis. Also a recent study demonstrated a significant and dose dependent inhibition of insulin-stimulated glycogen synthesis by leptin in isolated soleus muscle in *ob/ob* mice and a smaller inhibition in soleus muscle of lean mice (Liu et al., 1997). This supports the data obtained in part two of this chapter that leptin may directly attenuate insulin action in skeletal muscle. This mechanism has been further investigated in hepatic cells expressing the long form of the leptin receptor (Cohen et al., 1996). In this study leptin (at high concentrations) caused attenuation of several insulin-induced activities. These were shown to include tyrosine phosphorylation of IRS-1, association of the adapter molecule growth factor receptor-bound protein 2 with IRS-1, and down regulation of gluconeogenesis. Interestingly, leptin was also observed to increase the activity of IRS-1 associated PI 3-kinase in hepatic cells, a mechanism that could increase basal glucose transport as observed in L6 cells (as discussed above).

In conclusion, the mechanisms by which leptin mediates its multiple responses appears to be highly complex. The data obtained in this chapter alone demonstrate the numerous biological effects of leptin. Chronic repeated treatment of *ob/ob* mice with leptin appeared to attenuate the improvement in muscle glucose metabolism brought about by the decrease in feeding. Short-term administration of leptin of *ob/ob* mice appeared to have no effect on glucose metabolism and direct exposure of soleus muscles of *ob/ob* and lean mice to leptin had no effect on glucose uptake. In L6 cells, leptin acutely increased basal glucose transport, a signalling mechanism which appeared to be mediated via the short form of the leptin receptor which either directly or indirectly stimulated PI 3-kinase activity. This mechanism was not mediated by Jak2. In contrast high concentrations of leptin (comparable to those observed in obesity) attenuated insulin mediated glucose transport.

In non-insulin resistant states, leptin activates the insulin-signalling pathway. This mechanism is likely to be highly dependent on tissue type and leptin receptor expression in that tissue. This ultimately results in increased glucose uptake due to GLUT translocation. In insulin resistant or hyperleptinaemic states, leptin may attenuate insulin action possibly via an inhibition of autophosphorylation of IRS1, thereby causing a reduction in glucose uptake. An alternative hypothesis is the involvement of PI 3-kinase in an inhibitory feedback mechanism on earlier steps in the pathway. Again this mechanism of leptin action is likely to be highly dependent on the extent of the hyperleptinaemia and/or hyperinsulinaemia and the tissue expression of the leptin receptor. Thus in obese Type 2 diabetic patients leptin may play a role in the insulin resistance observed in muscle cells and other periphery target cells.

Chapter Five:
Sibutramine

Chapter Five: Sibutramine

5.0 Introduction

5.0.1 Obesity

As discussed previously (chapter 4), the prevalence of obesity is reaching epidemic proportions and there is major evidence to suggest that obesity constitutes an independent risk factor for a range of co-morbidities including Type 2 diabetes. The relationship between Type 2 diabetes and obesity is highly intricate and both are implicated with insulin resistance, impaired glucose tolerance, hyperglycaemia, dislipidaemia and hypertension. Type 2 diabetes can be largely prevented by the treatment of obesity (reviewed by Bray, 1999; reviewed by Carek and Dickerson, 1999; Scheen and Lefebvre, 1999).

Obesity is rapidly becoming a chronic health problem for modern society (Kurscheld and Lauterbach, 1998) but it is also one of the most ignored and untreated diseases. Conventional approaches to weight loss include diet and exercise, which in many cases is insufficient to maintain sustained weight loss (Atkinson, 1998).

5.0.2 Pharmacological Intervention

Several pharmacological agents are used to treat obesity. These have included serotoninergic compounds and a combination of serotoninergic and adrenergic compounds.

Fenfluramine and derivatives (dexfenfluramine, nordexfenfluramine) modify eating behaviour by increasing satiety, via an increase in serotonin release and by inhibiting reuptake (reviewed by Kosmiski and Eckel, 1997; Heal et al., 1998). There have been numerous clinical trials with dexfenfluramine, which have demonstrated significant weight loss in treated patients (Guy-Grand et al., 1989). Weight loss was shown to plateau at 6 months and was better maintained than placebo-treated patients (reviewed by Bray and Ryan, 1997). Phentermine is a sympathomimetic drug also approved and used as an appetite suppressant (reviewed by Kosmiski and Eckel., 1997; reviewed by Carek and Dickerson, 1999).

5.0.3 Sibutramine

Sibutramine (BTS 54 524; N-1{[-1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-N,N-dimethylamine hydrochloride monohydrate, appendix II) was primarily developed as a new antidepressant (Buckett et al., 1988). However during clinical trials it was observed that sibutramine produced significant weight loss in patients and was relatively ineffective as an antidepressant. Therefore it was decided that sibutramine should be developed as an antiobesity agent. It is a reuptake inhibitor of 5-hydroxytryptamine (serotonin), 5HT and noradrenaline, and is therefore known as a SNRI (Serotonin and Noradrenaline Reuptake Inhibitor) (Heal et al., 1998). Thus, it combines the beneficial effects of both serotoninergic and adrenergic agents, as regulators of appetite and satiety.

Sibutramine is a tertiary amine which is metabolised to a secondary amine (metabolite 1, M1) and a primary amine (metabolite 2, M2), see appendix II. The metabolites have been found to be the dominant active species in animals and Man (Hind et al., 1999).

5.0.3.1 Pre-Clinical Studies

Sibutramine induces satiety, stimulates thermogenesis and reduces weight gain in rodents (Buckett et al., 1988; Luscombe et al., 1989; Jackson et al., 1997; Halford et al., 1995; Connoley, et al., 1999; Day and Bailey, 1998; reviewed by Stock, 1997). This effect causes a reduction in food intake and this has been shown to be a CNS mediated effect due to its ability to enhance post-ingestive satiety, that is, animals treated with sibutramine stopped eating earlier than control animals. Sibutramine causes a dose-dependent inhibition (up to 70%) of 24h food intake in rats (reviewed by Stock, 1997). Other aspects of eating patterns and behaviour were identical in control and sibutramine treated animals (Halford et al., 1995). Unlike amphetamine, sibutramine does not stimulate locomotor activity. Various serotonergic and noradrenergic antagonists inhibit the effects of sibutramine on food intake, but prazosin, a α1-adrenoceptor antagonist is one of the most effective inhibitors (reviewed by Stock, 1997). The second biological effect of sibutramine, as demonstrated in animals is to increase energy expenditure through stimulation of thermogenesis (Connoley et al., 1999). In rats, it increases oxygen consumption 30% for periods of 6h or longer. It is thought that this effect is mediated via the β3 adrenoceptor due to blockade studies using various β adrenergic antagonists. As sibutramine and its metabolites cannot bind to the β3 adrenoceptor, the effect must be indirectly mediated via 5HT and noradrenaline reuptake inhibition.

It has recently been demonstrated that sibutramine has blood glucose lowering properties. This has been investigated in obese hyperglycaemic insulin resistant *ob/ob* mice. Sibutramine did not alter basal plasma glucose levels, although an acute glucose lowering effect has been noted after administration of metabolite 2. Nevertheless, chronic sibutramine administration causes the rate of insulin-induced glucose uptake to increase by 10%. Sibutramine also lowered both insulin levels and plasma non esterified fatty acids (Day and Bailey, 1998). A similar study has shown that after 28 days of treatment of sibutramine, plasma glucose of *ob/ob* mice was significantly decreased. Withdrawal of sibutramine resulted in an increase in glucose levels (Jones et al., 1997).

These studies indicate that sibutramine may be beneficial in the treatment of insulin resistance in obesity and Type 2 diabetes, especially the combination of the two conditions in the obese Type 2 diabetic patient.

5.0.3.2 Clinical Studies

In clinical studies, sibutramine promoted weight loss in obesity (Weintraub, 1991; Bray et al., 1999; Apfelbaum et al., 1999) and sibutramine has recently been used in phase 3 clinical trial as an antiobesity agent where results have shown significant dose-dependent weight loss and a decrease in waist:hip ratio with treatment (Hanotin et al., 1998). Plasma triglycerides, total and LDL cholesterol were also reduced and

HDL cholesterol levels raised (reviewed by Lean, 1997). Controlled trials with overweight Type 2 diabetic patients have demonstrated both weight loss and improved glycaemic control (Vargas et al., 1994; Griffiths et al., 1995; reviewed by McNeely and Goa, 1998; Fujioka et al., 1998). Sibutramine received approval by the Food and Drug Administration in 1998 (Seagle et al., 1998) and is under regulatory review in Europe.

Sibutramine is well absorbed following oral ingestion and the active metabolites 1 and 2 have elimination half lives of 14h and 16h respectively. Sibutramine is eliminated through hepatic metabolism of the active metabolites to inactive conjugated metabolites, which are excreted preferentially in urine (reviewed by Lean, 1997).

Side effects of sibutramine have so far been shown to be moderate, mainly a small dose related increase in blood pressure and heart rate, hence it is a promising treatment for both non-diabetic and diabetic obese patients (reviewed by Kolanowsky, 1999).

Part One: *In Vitro* Studies using the Rat L6 Skeletal Muscle Cell Line

Sibutramine has been demonstrated to reduce insulin resistance in obese-diabetic *ob/ob* mice to a greater extent than expected for the reduced rate of weight gain (Day and Bailey, 1998). Thus it is possible that sibutramine or its active metabolites (M1 and M2) might improve insulin action, at least in part, independently of the weight reducing effect. To investigate this possibility, sibutramine, its metabolites and various structurally and pharmacologically related agents were used to treat cultured

L6 muscle cells. Glucose uptake by the cells was assessed in the absence and presence of added insulin using the non-metabolised analogue 2-deoxy-[^{3}H]-glucose (as described in 3.3.1 and 2.4.1).

5.1.1 Experimental Design

5.1.1a Effect of Sibutramine, Metabolite 1 and Metabolite 2 on Basal and Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake

Sibutramine, M1 and M2 were a gift from Knoll Pharmaceuticals, Nottingham, UK, and were dissolved in PBS by sonication for 30min. Basal dose response curves were completed in L6 cells to determine the effect on glucose uptake of concentration and incubation time with sibutramine, metabolite 1 (M1) and metabolite 2 (M2). L6 skeletal muscle cells were grown to confluence in 24 well plates and differentiated to form myotubes as described in chapter 2 (2.2.2). Cells were incubated for either 24h, 48h or 72h with sibutramine (10^{-8}M – 10^{-6}M), M1 (10^{-8}M – 10^{-6}M) or M2 (10^{-8}M – 10^{-6}M) and glucose uptake measured as described previously (2.4.1).

To assess the effects of sibutramine, M1 or M2 on insulin-stimulated glucose transport, cells were incubated with sibutramine, M1 and M2 (at concentrations described above for basal glucose uptake) for 24h, 48h or 72h. Insulin (10^{-8}M) was added to the assay system for the final 24h prior to measuring glucose uptake (2.4.1). Experiments were completed on at least three separate occasions using multiples of 6 for each test compound or concentration. Data were analysed as % of control values, and were found to be significant if $p<0.05$. This was determined using Student's paired 't' test.

5.1.1b Mechanism of Action of Metabolite 2

The following experiments were undertaken to further characterise the effects of M2 on *in vitro* glucose transport in L6 cells.

Effect of M2 on the Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake Dose-Response

To determine the effect of M2 on the insulin dose-response curve, L6 cells were grown to confluence, differentiated to form myotubes (2.2.2) and incubated with M2 (10^{-6}M) for 24h with a range of insulin concentrations (10^{-9}M – 10^{-6}M). Glucose transport was determined as described previously (2.4.1). Experiments were completed on 3 separate occasions using multiples of 6 wells per test.

10^{-6}M M2 was found to produce the most potent effect in increasing both basal and insulin-stimulated glucose uptake. Thus 10^{-6}M M2 was utilised in the following investigations to identify the possible mechanisms by which M2 increases glucose transport. The experimental designs for the use of known inhibitors of intracellular signalling of glucose transport in rat L6 skeletal muscle cells can be found in chapter 3. The following experiments were based on the preliminary data (chapter 3) where the effects of the following compounds on basal and insulin-stimulated glucose transport were investigated.

Effect of LY-294,002 on M2-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake

L6 cells were incubated with M2 (10^{-6}M) and/or LY-294,002 (10^{-8}M – 10^{-5}M) for 24h. LY-294,002 has been previously discussed in detail in chapter 3 (3.4.2). It is a selective inhibitor of PI 3-kinase and can be used to identify intracellular signalling via PI 3-kinase. The effects of LY-294,002 on basal and insulin-stimulated glucose uptake in L6 cells were determined in chapter 3 (figure 3.6). It was found that cells can be exposed to this compound for 24h (at concentrations of 10^{-5}M or higher) with no loss of cell viability. Thus L6 cells were incubated with M2 and/or LY294,002 for 24h and glucose uptake measured (2.4.1). Data were analysed as described above.

Effect of Cytochalasin B on M2-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake

The effects of M2 on glucose transport in these cells were further characterised by incubating the cells with cytochalasin B. As described in chapter 3 (3.4.5), cytochalasin B is an inhibitor of glucose transporter function. The effects of cytochalasin B on basal and insulin-stimulated glucose transport were determined in 3.4.5.3. To investigate the effect of cytochalasin B on M2-stimulated glucose uptake, cells were incubated with M2 (10^{-6}M) and/or cytochalasin B (10^{-9}M – 10^{-6}M) for 24h. Glucose uptake was measured as described in 2.4.1.

Effect of Cycloheximide on M2-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake

The effects of M2 on glucose transport were not observed until the cells have been exposed to the compound for 24h or longer (data not shown). Therefore it can be postulated that M2 may alter the rate at which L6 cells take up glucose via the synthesis of new proteins. To investigate this, L6 cells were incubated with M2 (10^{-6}M) and/or cycloheximide (10^{-8}M – 10^{-5}M) for 24h and glucose uptake measured. Cycloheximide is an inhibitor of protein synthesis, and the effects of this compound on basal and insulin-stimulated glucose transport in L6 cells are discussed in chapter 3 (3.4.6).

5.1.1c Comparative Agents

A number of pharmacologically related compounds (appendix II) were used to compare their effects on glucose transport in L6 cells with those observed with sibutramine, M1 and M2.

Fluoxetine and nisoxetine (Sigma) are potent reuptake inhibitors of serotonin and noradrenaline (respectively). Fluoxetine is an antidepressant, which acts by selectively inhibiting serotonin reuptake: it does not however, stimulate serotonin release. It is approved for use to treat clinical depression, but has not been approved for use to treat obesity. Nevertheless, it has been shown to reduce weight in obese patients (Ferguson and Feighner, 1987). Clinical trials with fluoxetine demonstrated a dose-dependent weight loss over a 16 week period, however after this time many patients regained weight, even though medication was continued (reviewed by Bray and Ryan, 1997; Darga et al., 1991). In order to determine the effects of these compounds on metabolism *in vitro*, glucose uptake was measured after L6 cells had been exposed to fluoxetine (10^{-8}M – 10^{-6}M), nisoxetine (10^{-8}M – 10^{-6}M) or a combination of both compounds for 24h. The effects of fluoxetine and nisoxetine on insulin-stimulated glucose uptake were determined by culturing the cells as for basal glucose uptake with the addition of insulin (10^{-8}M) for 24h.

Serotonin, a monoamine neurotransmitter, is thought to be involved in the control of mood and behaviour, feeding and satiety, thermoregulation, motor activity, sleep and possibly some neuro-endocrine control mechanisms in the hypothalamus (reviewed by Kruk and Pycock, 1993). Dopamine (Sigma), a catecholamine neurotransmitter, acts in the CNS to control movement, and is part of the neuronal control of the hypothalamic-pituitary endocrine system and is concerned with cognitive, reward and emotional behaviour. Noradrenaline is a catecholamine neurotransmitter in postganglionic sympathetic nerves and CNS. To determine the effects of these neurotransmitters on glucose uptake, cells were incubated with each individual agent at concentrations $10^{-8}M - 10^{-6}M$ with and without added insulin ($10^{-8}M$) for 24h. Glucose uptake was measured as previously described (2.4.1).

Fenfluramine (Sigma) is used clinically as an anorectic agent (as described above and reviewed by Davis and Faulds, 1996), and acts by causing the direct release of serotonin and by inhibiting reuptake into the synaptic cleft. Nordexfenfluramine (a gift from Knoll Pharmaceuticals, UK) is the active metabolite of fenfluramine. Phentermine (Sigma) acts to release noradrenaline. These agents were investigated by incubating L6 cells with these agents ($10^{-8}M - 10^{-6}M$) for 24h with and without added insulin ($10^{-8}M$). Glucose uptake was assessed as described previously (2.4.1).

Data for these glucose uptake assays were expressed as % of control and were analysed by Student's unpaired 't' test (2.6) with the Bonferroni adjustment. Results were considered significant if $p<0.05$.

5.1.2 Results

5.1.2a Effect of Sibutramine, Metabolite 1 and Metabolite 2 on Basal and Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake

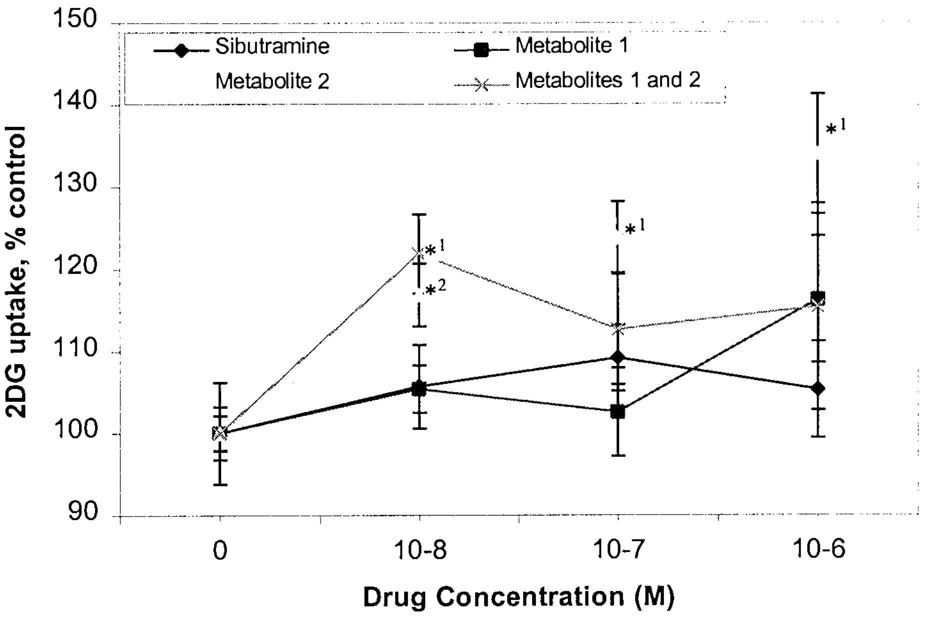
In the absence of added insulin, incubation for 24h-72h with sibutramine did not significantly alter basal glucose uptake in L6 cells (Figure 5.1a, 5.2a and 5.3a). However M2 (10^{-8}M, 10^{-7}M and 10^{-6}M) produced a small concentration-related increase in glucose uptake (by 17%, 24% and 34% respectively, $p<0.02$ 10^{-8}M versus control and $p<0.01$ 10^{-7}M and 10^{-6}M versus control) after 24h (figure 5.1a), but did not produce a significant effect after longer incubation times (figure 5.2a and 5.3a). In contrast, M1 (10^{-6}M) significantly increased basal glucose uptake (by 19%, $p<0.05$) only after 72h incubation (figure 5.3a). Incubation of L6 cells with M1 and M2 together increased basal glucose uptake by 22% after a 24h ($p<0.01$ 10^{-8}M versus control, figure 5.1a). A significant effect on basal glucose uptake was not observed at any other time point.

Addition of insulin (10^{-8}M for 24h) to the control incubation medium increased glucose uptake by approximately 40% ($p<0.01$). Addition of insulin (10^{-8}M) for the last 24h of incubations with sibutramine (24h - 72h) had no further effect on glucose uptake in L6 cells (figure 5.1b, 5.2b and 5.3b).

In the presence of added insulin, M2 produced a small further increase (by 13% and 16% respectively; $p<0.05$ 10^{-7}M M2 versus insulin and $p<0.02$ 10^{-6}M M2 versus insulin alone) in glucose uptake after 24h (figure 5.1b), but not after longer incubation times (figure 5.2b and 5.3b).

Figure 5.1 Effect of sibutramine, M1, M2 and M1 and M2 added together on 2DG uptake in L6 cells. Cells were exposed to sibutramine, M1, M2 or M1 and M2 at concentrations 10^{-8}M – 10^{-6}M and/or insulin (10^{-8}M) for 24h. In the absence of added insulin **(5.1a)**, sibutramine and M1 had no effect on 2DG uptake. M2 increased 2DG uptake by 17% - 34%. Addition of insulin **(5.1b)** for 24h increased 2DG uptake by approximately 40%. Sibutramine and M1 had no effect on insulin-stimulated 2DG uptake, but M2 increased 2DG uptake by 13% - 16% (10^{-7}M and 10^{-6}M). Data are expressed as % control, values are mean ± SEM, n=6. *Significance versus control, †significance versus insulin alone, $^{1}p<0.01$, $^{2}p<0.02$, $^{3}p<0.05$.

5.1a 24h without added insulin



5.1b 24h with added insulin

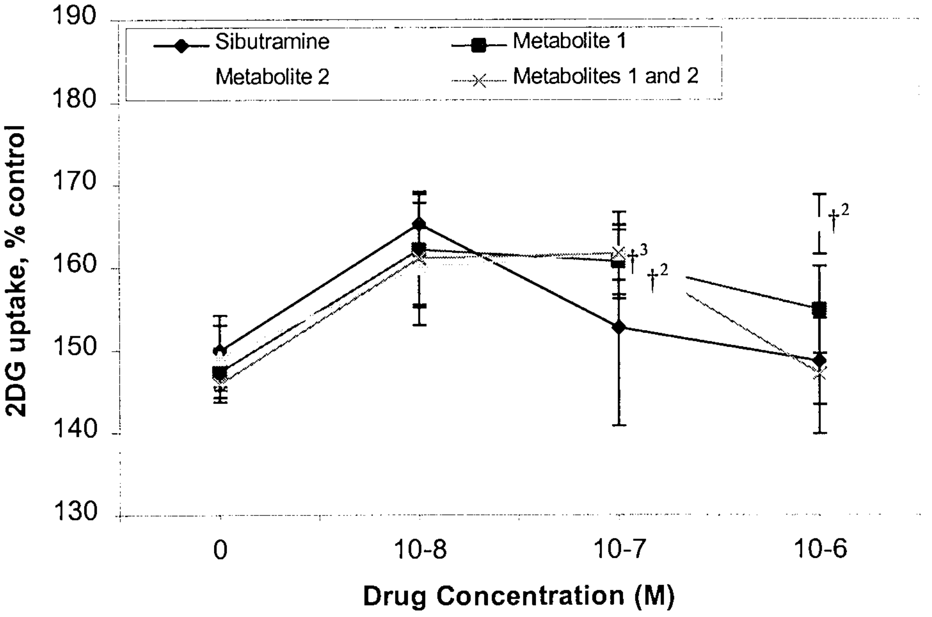
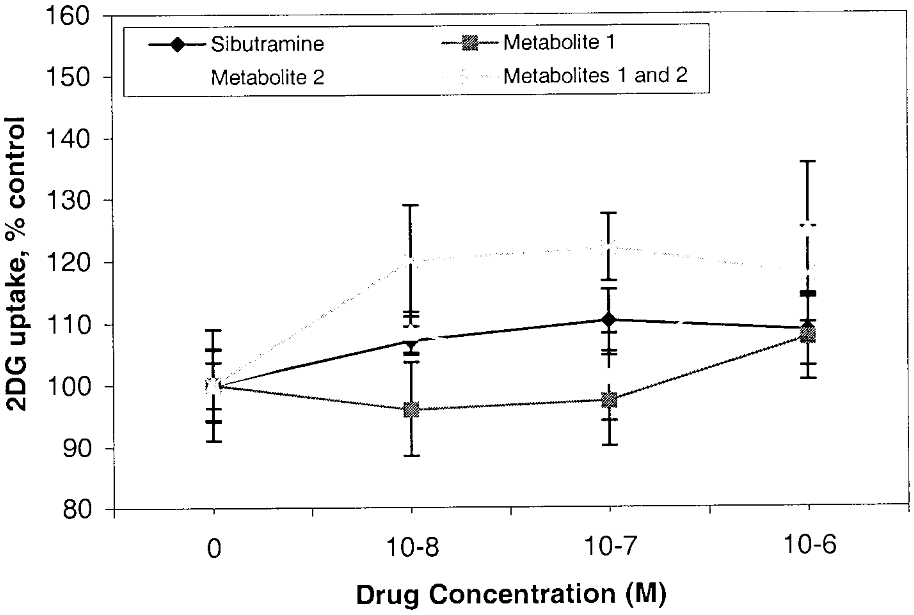


Figure 5.2 Effect of sibutramine, M1, M2 and M1 and M2 added together on 2DG uptake in L6 cells. Cells were exposed to sibutramine, M1, M2 or M1 and M2 at concentrations 10^{-8}M – 10^{-6}M for 48h and/or insulin (10^{-8}M) for 24h. In the absence of added insulin **(5.2a)**, there was no effect of any of the test compounds on basal glucose uptake. Addition of insulin **(5.2b)** for 24h increased 2DG uptake by approximately 40%. Addition of insulin for the last 24h of 48h incubations of sibutramine or M2 had no effect on insulin-stimulated 2DG uptake. M1 and M1 and M2 together increased insulin-stimulated 2DG uptake by 20% - 32%. Data are expressed as % control, values are mean ± SEM, n=6. *Significance versus control, †significance versus insulin alone, $^{1}p<0.01$, $^{2}p<0.02$, $^{3}p<0.05$

5.2a 48h without added insulin



5.2b 48h with added insulin

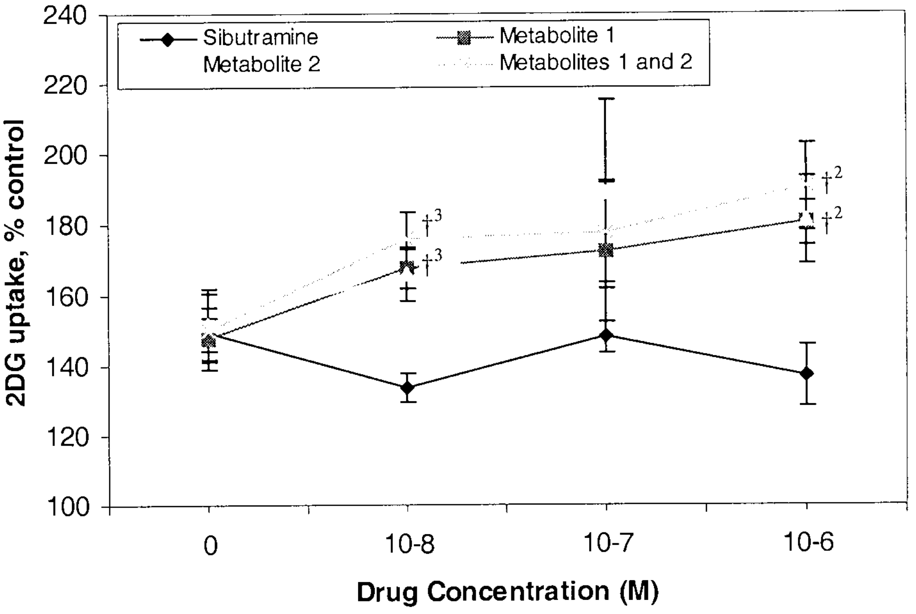
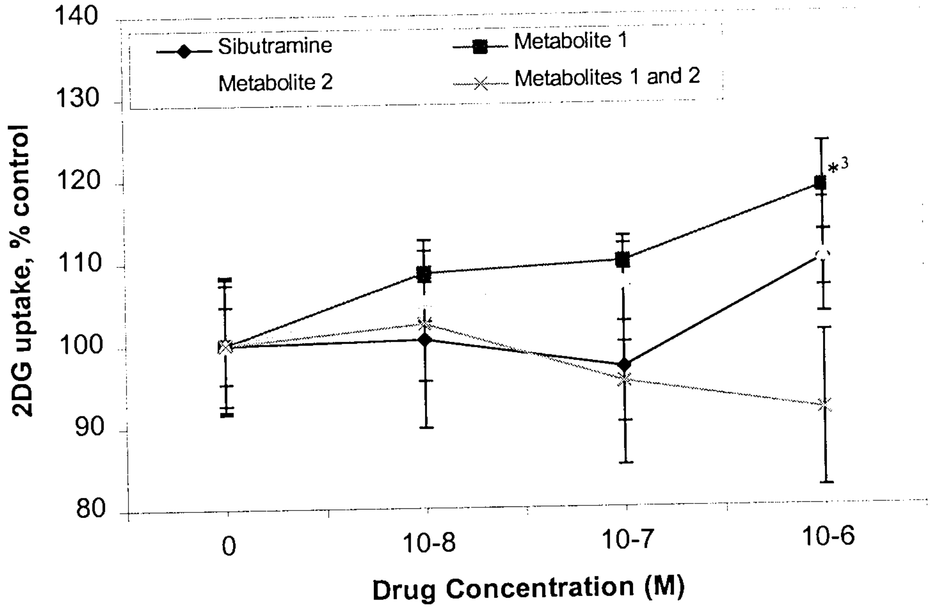
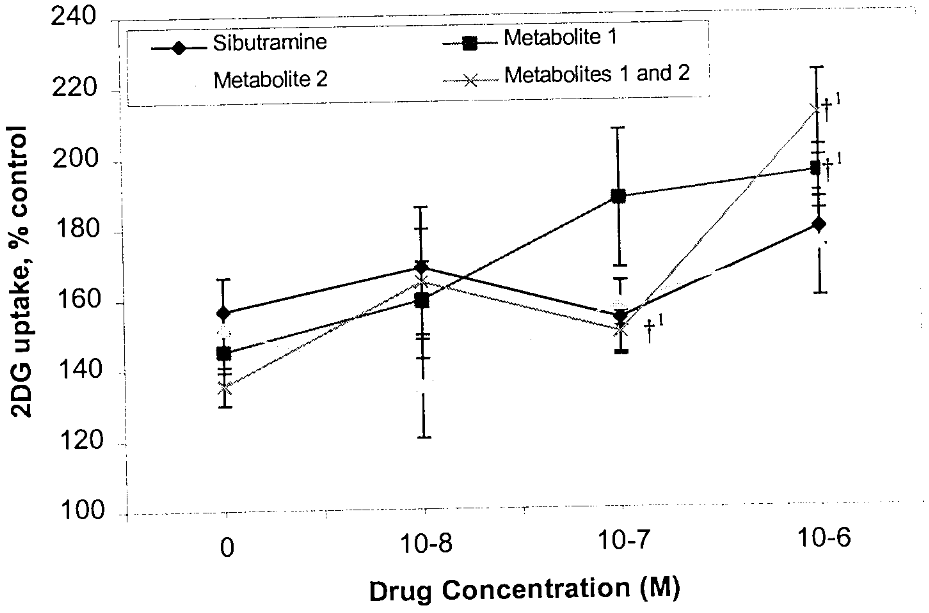


Figure 5.3 Effect of sibutramine, M1, M2 and M1 and M2 added together on 2DG uptake in L6 cells. Cells were exposed to sibutramine, M1, M2 or M1 and M2 at concentrations 10^{-8}M – 10^{-6}M for 72h and/or insulin (10^{-8}M) for 24h. In the absence of added insulin (**5.3a**), sibutramine and M2 had no effect on 2DG uptake. M1 (10^{-6}M) increased 2DG uptake by 19%. Addition of insulin (**5.3b**) for 24h increased 2DG uptake by approximately 40%. Incubation of the cells with Sibutramine and M2 for 72h and insulin for the final 24h had no effect on insulin-stimulated 2DG uptake, but M1 (10^{-6}M) and a combination of M1 and M2 (10^{-6}M and 10^{-7}M) increased 2DG uptake by 50%. Data are expressed as % control, values are mean ± SEM, n=6. *Significance versus control, †significance versus insulin alone, ^{1}p<0.01, ^{2}p<0.02, ^{3}p<0.05.

5.3a 72h without added insulin



5.3b 72h with added insulin



M1 had no effect on insulin-stimulated glucose uptake after 24h incubations. However, M1 increased insulin-stimulated glucose uptake by 20% - 33% after 48h incubations ($p<0.05$ 10^{-8}M M1 versus insulin and $p<0.02$ 10^{-6}M M1 versus insulin, figure 5.2b). High concentrations of M1 increased insulin-stimulated glucose uptake by 49% after 72h incubations of L6 cells ($p<0.01$ versus insulin alone, figure 5.3b). Incubation of L6 cells with M1 and M2 together increased insulin-stimulated glucose uptake by 16% (24h, 10^{-7}M $p<0.02$ versus insulin, figure 5.1b), 26% - 40% (48h, $p<0.05$ 10^{-8}M versus insulin, $p<0.02$ 10^{-6}M versus insulin, figure 5.2b), and 15% - 76% (72h, $p<0.01$ versus insulin, figure 5.3b).

5.1.2b Mechanism of Action of Metabolite 2

Incubation of M2 (10^{-6}M) for 24h with a range of insulin concentrations (10^{-9}M – 10^{-6}M) shifted the insulin concentration-response curve to the left (figure 5.4). Using near-physiological concentrations of insulin, M2 significantly increased insulin-stimulated glucose uptake by 28.9% and 13.2% ($p<0.05$ versus insulin alone). M2 did not significantly increase 10^{-7}M and 10^{-6}M insulin-stimulated glucose uptake.

M2 alone stimulated glucose uptake by 34%, this stimulatory effect of M2 on basal glucose uptake was not inhibited by LY-294,002 (figure 5.5). In comparison, basal glucose transport was inhibited by LY-294,002 in a dose-dependent manner. Maximal inhibition was observed using 10^{-5}M LY-294,002 which significantly decreased basal glucose transport by 48% ($p<0.01$).

Figure 5.4 Effect of M2 on insulin-stimulated 2DG uptake in L6 cells. Cells were incubated with a range of insulin concentrations (10^{-9}M – 10^{-6}M) and M2 (10^{-6}M) for 24h to demonstrate the effect of M2 on the insulin-dose response curve. M2 shifted the dose response to insulin to the left. Data are expressed as % control, values are mean ± SEM, n=6. *Significance versus control, †significance versus insulin alone, ^{1}p<0.01, ^{2}p<0.02, ^{3}p<0.05.

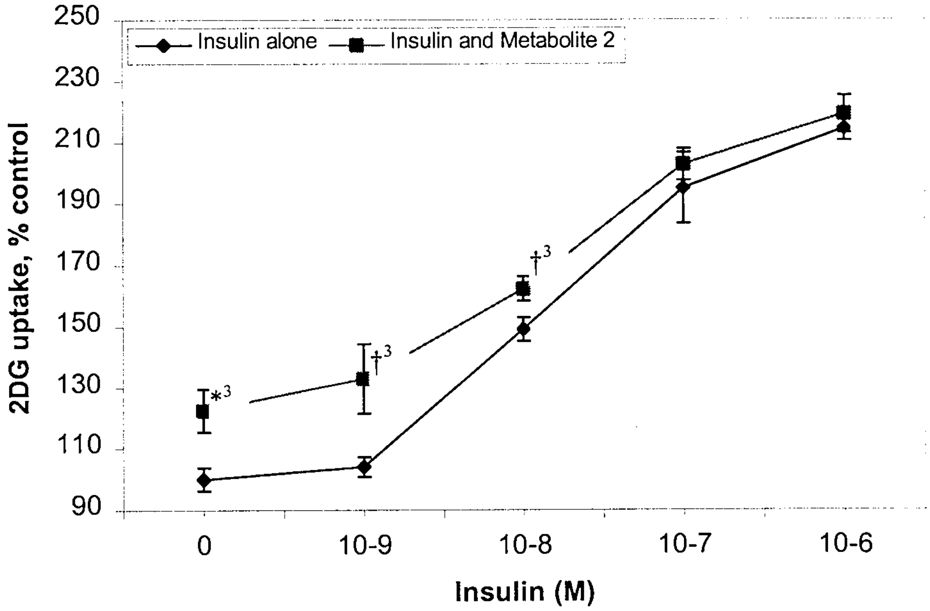


Figure 5.5 Effect of the PI 3-kinase inhibitor LY-294,002 on M2-stimulated 2DG uptake in L6 cells. Cells were cultured with LY-294,002 and/or M2 for 24h. LY-294,002 decreased basal 2DG uptake in a concentration-dependent manner, but had no effect on M2-stimulated 2DG uptake. Data are expressed as % control, values are mean ± SEM, n=6. *Significance versus no LY-294,002, $^1p<0.01$, $^2p<0.02$, $^3p<0.05$.

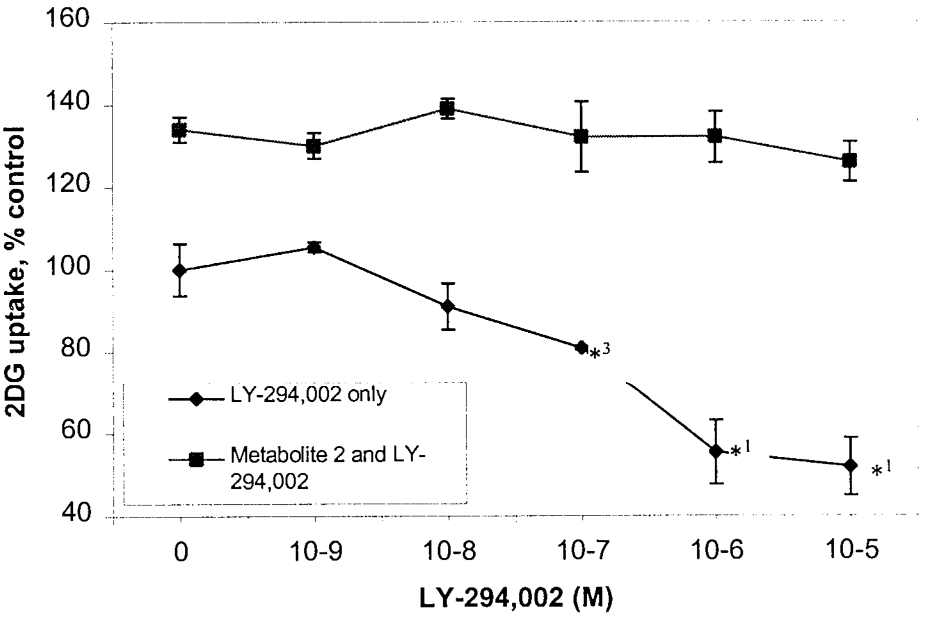


Figure 5.6 Inhibition of basal and M2-stimulated 2DG uptake by the high affinity inhibitor of glucose transporters cytochalasin B, in L6 cells. Cells were cultured with cytochalasin B (10^{-9}M – 10^{-6}M) and/or M2 for 24h. Cytochalasin B decreased both basal and M2-stimulated 2DG uptake in a concentration dependent manner. Data are expressed as % control, values are mean ± SEM, n=6. *Significance versus no cytochalasin B, ^{1}p<0.01, ^{2}p<0.02.

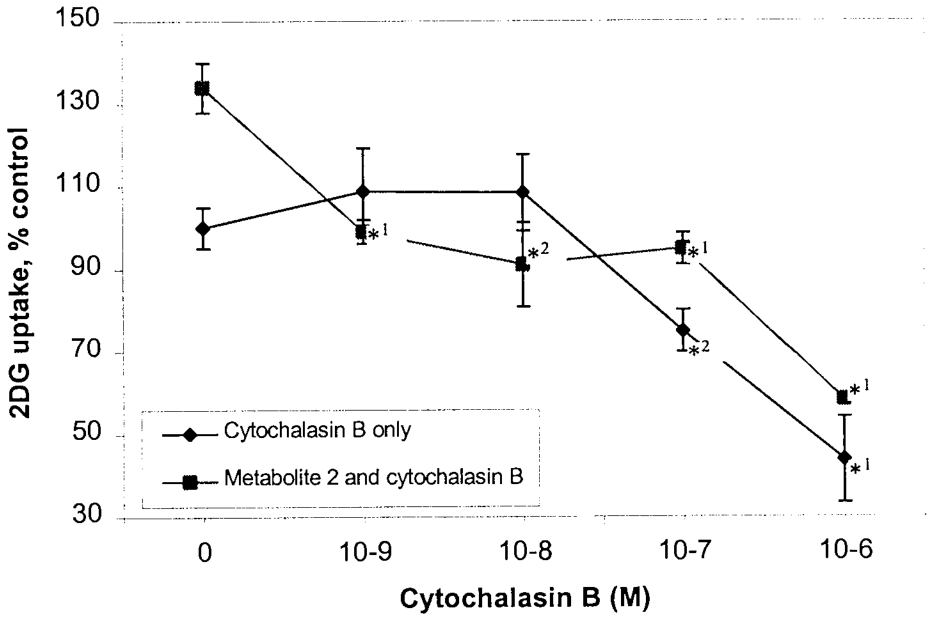
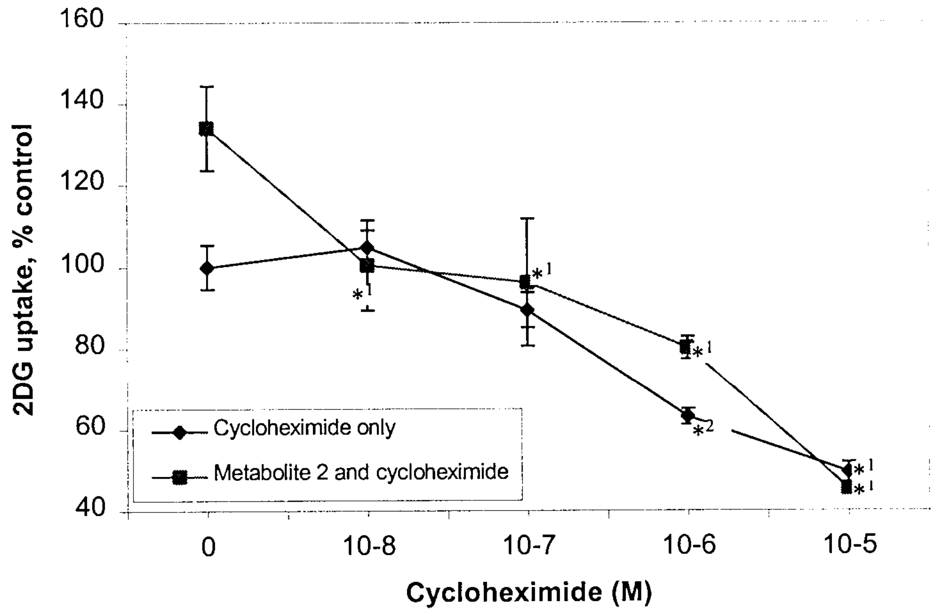


Figure 5.7 Inhibition of basal and M2-stimulated 2DG uptake by cycloheximide an inhibitor of protein synthesis in L6 cells. Cells were stimulated with cycloheximide (10^{-8}M – 10^{-5}M) and/or M2 (10^{-6}M) for 24h. Cycloheximide decreased both basal and M2-stimulated 2DG uptake in a concentration dependent manner. Data are expressed as % control, values are mean ± SEM, n=6, *Significance versus no cycloheximide, $^{1}p<0.01$, $^{2}p<0.02$.



Cytochalasin B reduced M2-stimulated glucose transport in a manner comparable to the effect of cytochalasin B on basal transport alone (figure 5.6). Both basal and M2-stimulated glucose transport was inhibited by cytochalasin B in a dose dependent manner. High concentrations of cytochalasin B decreased basal glucose uptake by 25% - 56% ($p<0.02$ 10^{-7}M cytochalasin B versus control, $p<0.01$ 10^{-6}M cytochalasin B versus control), however lower concentrations of cytochalasin B had no effect on basal glucose transport. Cytochalasin B decreased M2-stimulated glucose uptake at all concentrations investigated. High concentrations of cytochalasin B decreased M2-stimulated glucose uptake by 39% - 76% (10^{-7}M and 10^{-6}M $p<0.01$ versus M2 alone). 10^{-8}M cytochalasin B reduced M2-stimulated glucose uptake by 43% ($p<0.02$ versus M2 alone) and 10^{-9}M cytochalasin B reduced M2-stimulated glucose uptake by 35% ($p<0.01$ versus M2 alone).

Cycloheximide decreased both basal and M2-stimulated glucose uptake in a concentration-dependent manner (figure 5.7). 24h incubation of L6 cells with cycloheximide inhibited basal glucose transport by 37% - 50% (10^{-6}M – 10^{-5}M cycloheximide $p<0.02$ and $p<0.01$ versus control). 10^{-7}M cycloheximide reduced basal glucose transport by 11% (not statistically significant) and 10^{-8}M had no effect. Cycloheximide reduced M2-stimulated glucose uptake by 34% - 89%. The inhibitory effect was found to be highly significant at all concentrations of cycloheximide investigated (10^{-8}M – 10^{-5}M, $p<0.01$ versus M2 alone).

5.1.2c Comparative Agents

Fluoxetine and nisoxetine (10^{-8}M-10^{-6}M), (which are known to selectively inhibit the reuptake of serotonin and noradrenaline respectively), did not significantly alter glucose uptake after 24h incubations with and without added insulin (10^{-8}M, figure 5.8a and 5.8b). At 10^{-6}M, serotonin and dopamine reduced insulin-stimulated glucose uptake (by 24% and 45%, respectively, $p<0.02$ 10^{-6}M serotonin versus insulin and $p<0.01$ 10^{-6}M dopamine versus insulin, figure 5.9b), and dopamine (10^{-6}M) reduced glucose uptake (by 17%, $p<0.05$ versus control) after 24h incubation without added insulin (figure 5.9a). Lower concentrations of serotonin and dopamine, and the range of noradrenaline concentrations tested (10^{-8}M-10^{-6}M) did not significantly alter glucose uptake (figure 5.9a and 5.9b).

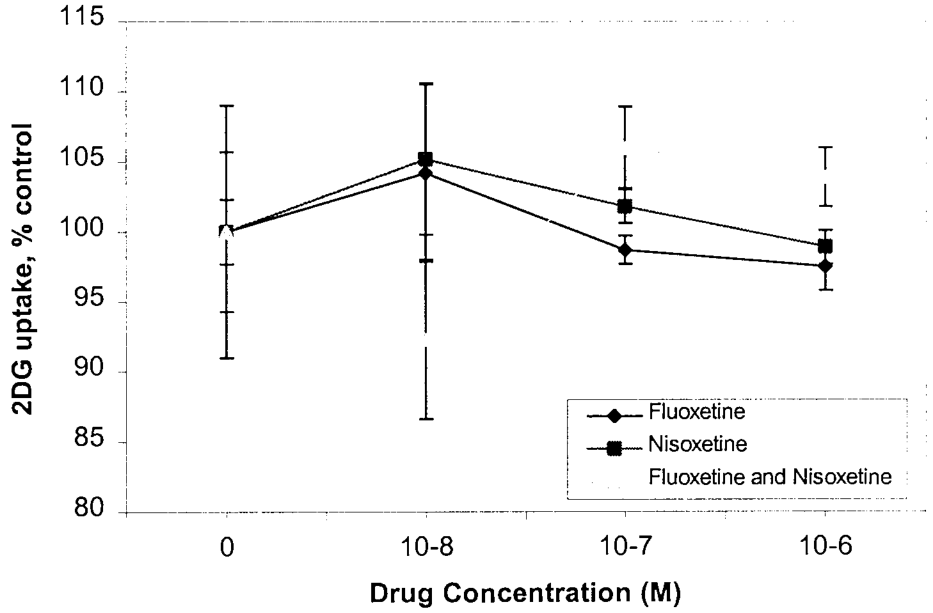
The serotonin releaser fenfluramine and its active metabolite nordexfenfluramine (10^{-8}M-10^{-6}M) did not significantly alter glucose uptake after 24h incubation without added insulin (figure 5.10a). However insulin-stimulated glucose uptake was increased by 10^{-8}M and 10^{-7}M nordexfenfluramine (by 25% and 21%, $p<0.02$, figure 5.10b). The noradrenaline releaser phentermine (10^{-8}M-10^{-6}M) did not affect glucose uptake after 24h incubations with and without added insulin (figure 5.10a and 5.10b).

5.1.3 Discussion

The results show that sibutramine metabolites can increase glucose uptake by cultured L6 muscle cells. Sibutramine is metabolised *in vivo* to the secondary amine metabolite M1, and then to the primary amine metabolite M2. M2 increased glucose uptake by 24h whereas M1 was not effective at 24h, but became effective by 72h. This suggests

Figure 5.8 Effect of fluoxetine and nisoxetine on 2DG uptake in L6 cells. Cells were exposed to fluoxetine, nisoxetine and fluoxetine and nisoxetine together at concentrations 10^{-8}M – 10^{-6}M and/or insulin (10^{-8}M) for 24h. In the absence of added insulin **(5.8a)**, fluoxetine and nisoxetine had no effect on 2DG uptake. Addition of insulin **(5.8b)** for 24h increased 2DG uptake by approximately 40%. Fluoxetine and nisoxetine had no effect on insulin-stimulated glucose uptake after 24h incubations in L6 cells. Data are expressed as % control, values are mean ± SEM, n=6.

5.8a without added insulin



5.8b with added insulin

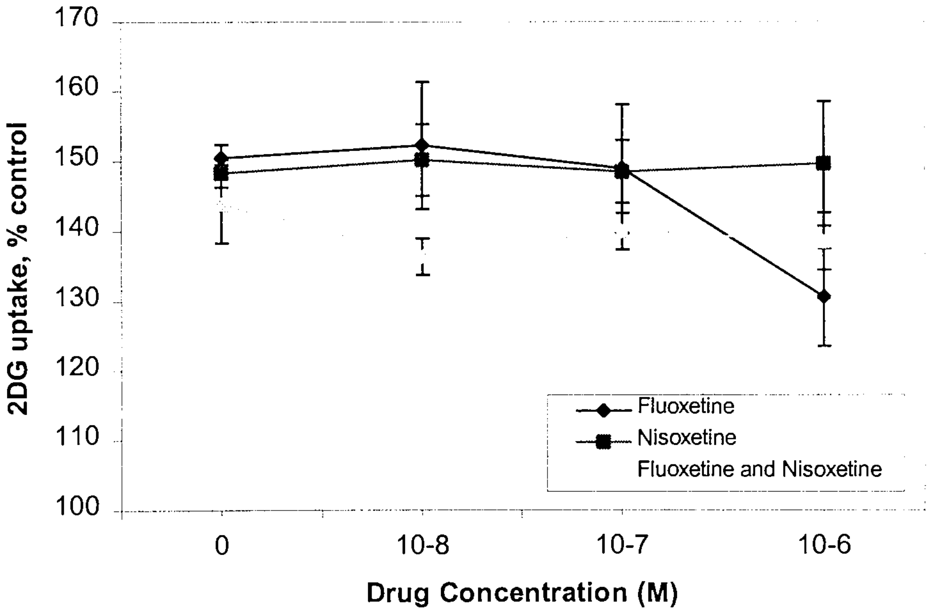
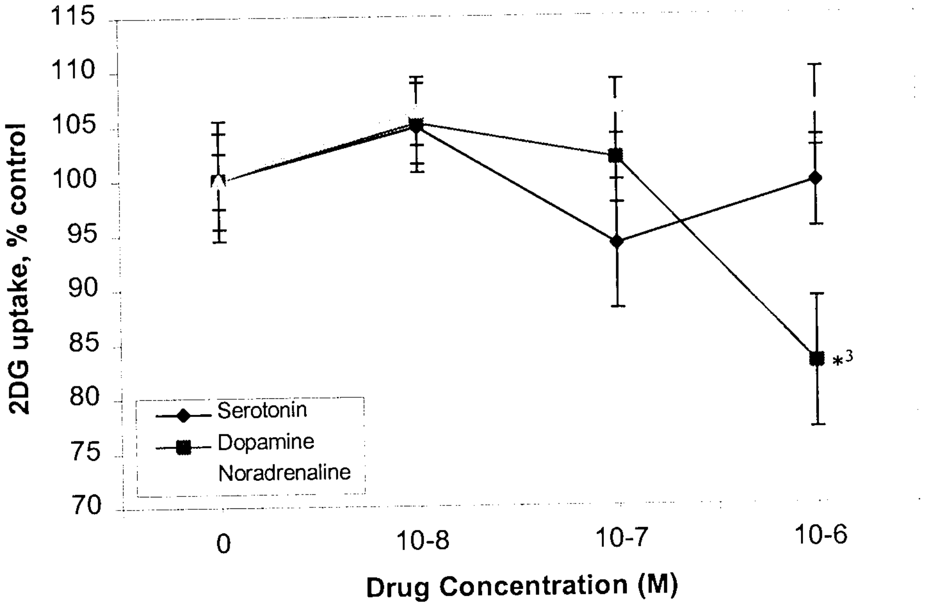


Figure 5.9 Effect of serotonin, dopamine and noradrenaline on 2DG uptake in L6 cells. Cells were exposed to serotonin, dopamine and noradrenaline at concentrations 10^{-8}M – 10^{-6}M and/or insulin (10^{-8}M) for 24h. In the absence of added insulin **(5.9a)**, serotonin and noradrenaline had no effect on 2DG uptake, however dopamine (10^{-6}M) significantly decreased basal 2DG uptake by 17%. Addition of insulin **(5.9b)** for 24h increased 2DG uptake by approximately 40%. Serotonin (10^{-6}M) significantly decreased insulin-stimulated 2DG uptake by 24% and dopamine (10^{-6}M) significantly reduced insulin-stimulated 2DG uptake by 45%. Noradrenaline had no effect on insulin-stimulated 2DG uptake in L6 cells. Data are expressed as % control, values are mean ± SEM, n=6. *Significance versus control, †significance versus insulin alone, $^{1}p<0.01$, $^{2}p<0.02$, $^{3}p<0.05$.

5.9a without added insulin



5.9b with added insulin

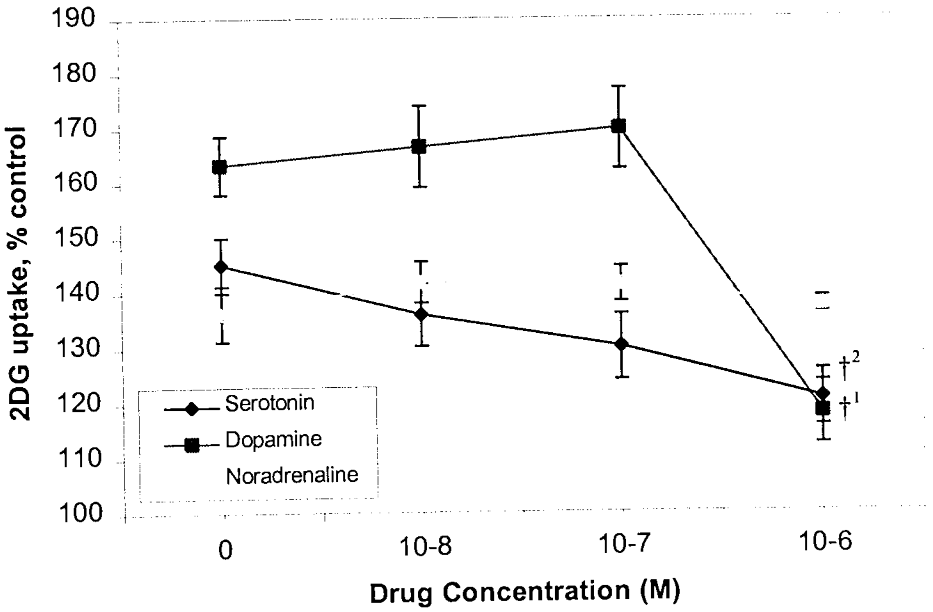
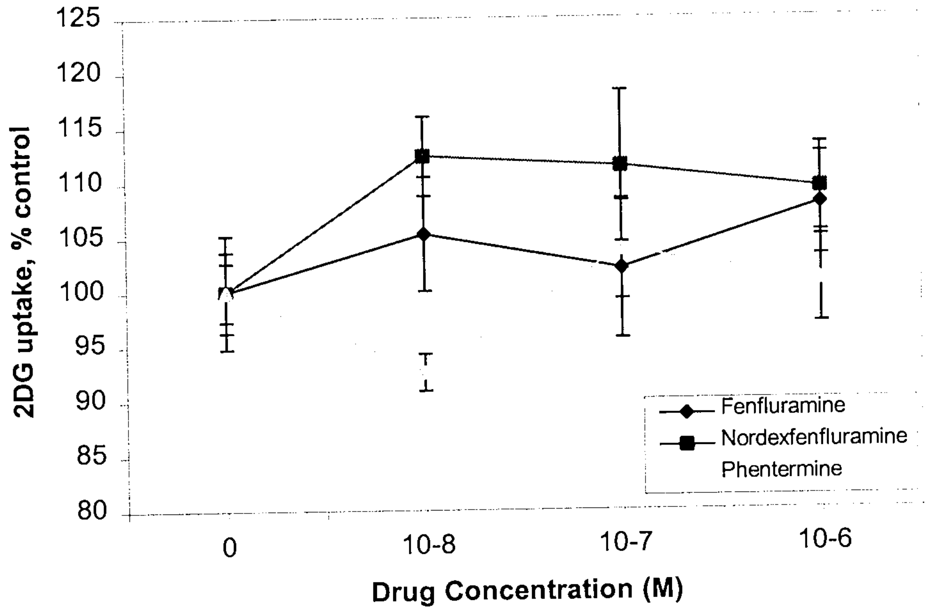
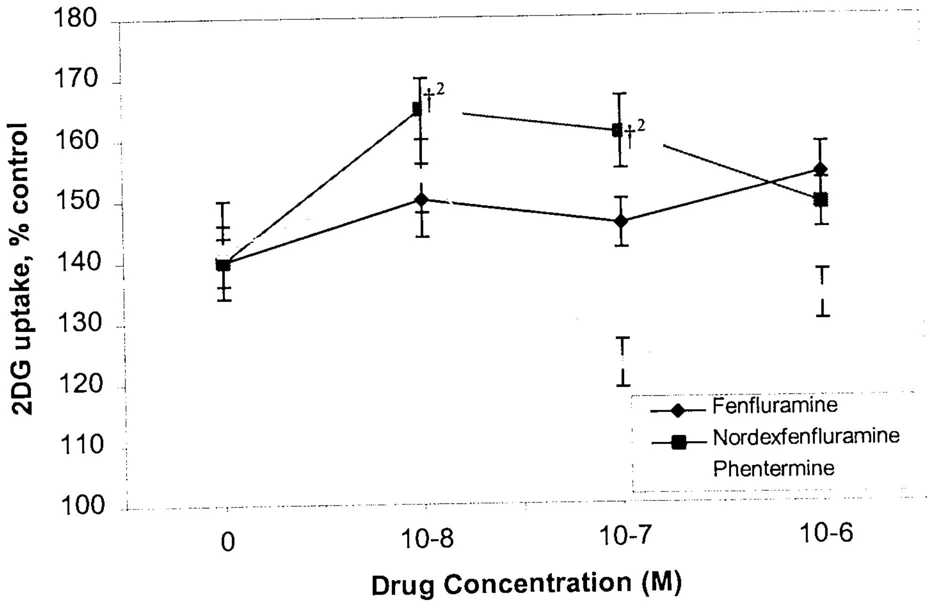


Figure 5.10 Effect of fenfluramine, nordexfenfluramine and phentermine on 2DG uptake in L6 cells. Cells were exposed to fenfluramine, nordexfenfluramine and phentermine at concentrations 10^{-8}M – 10^{-6}M and/or insulin (10^{-8}M) for 24h. In the absence of added insulin **(5.10a)**, fenfluramine, nordexfenfluramine and phentermine had no effect on 2DG uptake. Addition of insulin **(5.10b)** for 24h increased 2DG uptake by approximately 40%. Insulin-stimulated 2DG uptake was significantly increased by nordexfenfluramine (10^{-7}M and 10^{-6}M). Fenfluramine and phentermine had no effect on insulin-stimulated 2DG uptake in L6 cells. Data are expressed as % control, values are mean ± SEM, n=6. †significance versus insulin alone, $^{2}p<0.02$.

5.10a without added insulin



5.10b with added insulin



that gradual conversion of M1 to M2 could at least partly account for the effectiveness of M1 after M2.

Although M2 increased glucose uptake by muscle cells incubated without added insulin (basal uptake), this does not exclude insulin, since the cells were exposed to low insulin concentrations in the FCS. FCS contains approximately 5.96μIU/ml bovine insulin (112 batches were tested and the insulin concentrations ranged 2.56-12.11μIU information supplied by Gibco), it would be expected that this would be almost completely utilised from the medium in 24h. The greater/main increase in glucose uptake at submaximally stimulating insulin concentrations (of little compared with maximally stimulating insulin concentration) indicates that M2 is predominantly increasing insulin sensitivity, with little increase in total responsiveness to maximally-stimulating insulin concentration. This suggests that M2 may activate or enhance signalling pathways and/or the final biological effectors that mediate insulin action, rather than cellular mechanisms that are entirely independent of insulin (which might be expected to be additive to insulin). Improved insulin sensitivity by M2 in muscle cells is consistent with the amelioration of insulin resistance during sibutramine treatment in *ob/ob* mice (Day and Bailey, 1998), and the improved glycaemic control during sibutramine treatment in obese Type 2 diabetic patients (Vargas et al., 1994; Griffiths et al., 1995). Although decreased adiposity itself would be expected to reduce insulin resistance (Mingrone et al., 1997), the present study suggests that M2 also acts directly on muscle to improve insulin action. Skeletal muscle is quantitatively the major site of insulin stimulated glucose disposal (reviewed by DeFronzo et al., 1992), and an important focus of insulin resistance in obese Type 2 diabetic patients.

M2 was not inhibited by LY-294,002, therefore it is unlikely that the action of M2 in increasing glucose uptake in L6 cells is mediated via PI 3-kinase. Thus M2 acts to modify glucose uptake in skeletal muscle cells via a mechanism that is either separate from the insulin signalling pathway or at least distal to the action of PI 3-kinase. It is possible that M2 binds to a separate receptor and intracellular signalling is mediated by one or more unknown proteins that act upon the insulin-signalling cascade down stream from PI 3-kinase. The increase in glucose uptake observed is mediated by facilitated diffusion of glucose as the effect of M2 was completely inhibited by cytochalasin B. Thus M2 increases glucose uptake via a glucose transporter dependent mechanism. This action is not apparent until 24h after cell exposure to M2. Thus this effect is likely to be dependent upon glucose transporter synthesis rather than translocation or an increase in intrinsic activity (as an effect of M2 would have been observed at earlier time points). This is supported by the cycloheximide data obtained in this chapter. Cycloheximide is an inhibitor of protein synthesis at the level of translation, which was found to completely inhibit M2-stimulated glucose uptake. Thus it is likely that M2 acts independently of PI 3-kinase to increase glucose transport via the synthesis of glucose transporters.

Sibutramine acts as an SNRI to induce satiety in rodents, enhancing activation of 5HT2A/2C receptors and $\alpha 1$ and $\beta 1$ adrenoceptors (Jackson et al., 1997). To investigate whether these mechanisms could account for increased glucose uptake in muscle cells, experiments were undertaken with serotonin, noradrenaline, fluoxetine (serotonin reuptake inhibitor) and nisoxetine (noradrenaline reuptake inhibitor). None of these agents increased glucose uptake, suggesting that M2 is not acting on the muscle cells via serotonin or noradrenaline. Very high concentrations of sibutramine may act as dopamine receptor agonists, but there was no evidence that dopamine could stimulate glucose uptake in muscle. Indeed, high concentrations (10^{-6}M) of serotonin and dopamine decreased glucose uptake.

The serotonin-releasing anorectic agent fenfluramine has been reported to increase insulin action *in vivo* and *in vitro*, independently of its weight reducing effect. Although fenfluramine itself did not significantly alter glucose uptake into the muscle cells, its active metabolite nordexfenfluramine increased insulin-stimulated glucose uptake, presumably via a mechanism that is independent of serotonin. The lack of effect of the anorectic agent phentermine (a noradrenaline releaser) on glucose uptake by the muscle cells substantiates that an improvement of insulin sensitivity is not a general feature of amphetamine based anorectic agents (Kosmiski and Eckel, 1997).

Thus, sibutramine acts via its metabolites, principally by the M2 amine, to increase insulin-sensitive glucose uptake by cultured L6 muscle cells. This is independent of the SNRI properties of sibutramine, and provides a means of reducing insulin resistance, which is separate from that associated with decreased adiposity.

Part Two: *In Vitro* Studies Using Isolated Tissue and *In Vivo* Experimentation

The effects of M2 on glucose metabolism were investigated in the rat L6 skeletal muscle cell line in part 1 of this chapter. The second part of this chapter is focused upon the investigation of M2 in other models of skeletal muscle and to determine the effects of M2 *in vivo*.

5.2.1 Experimental Design

5.2.1a Acute Effect of Oral Administration of M2 on Basal Glycaemia in Normal Mice

To further evaluate M2, it was administered orally to normal mice so as to assess its effects on glycaemia. 60 adult male homozygous lean (+/+) mice from the colony at Aston University were housed as described previously (section 2.3.1). M2 was dissolved in PBS and administered orally at a dose of 10mg/kg to 30 mice. Control mice were similarly prepared but received vehicle only. Food was withheld during the test (at time zero), water was available *ad libitum.* Blood samples were taken from the tail tip for plasma glucose determination (as described in section 2.3.2) at 2h intervals for 8h and a 24h sample was also taken. At time intervals 4h, 6h, 10h, 18h and 24h a group of 10 mice (5 control and 5 M2 treated mice) were removed from the study and sacrificed for the determination of glucose uptake into soleus muscles as described below (5.2.1b).

Significance was assessed by Student's unpaired 't' test (2.6) and values were considered to be significant if $p<0.05$.

5.2.1b Acute Effect of M2 on 2-Deoxy-[^{3}H]-Glucose Uptake in Isolated Soleus Muscles of Lean Mice

60 adult male homozygous lean mice received M2 or vehicle only as described above. At timed intervals, 10 mice (5 control and 5 M2 treated mice) were killed by cervical dislocation. Soleus muscles were dissected (2.3.7 and appendix IV, illustration I) and glucose uptake measured as described in 2.4.2 with added insulin (10^{-8}M).

Significance was assessed by Student's paired 't' test (2.6) and values were considered significant if $p<0.05$.

5.2.1c Acute Effect of M2 on Basal Glycaemia in *ob/ob* Mice

Ten 20-week-old *ob/ob* mice from the colony at Aston University were housed as described previously (section 2.3.1) and separated into 2 groups according to basal glycaemia. M2 was dissolved in PBS and administered orally at a dose of 10mg/kg to 5 of the mice. The remaining 5 mice received PBS only. Food was withheld during the test (at time zero), water was available *ad libitum*. Blood samples were taken from the tail tip for plasma glucose determination (as described in section 2.3.2) at time zero, 18h after M2 administration and 24h after M2 administration. Data were analysed as described above (5.2.1a).

5.2.1d Acute Effect of M2 on 2-Deoxy-[^{3}H]-Glucose Uptake in Isolated Soleus Muscles and Hemi-diaphragms of *ob/ob* Mice

Ten 20-week-old *ob/ob* mice from the Aston colony were separated into 2 groups, control and M2-treated (10mg/kg po) as described above (5.2.1c). 24h after M2 administration, both groups of mice were killed by cervical dislocation, and soleus muscles and hemi-diaphragms dissected (2.3.7 and appendix IV, illustrations I and II). 2DG uptake into the tissues was measured (with added insulin 10^{-8}M) as described in 2.4.2. Data were analysed as 5.2.1b.

5.2.2 Results

5.2.2a Acute Effect of M2 on Basal Glycaemia in Lean Mice

Administration of M2 (10mg/kg po) to normal lean mice, resulted in a greater lowering of plasma glucose within 8h of treatment (4.89mmol/l in M2-treated versus 8.83 in mmol/l control, $p<0.01$, figure 5.11). The hypoglycaemic effect of M2 was both rapid and significant. 4h after administration of M2, the treated mice had plasma glucose concentrations of 6.24mmol/l versus control mice 8.47mmol/l ($p<0.01$). Within 6h of M2 treatment, test mice had mean plasma glucose concentrations of 5.29mmol/l and control mice 8.79mmol/l ($p<0.01$). However, after an overnight fast (24h after M2 administration) both control and M2 treated mice had similar plasma glucose concentrations of approximately 4.5mmol/l.

5.2.2b Acute Effect of M2 on 2-Deoxy-[^{3}H]-Glucose Uptake in Isolated Soleus Muscles of Lean Mice

Over 24h, glucose uptake by soleus muscles of lean, normal control mice decreased by 50% (figure 5.12). This was a gradual decrease that began approximately 6h after food was withdrawn. M2 treated mice were also starved at time zero, however glucose uptake in soleus muscles of these mice did not decrease over 24h, or at any of the time intervals investigated. Glucose uptake in soleus muscles of these mice remained at levels observed prior to administration of M2. Thus 24h after M2 administration glucose uptake was significantly increased ($p<0.01$ versus control).

Figure 5.11 Effect of oral administration of M2 (10mg/kg) on basal glycaemia in normal mice. Mice received either M2 or vehicle-only (control group). Food was withheld during the test but drinking water was available *ad libitum*. Plasma glucose concentration was determined at timed intervals for 24h. Administration of M2 to normal lean mice, resulted in a significant decrease in plasma glucose concentration within 8h of treatment compared to control mice. After an overnight fast, both M2 treated and control mice had similar plasma glucose concentrations. Data are expressed as mean plasma glucose concentrations (mmol/l) ± SEM. *Significance versus control mice plasma glucose concentrations, $^1p<0.01$, n=30.

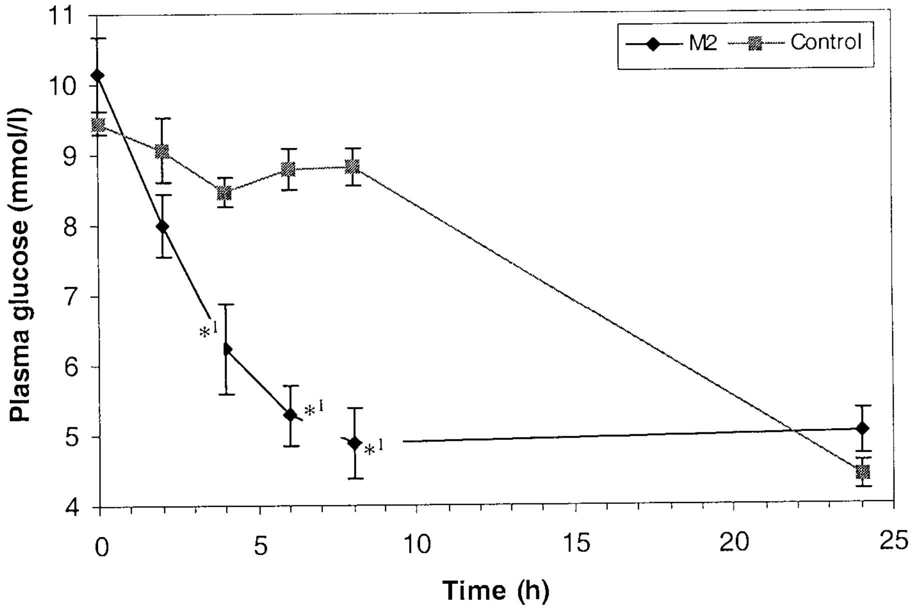
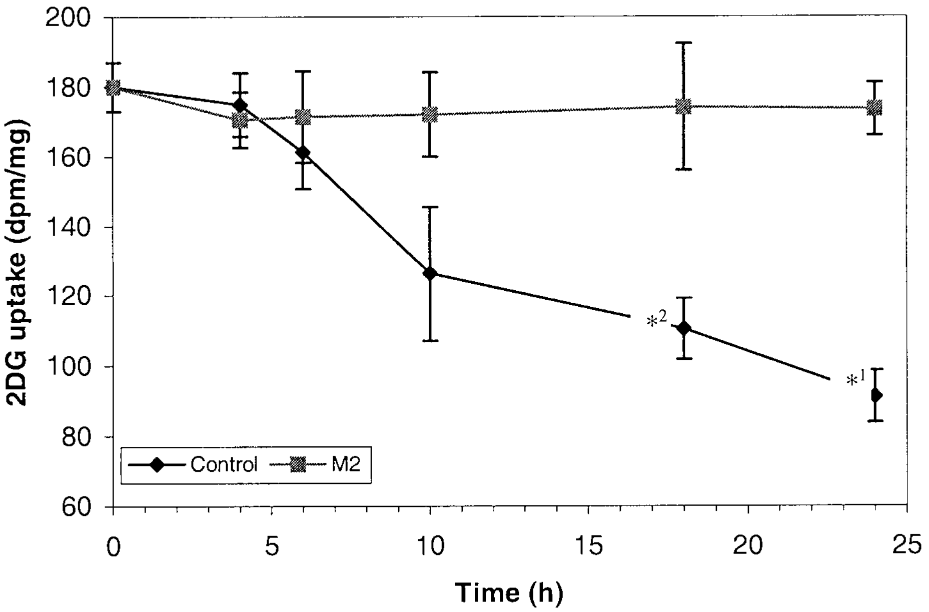


Figure 5.12 Effect of oral administration of M2 (10mg/kg) on 2DG uptake in soleus muscles of normal mice. Mice received either M2 or vehicle-only (control group). Food was withheld during the test but drinking water was available *ad libitum*. Mice were sacrificed at timed intervals and 2DG uptake into soleus muscles measured. 2DG uptake into soleus muscles of lean control mice decreased over the 24h fasted period, however 2DG uptake in soleus muscles of M2-treated mice remained increased throughout the period of study. Data are expressed as mean dpm/mg ± SEM. *Significance versus control mice plasma glucose concentrations, $^{1}p<0.01$ $^{2}p<0.02$, n=5.



5.2.2c Acute Effect of M2 on Basal Glycaemia in *ob/ob* Mice

The *ob/ob* mice were severely hyperglycaemic with nonfasted plasma glucose concentrations of approximately 24mmol/l. At time zero, the mice were fasted and in control mice plasma glucose concentrations did not significantly decrease over a 24h period (23.6mmol/l at time zero versus 22.2mmol/l after 24h fast). M2-treated mice had nonfasted plasma glucose concentrations of 25.5mmol/l (time zero). 18h after M2 administration and the removal of food, plasma glucose concentrations had fallen to 19.6mmol/l (not statistically significant versus control mice). However 24h after M2 administration, plasma glucose levels of M2-treated mice had decreased to 12.8mmol/l versus 22.2mmol/l control mice. This was not statistically significant versus control mice but was found to be a significant decrease compared to M2-treated mice at time zero ($p<0.02$, figure 5.13).

5.2.2d Acute Effect of M2 on 2-Deoxy-[^{3}H]-Glucose Uptake in Isolated Soleus Muscles and Hemidiaphragms of *ob/ob* Mice

Administration of a single dose M2 to hyperglycaemic *ob/ob* mice had no effect on glucose uptake, 24h after M2 administration (figure 5.14). Glucose uptake into soleus muscles and hemidiaphragms isolated from *ob/ob* mice remained at levels comparable to control mice.

Figure 5.13 Effect of oral administration of M2 (10mg/kg) on basal glycaemia in *ob/ob* mice. Mice received either M2 or vehicle-only (control group). Food was withheld during the test but drinking water was available *ad libitum*. Plasma glucose concentration was determined 18h and 24h after M2 administration. Administration of M2 to normal lean mice, resulted in a significant decrease in plasma glucose concentration after 24h of treatment compared to control mice. Data are expressed as mean plasma glucose concentrations (mmol/l) ± SEM. *Significance versus time zero plasma glucose concentrations, $^2p<0.01$, n=5.

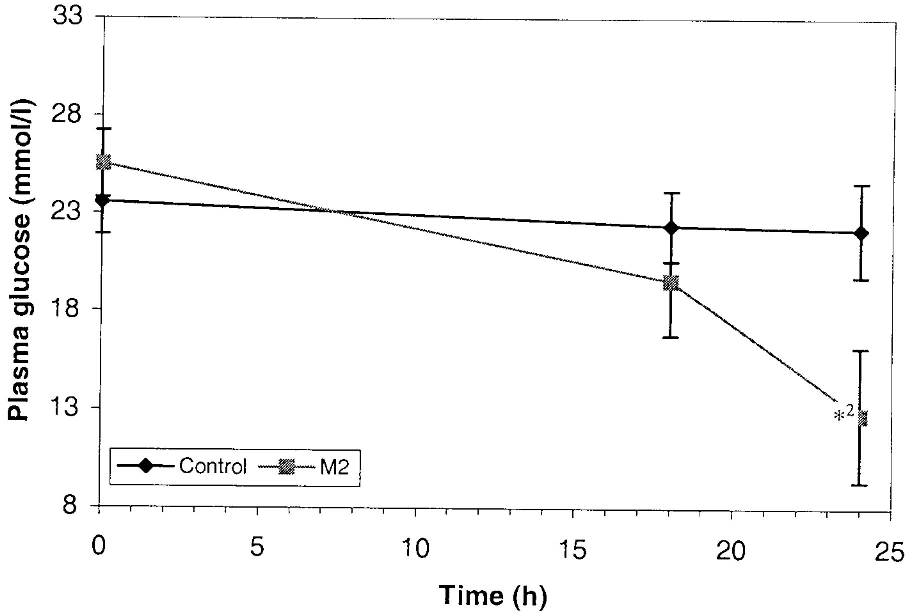
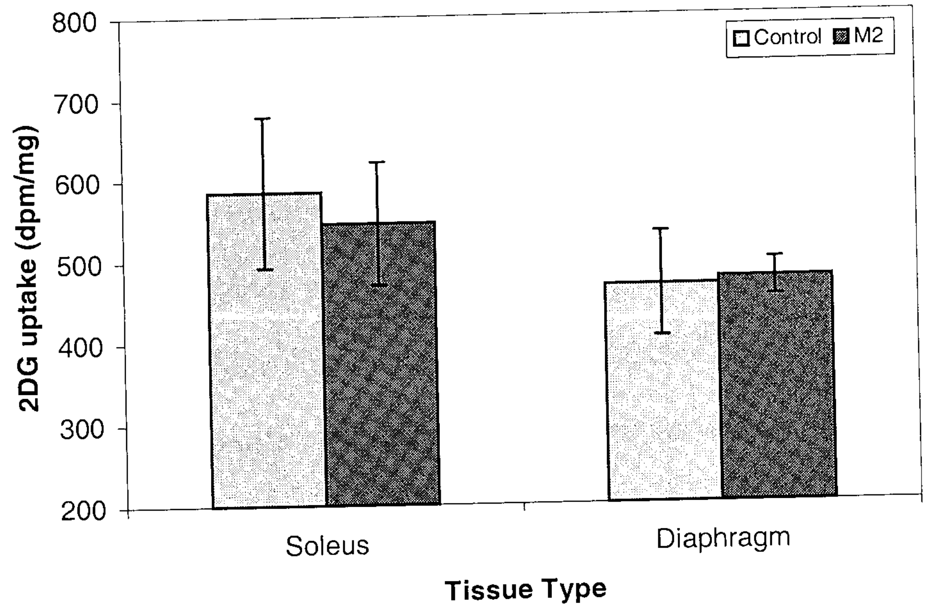


Figure 5.14 Effect of oral administration of M2 (10mg/kg) on 2DG uptake in soleus muscles and hemidiaphragms of *ob/ob* mice. Mice received either M2 or vehicle-only (control group). Food was withheld during the test but drinking water was available *ad libitum*. Mice were sacrificed 24h after M2 administration and 2DG uptake into soleus muscles and hemidiaphragms measured. M2 did not alter 2DG uptake into soleus muscles or hemidiaphragms of *ob/ob* mice. Data are expressed as mean dpm/mg ± SEM, n=5.



5.2.3 Discussion

To further investigate the evidence of M2 actions obtained in Part One of this chapter, the effects of M2 *in vivo* were determined. M2 was demonstrated to rapidly decrease plasma glucose concentrations of lean non-diabetic mice. This effect was at least partly due to a significant increase in peripheral glucose transport as observed in isolated soleus muscles of M2 treated mice. This observation is in agreement with the effects of M2 in L6 skeletal muscle cells, thus indicating a direct effect of M2 to increase glucose transport.

The effects of M2 were observed in mice that were fasted throughout the duration of the experiment, therefore the action of M2 is likely to be independent of any satiety inducing effects. The thermogenic effects of sibutramine and its metabolites have recently been investigated by measuring oxygen consumption in rats treated with sibutramine, M1 or M2. Sibutramine and its metabolites caused a dose dependent increase in oxygen consumption that were sustained for at least 6h, accompanied by significant increases in body temperature. The use of specific adrenoceptor antagonists and ganglionic blockers demonstrated that stimulation of thermogenesis by sibutramine and its metabolites requires central reuptake inhibition of both serotonin and noradrenaline, resulting in increased efferent sympathetic activation of BAT thermogenesis via beta(3)-adrenoceptor (Connoley et al., 1999). Therefore the glucose lowering effect of M2 in lean mice observed in this chapter were not independent of possible thermogenic effects of the compound. A combination of increased thermogenesis and increased peripheral glucose transport in non-diabetic lean mice may be the mechanism by which M2 acts to decrease glycaemia.

The effects of M2 were further investigated in *ob/ob* mice. M2 modestly decreased plasma glucose concentrations in these mice over a period of 24h. The effects were not as rapid as those demonstrated in the lean non-diabetic mice, but were significant when compared to the plasma glucose concentrations determined prior to M2 administration. The glucose lowering effect was independent of the satiety-inducing actions, because mice were fasted throughout the duration of the test; thus the effects must be mediated by a different mechanism. M2 had no effect on glucose transport in either soleus muscles or diaphragms isolated from M2-treated mice. Hence the direct effect of M2 on glucose transport appears to be lost in the obese, insulin resistant state. Therefore the effect of M2 in lowering glycaemia in *ob/ob* mice cannot be attributed to a direct action of M2 on peripheral glucose transport and must be mediated by a different mechanism. Sibutramine (but not M2) has previously been investigated in *ob/ob* mice (Day and Bailey 1998). Chronic administration of sibutramine reduced weight gain, lowered non esterified fatty acid concentrations and decreased hyperinsulinaemia. Therefore the acute effect of M2 in *ob/ob* mice may be mediated via thermogenic effects and alternative mechanisms to ameliorate insulin resistance but not via a direct action on skeletal muscle.

It was established in Part One of this chapter that M2 increases glucose transport in non-insulin resistant skeletal muscle. The mechanism of action was observed to be dependent upon protein synthesis and facilitated glucose transport but not via a PI 3-kinase dependent pathway. Thus it was hypothesised that M2 signals via the insulin signalling pathway, but acts on components in the cascade down stream of PI 3-kinase. Thus M2 may signal via an alternative pathway prior to its action on the insulin-signalling pathway. In insulin resistant skeletal muscle of *ob/ob* mice the effect of M2 on glucose transport is lost, therefore the lesion in the pathway that causes the insulin resistant state is also preventing the action of M2. This is consistent with the earlier hypothesis that M2 acts via the insulin signalling pathway to increase glucose transport in non insulin resistant skeletal muscle. The effectiveness of M2 to directly alter glucose transport is therefore dependent upon the degree of insulin resistance. In a clinical setting, the severity of insulin resistance varies from patient to patient and depends on the severity of the obesity and the length of time the patient has been overweight. Thus, speculatively, the hypoglycaemic effect of M2 is unlikely to ameliorate insulin resistance in obese Type 2 diabetic patients but might delay the onset of Type 2 diabetes in obese patients by deferring the progression from impaired glucose tolerance (IGT) to Type 2 diabetes.

The impairment(s) in the insulin signalling cascade that contributes to insulin resistance is unknown. In *ob/ob* mice there may be several lesions in the signalling pathway which result in severe insulin resistance. It is possible that this impairment may be upstream of M2 action, thus rendering the *ob/ob* mouse an unsuitable model to detect the site of M2 signalling and glycaemic action. In order to determine if M2 has antidiabetic properties, clinical studies would need to be completed in patients with different insulin resistant states to identify the full range of actions of this compound.

Chapter Six:
Plant-Derived Compounds

Chapter Six: Plant-Derived Compounds

6.0 Introduction

Many plants have been used for centuries for their hypoglycaemic properties (Bailey and Day, 1989) and more than 400 plants with suspected glucose-lowering potential are known (Day, 1995; Ernst, 1997). Most of these plants have been claimed to possess antidiabetic properties, however very few have received scientific and medical evaluation to assess their efficacy and mechanism of action. Many plant materials and extracts of plants and leaves have been described for the treatment of diabetes throughout the world, and many continue to be used in underdeveloped regions. Traditional antidiabetic plants may provide a useful source of new oral hypoglycaemic compounds or as dietary supplements to existing therapies (Day, 1998).

Plants with potential hypoglycaemic activity have been identified around the world and some have been subjected to preliminary investigation in animal models of diabetes. Recently the hypoglycaemic activity of four plants used in Chilean popular medicine were investigated in alloxan and streptozotocin induced hyperglycaemic rats (Lemus et al., 1999), and plants used to treat diabetes in Taiwan have been investigated in STZ-induced diabetic rats (Hsu and Cheng, 1992). Preliminary studies on the inorganic constituents of some hypoglycaemic herbs have been investigated in fasted albino rats. The herbs were selected from indigenous folk medicines, Ayurvedic, Unani and Siddha systems of medicine (Kar et al., 1999). Many of these plants have been effective in reducing hyperglycaemia in diabetic animal models and the results suggest some validity for their clinical use.

Several clinical trials have been undertaken to determine the effects of various potential antidiabetic agents. Early studies are reviewed by Bailey and Day (1989). More recently the use of traditional plant medicines for Type 2 diabetes was investigated in South Texas, where 662 Type 2 diabetic patients reported using alternative treatments for their diabetes with beneficial effects (Noel et al., 1997). A multicentre randomised double blind study of glibenclamide versus a traditional Chinese treatment based on 3 plants in Type 2 diabetic patients has evaluated the efficacy of the Chinese treatment (Vray and Attali, 1995). Patients included were Type 2 diabetic outpatients, 40-70 years old, who were assessed for HbA1, blood glucose and plasma insulin. The Chinese treatment significantly decreased blood glucose and appeared to work synergistically with the sulphonylurea. However, some reviews of the literature pertaining to the use of traditional medicines in diabetes have reported that there is only limited information about the effects of these agents in a clinical setting. These conclude that the use of hypoglycaemic plant remedies is not supported by results from rigorous clinical trials and requires further investigation (Ernst, 1996; 1997).

There are many antidiabetic plant treatments from which a purportedly hypoglycaemic compound or fraction has been obtained. Various molecular species have been identified including alkaloids, glycosides and polysaccharides, which possess hypoglycaemic activity. These compounds have been extracted from numerous plants, fruits, leaves, and seeds including onion bulbs, garlic cloves, periwinkle, lupin, fenugreek, karela and bilberry leaves. For a detailed review of hypoglycaemic agents from plants see Bailey and Day (1989).

Part One: Pinitol

6.1.0 Introduction

Pinitol is a form of chiroinositol, a methyl inositol found in the leaves of *Bougainvillaea spectabilis*, in legumes and in soy products. Early reports and folk remedies have stated that the leaves of *Bougainvillaea* can cure diabetes mellitus (Narayanan et al., 1987), and more recently, chiroinositol deficiency has been implicated in insulin resistant states (Ortmeyer et al., 1993a; Ortmeyer et al., 1993b).

Pinitol was first isolated in 1953 from sugar pine stump (Anderson, 1953) but has since been isolated by repeated extraction with alcohol and water from the leaves of *Bougainvillaea spectabilis* (Narayanan et al., 1987). The active crystalline compound was characterised as pinitol by its melting point, specific rotation, NMR, IR and mass spectral characteristics. It has since been shown to be the 3-O-methyl ether of chiroinositol and it is this compound upon which the present research is focussed.

6.1.0.1 Hypoglycaemic Activity

In vivo studies have been conducted in both normal and diabetic models and have reported a potential anti-diabetic action of pinitol. One of the earlier studies reported a significant hypoglycaemic effect of pinitol (10mg/kg) in fasted normal albino mice and also in alloxan-induced diabetic albino mice (Narayanan et al., 1987). Furthermore, a 'chiroinositol glycan insulin putative mediator' has been shown to normalise plasma glucose in streptozotocin induced diabetic rats at a dose equivalent to insulin without inducing hypoglycaemia (Fonteles et al., 1996). Humanetics Corp. Minnesota submitted Pinitol to the FDA in December 1997 as a potential treatment to enhance insulin function.

6.1.0.2 Chiroinositol Deficiency

Chiroinositol deficiency in urine has been associated with insulin resistance in rhesus monkeys (Ortmeyer et al., 1993a), G/K(Goto/Kakizaki) non-obese Type 2 diabetic rats (Suzuki et al., 1991) and humans with insulin resistance and diabetes (Suzuki et al., 1994) The urinary excretion rate of chiroinositol has been found to be directly inversely correlated with insulin resistance indicating that chiroinositol may play a unique role in insulin action and may possibly provide a biochemical indicator of insulin resistance. To further investigate the biological significance of chiroinositol, Ortmeyer (1993b), studied the acute effects of this compound in streptozotocin diabetic rats, normal rats given a glucose load and in spontaneously insulin resistant rhesus monkeys. In all three groups of animals a single dose of chiroinositol increased glucose disappearance and sustained a lower plasma glucose concentration (Ortmeyer et al., 1993b).

6.1.0.3 Mechanism of Action

Inositol glycans are known to play important roles in the regulation of cell proliferation and as second messengers of biological receptors. Binding of insulin to its receptor has been shown to release certain specific inositol phosphoglycans to mediate insulin action thereby leading to hypoglycaemia (Huang and Larner, 1993). Several preparations of phosphoglycan molecules that appear to mediate insulin action as second messengers contain chiroinositol, which can be obtained from the diet as pinitol. Thus the focus of Part 1 of this chapter is to identify the action of pinitol both *in vitro* and *in vivo.* L6 skeletal muscle cells will be used to determine whether pinitol affects insulin action *in vitro* and thus establish if pinitol can directly alter glucose transport. Pinitol will be further investigated in a number of animal models of diabetes, insulin resistance and obesity, to identify any 'direct' or 'indirect' effects of pinitol on mammalian physiology.

6.1.1 Acute and Sub-chronic Effect of Pinitol on Basal and Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

6.1.1.1 Experimental Design

To determine the effect of pinitol on glucose metabolism *in vitro*, L6 skeletal muscle cells were grown to confluence, differentiated to form myotubes and incubated for 10min, 4h or 24h with 10^{-3}M pinitol (Aldrich). Basal and insulin-stimulated glucose transport by the muscle cells was assessed using the method of 2DG uptake with and without the presence of added insulin (10^{-8}M) as described in section 2.4.1 (chapter 2). The experiment was completed on multiples of 6 wells per test on 3 separate occasions; data are expressed as % of control values, mean±SEM.

6.1.1.2 Results

Cell exposure to insulin (10^{-8}M) for 10min, 4h or 24h increased glucose uptake in a time-dependent manner as described previously in chapter 3. Pinitol (10^{-3}M) alone increased basal glucose transport in a time dependent manner, the most potent effect observed was after 10min stimulation (figure 6.1). The stimulatory effect of pinitol

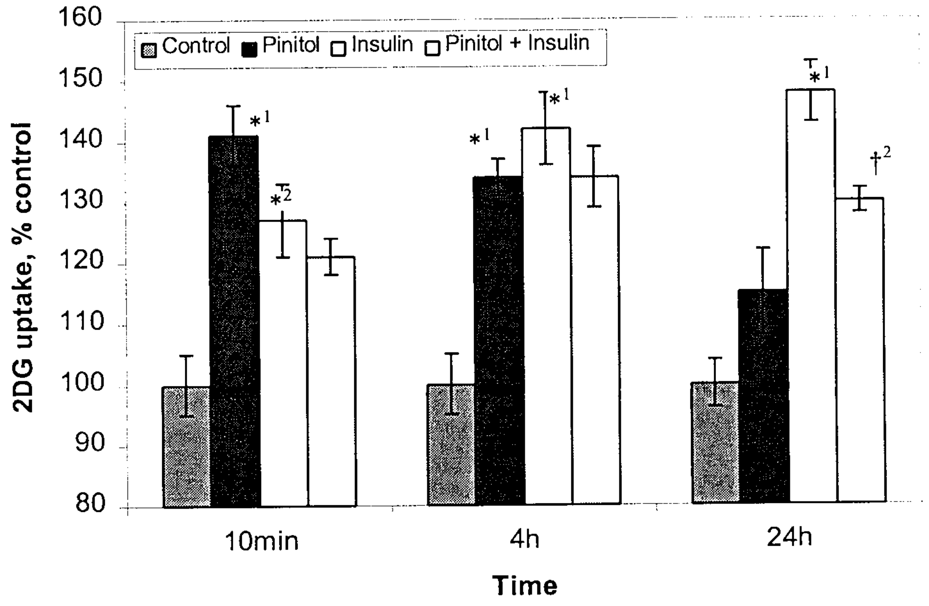


Figure 6.1 Effect of pinitol on basal and insulin-stimulated 2DG uptake by L6 skeletal muscle cells. Cells were stimulated with pinitol (10^{-3}M) for 10min, 4h and 24h with and without added insulin (10^{-8}M). Insulin significantly increased 2DG uptake in a time-dependent manner. Pinitol alone increased 2DG uptake in a time dependent manner, the most potent effect was after 10min cell exposure to pinitol (41% increase). Pinitol did not increase insulin-stimulated 2DG uptake but significantly decreased the insulin effect after 24h incubation (by 18%). Data are expressed as % control, values are mean ± SEM, n=12, *Significance versus control, †significance versus insulin alone, $^1p<0.01$, $^2p<0.02$.

was significant after 10min incubation (41%, p<0.01 versus control) and 4h incubation times (34%, p<0.01), after 24h incubation, pinitol was without effect.

Unlike the situation without added insulin, pinitol did not increase insulin-stimulated glucose uptake at any of the incubation times. Pinitol lowered mean insulin-stimulated glucose transport into these cells (by 6% after 10min, by 8% after 4h). This was found to be statistically significant after 24h incubation of pinitol, when insulin-stimulated glucose uptake was decreased by 18% (p<0.02 versus insulin alone).

6.1.2 Antidiabetic Effect of Pinitol in Normal and Diabetic Mice

6.1.2.1Experimental Design

A. Acute Effect of Pinitol on Glycaemia in Lean Mice

Normal, lean, six week old male mice (mean weight 30g) were housed in groups of 6-7 per cage, maintained at 22±2°C. Food was withdrawn at the beginning of the experiment (time zero), while drinking water was available *ad libitum.* Two groups of mice were matched for body weight and non-fasted plasma glucose concentration, cage 1 (control group) and cage 2 (test group) for both 5mg/kg and 100mg/kg studies. The test group received pinitol either 5mg/kg or 100mg/kg given by oral gavage at time zero, and the placebo control group received vehicle only (PBS). Blood samples were taken from the tail tip into heparinised tubes at time zero (basal plasma glucose concentration), and at 2h intervals for 4h. Plasma was separated and assayed for glucose immediately (section 2.4.5). An intraperitoneal glucose tolerance test (ipgtt) was conducted at the 4h time point (section 2.3.5). Glucose 2g/kg, ip, was given in a

40% w/v solution. Blood samples for plasma glucose were then taken at 45min and 90min. Food was replaced after the period of study. Data are expressed as % change in plasma glucose concentration (mmol/l), mean±SEM, n=10.

B. Acute Effect of Pinitol on Glycaemia in *ob/ob* Mice

Obese-hyperglycaemic *ob/ob* mice were housed in groups of 4-5 per cage, maintained at 22±2°C. Four groups of mice were matched for body weight. In cage 1 (control group) and cage 2 (test group) food was withdrawn at the beginning of the experiment. Cage 3 (control) and cage 4 (test group), food was withdrawn 16h previously. In all experiments drinking water was available *ad libitum*. The test groups received pinitol 100 mg/kg given by oral gavage at time zero, and the placebo control group received vehicle only. Blood samples were taken as previously described (above 6.1.2.1A and in detail in chapter 2 section 2.3.2), at 2h intervals for 6h. Plasma was assayed for glucose as described in chapter 2 section 2.4.5. Food was replaced after the period of study. Data are expressed as % change in plasma glucose concentration (mmol/l), mean±SEM, n=10.

C. Acute Effect of Pinitol on Glycaemia in STZ-induced Diabetic Mice

50 male, lean, 6 week old mice were administered STZ as described previously (chapter 2 section 2.3.3). These 'diabetic' animals were matched for body weight and plasma glucose, into 5 separate groups. Animals were grouped into cages of 3-8 animals, and food was removed at the beginning of the experiment (time zero) although water was freely available. The test groups received pinitol, either 5mg/kg, 10mg/kg, 100mg/kg or 200mg/kg by oral gavage, and placebo control group was given vehicle only. Blood samples were taken at time zero and at 2h intervals for 6h,

and analysed for plasma glucose as described above. Blood samples were also taken at time zero and 6h from control and pinitol treated mice. Data are expressed as % change in plasma glucose concentration (mmol/l), mean±SEM, n=10 and insulin concentration (pmol/l) mean±SEM.

D. Acute Effect of Pinitol on Glucose Disappearance in STZ-induced Diabetic Mice

12 lean, 6 week old male mice were administered STZ as described above. These animals were matched for body weight and plasma glucose concentrations into 2 groups of 6 animals. Food was removed at time zero, but water was freely available. The test group received pinitol (100mg/kg po) and the placebo group was given vehicle only. Blood samples were taken at time zero and 6h after administration. At the 6h time point, an insulin hypoglycaemia test was performed (see section 2.3.6) and blood samples then taken at 20, 40, 60 and 150min after this time. Blood was analysed for plasma glucose as described above. Data are expressed as % change as described above, mean±SEM, n=6.

E. Acute Effect of Two Separate Doses of Pinitol on Glycaemia in STZ-induced Diabetic Mice

12 lean, 6 week old mice were administered STZ, and were matched for body weight and plasma glucose into 2 groups. Food was removed at time zero, when the test group received pinitol by oral gavage (100mg/kg,) and the placebo group was given vehicle only. Blood samples were taken from the tail tip at time zero and 6h after administration. At the 6h time point, a second dose of pinitol was given intraperitoneally (100mg/kg,). Blood samples were then taken at 8h after the first dose

was administered. Plasma samples were analysed for glucose and insulin as described previously. Data are expressed as described above, n=6.

F Chronic Effect of Bi-daily Administration of Pinitol on Body Weight, Food Intake and Glycaemia of STZ-Induced Diabetic Mice

20 lean, 6 week old mice were dosed with STZ (160mg/kg ip) to induce diabetes (2.3.3) and were monitored for 10 days prior to pinitol administration. On day 0 mice were separated into two groups based on plasma glucose concentration (for ethical reasons mice with highest plasma glucose concentrations were selected for treatment). Mice were removed from the study if plasma glucose concentrations were lower than 10mmol/l. Mice received the first injection of either pinitol (100mg/kg ip) or vehicle only (control group) and food was removed for an overnight fast. Thereafter on days 1-11 inclusive, mice received either pinitol or vehicle twice per day, a single dose early morning and a single dose early evening. Food and water were available *ad libitum*. After the 2nd dose on day 11, treatment was withdrawn and mice were monitored for a further 10 days. Food was freely available all days throughout the study except day –9 (food was withdrawn for 6h during STZ administration) and day 0 (food was withdrawn for 16h for an overnight fast). Blood samples were taken from the tail tip (2.3.2) 2-3 times per week to assay for plasma glucose concentration (2.4.4) and food intake and body weight were monitored daily.

6.1.2.2 Results

A. Acute Effect of Pinitol on Glycaemia in Lean Mice

In the placebo treated non-diabetic lean mice, the plasma glucose concentrations significantly decreased over the 4h after placebo administration and food removal (15%, $p<0.05$, versus time zero). Administration of 5mg/kg or 100mg/kg pinitol decreased plasma glucose concentrations by 17% and 20% respectively, which was not significantly different from the decrease observed in control mice.

Glucose (2g/kg ip) increased plasma glucose of control animals by 61%, and test animals by 77% (5mg/kg pinitol) and 96% (100mg/kg pinitol) within 45min of administration. Plasma glucose concentrations of all animals had returned to basal levels within 1.5h of glucose administration. Thus pinitol had no effect on glucose tolerance in lean non-diabetic mice (figure 6.2).

B. Acute Effect of Pinitol on Glycaemia in *ob/ob* Mice

Administration of pinitol (100mg/kg) did not procure a significant effect on plasma glucose concentrations of either non-fasted (food was removed at time zero) or fasted (16h prior to pinitol administration) *ob/ob* mice (figure 6.3). In non-fasted mice, administration of pinitol produced an insignificant decrease in plasma glucose concentration over the 0-6h period when food was withheld (14%), which was less than the decrease observed in control mice (30%). In the overnight (16h) fasted *ob/ob* mice, the pinitol-treated group of mice had plasma glucose concentrations similar to that of the non-treated mice. A 7% decrease in plasma glucose concentrations was observed in the control animals and a 6% decrease in pinitol-treated mice.

Figure 6.2 Acute effect of pinitol on basal glycaemia and glucose tolerance in lean non-diabetic mice. Mice were dosed with pinitol (5mg/kg or 100mg/kg po), a glucose tolerance test 2g/kg ip) was performed 4h after initial dose was administered. Administration of 5mg/kg or 100mg/kg pinitol had no effect on plasma glucose concentration over 4h compared to control mice and had no effect on glucose tolerance. Data are expressed as % change, values are mean ± SEM, n=10. Plasma glucose concentrations (mmol/l) at time zero were 9.1 ± 0.4, 9.0 ± 0.5 and 8.6 ± 0.3 for control and 5 and 100mg/kg pinitol respectively.

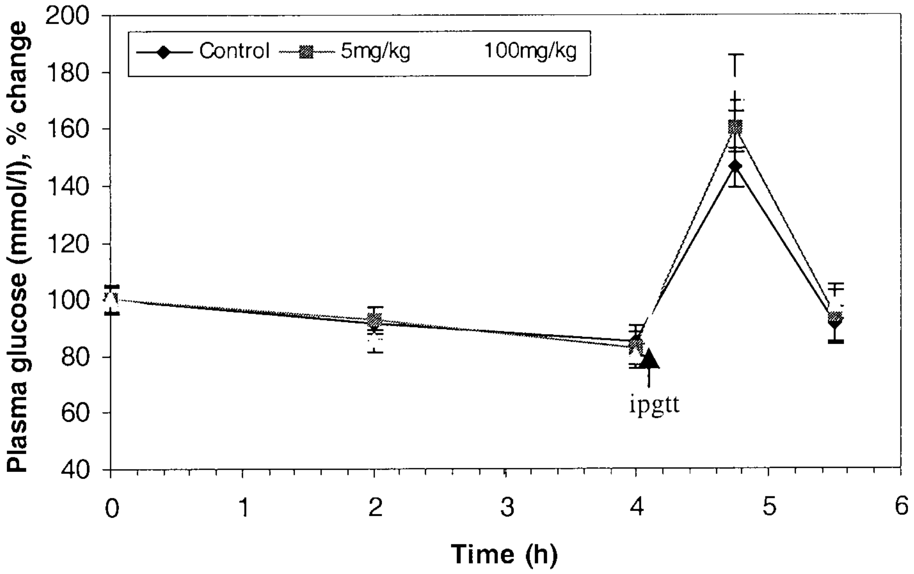
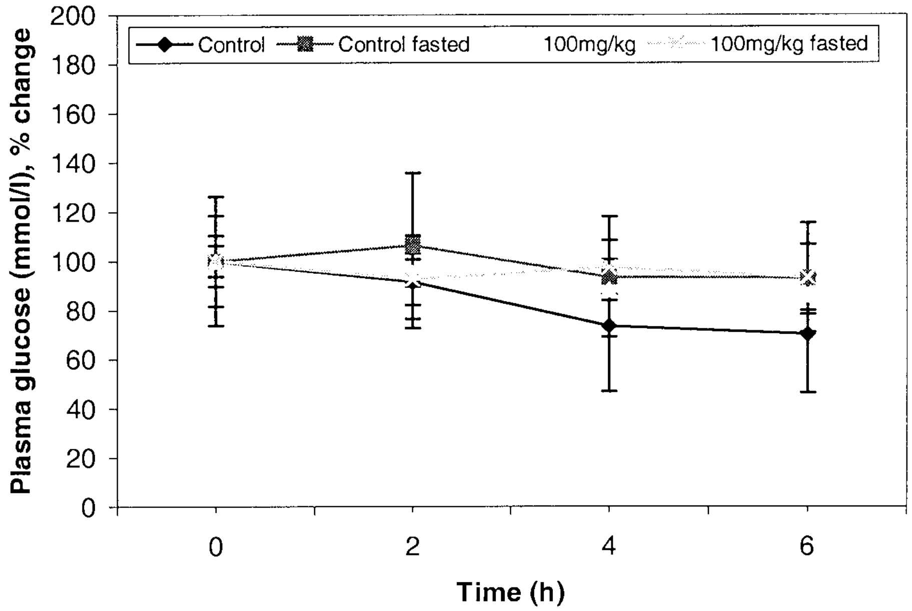


Figure 6.3 Acute effect of pinitol on basal glycaemia in *ob/ob* mice. Fasted and nonfasted *ob/ob* mice were dosed with pinitol (100mg/kg po) and plasma glucose concentrations determined over 6h. Pinitol had no effect on plasma glucose concentrations of fasted or nonfasted *ob/ob* mice compared to control mice over the 6h test period. Data are expressed as % change, values are mean ± SEM, n=10.



C. Acute Effect of Pinitol on Glycaemia in STZ-induced Diabetic Mice

In STZ-induced diabetic mice, pinitol was observed to decrease plasma glucose concentrations at a rate faster than observed in control mice (figure 6.4).

In control (non treated STZ-induced diabetic mice) plasma glucose concentrations decreased by 6% over the 6h test period as all mice were fasted at time zero.

Administration of 5mg/kg pinitol decreased plasma glucose concentrations by 21% and 10mg/kg by 22%.

Administration of 100mg/kg pinitol had the most potent glucose lowering effect. 2h after administration, plasma glucose concentrations had decreased by 15% ($p<0.05$ versus time zero), compared to 3% in control mice. 4h after administration of pinitol, plasma glucose concentrations had decreased by 23% ($p<0.02$ versus time zero) compared to 5% in control mice. Within 6h of administration plasma glucose concentrations of mice that received 100mg/kg pinitol had decreased by 28% ($p<0.02$ versus time zero) compared to 6% in control mice.

200mg/kg pinitol did not affect plasma glucose concentrations in STZ-induced diabetic mice (9% decrease over test period).

Figure 6.4 Acute effect of pinitol on basal glycaemia in STZ-induced diabetic mice. Mice were dosed with pinitol (5mg/kg, 10mg/kg, 100mg/kg or 200mg/kg po) and plasma glucose concentrations determined over 6h. Pinitol decreased plasma glucose concentrations by 21% - 28% compared to 6% in control mice. Data are expressed as % change, n=10, values are mean ± SEM. *Significance versus time zero, †significance versus control. $^1p<0.01$, $^2p<0.02$, $^3p<0.05$.

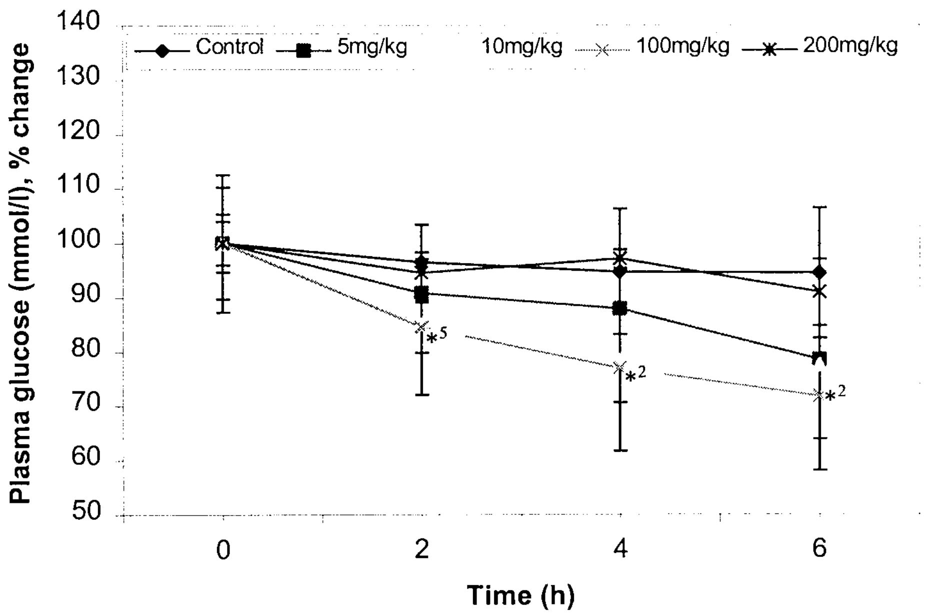
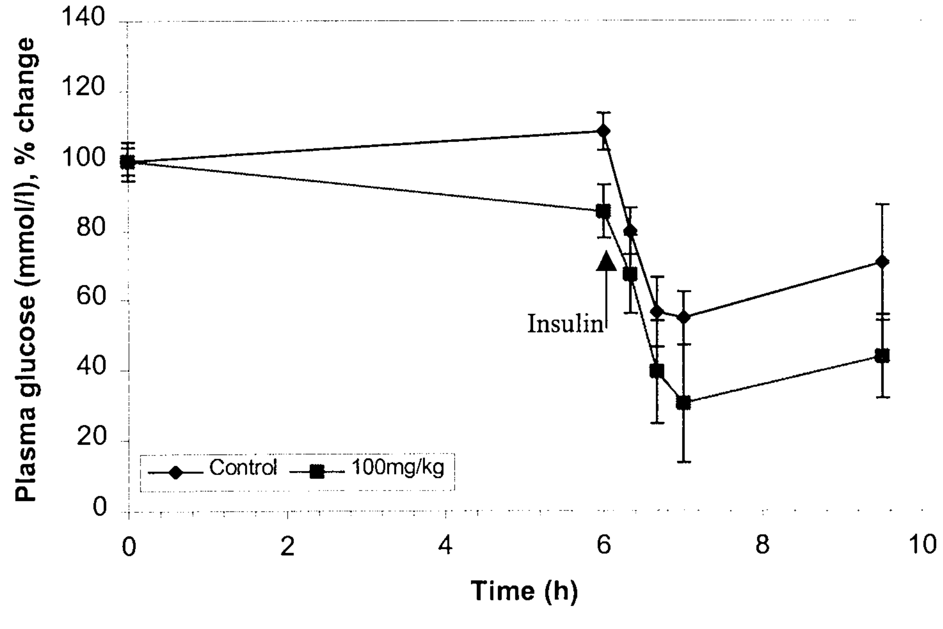


Figure 6.5 Acute effect of pinitol on glucose disappearance in STZ-induced diabetic mice. Mice were dosed with pinitol (100mg/kg po) and after 6h an insulin hypoglycaemia test was performed (human actrapid, 1U/kg, ip) plasma glucose concentrations determined over a further 4h. Pinitol did not alter the rate of glucose disappearance. Data are expressed as % change, n=6, values are mean ± SEM.



D. Acute Effect of Pinitol on Glucose Disappearance in STZ-induced Diabetic Mice

Administration of 100mg/kg pinitol (po) to STZ-induced diabetic mice, resulted in a 15% decrease in plasma glucose concentration over 6h (figure 6.5). A decrease in plasma glucose concentration of control animals was not observed (8% increase over 6h). Administration of insulin to control mice caused a 53% decrease in plasma glucose concentration within 1h of administration. Administration of the same concentration of insulin to pinitol treated mice resulted in a 55% decrease in plasma glucose concentration. Thus the rate of glucose disappearance was not altered by pinitol administration.

E. Acute Effect of Two Separate Doses of Pinitol in STZ-induced Diabetic Mice

The first dose of pinitol (100mg/kg given orally) produced a 19% decrease in plasma glucose concentrations after 6h in STZ-induced diabetic mice compared with 5% decrease in control STZ-induced diabetic mice ($p<0.05$ versus time zero and control mice, figure 6.6). A further dose of pinitol (100mg/kg, intraperitoneally) administered to the treated animals 6h after the initial dose resulted in a further 23% decrease in plasma glucose concentration within 2h ($p<0.02$ versus time zero and $p<0.05$ versus control mice). In comparison the plasma glucose concentrations of the control diabetic mice did not significantly alter (6% decrease). After a further 16h (24h after the initial dose was administered and 18h after the second dose), the plasma glucose concentrations of the treated group had decreased by a further 12%, a total decrease of 54% within 24h ($p<0.02$ versus time zero, not significant versus control). The plasma glucose concentrations of the control group had also decreased by a further 12%

Figure 6.6 Acute effect of two separate doses of pinitol on basal glycaemia in STZ-induced diabetic mice. Mice were dosed with pinitol (100mg/kg po) and after 6h a further dose was administered (100mg/kg ip). Plasma glucose concentrations were determined over a further 2h and overnight. Pinitol decreased plasma glucose concentrations by 19% 6h after initial administration (5% in control mice). Administration of a second dose of pinitol decreased plasma glucose concentrations by 23% within 2h. 24h after initial dose, plasma glucose concentrations of pinitol-treated mice had decreased by 54% compared to 23% in control mice. Data are expressed as % change, n=6, values are mean ± SEM. *Significance versus control mice, †significance versus time zero, $^1p<0.01$, $^2p<0.02$, $^3p<0.03$.

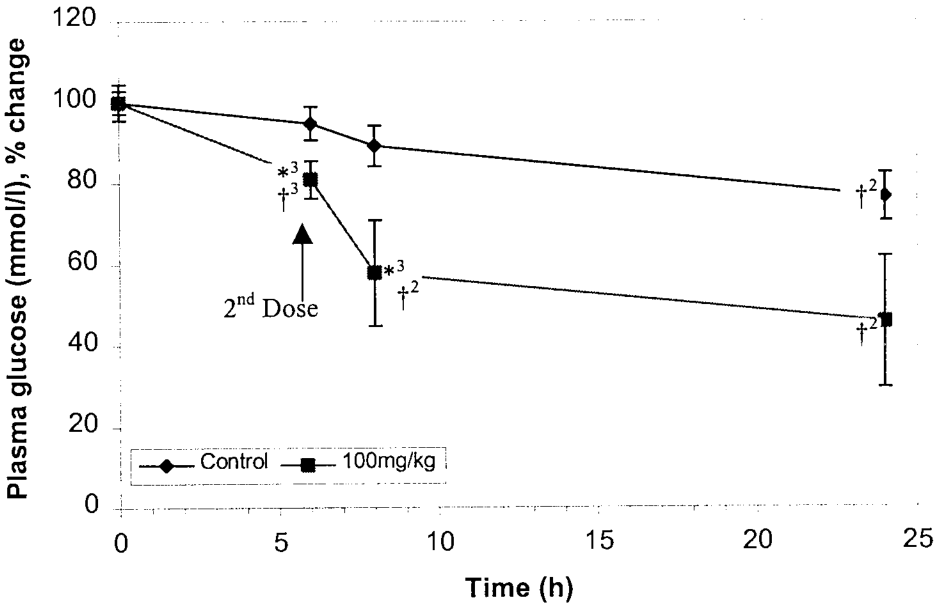


Table 6a Acute effect of two separate doses of pinitol on plasma insulin concentration in STZ-induced diabetic mice. Mice were dosed with pinitol (100mg/kg po) and after 6h a further dose was administered (100mg/kg ip). Plasma insulin concentrations were determined over a further 2h and overnight. Plasma insulin concentrations of lean STZ-induced diabetic mice were characteristically low. Pinitol had no effect on plasma insulin concentrations of STZ-induced diabetic mice when administered either orally or by ip injection. Data are expressed in plasma insulin concentration (pmol/l), values are mean±SEM, n=6.

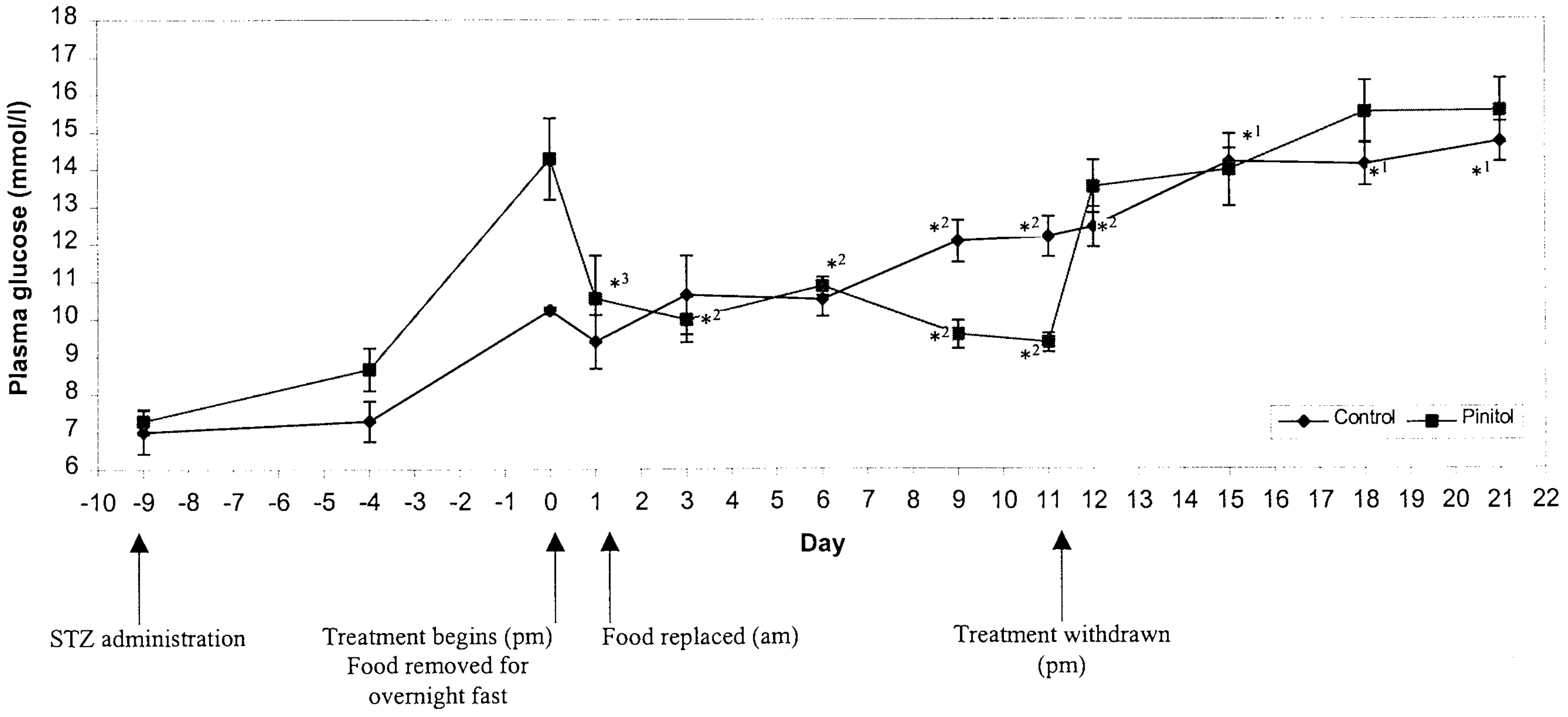
Time (h)	Control (n=6)	SEM	Pinitol (n=6)	SEM
0 - 1st Dose	153	34	168	68
6 - 2nd Dose	203	34	153	17
8	136	34	169	51
24	237	68	170	16

overnight, a total decrease of 23% over a 24h period ($p<0.02$ versus time zero). Pinitol did not affect plasma insulin concentrations (table 6a).

F Chronic Effect of Bi-daily Administration of Pinitol on Body Weight, Food Intake and Glycaemia of STZ-Induced Diabetic Mice

Administration of STZ increased plasma glucose concentrations from 7mmol/l to approximately 12mmol/l within 9 days (figure 6.7). Mice selected for pinitol treatment had mean plasma glucose concentrations of 14.28mmol/l on day 0 and control mice 10.22mmol/l. After an overnight (16h) fast and 16h after the 1st dose of pinitol, the plasma glucose concentrations of treated mice had significantly decreased to 10.54mmol/l ($p<0.05$) compared to control which had decreased plasma glucose concentrations to 9.4mmol/l (not significant). Bi-daily pinitol administration significantly lowered plasma glucose concentrations by 4.92mmol/l to 9.36mmol/l (day 11 $p<0.02$) over the 10 day test period. Administration of vehicle-only did not lower plasma glucose concentrations of STZ-induced diabetic mice; conversely plasma glucose concentrations in these mice had significantly increased by day 9 of the study ($p<0.02$). Treatment was withdrawn after day 11 of the study. Plasma glucose concentrations of control mice continued to increase throughout the duration of the study to 14.72mmol/l, a significant increase of 4.5mmol/l ($p<0.01$). Withdrawal of pinitol treatment from the test group of mice resulted in an immediate and significant rise in plasma glucose concentration to 13.52mmol ($p<0.01$ day 12 versus 9.36mmol/l day 11). Plasma glucose concentrations of test mice without treatment continued to rise to 15.54mmol/l throughout the study. Treatment with pinitol twice daily had no effect on body weight (which remained at approximately 32g/mouse/day throughout the study compared to control mice: 30g/mouse/day), or food intake

Figure 6.7 Chronic effect of pinitol on plasma glucose concentrations in lean STZ-induced diabetic mice. Mice were grouped according to plasma glucose concentrations and received either pinitol (100mg/kg ip) or vehicle only twice daily for 11 days. Pinitol significantly decreased plasma glucose concentrations in STZ-induced diabetic mice. Withdrawal of pinitol treatment caused plasma glucose concentrations to increase to elevated levels observed prior to treatment. Data are mean plasma glucose concentration (mmol/l)±SEM, n=5. *Significance versus day 0 values, $^{1}p<0.01$, $^{2}p<0.02$, $^{3}p<0.05$.



(approximately 7.35g/mouse/day in the study group compared to 6.61g/mouse/day in the control group) in lean STZ-diabetic mice.

6.1.3 Discussion

In rat L6 skeletal muscle cells both pinitol and insulin acutely stimulated basal glucose transport. The stimulatory effect of pinitol was time-dependent and most potent after 10min incubation but the effect was not maintained over 24h. Thus pinitol may exert a 'direct' effect on skeletal muscle which causes an immediate increase in glucose transport. As this effect was not observed after prolonged incubation of the cells with pinitol, this effect is unlikely to be caused by protein synthesis and may involve an alteration in the intrinsic activity of glucose transporters already at the cell surface. Pinitol is the 3-O-methyl ether of chiroinositol and as described previously, may act as a second messenger in intracellular signalling and thus acts in this manner to mediate glucose transport (6.1.0.3). The exact mechanism of action of pinitol is presently unknown. However it is possible that it is being incorporated into an intracellular precursor of insulin signalling and is being processed to a chiroinositol containing mediator, possibly glycosyl-phosphatidylinositol anchors of plasma membrane lipids or proteins (Ortmeyer et al., 1993; Low and Saltiel, 1988; Romero et al., 1988).

In vivo studies demonstrated that pinitol acutely decreased hyperglycaemia in STZ-induced diabetic mice. The effect was concentration dependent up to 100mg/kg, but was reduced at 200mg/kg, indicating a 'bell' shaped dose-response curve. This might imply that large dosages of pinitol exert an antagonistic or inhibitory influence upon insulin signalling. For example, whereas a low dose of pinitol could activate or facilitate an insulin-signalling step; a high dosage might bind for a protracted period and prohibit the normal insulin-stimulated pathway from normal operation. Interestingly, during insulin hypoglycaemia tests *in vivo*, pinitol did not acutely alter the rate of insulin-induced glucose disappearance. Chronic administration of pinitol normalised blood glucose concentrations in STZ-induced diabetic mice whereas withdrawal of pinitol resulted in the return of hyperglycaemia to levels observed prior to treatment. Pinitol did not affect plasma insulin concentrations, therefore the hypoglycaemic activity of pinitol is unlikely to be mediated via an action on β-cells of the islets of Langerhans to increase insulin production and subsequent increased glucose utilisation. Pinitol also did not affect body weight or food intake and therefore is unlikely to enhance thermogenesis or satiety. Interestingly, pinitol did not significantly affect plasma glucose concentrations in normal lean, non-diabetic and severely insulin resistant, obese diabetic *ob/ob* mice.

Pinitol is an effective glucose lowering agent in STZ mice and enhances glucose transport in L6 muscle cells. Pinitol does not however acutely alter insulin resistance in severely insulin resistant *ob/ob* mice. Thus in the absence of adequate insulin, pinitol may modulate a non-insulin stimulated glucose uptake mechanism. This suggests that pinitol has insulin-like activity and thus mimics the action of insulin both *in vitro* and *in vivo*. This activity however, does not enhance the action of insulin, as pinitol is most effective in the absence of adequate insulin. Indeed, pinitol is not effective as a hypoglycaemic agent if there is adequate insulin present. This indicates that pinitol may act, at least in part, via the insulin signalling pathway but that the initial signalling elements upon which pinitol acts are more efficacious for insulin than for pinitol. Thus in the presence of insulin, pinitol has little or no effect.

Research into pinitol is in its infancy and further work will be required to determine the mechanism of chiroinositol incorporation into inositol phospholipid precursers, and its action on insulin resistance. However the potential for therapy using pinitol in insulin deficiency is evident, and it may become a useful tool in elucidating lesions in the insulin signalling pathway, which cause insulin resistance.

Part Two: Chamaemeloside

6.2 Introduction

Recent evidence has demonstrated that 3-hydroxy-3-methylglutaric acid (HMG) isolated from Spanish moss (*Tillandsia useneoides* L., Bromeliaceae) has hypoglycaemic activity and may be responsible for the purported antidiabetic effect of this plant in folklore herbal medicine (Witherup et al., 1995). Chamaemeloside is a new apigenin compound, an unusual feature of which is that the 3-hydroxy-3-methylglutaric acid moiety is associated with glucose (Tscan et al., 1996).

Roman chamomile, (*Chamaemelum nobile*, (L.) All., synonym: *Anthemis nobilis* (L.) is a popular herbal drug in Europe, (particularly France), and in the United States (Isaac, 1993). The dried flower heads (Chamomillae romanae flos) are monographed in many pharmacopoeias, e.g. DAB10, Belg. V, Helv. VII, and are used to prepare a herbal tea or an alcoholic tincture. Roman camomile is reputed to possess carminitive, antispasmodic, sedative and antimicrobial properties (Konig et al., 1998). It is often applied as a remedy against gastrointestinal disorders, or locally to treat skin lesions and inflammation. Roman chamomile has been the subject of numerous phytochemical studies which have revealed the presence of etheric oils, sesquiterpene lactones, hydroperoxides, coumarins, polyines and polyphenolic compounds (Konig et al., 1998).

6.2.1 Effect of Chamaemeloside on 2-Deoxy-[^{3}H]-Glucose Uptake in Rat L6 Skeletal Muscle Cells

6.2.1.1 Experimental Design

Chamaemeloside was a gift from Professor Gabriele M Konig and Dr Anthony D Wright of the Institute for Pharmaceutical Biology, Technical University of Braunschweig, Germany. The initial plant material, chamomillae romanae flos DAB 10,1Ntr./Ph.Eur., was obtained from Caesar and Loretz GMBH, Germany. Chamaemeloside was extracted and isolated from the plant as described in Konig et al., (1998).

Chamaemeloside was tested for its effect on glucose uptake in cultured L6 muscle cells. As in previous experiments L6 cells were grown to confluence in 24 well plates, and were differentiated to form myotubes by reducing the foetal calf serum concentration to 0.5% (2.2.2). Cells were incubated for 24h with chamaemeloside at concentrations 10^{-9}M, 10^{-7}M, and 10^{-5}M in 24 well plates. Cells were also incubated with 10^{-8}M insulin alone as a positive control. Glucose uptake was measured as described previously using the method of 2-deoxy-[^{3}H]-glucose uptake (2.4.1). Experiments were completed using 6 wells per test on 3 separate occasions. Data are expressed as % of control values, mean±SEM.

6.2.1.2 Results

Chamaemeloside was tested for its effect on glucose uptake in cultured L6 muscle cells. These cells are sensitive to insulin stimulated glucose uptake and are described in detail in chapter 3. The results indicate that under the test conditions in this model, chamaemeloside at concentrations in the 10^{-9}M – 10^{-5}M range did not significantly alter glucose uptake in L6 cells. Under the same conditions, insulin caused a significant increase in glucose uptake (table 6b).

As considered in chapter 3, *in vitro* test systems are not always predictive of activity *in vivo*, however they do provide a useful indication of whether a test compound is likely to affect glucose uptake by muscle. Thus the present study suggests that chamaemeloside is unlikely to have a potent effect on muscle glucose uptake *in vivo*.

6.2.2 Effect of Oral Administration of Chamaemeloside on Interprandial Glycaemia and Oral Glucose Tolerance in Normal Mice

The results obtained in the L6 muscle cell assay did not preclude the possibility that chamaemeloside could lower blood glucose concentrations *in vivo*, either by an effect on the liver, to reduce hepatic glucose output, or via some other mechanism.

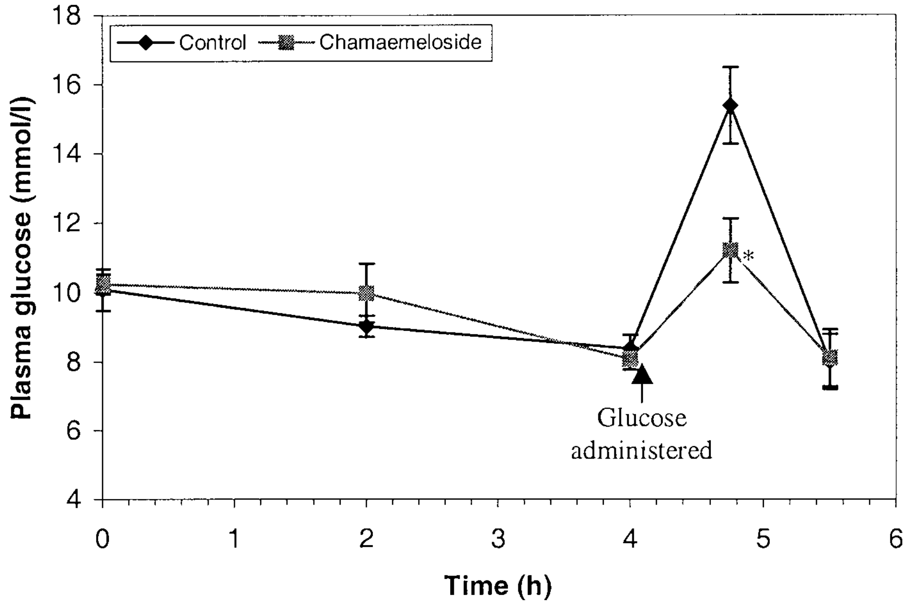
6.2.2.1 Experimental Design

To further evaluate chamaemeloside, it was administered orally to normal mice so as to assess its effects on interprandial glycaemia and oral glucose tolerance. 10 adult male homozygous lean (+/+) mice from the colony at Aston University were housed as described previously (section 2.3.1). Chamaemeloside was dissolved in water and administered orally at a dose of 100mg/kg to fed mice, time zero. 4h later the mice

Table 6b Effect of chamaemeloside on 2DG uptake in cultured L6 skeletal muscle cells. Cells were incubated with chamaemeloside (10^{-9}M – 10^{-5}M) for 24h without added insulin. Chamaemeloside had no effect on 2DG uptake in these cells, this was confirmed utilising 10^{-8}M insulin as a positive control. Data are expressed as mean dpm/well ±SEM, n=18.

	Chamaemeloside Concentration (M)			
	0	10-5	10-7	10-9
Mean dpm/well (n=18)	103.67	95.33	104	100.6
SEM	14.51	12	13.5	17

Figure 6.8 Acute effect of chamaemeloside (100mg/kg po time zero) on basal glycaemia and glucose tolerance (2g/kg po immediately after 4h blood sample) in normal mice. Administration of chamaemeloside to fed lean mice did not affect interprandial glycaemia, however 4h after administration it significantly improved glucose tolerance. Data are expressed as plasma glucose (mmol/l), values are mean ± SEM, n=5. *p<0.05 compared with control.



were given an oral glucose challenge (glucose 2g/kg body weight), as section 2.3.5. Blood samples were taken at intervals from the tail tip for plasma glucose determination as described in section 2.3.2. Control mice were similarly prepared but received vehicle only. Food, but not water, was withheld during the test. Data were evaluated using Student's 't' test (2.6.1). Values were considered to be significant if $p<0.05$. Data are expressed as actual plasma glucose concentration (mmol/l), mean±SEM, n=5.

6.2.2.2 Results

Administration of chamaemeloside to fed lean mice did not affect interprandial glycaemia. 4h after treatment, plasma glucose concentrations of test mice and control mice had decreased from 10mmol/l to 8mmol/l. However, 4h after administration, chamaemeloside significantly improved glucose tolerance in these mice (figure 6.8). Administration of glucose increased plasma glucose concentrations in control mice from 8mmol/l to 15mmol at 45min. In chamaemeloside treated mice the plasma glucose concentrations increased to 11mmol/l 45 min after the glucose load, which was significantly ($p<0.05$) less than in control mice. 1.5h after administration of glucose, plasma glucose concentrations had returned to basal levels in both control and chamaemeloside treated mice.

6.2.3 Discussion

Chamaemeloside had no effect on basal glucose uptake in L6 skeletal muscle cells; thus it is unlikely that any antidiabetic properties of the compound are mediated via a direct mechanism on peripheral tissues to increase glucose utilisation. However the study did not determine any effect on insulin-stimulated glucose uptake, through which there may have been an effect. Previous studies have evaluated chamaemeloside using Swiss-Webster mice to determine the effects on plasma glucose levels *in vivo* (Konig et al., 1998). The results of these tests showed that chamaemeloside at dosages of 125 and 250mg/kg reduced plasma glucose levels by 19.2% and 31.9% respectively ($p<0.05$), at 4h after administration (ip). To further investigate this observation chamaemeloside was administered orally to normal mice so as to assess its effects on interprandial glycaemia and oral glucose tolerance. Although chamaemeloside did not alter interprandial plasma glucose concentrations, its ability to improve glucose tolerance suggests that it might influence glucose homeostasis possibly via more than one mechanism. Reduced basal glycaemia could reflect a decrease in hepatic glucose production e.g. reduced gluconeogenesis, while improved glucose tolerance could involve effects in intestinal glucose absorption, hepatic or peripheral glucose disposal. While the lack of effect of chamaemeloside on glucose uptake by L6 muscle cells suggests that it is unlikely to act directly on muscle to increase glucose disposal, this however does not preclude an indirect effect, e.g. via increased glucose-induced insulin secretion.

Since chamaemeloside contains an HMG moiety, it is possible that it might act in a similar way to that proposed for HMG itself (Witherup et al., 1995). That is, hypoglycaemia may be caused by inhibition of HMG CoA lyase, which converts HMG CoA to acetyl CoA and acetoacetate, thus reducing energy production. This results in an increased metabolism of glucose, and not a stimulation of insulin receptors, insulin postreceptor signalling or insulin secretion. Furthermore it is possible to speculate that in the *in vivo* assay, HMG is being liberated from chamaemeloside, and the observed activity is due to free HMG, with the chamaemeloside being viewed as a potential prodrug.

Chapter Seven:
Pharmacological Agents Known to Modify Insulin Action

Chapter Seven: Pharmacological Agents Known to Modify Insulin Action

This chapter focuses on compounds that are already known to affect insulin resistance. Some of these are oral therapies presently used to treat Type 2 diabetes (metformin, tolbutamide, thiazolidinediones) and some are potential new therapies currently under investigation (vanadyl sulphate and thioctic acid). Some of the compounds investigated in this chapter have been widely used and knowledge of their mechanisms of action are already well established. Also some have previously been investigated using the rat L6 skeletal muscle cell line, these experiments have been repeated and are included here for comparative purposes.

Part One: Metformin

7.1.1 Introduction

Metformin (appendix II) is the main biguanide used for treating Type 2 diabetes (Bailey and Turner, 1996). It acts by increasing peripheral uptake of glucose whilst reducing hepatic glucose output, thus lowering blood glucose concentrations without stimulating insulin secretion (Stumvoll et al., 1995). Metformin is not however effective in the absence of insulin (Bailey, 1992). The study of metformin has revealed that *in vitro* it increases translocation of glucose transporters GLUT1 and GLUT4 (Matthaei et al., 1991; Hundal et al., 1992) increases glucose uptake and oxidation (Bailey and Puah, 1986; Galuska et al., 1994) and prevents the development of insulin resistance (Melin et al., 1990).

Metformin is taken in two or three doses daily and can be used on its own or in combination with sulphonylureas. Side effects include gastrointestinal upsets and nausea, and there is a rare risk of precipitating lactic acidosis (Bailey and Nattrass, 1988). Metformin is not metabolised in humans or rats, and its circulating levels typically range from 10 - 50μM in humans after the standard oral dose of 1.5g/day (Pentikainen et al., 1979; Sirtori et al., 1978).

The observation that in metformin-treated Type 2 patients, oral glucose tolerance was improved significantly relative to placebo-treated patients triggered more in-depth studies of metformin action on glucose utilisation (Prager et al., 1983). Metformin has been shown to increase glucose utilisation of peripheral tissues by 50% at a high insulin infusion rate and by 25% at a low infusion rate using the euglycaemic clamp technique (Nosadini et al., 1987; Prager et al., 1986)

The effects of metformin on glucose uptake have been studied in numerous cell and tissue systems, either after *in vivo* treatment with the drug or by direct exposure *in vitro*. Studies using rat skeletal muscle have indicated that *in vitro* treatment with metformin also increases glucose uptake in this tissue. 10μg/ml metformin has been shown to increase insulin-dependent glucose uptake by 15-35% *in vitro* (Frayn and Adnitt, 1972). In nondiabetic rat adipocytes and guinea pig fat pads, metformin added *in vitro* increased basal glucose oxidation in a concentration dependent manner, with maximal effects at 5mM and half-maximal effects at 0.3mM (Fantus and Brosseau, 1986; Jangaard et al., 1968). There was no further effect of metformin on glucose oxidation in the presence of added insulin. Although, addition of metformin to isolated adipose and skeletal muscle tissue fragments from non diabetic humans had no effect on glucose oxidation, it increased insulin responsiveness by 20% (Lord et al., 1983). Metformin was reported to have no effect on glucose disposal in normal human muscle *in vitro* (Frayn et al., 1973), but increased glucose uptake in other studies with insulin resistant human muscle (Galuska et al., 1991).

The effect of metformin on glycogen synthesis has not been explored in great detail. In cultured rat hepatoma cells metformin increased both basal and insulin-stimulated glucose incorporation into glycogen, but in rat hepatocyte monolayers metformin reduced basal and insulin-stimulated glycogen synthesis (Purrello et al., 1988; Alengrin et al., 1987). In soleus muscles of streptozotocin-induced diabetic mice, metformin was found to increase glucose uptake by about 20% at both submaximal and maximally stimulating insulin concentrations (Bailey and Puah, 1986).

In vivo work has demonstrated that metformin increases insulin-stimulated glucose uptake in skeletal muscle, causing increased glucose oxidation and glycogen synthesis (Rossetti et al., 1990). It has also been shown to increase insulin receptor binding and to increase insulin receptor phosphorylation and tyrosine kinase activity (reviewed by Bailey and Turner, 1996).

In 1992, Klip, et al., reported the stimulation of hexose transport by metformin in L6 cells. Metformin increased the initial rate of uptake of 2-deoxyglucose and 3-O-methylglucose. This was time dependent, with half-maximal stimulation at 5-6h and maximal stimulation by 16h. The stimulation of hexose uptake was not prevented by cycloheximide. In 5mM glucose medium, half maximal stimulation was obtained with 800μM metformin when tested for 24h. The stimulation of hexose transport by metformin was only detectable in fused myotubes and not in prefusion myoblasts. No significant changes were observed in glucose transporter levels in total cell membranes or in immunoreactive glucose transporter isoforms GLUT1 & 4 (Klip et al., 1992).

7.1.2 Investigation into the Mechanism of Metformin Action in Cultured L6 Skeletal Muscle Cells

7.1.2.1 Experimental Design

Metformin (Sigma) was dissolved in PBS at 1M concentration, filter sterilised and stored at –20°C in 100μl aliquots. L6 cells were grown to confluence in 24 well plates and serum starved for 24h as described in 2.2.2.

Metformin was diluted and added to the cell incubation medium to give a final concentration of 10^{-5}M to 10^{-2}M. To determine the effect of metformin on basal glucose transport, L6 cells were incubated with metformin for 24h, and glucose uptake assessed as described in 2.4.1. Acute studies were performed as a prelude to signalling experiments and cells were exposed to metformin for 4h or 40min prior to 2DG uptake. Experiments were undertaken in multiples of six wells on at least three occasions. Six control wells were included in each 24 well plate.

To determine the effects of metformin on insulin stimulated glucose uptake, insulin was diluted as described in 3.4 and added to the incubation medium at a final concentration of 10^{-8}M for 24h in addition to metformin over the concentration range 10^{-5}M – 10^{-2}M. Glucose uptake was measured as described above using multiples of six wells.

In order to investigate the mechanism of action of metformin, a number of agents that are known to inhibit insulin signalling were utilised to determine their effect on the action of metformin in L6 cells. These agents are described in detail in chapter 3 and their effects on insulin action are documented.

To investigate whether metformin acts via a PI 3-kinase mediated signalling pathway, metformin-stimulated L6 cells were exposed to LY-294,002. The effect of LY-294,002 on metformin stimulated glucose transport was investigated by incubating L6 cells with metformin at concentrations 10^{-4}M – 10^{-2}M and/or LY-294,002 over the concentration range 10^{-9}M – 10^{-5}M for 24h. The effects of LY-294,002 on insulin action are documented in chapter 3 where it was established that 10^{-4}M LY-294,002 results in a decrease in cell viability. Therefore for the purpose of this investigation cells were exposed to a lower dose range. Glucose uptake was measured using the 2DG uptake method as described in 2.4.1 Uptake of 2DG was expressed as described in section 3.4.

As demonstrated in chapter 3, wortmannin is highly toxic if the cells are exposed to this compound for longer than 4h. Preliminary experiments were undertaken to determine the acute effects of metformin in this assay system and 40min exposure of the cells was inadequate to elicit a significant response to metformin. Subsequently, the effect of wortmannin on metformin stimulated glucose transport was not established due to the poor response of metformin at the short time point necessary for wortmannin activity.

The effect of the GLUT inhibitor cytochalasin B (section 3.4.5) and the protein synthesis inhibitor cycloheximide (section 3.4.6) were investigated in L6 cells to further establish glucose transport by metformin action. Cytochalasin B was used to determine specific facilitated glucose uptake in these cells in order to establish if the nonspecific component of uptake was affected by metformin. Given that several hours cell exposure to metformin was required to increase glucose transport, it may be possible that metformin induces the synthesis of a protein relevant for the stimulation of glucose uptake, hence the protein synthesis inhibitor, cycloheximide was utilised as an agent to block potential translational effects.

Cells were exposed to metformin (10^{-4}M – 10^{-2}M) for 24h and/or cytochalasin B (10^{-9}M – 10^{-6}M) and/or cycloheximide (10^{-8}M – 10^{-5}M). Experimental design was identical to that of sections 3.4.5.2 and 3.4.5.5. 2DG uptake was assessed as described in 2.4.1 and expressed as described previously.

7.1.2.2 Results

7.1.2.2a Acute and Sub-Chronic Effect of Metformin on Basal and Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

Metformin increased basal glucose uptake in a time dependent and concentration dependent manner (figure 7.1). Acute stimulation of L6 cells for 40min had little effect on glucose uptake. Lower concentrations investigated (10^{-5}M – 10^{-3}M) had no effect or marginally increased glucose uptake by insignificant amounts (10%). 10^{-2}M metformin at high concentrations (10^{-2}M) significantly increased basal glucose uptake by 16% ($p<0.05$ versus control).

L6 cell exposure to metformin for 4h increased basal glucose transport by 20% - 51% (10^{-3}M – 10^{-2}M). This was concentration dependent, 10^{-5}M and 10^{-4}M had little/no effect and 10^{-3}M and 10^{-2}M significantly increased basal glucose uptake ($p<0.01$ versus control).

Culture of L6 cells for 24h with metformin increased basal glucose transport by 5% - 159% (10^{-5}M – 10^{-2}M). This effect was concentration dependent and was significant at concentrations 10^{-3}M and 10^{-2}M ($p<0.01$ versus control).

10^{-8}M insulin significantly increased glucose uptake in L6 cells by 42% (as described in chapter 3). Metformin increased insulin stimulated glucose uptake in a dose dependent manner by 17% (10^{-5}M) to 345% (10^{-2}M). See figure 7.2 for levels of significance.

Figure 7.1 Effect of metformin on basal 2DG uptake by L6 skeletal muscle cells. Cells were stimulated with metformin (10^{-5}M - 10^{-2}M) for 40min, 4h, and 24h. Metformin stimulated 2DG uptake in a time and concentration dependent manner. Maximal stimulation of 2DG uptake by L6 cells was after 24 cell exposure to 10^{-2}M metformin, (160% over basal values). Data are expressed as % control, values are mean ± SEM, n=18. *Significance versus control, $^{1}p<0.01$, $^{2}p<0.02$, $^{3}p<0.05$.

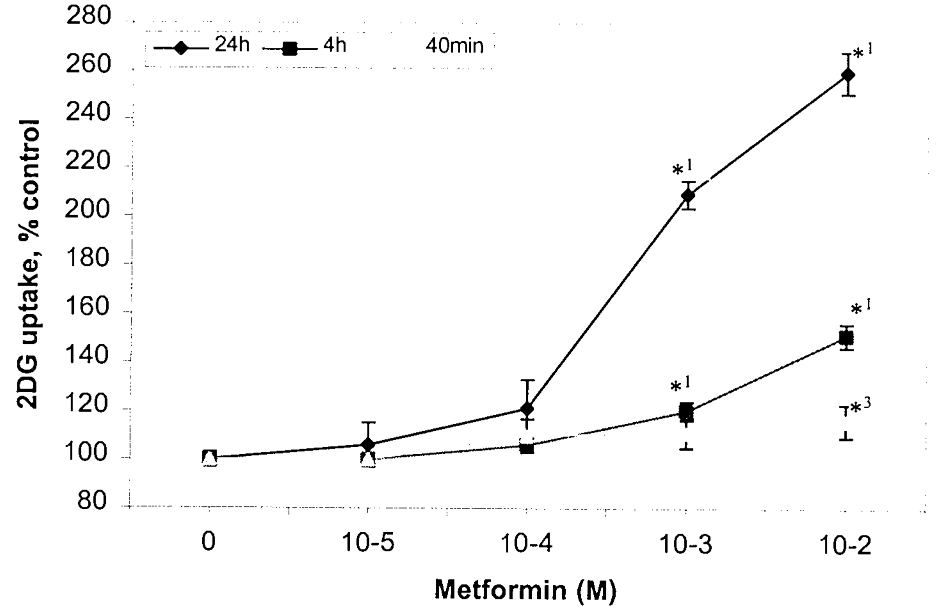
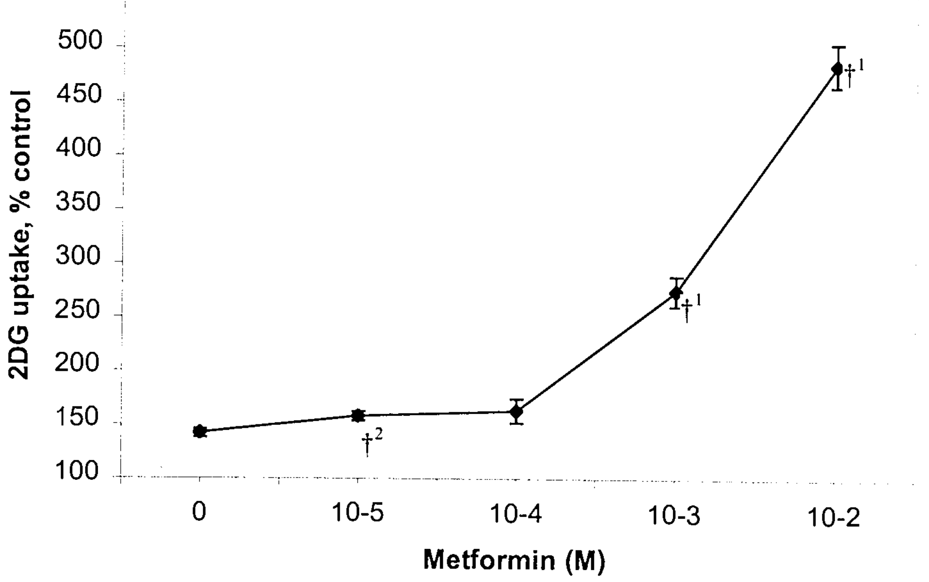


Figure 7.2 Effect of metformin on insulin-stimulated 2DG uptake by L6 skeletal muscle cells. Cells were stimulated with metformin (10^{-5}M - 10^{-2}M) and/or insulin (10^{-8}M) for 24h. Metformin increased insulin stimulated 2DG uptake in a concentration dependent manner. Maximal stimulation of insulin-stimulated 2DG uptake by L6 cells was after 24 cell exposure to 10^{-2}M insulin, (345% over insulin stimulated uptake). Data are expressed as % control, values are mean ± SEM, n=18. †Significance versus insulin alone, $^{1}p<0.01$, $^{2}p<0.02$, $^{3}p<0.05$.



7.1.2.2b Effect of LY-294,002 on Metformin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

Metformin increased basal glucose transport as described above 7.1.2.2a and figure 7.1. LY-294,002 decreased basal glucose transport in a concentration dependent manner as described in Chapter 3, section 3.4.2. LY-294,002 did not significantly reduce metformin-stimulated glucose transport in L6 cells at any concentrations investigated (figure 7.3).

7.1.2.2c Effect of Cytochalasin B on Metformin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

Metformin increased basal glucose uptake in a concentration dependent manner as described in 7.1.2.2a, and cytochalasin B decreased basal glucose transport as described in Chapter 3 section 3.4.5. Cytochalasin B dose-dependently decreased metformin stimulated glucose uptake. 10^{-9}M cytochalasin B had no effect on metformin-stimulated glucose transport but higher concentrations (10^{-8}M – 10^{-6}M) decreased both the basal and metformin-stimulated glucose uptake. 10^{-8}M cytochalasin B decreased metformin-stimulated glucose transport by 20% - 68% (10^{-4}M – 10^{-3}M metformin), 10^{-7}M cytochalasin by 41% (10^{-3}M metformin) and 22% (10^{-2}M metformin). 10^{-6}M cytochalasin B decreased metformin-stimulated glucose uptake by 45% - 115% (10^{-4}M – 10^{-2}M metformin). See figure 7.4 for levels of significance.

7.1.2.2d Effect of Cycloheximide on Metformin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

Metformin increased basal glucose transport as outlined above and cycloheximide inhibited this effect in a concentration dependent manner. 10^{-8}M cycloheximide had

Figure 7.3 Effect of the structurally distinct inhibitor of PI 3-kinase LY-294,002 on basal and metformin-stimulated 2DG uptake in L6 cells. Cells were cultured with LY-294,002 (10^{-9}M – 10^{-5}M) and/or metformin (10^{-4}M – 10^{-2}M) for 24h. Metformin stimulated 2DG uptake in these cells in a concentration dependent manner. LY 294,002 decreased basal 2DG uptake in a concentration dependent manner, but had no effect on metformin stimulated 2DG uptake in L6 cells. Data are expressed as % control, values are mean ± SEM, n=3. *Significance versus control, †significance versus metformin alone, $^1p<0.01$, $^2p<0.02$, $^3p<0.05$.

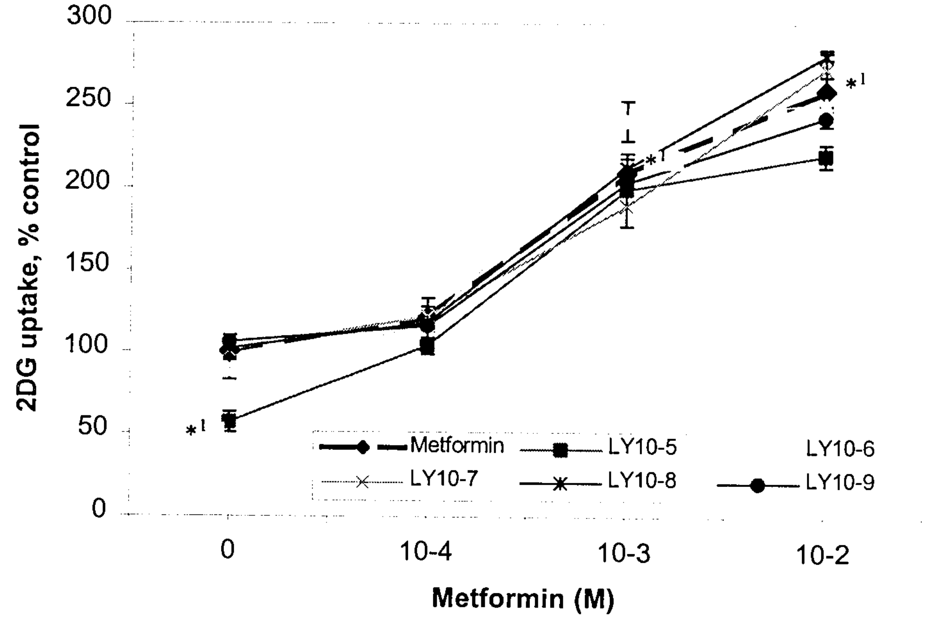


Figure 7.4 Inhibition of basal and metformin-stimulated 2DG uptake by the high affinity inhibitor of glucose transporters cytochalasin B (Cyt B), in L6 cells. Cells were cultured with cytochalasin B (10^{-9}M – 10^{-6}M) and/or metformin (10^{-4}M – 10^{-2}M) for 24h. Metformin stimulated 2DG uptake in these cells in a concentration dependent manner. Both basal and metformin stimulated 2DG uptake was inhibited by cytochalasin B in a concentration dependent manner. Data are expressed as % control, values are mean ± SEM, n=3. *Significance versus control, †significance versus metformin, $^{1}p<0.01$, $^{2}p<0.02$, $^{3}p<0.05$.

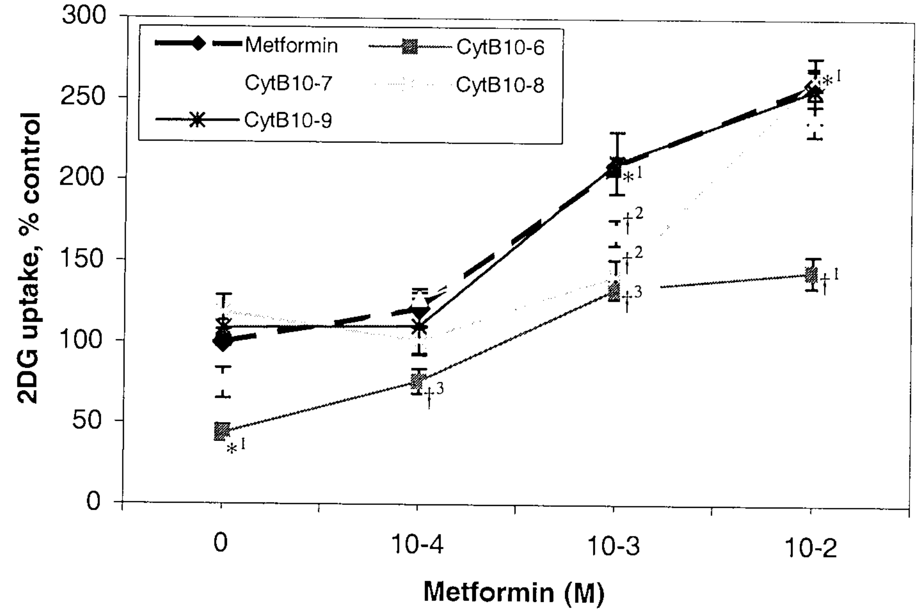
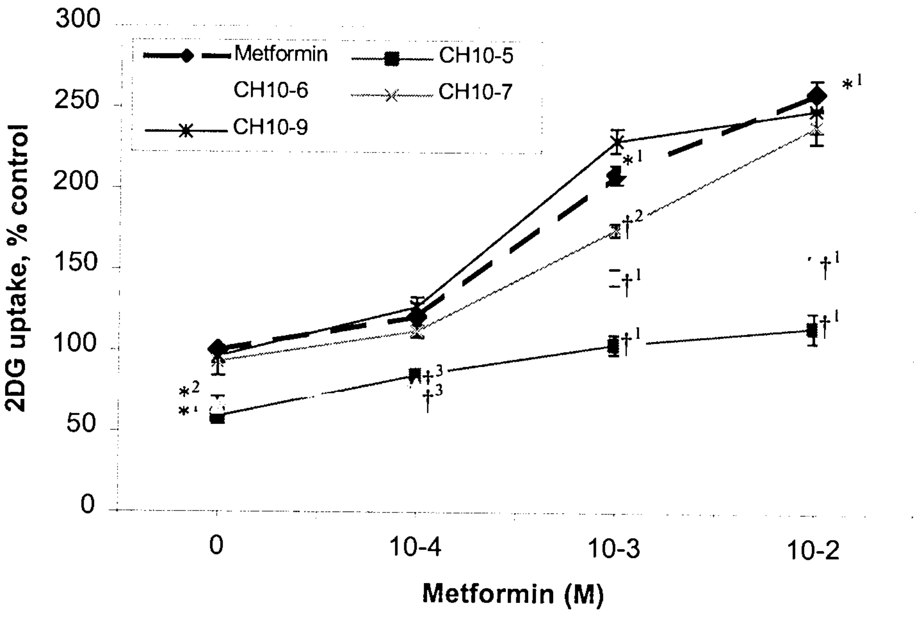


Figure 7.5 Inhibition of basal and metformin-stimulated 2DG uptake by cycloheximide (CH), an inhibitor of protein synthesis in L6 cells. Cells were stimulated with cycloheximide (10^{-8}M – 10^{-5}M) and/or metformin (10^{-4}M – 10^{-2}M) for 24h Metformin stimulated 2DG uptake in these cells in a concentration dependent manner. Both basal and metformin stimulated 2DG uptake was inhibited by cycloheximide in a concentration dependent manner. Data are expressed as % control, values are mean ± SEM, n=3. *Significance versus control, †significance versus metformin, ^{1}p<0.01, ^{2}p<0.02, ^{3}p<0.05.



no effect on metformin-stimulated glucose uptake in these cells, although higher concentrations significantly inhibited the effect of metformin. 10^{-7}M cycloheximide decreased metformin stimulated uptake by 9%, (10^{-4}M metformin), 34% (10^{-3}M metformin, and 10% (10^{-2}M metformin). 10^{-6}M cycloheximide inhibited the response by 42% - 100% (10^{-4}M – 10^{-2}M metformin) and 10^{-5}M cycloheximide inhibited by 36% - 144% (10^{-4}M – 10^{-2}M metformin). See figure 7.5 for levels of significance.

Part Two: Effect of Tolbutamide on 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

7.2.1 Introduction

Sulphonylureas lower blood glucose by stimulating insulin secretion (Groop, 1992). They stimulate the immediate and transient and also the slowly generated phases of insulin secretion. They act by binding to the plasma membrane of the β cells of the islets of Langerhans via ATP-sensitive potassium channels. By closing these channels they promote membrane depolarisation, thereby activating voltage dependent Ca+ channels (Bailey, 1998). The increase in cytosolic Ca2+ causes exocytosis of insulin granules (Malaisse and Lebrun, 1990).

The efficacy of these agents depends on the presence of enough β-cells with sufficient functional reserve, hence, if the pancreas lacks adequate β-cells, then sulphonylureas will not work and therefore their use is restricted to Type 2 diabetes.

There is a large selection of sulphonylurea preparations, which are either long acting or short acting. Each has been developed with different pharmacokinetic properties with the tailoring of drug therapy to meet different needs.

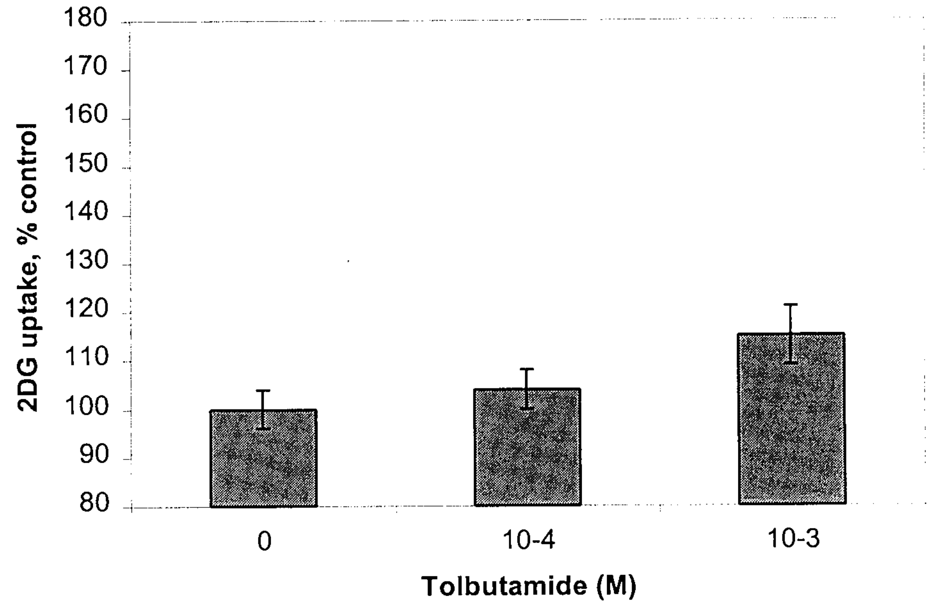
Side effects with this treatment are rare, although all preparations can cause weight gain and are prone to cause hypoglycaemia if there is overdose or a missed meal. However, the use of sulphonylureas is widespread and they have been reported to produce acceptable glycaemic control in more than two thirds of Type 2 diabetic patients (Groop, 1992).

As well as stimulating insulin release, sulphonylureas exert some direct effects on glucose metabolism in liver, muscle and fat. These effects are thought to reinforce the actions of insulin, but it is thought to be unlikely that they are sufficiently potent to sustain any substantive benefit to glycaemic control if acting without the support of an insulin-releasing effect (reviewed by Bailey and Flatt, 1996). This study was undertaken to investigate whether tolbutamide can alter basal glucose uptake by cultured muscle cells.

7.2.2 Experimental Design

Tolbutamide (Sigma) (appendix II) was dissolved in PBS and diluted to 10^{-1}M and 10^{-2}M concentrations. Experiments were undertaken on L6 skeletal muscle cells taken from preconfluent flasks and seeded in 24 well plates. The cells were grown to confluence before serum starving as described previously (2.2.2). Cells were incubated with tolbutamide at 10^{-3}M and 10^{-4}M for 24h prior to the glucose uptake assay. Glucose uptake was measured as described in 2.4.1 and experimental design and statistical analyses performed as described above for metformin studies.

Figure 7.6 Effect of tolbutamide on basal 2DG uptake by L6 skeletal muscle cells. Cells were stimulated with tolbutamide (10^{-4}M - 10^{-3}M) for 24h. Tolbutamide had no effect on basal 2DG uptake in L6 cells. Data are expressed as % control, values are mean ± SEM, n=6.



7.2.3 Results

Tolbutamide at 10^{-3}M caused a trivial increase in the mean basal glucose uptake (not significant). At 10^{-4}M, it was without effect (figure 7.6).

Part Three: Effect of the Thiazolidinediones Troglitazone and Rosiglitazone on 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

7.3.1 Introduction

Thiazolidinediones (TZDs) are a new class of oral antidiabetic agents which partially mimic or selectively enhance certain actions of insulin. This causes an antihyperglycaemic effect in Type 2 diabetics, which is slowly generated and is often accompanied by a reduction in circulating concentrations of insulin, triglycerides and NEFAs (nonesterified fatty acids). The effect of TZDs in lowering glucose levels is attributed to increased peripheral glucose disposal and decreased hepatic glucose output. Thiazolidinediones have been shown to enhance the action of insulin to reduce hyperglycaemia without increasing the release of insulin (Bailey and Flatt, 1996) and have been demonstrated to act additively with other types of oral antidiabetic agents and reduce the dosage of insulin required in insulin treated diabetics. Two TZDs have been introduced into clinical use: troglitazone (in USA, UK and Japan use was suspended after 2 months in the UK pending further investigation of adverse effects on liver function) and rosiglitazone which has recently been introduced in the USA (reviewed by Day, 1999).

7.3.1.1 Mechanism of Action

The mechanism of molecular action of TZDs is known to be via the peroxisome proliferator-activated receptor gamma (PPARγ) (Spiegelman, 1998; Saltiel and Olefsky, 1996). PPARs are members of the steroid-thyroid-retinoid superfamily of nuclear receptors of which PPARγ is mainly expressed in white and brown adipocytes, where it is complexed to the retinoid X receptor (RXR) within the nucleus (Keller et al., 1993; Lemberger et al., 1996). However, skeletal muscle is the main site of impaired insulin-stimulated glucose uptake in Type 2 diabetes and expression of PPARγ is very weak in skeletal muscle. Nevertheless, the blood glucose-lowering efficacy of TZDs in animal models is associated with improved glucose disposal into skeletal muscle.

TZDs are lipophilic, they enter cells and bind to PPARγ with high affinity causing a conformational change in the PPARγ-RXR complex (Lehmann et al., 1995). This action displaces a co-repressor and allows activation of regulatory sequences of DNA, which control the expression of genes, some of which are also controlled by insulin. Therefore, the genomic effects of insulin on adipocytes are amplified or mimicked by TZDs also causing an increase in fatty acid uptake and lipogenesis.

7.3.1.2 *In Vivo* Studies

In vivo studies have demonstrated that TZDs reduce hyperglycaemia in a range of diabetic and obese animal models (reviewed by Day, 1999). They reduce plasma insulin concentrations by increasing the effects of insulin on glucose and lipid metabolism in liver, muscle and adipose (Colca and Morton, 1990; Iwanishi and Kobayashi, 1993). Consequently, skeletal muscle and adipose tissue glucose uptake is enhanced and hepatic glucose production lowered (Bailey and Flatt, 1996). Within 2-10 days, near-normal glycaemia was reinstated in hyperglycaemic animal models and hyperinsulinaemia reduced in obese diabetic models indicating a reduction in insulin resistance (Bowen et al., 1991). In streptozotocin diabetic animals, TZDs have little effect on hyperglycaemia, indicating that their glucose-lowering action is dependent upon the presence of insulin (Chang et al., 1983). In normal nondiabetic animals TZDs exert little or no effect on basal glycaemia (Fujiwara et al., 1988) but do improve insulin sensitivity (Lee and Olefsky, 1995).

7.3.1.3 Troglitazone

Troglitazone (appendix II) is a TZD linked to an α-tocopherol (vitamin E) moiety. Bioavailability of troglitazone is approximately 50% and absorption is rapid, the plasma half life is usually 10-20h and steady state is achieved in about 4 days (Prescribing Information, Warner-Lambert Co). Clinical studies in Europe have recently found that in Type 2 patients basal glycaemia was significantly reduced by troglitazone and was also well tolerated. Initial clinical trials suggesting no weight gain have been superseded by long term studies suggesting weight gain in Type 2 diabetic patients. The hepatic metabolites of troglitazone are a sulphate and glucuronide (both inactive) and a quinone (active). The main clinical concern with troglitazone is the reported hepatotoxicity that has given rise to the suspension of treatment with troglitazone in the UK. In the USA, liver function is checked monthly for the first year of treatment and regularly thereafter.

7.3.1.4 Rosiglitazone

Rosiglitazone maleate (appendix II), (±)-[[4-[2-(methyl-2-pyridinylamino)-ethoxy]phenyl]methyl]-2,4-thiazolidinedione, (Z)-2-butenedioate (1:1) has been developed as an oral antidiabetic agent which acts primarily by increasing insulin sensitivity and is presently used in the USA for the management of Type 2 diabetes. The absolute bioavailability is 99%, and peak plasma concentrations are observed approx. 1 h after dosing. It is extensively metabolised with no unchanged drug excretion in the urine. The major routes of metabolism were N-demethylation and hydroxylation, followed by conjugation with sulphate and glucuronic acid. In clinical studies, treatment resulted in an improvement in glycaemic control, as measured by fasting plasma glucose and haemaglobin A1c, with a concurrent reduction in insulin and C-peptide. The maximum daily dose is 8mg. Reduction in hyperglycaemia was associated with increases in weight. Clinical data to date show no evidence of rosiglitazone-induced hepatotoxicity, however as it is structurally very similar to troglitazone, patients treated with rosiglitazone undergo periodic monitoring of liver enzymes (Prescribing Information, SmithKline Beecham Pharmaceuticals).

This study was undertaken to investigate whether rosiglitazone can alter basal or insulin-stimulated glucose uptake by cultured muscle cells. Such information would indicate whether rosiglitazone could act directly on muscle, and whether such an effect might contribute to its therapeutic blood glucose lowering effects.

It is not established whether rosiglitazone could act directly on skeletal muscle to increase glucose uptake and metabolism. Studies using another thiazolidinedione, troglitazone have suggested that this agent can increase glucose transport into muscle *in vivo* and *in vitro* more rapidly than would be anticipated for a transcriptionally mediated effect. Thus, the present study was undertaken using cultured L6 muscle cells to investigate whether rosiglitazone or troglitazone could alter basal or insulin-stimulated glucose uptake.

7.3.2 Experimental Design

Rosiglitazone and troglitazone were a kind gift from SmithKline Beecham. They were dissolved in DMSO and PBS (1:1 vol) at 10^{-2}M concentration; filter sterilised and stored at –20°C in 300μl aliquots. L6 cells were grown to confluence in 24 well plates and serum starved for 24h to promote differentiation and diffusion of myotubes as described in 2.2.2.

The thiazolidinediones were diluted and added to the cell incubation medium to give a final concentration over the range 10^{-9}M to 10^{-5}M. Rosiglitazone was studied at concentrations 10^{-9}M – 10^{-6}M as the clinical dose is up to 8mg/day, likely to give maximum circulating concentrations in the range 10^{-7}M – 10^{-8}M. Troglitazone was studied at concentrations 10^{-5}M – 10^{-9}M as the clinical dose is higher (up to 800mg/day) which is likely to give maximum circulating concentrations in the range of 10^{-5}M – 10^{-6}M.

To determine the effects of the thiazolidinediones on basal glucose transport, L6 cells were incubated with rosiglitazone or troglitazone for 24h, 48h, 72h and 96h and glucose uptake assessed as described in 2.4.1. Experiments were undertaken in multiples of six wells and six control wells were included in each 24 well plate.

To determine the effects of rosiglitazone and troglitazone on insulin stimulated glucose uptake; insulin was diluted as described in 3.4 and added to the incubation medium at a final concentration of 10^{-8}M for the final 24h of the incubation period. Glucose uptake was measured as described above using multiples of six wells.

7.3.3 Results

L6 muscle cells were incubated for 24h, 48h, 72h and 96h with rosiglitazone at concentrations 10^{-9}M – 10^{-6}M. Under all of these conditions there was no significant effect of rosiglitazone on basal glucose uptake (figure 7.9).

As shown in figure 7.7, troglitazone (10^{-9}M – 10^{-5}M for 24h - 96h) did not significantly affect basal glucose uptake, except that 10^{-7}M and 10^{-8}M troglitazone increased basal glucose uptake at 96h (by 31% and 32% respectively; $p<0.01$ and $p<0.05$ respectively). [The effect was confirmed in repeat experiments].

Insulin at 10^{-8}M for 24h produced a sub-maximal stimulation of glucose uptake. The effect of insulin (10^{-8}M for the final 24h) was not significantly changed by exposure to rosiglitazone (10^{-9}M – 10^{-6}M) or troglitazone (10^{-9}M – 10^{-5}M) for 24h - 96h (figure 7.8 and figure 7.10).

Figure 7.7 Effect of troglitazone on basal 2DG uptake by L6 skeletal muscle cells. Cells were stimulated with troglitazone (10^{-9}M – 10^{-5}M) for 24, 48, 72 or 96h. Troglitazone (10^{-8}M and 10^{-7}M) increased basal glucose transport after 96h cell exposure, but had little effect at any other concentration or period of exposure. Data are expressed as % control, values are mean ± SEM, n=6. *Significance versus control, $^1p<0.01$, $^2p<0.02$, $^3p<0.05$.

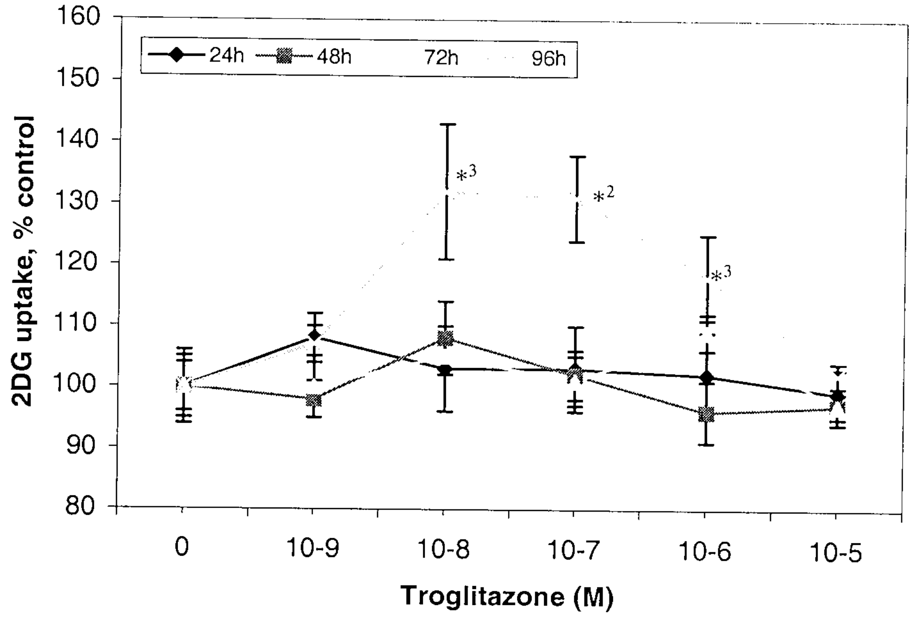


Figure 7.8 Effect of troglitazone on insulin-stimulated 2DG uptake by L6 skeletal muscle cells. Cells were stimulated with troglitazone (10^{-9}M – 10^{-5}M) for 24, 48, 72 or 96h and/or insulin (10^{-8}M). Troglitazone had no effect on insulin-stimulated glucose transport in L6 cells. Data are expressed as % control, values are mean ± SEM, n=6.

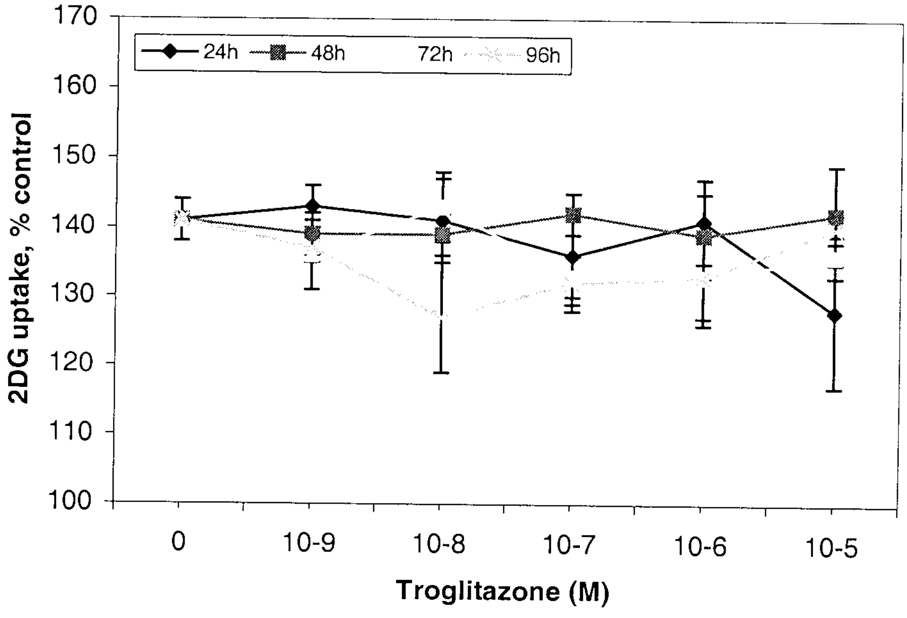


Figure 7.9 Effect of rosiglitazone on basal 2DG uptake by L6 skeletal muscle cells. Cells were stimulated with rosiglitazone (10^{-9}M – 10^{-6}M) for 24, 48, 72 or 96h. Rosiglitazone had no significant effect on basal glucose transport in L6 cells. Data are expressed as % control, values are mean ± SEM, n=6.

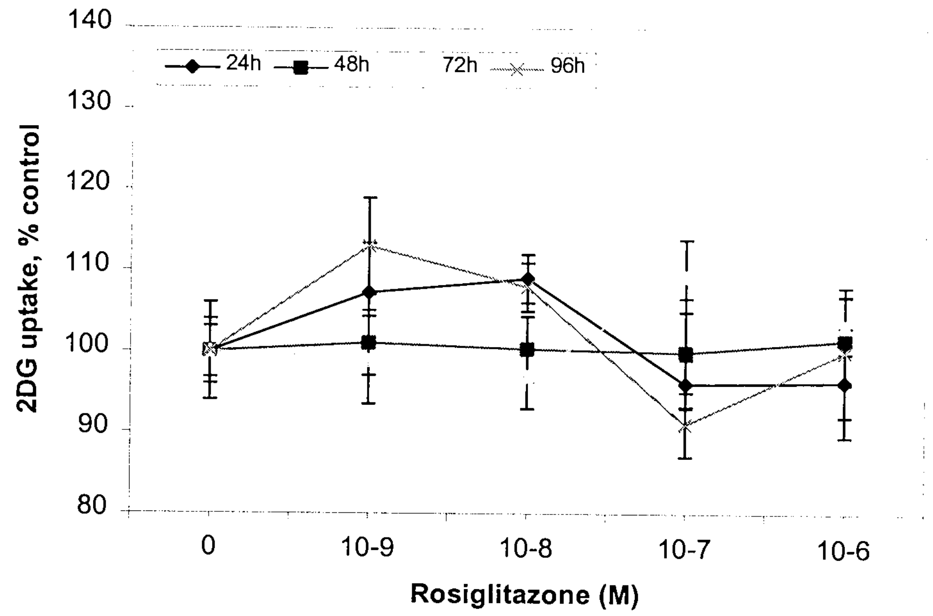
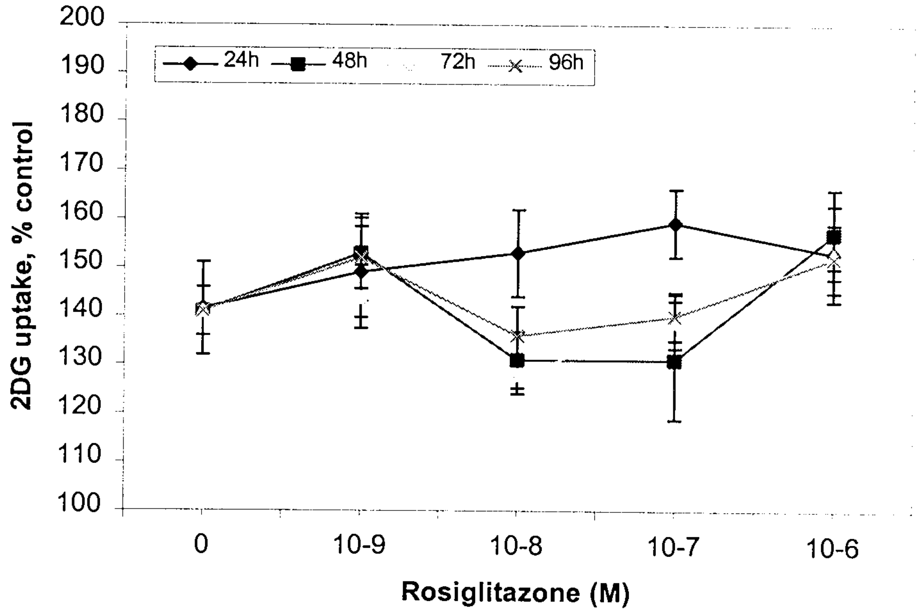


Figure 7.10 Effect of rosiglitazone on insulin-stimulated 2DG uptake by L6 skeletal muscle cells. Cells were stimulated with rosiglitazone (10^{-9}M – 10^{-6}M) for 24, 48, 72 or 96h and/or insulin (10^{-8}M). Rosiglitazone had no effect on insulin-stimulated glucose transport in L6 cells. Data are expressed as % control, values are mean ± SEM, n=6.



Part Four: Acute and Sub-chronic Effect of Vanadyl Sulphate on Basal and Insulin-Stimulated 2-deoxy-[^{3}H]-Glucose Uptake in L6 Cells

7.4.1 Introduction

Vanadium is an ultratrace element in mammals, which was discovered in 1830. Although it's exact role is unknown, oxidised forms of vanadium have been shown to possess insulin-like activity (reviewed by Tsiani and Fantus, 1997) the activity of which has been demonstrated *in vitro*, in rodents and in human diabetic subjects (Brichard and Henquin, 1995; Tsiani and Fantus, 1997). Vanadates, vanadyl salts and oxovanadiums have been extensively shown to lower blood glucose concentrations in both Type 1 and Type2 diabetic models by mimicking the action of insulin (Posner et al., 1990; Shechter, 1990).

Heyliger et al., (1985) first reported the successful treatment of STZ-induced diabetic rats with oral vanadate. Since then, numerous studies have demonstrated that oral administration of vanadate or vanadyl sulphate to STZ–induced diabetic rats and other animal models of insulin resistance normalises blood glucose levels (Heyliger et al., 1985; Meyerovitch et al., 1987 and 1991; Brichard et al., 1989 and 1992; Pederson et al., 1989; Blondel et al., 1989; Battell et al., 1992). More recently, a preliminary study investigated the acute and chronic oral administration of bis(maltolato)oxovanadium (BMOV) in the Zucker diabetic fatty rat (Yuen et al., 1999). BMOV was shown to significantly decrease plasma glucose levels and effectively preserved pancreatic β-cell function. Unfortunately, many studies have not measured food intake in these *in vivo* investigations and thus the glucose lowering effect of vanadate in STZ-induced diabetic rats may attributable, at least to some extent, to suppression of feeding (Malabu et al., 1994).

Vanadium has been shown to possess therapeutic potential in small-scale clinical studies of both Type 1 and Type 2 diabetic patients. Although vanadium has a significant biological potential, it has a poor therapeutic index and attempts have been made to reduce the dose of vanadium required for therapeutic effectiveness (reviewed by Badmaev et al., 1999). Organic forms of vanadium are recognised as safer and more absorbable. Thus numerous organic complexes of vanadium have been developed including BMOV as discussed above. Indeed, it has been reported that some vanadium compounds are more powerful phosphotyrosine phosphatase inhibitors than vanadate (Posner et al., 1994)

Unfortunately vanadate has the potential to disrupt normal metabolism by enzyme inhibition. Many studies have used high concentrations of vanadate to obtain significant hypoglycaemic effects without consideration of toxicological effects (Battell et al., 1992). However other investigations have reported loss of body weight, kidney failure and death (Saxena et al., 1992; Miralpeix et al., 1992). Hence, research into the use of vanadium compounds as agents to treat insulin resistance continues in order to seek solutions to overcome these problems.

7.4.2 Experimental Design

Vanadyl sulphate (Aldrich) was dissolved in PBS and diluted and aliquoted over the concentration ranges 10^{-2}M – 10^{-6}M.

In order to investigate the direct effect of vanadyl sulphate on glucose uptake in skeletal muscle *in vitro*, L6 cells were incubated with a range of concentrations of the compound at different time points. L6 skeletal muscle cells were grown to confluence in 24 well plates and serum starved to induce differentiation into myotubes as previously described (2.2.2). Cells were incubated for 10min, 1h, 4h or 24h with vanadyl sulphate (10^{-8}M – 10^{-4}M) and/or insulin (10^{-6}M). Glucose uptake was measured using the 2-deoxy-[^{3}H]-glucose uptake method as described previously (2.4.1). Cell viability was assessed by trypan blue exclusion as detailed in 2.4.5. Data are expressed as % of control, mean ± SEM, n=6. Significance was determined by Student's unpaired 't' test.

7.4.3 Results

After 10min incubations of L6 cells with (10^{-6}M – 10^{-4}M) vanadyl sulphate, basal glucose uptake was significantly increased by 21% - 28% ($p<0.05$ versus control, figure 7.11). 10^{-8}M vanadyl sulphate had no effect on glucose uptake. After 1h incubations, high concentrations of vanadyl sulphate (10^{-4}M) increased basal glucose uptake by 25% ($p<0.02$ versus control), however lower concentrations had no effect. Vanadyl sulphate had no effect on glucose uptake in L6 cells after 4h incubation periods, however after 24h it decreased glucose uptake in L6 cells. As shown in figure 7.11, the observed decrease in glucose uptake was concentration dependent and was highly significant when used at 10^{-4}M ($p<0.01$ versus control).

Insulin stimulated glucose uptake in L6 cells in a concentration and time dependent manner as described in 3.3.3. After 10min incubations, insulin stimulated glucose transport by 67% (figure 7.12). 10^{-8}M and 10^{-7}M vanadyl sulphate enhanced insulin-stimulated glucose uptake by a further 12% - 20% ($p<0.02$ versus insulin alone). 10^{-4}M vanadyl sulphate was without effect. Incubation of L6 cells with insulin for 1h increased glucose transport by 88%. Lower concentrations of vanadyl sulphate (10^{-8}M) increased insulin-stimulated glucose uptake by 23% ($p<0.05$ versus insulin alone). Higher concentrations of vanadyl sulphate decreased insulin-stimulated glucose uptake. 10^{-6}M reduced glucose uptake by 19% ($p<0.05$ versus insulin alone) and 10^{-4}M by 38% ($p<0.01$ versus insulin alone). Cell exposure to vanadyl sulphate for 4h or 24h decreased insulin-stimulated glucose uptake. After 4h incubations, 10^{-6}M decreased the response by 38% ($p<0.02$ versus insulin alone) and 10^{-4}M decreased glucose uptake by 62% ($p<0.01$ versus control). 10^{-8}M vanadyl sulphate was without effect. The inhibitory effect was more pronounced after 24h incubation periods, 10^{-6}M and 10^{-4}M vanadyl sulphate inhibited insulin-stimulated glucose uptake by 53% - 210% ($p<0.01$ versus insulin alone). 10^{-8}M vanadyl sulphate was without effect (figure 7.12).

Analysis of cell morphology after exposure to vanadyl sulphate demonstrated that it had a significant effect on cell viability and thus is likely to be highly toxic. At concentrations and time points where vanadyl sulphate was observed to decrease basal or insulin stimulated glucose uptake this effect was found to be caused by the toxicity effects of the compound (figure 7.13 and appendix III).

Figure 7.11 Effect of vanadyl sulphate on basal 2DG uptake by L6 skeletal muscle cells. Cells were stimulated with vanadyl sulphate (10^{-8}M – 10^{-4}M) for 10min, 1h, 4h or 24h. After 10min and 1h incubations vanadyl sulphate (10^{-4}M – 10^{-6}M) increased basal 2DG uptake (10^{-8}M was without effect). After 4h incubations vanadyl sulphate was without effect however after 24h 10^{-4}M vanadyl sulphate decreased basal 2DG uptake. Data are expressed as % control, values are mean ± SEM, n=12. *Significance versus control, $^{1}p<0.01$, $^{2}p<0.02$, $^{3}p<0.05$.

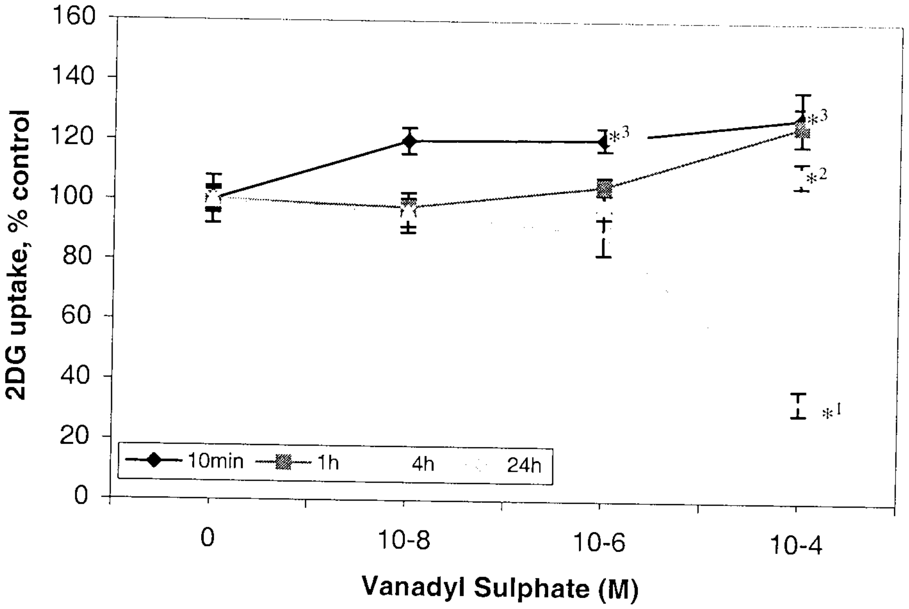


Figure 7.12 Effect of vanadyl sulphate on insulin-stimulated 2DG uptake by L6 skeletal muscle cells. Cells were stimulated with vanadyl sulphate (10^{-8}M – 10^{-4}M) for 10min, 1h, 4h or 24h, and/or insulin (10^{-6}M). After 10min incubations, vanadyl sulphate increased insulin-stimulated 2DG uptake. After 1h, 10^{-8}M vanadyl sulphate increased 2DG uptake but 10^{-6}M – 10^{-4}M decreased uptake. After 4h or 24h incubations vanadyl sulphate did not increase 2DG uptake, but 10^{-6}M and 10^{-4}M decreased the insulin-stimulated effect. Data are expressed as % control, values are mean ± SEM, n=12. †Significance versus insulin alone, $^{1}p<0.01$, $^{2}p<0.02$, $^{3}p<0.05$.

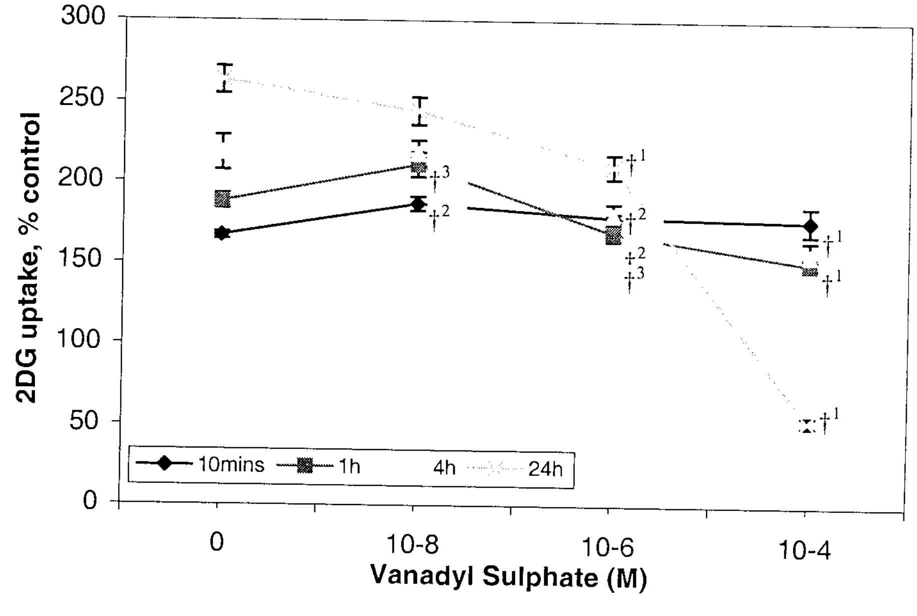
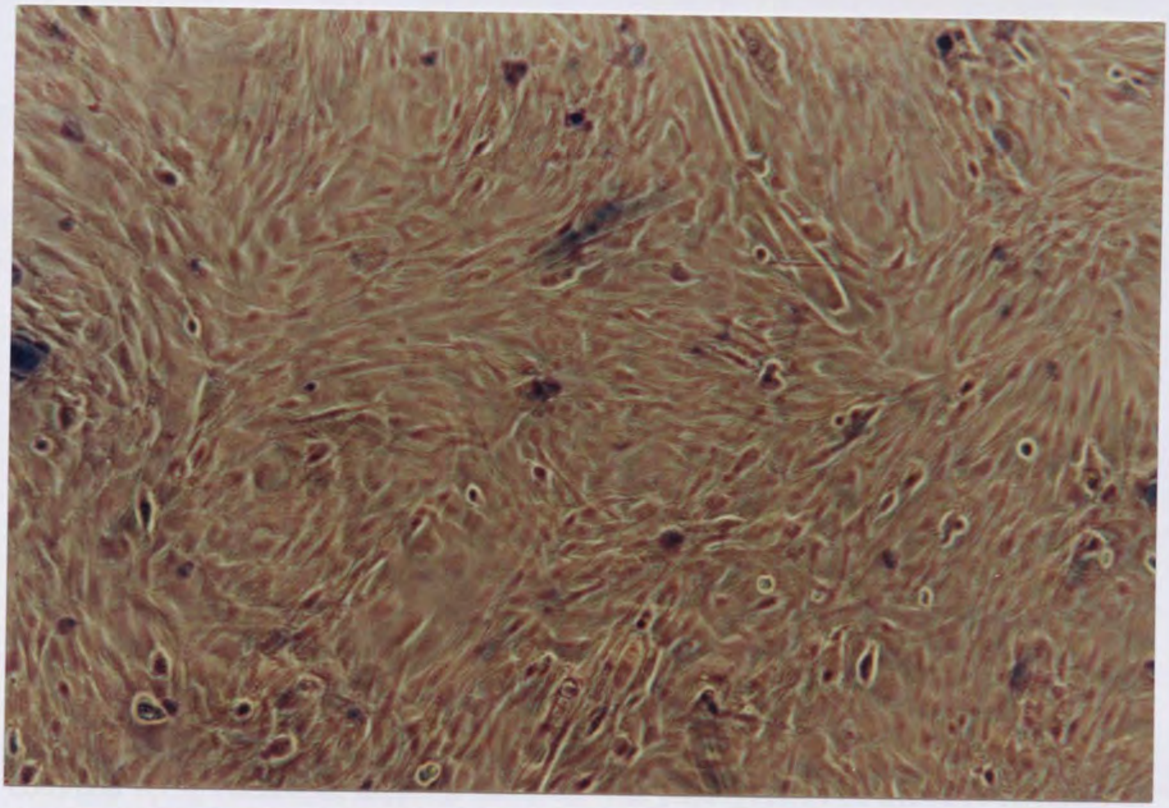
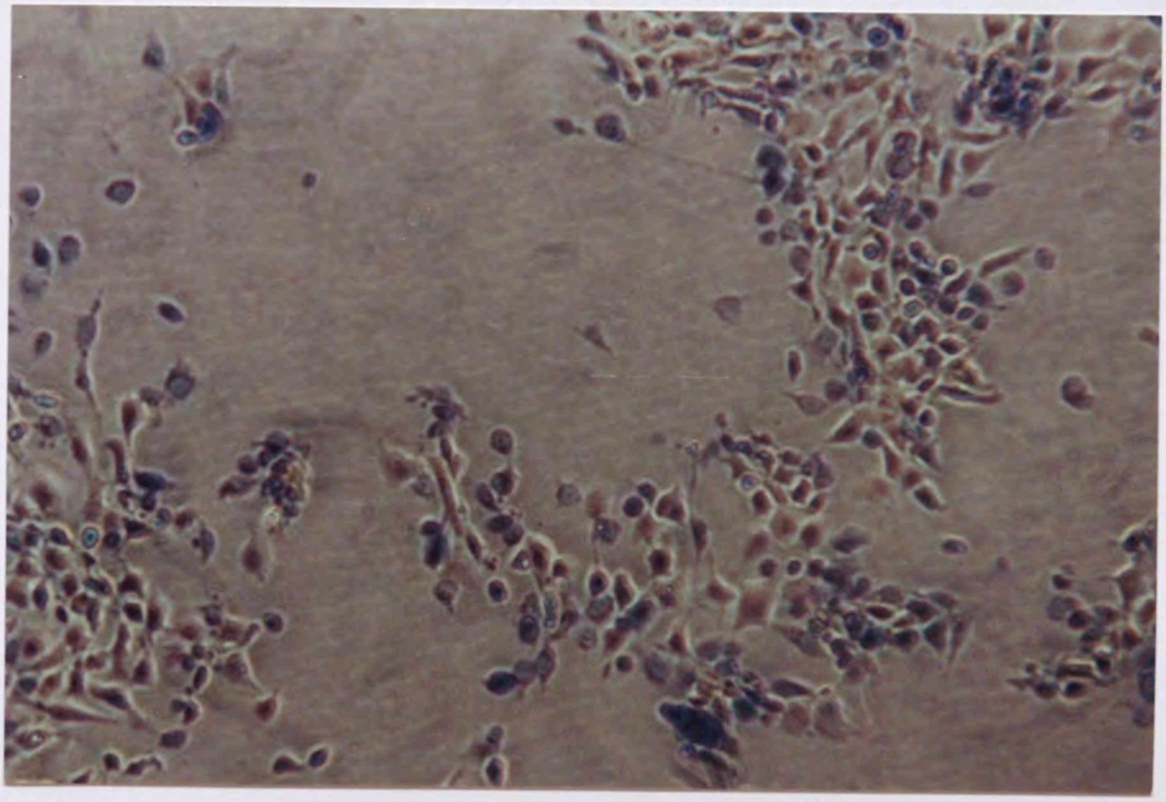


Figure 7.13 Photographs to demonstrate the effect of vanadyl sulphate on viability of L6 skeletal muscle cells. **7.13a.** Healthy cells stained with trypan blue. **7.13b** Cells were incubated with 10-4M vanadyl sulphate for 24h (as for glucose uptake studies) and stained with trypan blue. Photograph **7.13b** demonstrates that incubation of the cells with a high concentration of vanadyl sulphate for 24h caused damage to the majority of cells. Many cells (approximately 60%) had become detached from the surface of the plate and the remaining cells had taken up the blue dye indicating that vanadyl sulphate at this concentration is highly toxic. The effect of lower concentrations of vanadyl sulphate in L6 cells can be found in appendix III.

7.13a Healthy Control Cells



7.13b Cells Exposed to Vanadyl Sulphate (10^{-4}M for 24h)



Part Five: Acute and Sub-Chronic Effect of Thioctic Acid on Basal and Insulin-Stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 Cells

7.5.1 Introduction

Thioctic acid (α-lipoic acid) (appendix II) a natural cofactor in dehydrogenase complexes, is synthesised by the liver and has recently been implicated in insulin resistance. It is a natural cofactor in several enzyme complexes important in the regulation of carbohydrate metabolism, particularly pyruvate dehydrogenase.

Thioctic acid (TA) is a naturally occurring free radical scavenger and transition metal chelator known to exist as two different enantiomers: the biologically active (R)-isomer, and the (S)-isomer, which is part of the synthetic racemic mixture but is found minimally in biological tissues.

7.5.1.1 Diabetic Neuropathy and Glycation

Peripheral neuropathy is a process that affects the function of peripheral nerves, a common complication of diabetes mellitus. The pathogenesis of nerve fibre injury in diabetes is unknown, although it has long been held that the metabolic alterations of diabetes are responsible.

A peripheral, symmetric sensorimotor neuropathy is the most common form of diabetic neuropathy. Other forms include cranial and peripheral motor neuropathies and autonomic neuropathy (reviewed by Nathan, 1993).

Symptoms of distal sensorimotor neuropathy include paraesthesia, a tingling and numbness of the legs and sometimes the hands and less common, dysaesthesia with burning pains upon touching known as painful neuropathy. Distal neuropathy is identified by decreased or absent reflexes, decreased sensation to touch and vibration and wasting of interosseous muscles with flexion deformity. There is no treatment, other than foot care to prevent trauma and the prevention of foot ulcers (Nathan, 1993; Litzelman et al., 1991).

Brownlee (1994), reviewed the relationship between hyperglycaemia and diabetic complications, suggesting that advanced glycation end products (AGEs) play a central role via different pathways. These include the alteration of signal transduction pathways involving ligands on extracellular matrix and intracellular glycation by glucose, fructose and metabolic pathway intermediates, which can directly alter protein function in target tissues. A further mechanism suggested was that AGEs alter the level of soluble signals such as cytokines, hormones, and free radicals, through interactions with AGE-specific cellular receptors. When AGE binds to its macrophage receptor, it induces production of interleukin-1, insulin-like growth factor 1 and TNFα (Vlassara et al., 1988; Kirstein et al., 1992). Signal transduction by the AGE receptor appears to involve generation of oxygen free radicals (Yan et al., 1994).

Oxidative stress contributes to the vascular and neuropathic complications of experimental diabetes mellitus (Low and Nickander, 1991). Functional effects of antioxidant treatment include protection of vascular endothelium function, improved nerve flow, conduction velocity, and regenerative capacity (Nagamatsu et al., 1995; Cameron et al., 1994; Cotter et al., 1995; Love et al., 1996).

Thioctic acid (TA) has been investigated as a potential antioxidant treatment in diabetic rats. Treatment of diabetic rats with TA prevented the development of digital sensory nerve conduction velocity deficits, impaired sciatic nerve blood flow, diminished nerve glutathione and deficient glucose uptake by neural tissues (Nagamatsu et al., 1995; Low et al., 1996). TA was shown to act synergistically with the n-6 essential fatty acid γ-linoleic acid in its action against experimental diabetic neuropathy (Cameron et al., 1998; Hounsom et al., 1998). The effects of TA on symptomatic diabetic peripheral neuropathy and cardiac autonomic neuropathy have been investigated in short term clinical trials where it was demonstrated that TA may have beneficial effects (Ziegler et al., 1995; Ziegler et al., 1997). It has been successfully used clinically for the treatment of diabetic polyneuropathies (Ziegler and Gries, 1997; Ziegler et al., 1995). Most notably it has been widely used in Germany for the relief of symptoms of diabetic neuropathy (Estrada et al., 1996), and evidence suggests that an improvement in overall metabolic control occurs in patients treated with this cofactor. Indeed recently it was demonstrated that when TA was fed to old rats, it improved mitochondrial function, decreased oxidative damage and increased metabolic rate (Hagen et al., 1999).

7.5.1.2 Antidiabetic properties

Numerous studies have shown that thioctic acid may have potential therapeutic value in the treatment of insulin resistance. Acute and chronic administration of TA has been demonstrated to augment insulin-stimulated glucose transport into skeletal muscle of Zucker obese diabetic rats (Henriksen et al., 1994). It has been shown to stimulate glucose utilisation in rat hemidiaphragm (Haugaard et al., 1990), and in various tissue culture systems such as in skeletal muscle cells (L6 muscle cells) and in adipocytes (3T3-L1 cells), where thioctic acid was demonstrated to enhance glucose uptake (Estrada et al., 1996).

Khamaisi et al., (1997) demonstrated that prolonged thioctic acid treatment in STZ-diabetic rats reduced blood glucose, elevated GLUT 4 protein in gastrocnemius muscle, and normalised muscle lactate concentration. These changes were associated with a reversal of the diabetes-induced impairment in insulin-dependent 2DG uptake into isolated soleus muscle.

Conversely, the metabolic effects of TA were evaluated in rodent models of insulin resistance (fructose-fed Sprague-Dawley rats) and insulin deficiency (STZ-induced diabetic rats). Treatment of fructose-fed rats with TA had no significant effect on fasting or stimulated glucose levels. TA was shown to have no significant glucose lowering effects in STZ-diabetic rats. This demonstrated that after short-term oral therapy there were no significant improvements in glucose tolerance in rodent models of insulin resistance and insulin deficiency (Black et al., 1998).

In an acute glucose clamp study it was shown that the infusion of TA significantly increased glucose disposal in Type 2 diabetic patients (Jacob et al., 1995). Further clinical work has demonstrated that repeated parenteral administration of TA improves glucose disposal in patients with Type 2 diabetes (Jacob et al., 1996). More recently TA was shown to decrease serum lactate and pyruvate concentrations and improve glucose effectiveness in lean and obese Type 2 diabetic patients (Konrad et al., 1999).

7.5.1.3 Mechanism of Action

The exact mechanism of action of thioctic acid is unknown, however the insulin pathway has recently been investigated using this cofactor. Thioctic acid induced glucose uptake is abolished by wortmannin, an inhibitor of PI 3-kinase in adipocytes and muscle cells *in vitro*. Measurement of glucose transporters in these cell lines showed that upon stimulation with thioctic acid, the transporters were redistributed, suggesting that the mechanism of action of thioctic acid is dependent upon PI 3-kinase activity and the redistribution of glucose transporters (Estrada et al., 1996).

A recent study investigated the role of thioctic acid in cytokine induced glucose uptake in skeletal muscle (Khanna et al., 1999). It was demonstrated that interferon (IFN), lipopolysaccharides (LPS) and a combination of IFN, LPS and TNFα increased glucose uptake in L6 myotubes. This effect was demonstrated to be associated with an increased level of intracellular antioxidants and inhibited by the antioxidant pyrrolidinedithiocarbamate. Thioctic acid was able to stimulate glucose uptake in cytokine treated cells that are insulin resistant (Khanna et al., 1999).

Recently, the potential of TA to protect 3T3-L1 adipocytes against the induction of insulin resistance has been investigated when administered prior to exposure to oxidative stress (Bashan et al., 1999). TA was shown to protect against impaired insulin-stimulated GLUT 4 translocation and PKB activation induced by oxidative stress.

7.5.2 Experimental Design

The synthetic racemic mixture of thioctic acid (Sigma) was dissolved in PBS with 10μl DMSO and sonicated for 20min. To determine the effects of thioctic acid on glucose metabolism *in vitro*, L6 skeletal muscle cells were grown to confluence and differentiated to form myotubes as described previously (2.2.2). Cells were incubated for 10min, 1h, 4h or 24h with a range of concentrations of thioctic acid (10^{-6}M – 10^{-3}M) and/or insulin (10^{-6}M). Glucose uptake was measured as detailed in 2.4.1 and data analysed by Student's unpaired 't' test (2.6.1), n=6. Cell viability was assessed by trypan blue exclusion as previously described in 2.4.5.

7.5.3 Results

Incubations of L6 cells for 10mins with thioctic acid did not increase basal glucose transport at concentrations 10^{-6}M – 10^{-4}M (figure 7.14). However 10^{-3}M thioctic acid significantly decreased basal glucose transport by 39% ($p<0.01$ versus control). Thioctic acid produced a similar effect in L6 cells after incubations for 1h. 10^{-6}M – 10^{-4}M did not alter basal glucose uptake, however 10^{-3}M decreased glucose uptake by 23% ($p<0.01$ versus control). However, after 4h and 24h incubation of cells with 10^{-4}M thioctic acid, a significant increase in basal glucose uptake was observed; by 10% after 4h ($p<0.02$ versus control) and by 34% after 24h ($p<0.01$ versus control). 10^{-6}M, 10^{-5}M and 10^{-3}M thioctic acid did not affect basal glucose transport after 4h or 24h (figure 7.14).

Insulin stimulated glucose uptake as previously described (3.3.3). After 10mins, insulin increased glucose transport by 67%. As shown in figure 7.15, thioctic acid (10^{-6}M – 10^{-4}M) did not alter insulin stimulated glucose uptake, however 10^{-3}M thioctic

Figure 7.14 Effect of thioctic acid on basal 2DG uptake by L6 skeletal muscle cells. Cells were stimulated with thioctic acid (10^{-6}M – 10^{-3}M) for 10min, 1h, 4h or 24h. 10^{-6}M and 10^{-5}M thioctic acid had no effect on basal 2DG uptake in L6 cells. 10^{-4}M thioctic acid increased 2DG uptake after 4h and 24h incubations; conversely 10^{-3}M decreased 2DG uptake. Data are expressed as % control, values are mean ± SEM, n=12. *Significance versus control, $^1p<0.01$, $^2p<0.02$, $^3p<0.05$.

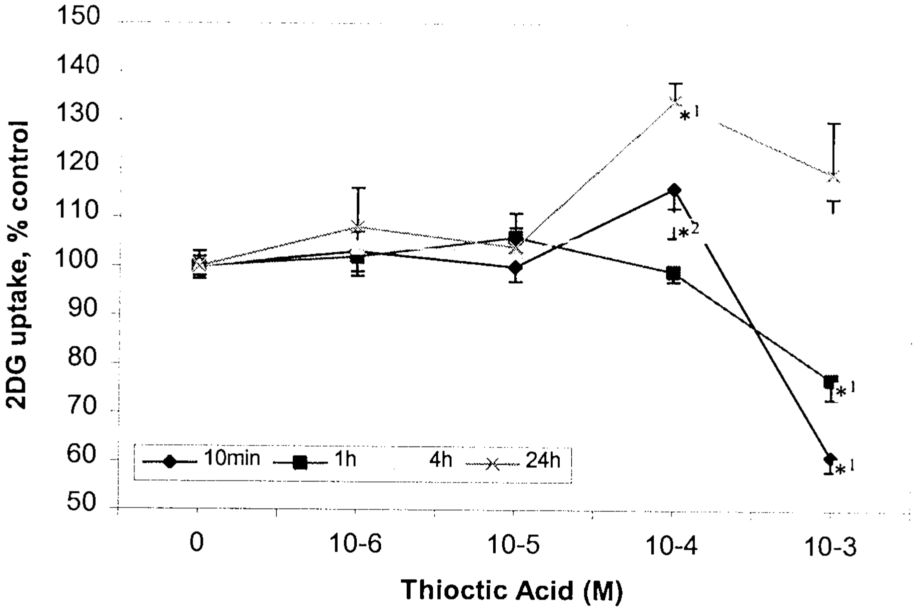


Figure 7.15 Effect of thioctic acid on insulin-stimulated 2DG uptake by L6 skeletal muscle cells. Cells were stimulated with thioctic acid (10^{-6}M – 10^{-3}M) for 10min, 1h, 4h or 24h, and/or insulin (10^{-6}M). At high concentrations (10^{-3}M) thioctic acid reduced insulin-stimulated 2DG uptake at all time points. Lower concentrations increased insulin-stimulated 2DG uptake after 1h incubations only. Data are expressed as % control, values are mean ± SEM, n=12. †Significance versus insulin, $^1p<0.01$, $^2p<0.02$, $^3p<0.05$.

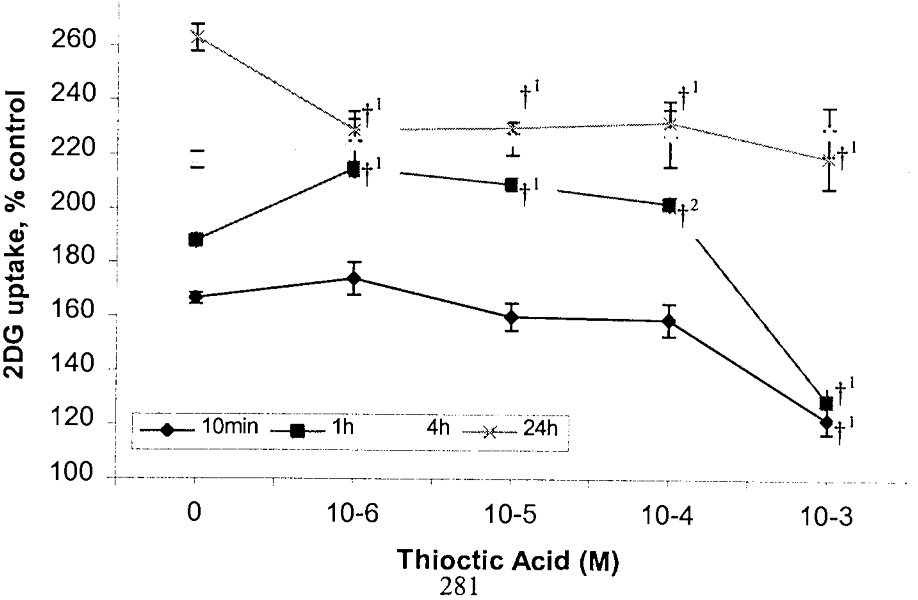
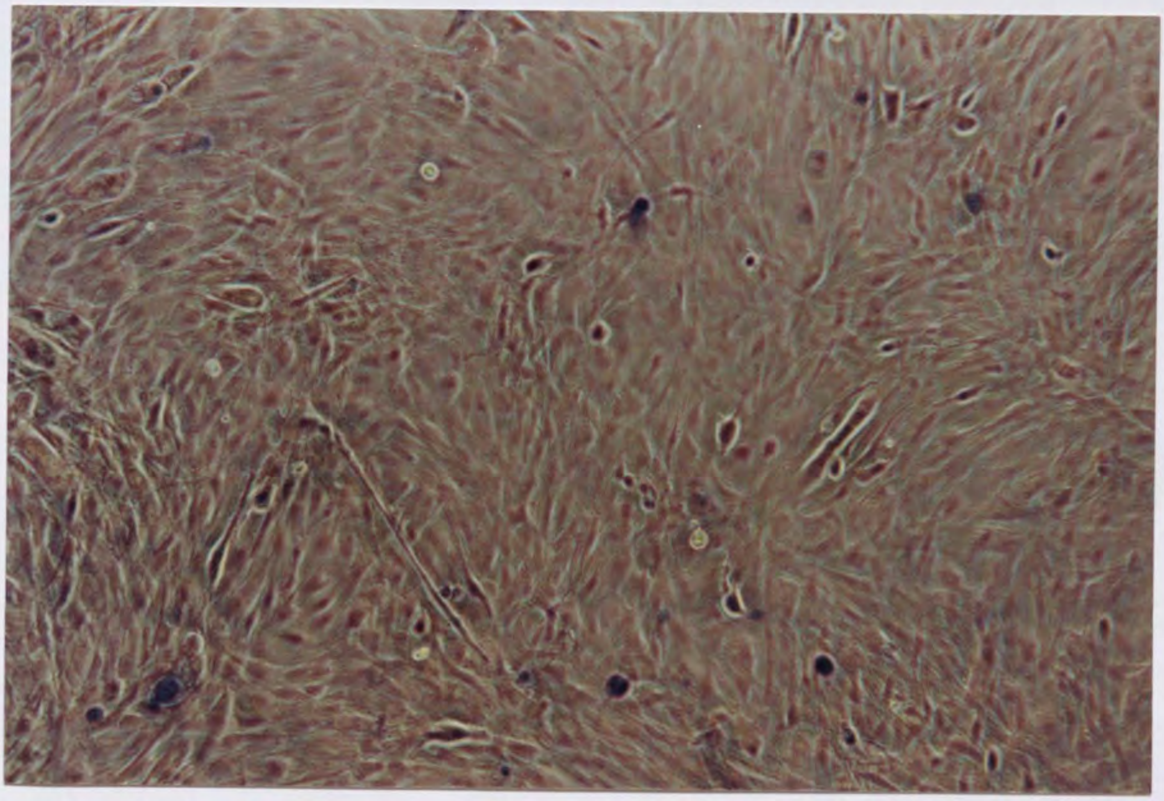
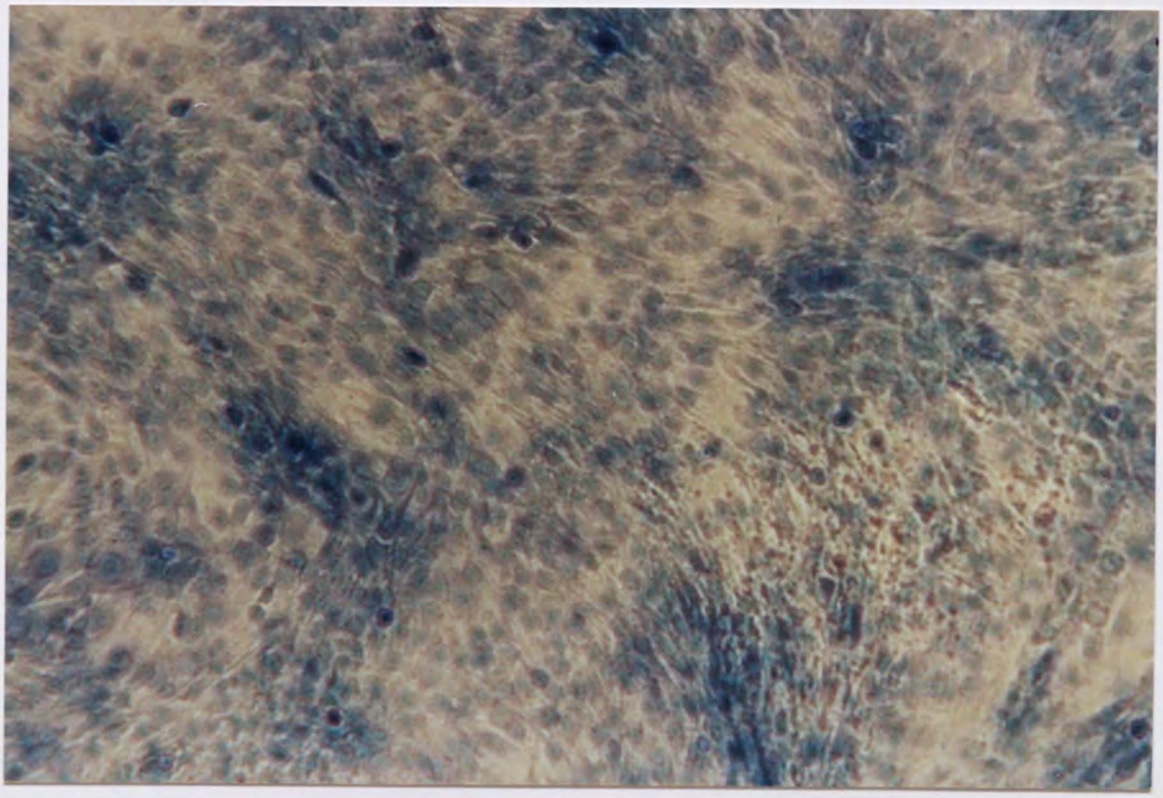


Figure 7.16 Photographs to demonstrate the effect of thioctic acid on viability of L6 skeletal muscle cells. **7.16a** Healthy cells stained with trypan blue. **7.16b** Cells were incubated with 10^{-3}M thioctic acid for 24h (a for glucose uptake studies) and stained with trypan blue. Photograph **7.16b** demonstrates that incubation of cells with 10^{-3}M thioctic acid caused the cells to take up the blue dye. Approximately 95% cells were stained blue and the remaining cells had become detached from the surface of the culture plate thus indicating that thioctic acid is highly toxic to L6 cells at this concentration. The effect of thioctic acid at lower concentrations can be found in appendix III.

7.16a Healthy Control Cells



7.16b Cells Exposed to Thioctic Acid (10^{-3}M for 24h)



acid significantly decreased glucose transport by 45% ($p<0.01$ versus control). After 1h incubations, insulin stimulated glucose uptake by 88% and thioctic acid significantly altered glucose transport at each concentration investigated. At lower concentrations (10^{-6}M – 10^{-4}M) thioctic acid increased insulin stimulated glucose uptake by 14% - 27% ($p<0.01$ 10^{-6}M and 10^{-5}M thioctic acid versus insulin alone, $p<0.02$ 10^{-4}M thioctic acid versus insulin alone). 10^{-3}M thioctic acid significantly decreased insulin-stimulated glucose uptake by 59% ($p<0.01$ versus insulin). 4h incubation of L6 cells with insulin increased glucose transport by 118% and thioctic acid had no effect on this response. 24h incubation of cells with insulin increased glucose uptake by 163%. Thioctic acid decreased insulin-stimulated glucose uptake at each concentration investigated (10^{-6}M – 10^{-4}M) by 31% - 50% ($p<0.01$ versus insulin alone).

Incubation of the cells with 10^{-3}M thioctic acid followed by staining with trypan blue demonstrated that thioctic acid potently affects cell viability at this concentration (figure 7.16 and appendix III).

7.6 Discussion

7.6.1 Metformin

Metformin increases both basal and insulin-stimulated glucose transport in L6 cells. This is in agreement with numerous studies, which have also demonstrated a stimulatory effect of metformin in L6 cells (Klip et al., 1992; Klip and Leiter, 1990), however the mechanism by which metformin mediates this effect intracellularly is largely unknown. Previous attempts to elucidate the cellular mechanism of action of metformin have primarily focussed on the effect of metformin on the insulin receptor and insulin receptor kinase activity (Wiernsperger N, 1996). However many studies, which have investigated the effects of metformin on the insulin receptor, have been inconsistent. Early studies found that the drug increased the number of insulin receptors in circulating cells by 20% in patients with either Type 1 or Type 2 diabetes (Pagano et al., 1983; Trischita et al., 1983; Lord et al., 1983). However, other studies have observed no effect of metformin on insulin binding in monocytes from Type 2 patients (Nosadini et al., 1987) or *in vitro* (Pedersen et al., 1989). Overall metformin appears to increase the number of insulin receptors when they are reduced in insulin resistant states. However the increased number of receptors does not account for any significant change in insulin action as due to the large number of 'spare' receptors already available in insulin-responsive cell membranes.

The effects of metformin on insulin-receptor kinase activity and receptor internalisation have been investigated in two major studies. A study by Rossetti et al., (1990), observed a decrease in kinase activity of streptozotocin-induced diabetic rat muscle that increased in response to metformin treatment. In contrast, it was found that in non-diabetic rat adipocytes that metformin had no effect on either receptor autophosphorylation or its exogenous kinase activity (Jacobs et al., 1986).

This study demonstrates that LY-294,002 has no effect on metformin-stimulated glucose transport in L6 cells. Thus the stimulatory effect of metformin on basal glucose transport (at least in L6 cells) is unlikely to be mediated via PI 3-kinase and thus must be mediated via a different mechanism. The intracellular mechanism of metformin action may be potentiated via components known to be integral for insulin signalling, however the results obtained in this study suggest that metformin is likely to act downstream from PI 3-kinase. This is in contrast to a number of recent studies, which have investigated the mechanism of action of metformin in a number of different models. Metformin has been shown to elevate receptor tyrosine kinase activity and inositol 1,4,5-trisphosphate mass in Xenopus oocytes (Stith et al., 1996). Stith et al., (1996) demonstrated that metformin (10^{-5}M – 10^{-4}M) increased insulin-stimulated meiotic cell division. Metformin was also demonstrated to elevate receptor tyrosine kinase activity and through this activation increased inositol 1,4,5-trisphosphate (IP_3) production. The authors noted that utilising whole cells required an incubation time of approximately 1h to induce an effect of metformin, however utilising a membrane preparation metformin was active within minutes thus suggesting that metformin may act directly at an intracellular site affecting the insulin signalling pathways.

The effect of metformin on early steps of insulin signal transduction has also been demonstrated in the liver and muscle of ageing rats (Nakamura et al., 1998). After 8h of metformin administration, there was a 2-3-fold increase in IRS-1/SHPTP2 association in muscle accompanied by a 50% decrease in IRS-1 phosphorylation, without any change in the liver. After 3 days of metformin administration there was a 3-4-fold increase in IRS-1 tyrosine phosphorylation and association with PI 3-kinase in both tissues of ageing rats (Nakamura et al., 1998). In addition, metformin has been shown to potentiate PI 3-kinase by IRS-2 recruitment in rat hepatic tissue (Grigorescu et al., 1998). In this study, intraportal bolus injection of insulin (1.5IU) was performed in Wistar rats, 90mins after oral administration of 4-400mg/kg metformin. Hepatic tissue was excised and various intracellular parameters measured. Metformin stimulated PI 3-kinase activity and the association of the insulin receptor with IRS-2 (Grigorescu et al., 1998).

This study demonstrated that the action of metformin is dependent on facilitated diffusion to increase glucose transport in skeletal muscle as the effect was inhibited by cytochalasin B, an inhibitor of glucose transporters. Klip et al., (1992) demonstrated that GLUT 1 and GLUT 4 levels were not significantly changed by metformin stimulation in L6 cells. This is in agreement with a recent study by Thomas et al., (1998) which demonstrated that metformin did not increase either the mRNA levels or total cellular membrane abundance of GLUT 1 and 4 in quadriceps isolated from metformin treated dexamethasone-induced insulin resistant mice. Thus it is likely that metformin stimulated glucose transport is mediated via an increase in intrinsic activity of glucose transporters already present at the cell membrane.

The results of the present study demonstrated that maximal stimulation of metformin was achieved after 24h. This effect was inhibited by cycloheximide an inhibitor of protein synthesis. This contrasts with an early study by Klip et al., (1992) which found that metformin-stimulated glucose uptake was not inhibited by cycloheximide. The results obtained in this study were of cycloheximide used over the range 10^{-8}M – 10^{-5}M and metformin was used over the range 10^{-4}M – 10^{-2}M. L6 cells were cultured in media containing 5% FCS, which was reduced to 0.5% FCS 24h prior to the addition of the test compounds. In contrast Klip et al., (1992) used a single concentration of cycloheximide (1μg/ml) and a single concentration of 400μM ($4x10^{-4}$M) of metformin (which caused half-maximal stimulation). In addition, the cells were cultured in serum free medium. It is established that metformin is not effective in the absence of insulin (Bailey, 1992), therefore serum free culture conditions are unlikely produce an environment in which the effects of metformin can be accurately studied. Thus the effect of cycloheximide on metformin stimulated glucose transport may be affected by determination in serum free media. These investigative differences may give rise to the discrepancies between the data presented here and those previously obtained by Klip et al., (1992).

As described previously, numerous studies have demonstrated that metformin does not cause translocation of GLUT to mediate glucose transport and thus is due to intrinsic GLUT activity. Therefore if metformin is inhibited by cycloheximide then the process is mediated at least in part, by protein synthesis. Therefore, the mechanism by which metformin stimulates glucose transport in L6 cells is likely to be via the synthesis of integral signalling proteins which directly or indirectly act to increase the intrinsic activity of glucose transporters.

Thus, metformin acts to increase both basal and insulin stimulated glucose transport. This effect may be mediated via a mechanism alternative to PI 3-kinase mediated signalling and is likely to be dependent on the synthesis of signalling proteins, which act to increase the intrinsic activity of glucose transporters at the cell membrane.

7.6.2 Tolbutamide

There are conflicting data on the existence of extrapancreatic effects brought about by the sulphonylureas. In this study 10^{-3}M tolbutamide caused a trivial increase in basal glucose uptake in L6 cells but was without effect at 10^{-4}M. This is consistent with recent studies suggesting that significant effects of sulphonylureas on muscle cells are only evident at inordinately high concentrations. Gliclazide has been demonstrated to significantly increase rates of insulin-stimulated glycogen synthesis in rat soleus muscle strips over a 8h period, however acute incubation of human rectus abdominal muscle strips resulted in a slight but non significant rise in the rate of glycogen synthesis (Webster and Taylor, 1996). Therefore it is likely that sulphonylureas exert some extrapancreatic effects, but the hypoglycaemic activity of these agents is primarily exerted via the stimulation of insulin secretion.

7.6.3 Thiazolidinediones

The results indicate that rosiglitazone does not act on cultured L6 muscle cells to significantly alter basal or insulin-stimulated glucose transport over 96h, which would allow ample time for a transcriptionally mediated effect.

Although troglitazone did not alter glucose uptake under most of the experimental conditions tested, 10^{-7}M and 10^{-8}M troglitazone increased basal glucose uptake after 96h. This might be dismissed as an anomalous finding in need of verification, especially since it occurred at only two concentrations within the range of five tested. However the observed increase was of the order of 30% and was reproduced in an independent set of experiments, leaving open the possibility that this is a ‘real’ effect. One possible explanation for this is that troglitazone contains a vitamin E moiety as part of its structure and this may be responsible for increasing glucose uptake independent of the TZD moiety.

Whether L6 cells can significantly metabolise rosiglitazone or troglitazone is unknown, but is considered unlikely since *in vivo* metabolism of these thiazolidinediones occurs almost entirely in the liver *in vivo*. Thus the present lack of effect of rosiglitazone cannot be explained by the rapid degradation of the thiazolidinedione.

A previous study reported that troglitazone doubles basal glucose uptake by L6 muscle cells after 72h (Cirialdi et al., 1995). However, the M3 metabolite of troglitazone (which is an active quinone) increased basal glucose uptake 5-fold after 72h, and there was no further effect of insulin stimulation (Cirialdi et al., 1995). This was accompanied by increased amounts of GLUT1 and to a lesser extent GLUT 4 in the plasma membrane, as measured by western blotting. The authors concluded that the M3 metabolite directly increases glucose uptake into cultured muscle cells not necessarily due to potentiation of insulin action (Cirialdi et al., 1995). Thus the possibility is raised that a metabolite of troglitazone can increase glucose uptake by muscle independently of any effect of the parent drug itself. The only clue to the mechanism is that M3 was not effective after 1h. Unfortunately the study of Cirialdi et al., (1995) only looked at two time points (1h and 72h) and only used one concentration of each compound (troglitazone at 2μg/ml, approx. 5×10^{-7}M; M3 at 15μg/ml, approx. 4×10^{-6}M). In this study, troglitazone (10^{-5}M-10^{-9}M) was not effective at 72h, but 10^{-7}M and 10^{-8}M increased basal glucose uptake at 96h. This could represent minor variability between the passages of L6 muscle cells or other differences in experimental procedure. Nevertheless, the present study provides some evidence that troglitazone may have a modest direct effect on basal glucose transport during long term exposure to cultured muscle cells. Furthermore, troglitazone has been reported to rapidly (<1h) increase glucose utilisation by muscle *in vivo* and *in vitro* (Lee and Olefsky, 1995; Okuno et al., 1997; Furnsinn et al., 1997). One of these studies, in isolated rat soleus muscle, suggested that troglitazone (unlike insulin) directly enhanced anaerobic glycolysis but suppressed glycogen synthesis (Furnsinn, 1997). This superficially resembles the effects of hypoxia and muscle contraction.

7.6.4 Vanadyl Sulphate

Vanadyl sulphate was observed to exert a stimulatory effect on basal glucose uptake in L6 cells only at short time points using inordinately high concentrations. At lower concentrations, effects were not observed, and at high concentrations for longer incubation time points vanadyl sulphate reduced cell viability. A similar effect of vanadyl sulphate was observed on insulin-stimulated glucose uptake, except that lower concentrations of the compound were capable of producing a response compared to concentrations required to stimulate basal glucose transport.

Previous *in vitro* studies have demonstrated that vanadium augments glucose uptake and utilisation in muscle (Venkatesan et al., 1991). Glucose uptake, skeletal muscle glycogenesis and oxidation, adipose tissue lipogenesis and decreased lipolysis are known effects of these trace elements (Bailey and Flatt, 1996). The exact mechanism of action of vanadium and related compounds are unknown, however the insulin-like effect has been associated with increased levels of phosphorylated tyrosine proteins. Indeed, a stimulatory effect of oxovanadiums on both receptor tyrosine kinase and cytosolic protein tyrosine kinase activities has been demonstrated (Fantus et al., 1989; Evans et al., 1994). This appears to result from their inhibitory effect on phosphotyrosine phosphatases (Swarup et al., 1982). The inhibitory effect of vanadate on protein tyrosine phosphatases is both potent and selective (Swarup et al., 1982) and early studies suggested that its biological effects may result from maintaining membrane receptors and other proteins in an a state of phosphorylation by inhibiting PTPases or activating tyrosine kinases. Vanadium has since been shown to mimic the action of insulin through alternative signalling pathways which involve the inhibition of phosphotyrosine phosphatases and the interplay between two non-insulin receptor tyrosine kinases (Elberg et al., 1997; Tsiani and Fantus, 1997). Vanadate is structurally analogous to phosphate, and this may partly account for its inhibitory effects on phosphatases (Lui et al., 1997).

The results indicate that vanadyl sulphate possesses potent insulin mimetic properties in L6 skeletal muscle cells. Unfortunately the increase in glucose transport elicited by vanadyl sulphate can only be observed for less than 1h before the element reduces cells viability, thus it is likely that it is highly toxic. Reduction of the concentration of vanadyl sulphate reduces the toxic effects of the compound although lower doses did not produce a response.

As the insulin-mimetic properties of vanadyl sulphate have been well documented, it has been previously considered for investigation as an oral therapy for the replacement of insulin in Type 1 diabetes. The results obtained in this study demonstrate that vanadyl sulphate appears to produce a bigger response in the presence of high concentrations of insulin thus removing the appropriateness of this compound as a treatment of Type 1 diabetes.

Vanadyl sulphate is unlikely to be developed as a suitable candidate for the treatment of diabetes, as the insulin-mimetic properties are not apparent at non-toxic concentrations. Thus the development of organic alternatives with increased efficacy and better therapeutic index may alleviate these problems and provide a more suitable candidate for a vanadium-derived insulin-mimetic therapy.

7.6.5 Thioctic Acid

The results obtained in this chapter demonstrated that thioctic acid increases basal glucose uptake between 4h and 24h cell exposure. A range of concentrations of the compound was tested and it was found to be active at 10^{-4}M only. Lower concentrations of thioctic acid were found to be ineffective and higher concentrations were found to affect cell viability. In the presence of added insulin, the response to thioctic acid was more acute (within 1h of addition of insulin and thioctic acid) and required lower concentrations to produce a response. It may be possible that in the absence of added insulin TA stimulates glucose transport, however if insulin is already maximally stimulating glucose uptake (i.e. after 24h) then TA is without effect.

These results are in keeping with those obtained by Estrada et al., (1996) who reported a significant increase in glucose uptake in L6 myotubes after incubations of 1h. As described previously, Estrada et al., (1996) reported that thioctic acid-stimulated glucose uptake via GLUT 1 and 4 translocation and that this was inhibited by wortmannin, thus demonstrating that thioctic acid is likely to increase glucose uptake via a PI 3-kinase dependent intracellular pathway.

Unfortunately it was difficult to accurately interpret the results of chronic exposure of cells to thioctic acid due to the confounding effects of toxicity. Significant increases in glucose transport after longer incubation periods may have been masked by a decrease in cell viability. The problems of toxicity may be alleviated in time by further analysis of the different isomers of thioctic acid.

Estrada et al., (1996) determined the effects of the different isomers of thioctic acid on glucose transport in L6 cells. All three formulations (R)-(+), (S)- and their racemic mixture stimulated glucose uptake reaching a plateau within 1h. Glucose uptake was reported to be higher after exposure to (R)-(+) than it was after incubation with (S)- or racemic mixture. The authors also described optimum stimulation of glucose transport using 2.5×10^{-3}M thioctic acid within 1h incubations. Thus their remaining study focussed on the use of (R)-(+) under optimum stimulatory conditions for the remainder of the study. In this chapter, such high concentrations of thioctic acid were found to decrease cell viability. However Estrada et al., (1996) did not report any observations of an adverse effect of thioctic acid on cell toxicity. In this chapter the racemic mixture of thioctic acid was used to stimulate glucose transport and this may be the reason there are discrepancies between the data described here and the results obtained by Estrada et al., (1996).

There have been some claims regarding the potential therapeutic utility of thioctic acid. Thioctic acid is presently sold as 100mg tablets (Natural Health Consultants, NHC) with the recommendation that healthy individuals need 20mg-50mg/day and that diabetic patients (Type not specified) should receive 200mg. Thioctic acid is advertised by NHC as a treatment for complications associated with diabetes such as neuropathy, macular degeneration and cataracts. It is claimed to regenerate damaged nerves and prevent glycation. Thioctic acid is also alleged to exert weight loss, protect against radiation, prevent kidney stone formation, remove toxic heavy metals and is hepatoprotective. In addition it is thought to be a strong antiviral agent and is said to prevent viral replication in many kinds of agents including human immunodeficiency virus (HIV).

Whilst it is unlikely that thioctic acid possesses such pronounced effects (bottles of thioctic acid sold as supplements for diabetic patients warn about thioctic acid-induced hypoglycaemic episodes), thioctic acid acutely increases glucose transport in skeletal muscle and therefore may possess antidiabetic properties due to an action on peripheral tissue. This clearly needs to be investigated further in clinical trials to assess the efficacy, therapeutic index and potential side effects.

Chapter Eight:
General Discussion

Chapter Eight: General Discussion

8.1 Introduction

Type 2 diabetes mellitus is caused by insulin resistance and abnormal β-cell function, which together contribute to a series of metabolic disturbances, the pathophysiology of which remains incompletely understood. The prevalence of Type 2 diabetes is increased in populations where high energy intake and obesity are common and approximately 60-90% of Type 2 diabetic patients are or have been obese (DeFronzo, 1992).

In Type 2 diabetes, the reduced biological response of insulin target tissues is believed to initiate a compensatory increase in insulin secretion, which eventually gives way to β-cell failure (Reaven, 1988). The defect in insulin action is the focus of this thesis as current evidence favours the view that insulin resistance is a primary underlying cause of most cases of Type 2 diabetes.

At the molecular level, insulin action is mediated via a complex branching cascade of kinase and phosphatase reactions that link the insulin receptor to the biological effects (Kahn, 1994). This thesis focuses on the pathway that mediates the acute metabolic effects of insulin, which ultimately result in glucose uptake. In brief, the initial event is insulin binding to its receptor, which results in stimulation of the kinase activity causing receptor autophosphorylation thought to be essential for insulin signalling. This in turn causes phosphorylation of a number of intracellular substrate proteins including insulin receptor substrate protein 1 (IRS-1) thus binding intracellular proteins including PI 3-kinase. PI 3-kinase phosphorylates the D-3 position of inositol ring of phosphatidylinositol to produce PI-3-P, PI-3,4-P_2 and PI-3,4,5-P_3 of which PI-3,4,5-P_3 is thought to be the main messenger in this pathway. The next component of this pathway has been named 3-phosphoinositide-dependent protein kinase (PDK-1) as it is only active in the presence PI-3,4-P_2 or PI-3,4,5-P_3. Down stream from PDK-1 lies protein kinase B (PKB) which is thought to activate glucose transporters (GLUTS) to stimulate glucose uptake (figures 1.1 an 1.2)

This thesis explores the use of a number of pharmacological agents to modify insulin action in cultured skeletal muscle cells and in animal models of diabetes, insulin resistance and obesity. Potential mechanisms of action of these compounds were investigated using known inhibitors of proteins that mediate intracellular signalling. This work was undertaken to gain a better understanding of insulin action in skeletal muscle and to investigate potential new therapies for the treatment of insulin resistance in diabetes and obesity.

8.2 Overview of Results

8.2.1 Characterisation of the Rat L6 Skeletal Muscle Cell Line

Skeletal muscle is quantitatively the major site responsible for glucose transport (DeFronzo, 1988). Thus the rat L6 skeletal muscle cell line was utilised for the majority of *in vitro* investigations in this thesis. There are several advantages for using a cell culture system over intact organ preparations or *in vivo* studies; cell type homogeneity, measurements are not complicated by surgical damage, even and rapid accessibility of all cells to substrates. Also there are no confounding effects of weight loss to take into consideration, no sympathetic nervous system involvement, tight, even control of insulin levels can be achieved and any observed effect is likely to be due to a direct action.

L6 cells have previously been shown to possess several characteristics of skeletal muscle *in vivo* (Shainberg et al., 1971; Kidboro, 1975), and this is supported by the data obtained in chapter 3. The results demonstrated that the cells divide in culture a finite number of times before subsequent fusion to form multinucleated muscle fibres. This was originally described by Yaffe (1968) and demonstrates that multiple sub-cloning, freeze/thaw cycling and repeated passaging has not destroyed the original phenotypical observations of the cell line.

Insulin action in L6 cells was investigated in chapter 3. The significant increase in glucose uptake observed upon cell exposure to insulin suggested that this cell line was a suitable system in which the response of glucose transport to insulin could be studied. This response was further examined utilising inhibitors of insulin mediated intracellular signalling. The role of PI 3-kinase in basal and insulin-stimulated glucose transport was investigated using wortmannin and LY-294,002. The data demonstrated that both wortmannin and LY-294,002 decreased both basal and insulin-stimulated glucose uptake. However toxicological investigations into the effect of wortmannin on cell viability demonstrated that incubation of the cells for protracted periods of time or with high concentrations of wortmannin decreased cell viability. Therefore in order to determine the effects of chronic stimulation of L6 cells with insulin, LY-294,002 was employed as a potential inhibitor of PI 3-kinase as it did not decrease cell viability after protracted exposure in L6 cells. Thus the compound could potentially be utilised in place of wortmannin for investigating insulin-stimulated protein synthesis.

Jak2 has not previously been implicated in insulin signalling, however it is thought to play a significant role in the effect of leptin on mammalian physiology. Intracellular signalling via the long isoform of the leptin receptor is thought to act directly via Jak2 and a Jak2 binding site has recently been mapped on the leptin receptor tail (Banks et al., 1999). Thus the investigations reported in chapter 3 determined whether Jak2 plays a role in insulin signalling, and also as a prelude to investigations in chapter four (leptin). Tyrphostin AG490 is an inhibitor of Jak2, and L6 cell exposure to this compound did not inhibit basal or insulin-stimulated glucose uptake thus indicating that insulin signalling is not mediated via Jak2.

The kinetics of glucose transport in L6 cells have previously been characterised (Klip et al., 1982) and much is already known about the nature of glucose transporters in L6 cells (Klip, 1982; Wilson et al., 1995). In chapter 3, glucose uptake in L6 cells was inhibited by cytochalasin B as glucose competes with cytochalasin B for reversible binding to the glucose transporter during facilitated diffusion of glucose. However analysis of cytochalasin B inhibition of glucose transport does not distinguish between the GLUT isoforms present on the cell surface. This has previously been completed to determine the ratios of each isoform and the quantities of functional transporters on the cell surface of L6 cells (Wilson et al., 1995). Thus it has been stated that L6 cells express saturable glucose transport kinetics of similar pharmacology as adult skeletal muscle (Klip et al., 1982; Klip et al., 1987).

Insulin may regulate the number of GLUTS at the cell surface via a number of different mechanisms, i.e. via GLUT protein synthesis, via GLUT translocation from an intracellular pool to the cell surface and via an increase in the intrinsic activity of GLUTS already present at the cell surface. Chapter 3 further documented an inhibitory effect of cycloheximide glucose uptake in L6 cells. This was shown to be significantly dependent upon time. Thus in L6 cells both GLUT1 and GLUT4 appear to be regulated by both protein synthesis and translocation/intrinsic activity (James et al., 1988).

8.2.2 Leptin

Leptin was identified in 1994 as the product of a gene that is defective in *ob/ob* mice (Zhang et al., 1994). It is a hormone secreted by adipocytes and is known to act via the hypothalamus to mediate behaviour including decreased voluntary feeding and metabolic efficiency. Obese individuals have high leptin circulating levels due to leptin resistance although the mechanism of this resistance state is unknown (Montague et al., 1997). The results obtained in chapter 4 demonstrated that chronic repeated treatment of *ob/ob* mice (which lack endogenous leptin) with exogenous leptin significantly decreased food intake and body weight. Leptin treatment reinstated normal circulating leptin concentrations, which subsequently reinstated an increase in glucose uptake by soleus muscle in response to insulin. The long-term improvement in glucose uptake by leptin was not as great as pair feeding, suggesting leptin may attenuate the improvement in muscle glucose metabolism brought about by the decrease in feeding. The effects of leptin in decreasing food intake and body weight were found to be rapid (within 48h of treatment), however after 48h treatment there was no effect on glucose uptake in soleus muscles indicating that the effect of leptin on muscle insulin sensitivity may occur via indirect mechanisms. In addition, acute *in vitro* investigation indicated that leptin does not exert a direct acute effect on glucose uptake by soleus muscles of *ob/ob* mice.

Leptin effects on other cells involved in glucose homeostasis are controversial, this has given rise to numerous studies that have utilised a variety of cell lines to investigate potential effects. The results of chapter 4 showed evidence of considerable leptin action on glucose uptake in L6 cells, but the data do not suggest a simple mechanism of action. The data suggest that *in vitro,* leptin alone can acutely mimic the effects of insulin with regard to the stimulation of glucose uptake, indicating that leptin must possess an acute paracrine action. This stimulatory effect of leptin on basal glucose uptake in skeletal muscle has also been observed utilising the C_2C_{12} skeletal muscle cell line (Berti et al., 1997). Furthermore it was demonstrated that the effects of leptin on basal glucose transport were inhibited by the PI 3-kinase inhibitor wortmannin. This is in keeping with data obtained in chapter 4 which clearly demonstrate that the leptin-stimulated glucose transport is potently inhibited by wortmannin indicating that leptin directly activates the insulin signalling pathway possibly at the level of PI-3 kinase. Leptin has been demonstrated to activate PI 3-kinase in C_2C_{12} myotubes via a Jak2 and IRS2 dependent pathway (Kellerer et al., 1997), conversely data reported in this thesis demonstrate that the leptin-stimulated increase in glucose transport in L6 cells was not conducted via Jak2. Furthermore, L6 cells signal via the short form of the receptor which is unlikely to signal via Jak2 as this is known to be mediated via the phosphorylation of tyrosine residues present only on the intracellular tail of the long receptor form (Tartaglia 1997). The tissue expression of the leptin receptor is likely to play an important role in mediating the effects of leptin. It can be postulated that soluble leptin receptors e.g. OB-Ra are present in the cell membranes and may transport leptin into the cells in a similar mechanism to their postulated action in the choroid plexus.

A further direct effect of leptin in these cells was demonstrated in chapter 4. At high leptin concentrations comparable with those observed in obese individuals, inhibition of insulin-stimulated glucose transport was observed. A similar effect has been reported in isolated rat adipocytes (Muller et al., 1997), indicating that leptin may directly attenuate insulin action in skeletal muscle. This mechanism has been further investigated in hepatic cells expressing the long form of the leptin receptor (Cohen et al., 1996). Leptin inhibition of insulin-induced activities included tyrosine phosphorylation of IRS-1, association of the adapter molecule growth factor receptor-bound protein 2 with IRS-1, and down regulation of gluconeogenesis. Interestingly, leptin was also observed to increase the activity of IRS-1 associated PI 3-kinase in the hepatic cells, a mechanism that could increase basal glucose transport as observed in L6 cells.

8.2.3 Sibutramine

Sibutramine is a serotonin and noradrenaline reuptake inhibitor (SNRI), which induces satiety, stimulates thermogenesis and reduces weight gain (Jones et al., 1997). It is used clinically in the treatment of obesity and has recently been demonstrated to possess anti-insulin resistant properties in insulin resistant mice (Day and Bailey, 1998) thus indicating potential therapeutic effects as a treatment for insulin resistance. Data presented in chapter 5 of this thesis demonstrate that sibutramine metabolites can increase glucose uptake by cultured L6 muscle cells. Sibutramine is metabolised *in vivo* to the secondary amine metabolite M1, and then to the primary amine metabolite M2. M2 increased glucose uptake by 24h whereas M1 was not effective at 24h, but became effective by 72h. This suggests that gradual conversion of M1 to M2 could at least partly account for the effectiveness of M1 after M2. Further experimentation demonstrated that M2 predominantly increases insulin sensitivity, with little increase in total responsiveness to maximally-stimulating insulin concentration. This suggests that M2 may activate or enhance signalling pathways and/or the final biological effectors that mediate insulin action, rather than cellular mechanisms that are entirely independent of insulin (which might be expected to be additive to insulin). Although decreased adiposity itself would be expected to reduce insulin resistance, the present study suggests that M2 also acts directly on muscle to improve insulin action. M2 was not inhibited by LY-294,002, therefore it is unlikely that the action of M2 in increasing glucose uptake in L6 cells is mediated via PI 3-kinase. It is possible that M2 binds to a separate receptor and intracellular signalling is mediated by unknown proteins that act upon the insulin-signalling cascade down stream from PI 3-kinase. The increase in glucose uptake observed is mediated by facilitated diffusion of glucose as the effect of M2 was inhibited by cytochalasin B. Thus M2 increases glucose uptake via a glucose transporter dependent mechanism. This action is not apparent until 24h after cell exposure to M2 thus this effect is likely to be dependent upon glucose transporter synthesis rather than translocation or an increase in intrinsic activity (as an effect of M2 would have been observed at earlier time points). In addition, cycloheximide was found to completely inhibit M2-stimulated glucose uptake. Thus it is likely that M2 acts independently of PI 3-kinase to increase glucose transport via the synthesis of glucose transporters.

Sibutramine enhances the activation of 5HT2A/2C receptors and α1 and β1 adrenoceptors (Jackson et al., 1997). However the results obtained in chapter 5 demonstrate that M2 is not acting to increase glucose transport in L6 cells via serotonin or noradrenaline as serotonin, noradrenaline, fluoxetine, nisoxetine were without effect. Very high concentrations of sibutramine may act as dopamine receptor agonists, but there was no evidence that dopamine could stimulate glucose uptake in muscle. The serotonin-releasing anorectic agent fenfluramine has been reported to increase insulin action *in vivo* and *in vitro*, independently of its weight reducing effect. Although fenfluramine itself did not significantly alter glucose uptake into the muscle cells, its active metabolite nordexfenfluramine increased insulin-stimulated glucose uptake, presumably via a mechanism that is independent of serotonin. The lack of effect of the anorectic agent phentermine (a noradrenaline releaser) on glucose uptake by the muscle cells substantiates that an improvement of insulin sensitivity is not a general feature of amphetamine based anorectic agents (Kosmiski and Eckel, 1997). Furthermore, M2 was demonstrated to rapidly decrease plasma glucose concentrations of lean non-diabetic mice. This effect was at least partly due to a significant increase in peripheral glucose transport as observed in isolated soleus muscles of M2 treated mice. The effects of M2 were observed in mice that were fasted throughout the duration of the experiment, therefore the action of M2 is likely to be independent of any satiety inducing effects but not independent of possible thermogenic effects of the compound. A combination of increased thermogenesis and increased peripheral glucose transport in non-diabetic lean mice may be the mechanism by which M2 acts to decrease glycaemia. In insulin resistant skeletal muscle of *ob/ob* mice the effect of M2 on glucose transport is lost, therefore the lesion in the pathway that causes the insulin resistant state is also preventing the action of M2. This is consistent with the earlier hypothesis that M2 acts via the insulin signalling pathway to increase glucose transport in non insulin resistant skeletal muscle. The effectiveness of M2 to directly alter glucose transport is therefore dependent upon the degree of insulin resistance. Thus the increased glucose transport produced by M2 is unlikely to ameliorate insulin resistance in obese Type 2 diabetic patients but may delay the onset of Type 2 diabetes in obese patients.

8.2.4 Plant-Derived Compounds

Pinitol is a form of chiroinositol, a methyl-inositol found in the leaves of some plants, legumes and soy (Narayanan et al., 1987). It has a purportedly profound hypoglycaemic activity and this was investigated in chapter 6 of this thesis. In rat L6 skeletal muscle cells pinitol acutely stimulated basal glucose transport. Thus pinitol may exert a 'direct' effect on skeletal muscle which causes an immediate increase in glucose transport. As this effect was not observed after prolonged incubation of the cells with pinitol, this effect is unlikely to be caused by protein synthesis and may involve an alteration in the intrinsic activity of glucose transporters already at the cell surface. Pinitol is the 3-O-methyl ether of chiroinositol and thus may act as a second messenger in intracellular signalling to mediate glucose transport. *In vivo* studies demonstrated that pinitol acutely decreased hyperglycaemia in STZ-induced diabetic mice in a concentration dependent manner but did not acutely alter the rate of insulin-induced glucose disappearance. Chronic administration of pinitol normalised hyperglycaemia in STZ-induced diabetic mice and withdrawal of pinitol resulted in hyperglycaemia of levels observed prior to treatment. Pinitol did not affect plasma insulin concentrations therefore the hypoglycaemic activity of pinitol is unlikely to be mediated via an increase insulin production. Pinitol also did not affect body weight or food intake and therefore is unlikely to enhance thermogenesis or satiety. In the absence of adequate insulin, pinitol may modulate a non-insulin stimulated glucose uptake mechanism. Pinitol did not significantly affect plasma glucose concentrations in normal lean, non-diabetic and severely insulin resistant, obese diabetic *ob/ob* mice suggesting that pinitol has insulin-like activity and thus mimics the action of insulin both *in vitro* and *in vivo*. This activity however, does not enhance the action of insulin, as pinitol is most effective in the absence of adequate insulin, indicating that pinitol may act via the insulin signalling pathway but that the initial signalling elements upon which pinitol acts are more effective for insulin than for pinitol.

Chamaemeloside, a new apigenin compound isolated from the dried flower heads of Roman chamomile has purportedly hypoglycaemic properties (Konig et al., 1998). Investigation in L6 cells demonstrated that the effect was not mediated via a direct mechanism on peripheral tissues to increase glucose utilisation. However to confirm the proposed antidiabetic properties chamaemeloside was investigated in lean non diabetic mice. Chamaemeloside did not alter interprandial plasma glucose concentrations, but it significantly improved glucose tolerance. Chamaemeloside contains an 3-hydroxy-3-methylglutaric acid (HMG) moiety which has been implicated as a hypoglycaemic agent via the inhibition of HMG CoA lyase, which converts HMG CoA to acetyl CoA and acetoacetate, thus reducing energy production (Witherup et al., 1995). This results in an increased metabolism of glucose, and not a stimulation of insulin receptors or insulin secretion. Thus *in vivo*, HMG may be liberated from chamaemeloside, and the observed activity is due to free HMG.

8.2.5 Pharmacological Agents Known to Modify Insulin Action

Chapter seven focussed on agents already known to alter insulin action. The biguanide metformin increased both basal and insulin-stimulated glucose transport in L6 cells and this study demonstrated that this stimulatory effect of metformin on basal glucose transport (at least in L6 cells) is unlikely to be mediated via PI 3-kinase. The intracellular mechanism of metformin action may be potentiated via components known to be integral for insulin signalling, however the results obtained in this study suggest that metformin is likely to act downstream from PI 3-kinase. The action of metformin is dependent on facilitated diffusion to increase glucose transport in skeletal muscle as the effect was inhibited by cytochalasin B, an inhibitor of glucose transporters. A recent study by Thomas et al., (1998) demonstrated that metformin did not increase either the mRNA levels or total cellular membrane abundance of GLUT 1 and 4 in quadriceps isolated from metformin treated dexamethasone-induced insulin resistant mice. Thus it is likely that metformin stimulated glucose transport is mediated via an increase in intrinsic activity of glucose transporters already present at the cell membrane. Maximal stimulation of metformin activity was achieved after 24h in L6 cells. This effect was inhibited by cycloheximide therefore the process is mediated at least in part, by protein synthesis. The mechanism by which metformin stimulates glucose transport in L6 cells is likely to be via the synthesis of integral signalling proteins which directly or indirectly act to increase the intrinsic activity of glucose transporters. Thus, metformin acts to increase both basal and insulin stimulated glucose transport. The increase in basal glucose transport may be mediated via a mechanism alternative to PI 3-kinase mediated signalling and is likely to be dependent on the synthesis of signalling proteins, which act to increase the intrinsic activity of glucose transporters at the cell membrane.

Sulphonlyureas primarily lower blood glucose by stimulating insulin secretion (Groop, 1992), but there are conflicting data on the existence of extrapancreatic effects brought about by the sulphonylureas. In this study tolbutamide caused a trivial increase in basal glucose uptake in L6 cells, which was only evident at inordinately high concentrations. Whilst it is likely that sulphonylureas exert some extrapancreatic effects, the hypoglycaemic activity of these agents is primarily exerted via the stimulation of insulin secretion.

Thiazolidinediones (TZDs) are a novel group of oral antidiabetic agents which cause a blood glucose lowering effect by partially mimicking or selectively enhancing certain actions of insulin. The mechanism of action is known to be mediated via PPARγ resulting in the expression of insulin controlled genes (Day, 1999). Two TZDs were investigated in chapter 7 rosiglitazone and troglitazone. Rosiglitazone had no effect on glucose transport in L6 cells however after 96h troglitazone was found to possess a stimulatory effect. Troglitazone contains a vitamin E moiety as part of its structure and this may be responsible for increasing glucose uptake independent of the TZD moiety. Whether L6 cells can significantly metabolise rosiglitazone or troglitazone is unknown as *in vivo* metabolism of these thiazolidinediones occurs almost entirely in the liver. Therefore the present lack of effect of rosiglitazone cannot be accounted for by rapid degradation.

Vanadyl sulphate has been shown to lower blood glucose concentrations in both Type 1 and Type 2 diabetic models by mimicking the action of insulin (Posner et al., 1990; Schechter et al., 1990). In chapter 7, vanadyl sulphate was observed to exert an effect on glucose uptake in L6 cells only at short time points using inordinately high concentrations. At lower concentrations, effects were not observed, and at high concentrations for longer incubation time points vanadyl sulphate reduced cell viability. Previous *in vitro* studies have demonstrated that vanadium augments glucose uptake and utilisation in muscle (Venkatesan et al., 1991). The insulin-like effect has been associated with increased levels of phosphorylated tyrosine proteins as a result from their inhibitory effect on phosphotyrosine phosphatases (Swarup et al., 1982). Vanadium has been shown to mimic the action of insulin through alternative signalling pathways which involve the inhibition of phosphotyrosine phosphatases and the interplay between two non-insulin receptor tyrosine kinases (Elberg et al., 1997; Tsiani and Fantus, 1997). Vanadate is structurally analogous to phosphate, and it may be this that accounts for its inhibitory effects on phosphatases (Lui et al., 1997). The results from chapter 7 indicate that vanadyl sulphate possesses potent insulin mimetic properties in L6 skeletal muscle cells. Unfortunately the increase in glucose transport elicited by vanadyl sulphate can only be observed for less than 1h before the element reduced cell viability. Thus it is likely that vanadyl sulphate is highly toxic. Reduction of the concentration of vanadyl sulphate reduces the toxic effects of the compound, but since lower doses did not produce a response it will be necessary to produce more potent vandium salts such as oxovanadiums that can be used at lower concentrations, if vanadium salts are to be developed for clinical use.

Thioctic acid (lipoic acid), a free radical scavenger and known treatment for diabetic neuropathy has recently been implicated as a possible treatment for insulin resistance. Thioctic acid acutely increased glucose uptake in L6 cells, however the effects were lost within 24h. These results are in keeping with those obtained by Estrada et al.,

(1996) who reported a significant increase in glucose uptake in L6 myotubes after incubations of via PI 3-kinase-dependent GLUT 1 and 4 translocation. Chapter 7 also documents confounding effects of toxicity of thioctic acid as significant increases in glucose transport after longer incubation periods may have been masked by a decrease in cell viability. The problems of toxicity may be alleviated in time by further analysis of the different isomers of thioctic acid.

8.3 Concluding Remarks

In conclusion, insulin-mediated responses are controlled via numerous signalling pathways. This thesis has focussed on one mechanism by which insulin controls glycaemia and has demonstrated the complexity of the pathway by which insulin mediates glucose transport in skeletal muscle. There are also many factors that appear to modify insulin action. It is clear that obesity, and factors that control obesity and feeding behaviour are intricately linked to insulin action. This thesis has demonstrated that leptin can directly modify insulin action, thus in obese Type 2 diabetic patients leptin may play a role in the insulin resistance observed in muscle cells and other peripheral target cells. Treatments for obesity with sibutramine may also benefit insulin action, reinforcing the link between obesity, insulin resistance and Type 2 diabetes.

The search for new agents to treat insulin resistance is a necessity as many patients fail to respond to conventional therapy. There have been several claims to support a possible therapeutic utility for thioctic acid and vanadate-related compounds. These compounds may possess antidiabetic properties due to an action on peripheral tissue but their effects require further investigation in clinical trials to assess the efficacy, therapeutic index and potential side effects. Plants have been used for centuries to treat diabetes and this thesis demonstrates the validity of investigating traditional plant medicines. Research into pinitol and chamaemeloside are in their infancy and further work will be required to determine the mechanism of chiroinositol incorporation into inositol phospholipid precursers and its action on insulin resistance. However the potential for therapy using pinitol in insulinopenic diabetes is evident. Further investigations to determine the exact mechanism of action of 3-hydroxy-3-methylglutaric acid are also warranted.

The focus of this thesis was to gain a better understanding of insulin action and intracellular signalling and to identify targets and mechanisms of action of compounds that modify insulin resistance in skeletal muscle. The complexity of insulin action means that there is still far to go before we have sufficient answers to prevent the insulin resistance syndrome and reverse Type 2 diabetes. Hopefully this work will pave the way for future research to elaborate the intervention targets discussed herein, and to improve insulin action.

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Appendices

Appendix I: Solutions and Buffers

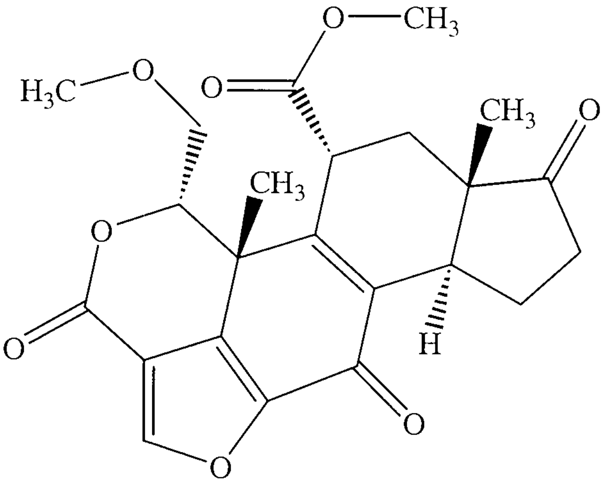
Buffers for Riboprobes	
A	1ml $MgCl_2$ (125mM), 1ml Tris (1.25M), 18μl β-ME, 5μl dATP (100mM), 5μl dTTP (100mM), 5μl dGTP (100mM)
B	2M Hepes pH 6.6
C	112Mg/ml random oligos
ABC	mix in ratio **A** 100, **B** 250, **C** 150
Citrate Buffer	0.1M $C_6H_8O_7.H_2O$ 2parts, 0.1M $C_6H_5O_7Na_3.2H_2O$ 3 parts, pH 4.8
Denaturing Buffer	100μl formamide, 20μl formaldehyde, 10μl 20X MOPS
Ethidium Bromide	Ethidium bromide (10mg/ml), 7.5μl per 100ml
HSD (10mg/ml)	Dissolve herring sperm DNA in water at 10mg/ml. Adjust concentration to 0.1M and phenol extract then phenol chloroform extract. Remove aqueous phase and pass rapidly 12 times through blunt 18-gauge needle. Precipitate by the addition of 2 vols ethanol, recover DNA, redissolve in GDW. Boil for 10 min then read OD_{260} and if necessary adjusts concentration to 10mg/ml store in aliquots at -20°C
KRB	Krebs Ringer Bicarbinate Buffer: Sodium chloride (118mM), potassium chloride (5mM), sodium hydrogen carbonate (25mM), magnesium sulphate (1.18mmM) and potassium dihydrogen orthophosphate (1.17mM). Pre-gassed with $5\%CO_2:95\%O_2$.
Loading Buffer for DNA	25% Glycerol, 0.1% Orange G, 25mM EDTA, GDW
Loading Buffer For RNA	As for DNA with the addition of 0.5% SDS
MOPS Buffer	0.2M MOPS, 0.05M Sodium Acetate, 0.01M EDTA
MOPS 20X	83.6g MOPS, 8.1g Sodium Acetate, 7.4g EDTA, 2.5ml Formaldehyde
PBS	137mM Sodium Chloride, 2.7mM Potassium Chloride, 10mM Na2 HPO4, 1.8mM KH2PO4, pH7.4, prepared by dissolving 1 PBS tablet (Mg2+ free, Ca2+ free) in 100ml distilled water

Prehybridisation Buffer	JAP buffer: 300ml 27g/l NaH_2PO_4, 83g/l Na_2HPO_4, 1.86g/l EDTA, 150ml 20% SDS, 4.5ml of 10mg/ml stock of denatured, sonicated herring sperm DNA
Reservoir buffer	900ml H_2O, 50ml MOPS, 75ml formaldehyde
RIA assay buffer	0.025M phosphate buffer, pH 7.5, containing 0.1% (w/v) sodium azide
Sephedex	8g G50, 2ml 100X TE, 0.1ml SDS (add 200ml GDW – autoclave to expand the resin)
Solution D	4M Guanidinium Thiocyanate, 25mM Sodium Citrate pH7.0, 0.5% Sarcosyl, 0.1M 2-Mercaptoethanol
SSC 20X	175.5g/l Sodium Chloride, 88.2g/l Sodium Citrate pH7.0
TE pH 8	10mM Tris-HCl pH 8, 1mM EDTA
TE 100X	121.1g Tris, 3.7g EDTA in 1l GDW

Appendix II: Chemical Structures

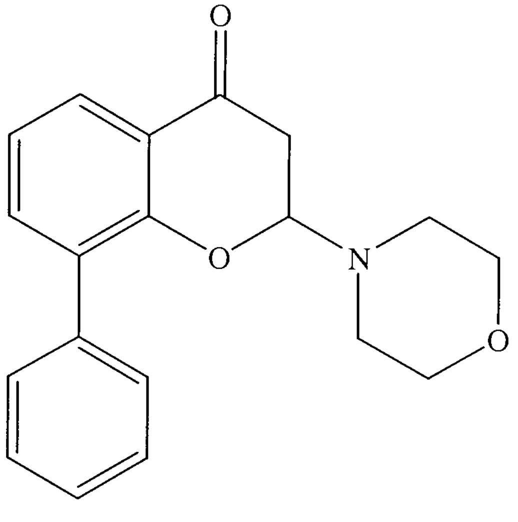
Wortmannin

A cell permeable, irreversible inhibitor of PI 3-kinase with an IC_{50} of 5nM. It blocks the catalytic activity of PI 3-kinase without affecting upstream signalling events such as insulin receptor tyrosine kinase activity. It also inhibits the activities of myosin light chain kinase and PI 4-kinase at concentrations a hundred times higher than those required to inhibit PI 3-kinase.



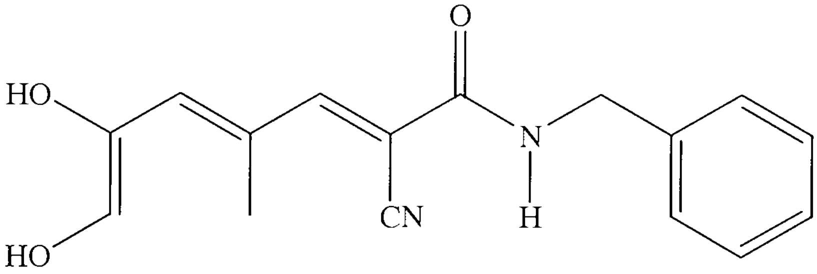
LY-294,002

A specific and potent inhibitor of PI 3-kinase with an IC_{50} of 1.4μM.

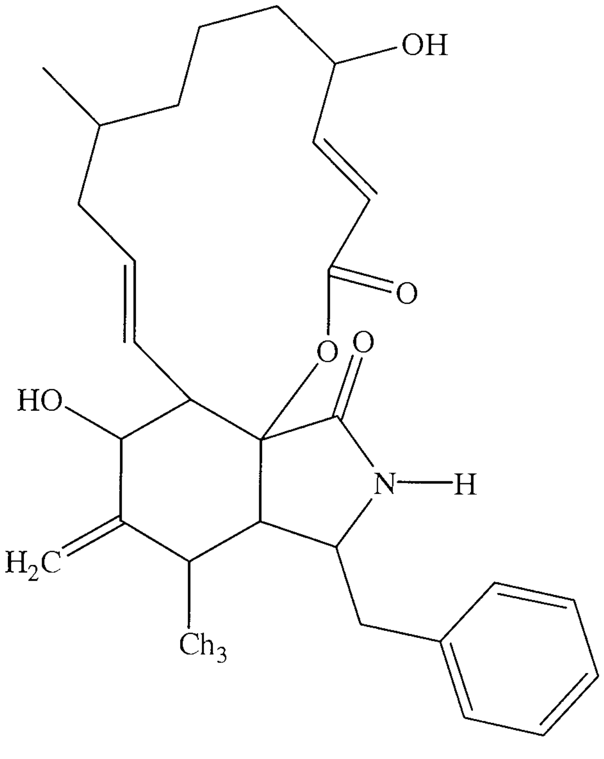


Tyrphostin AG490

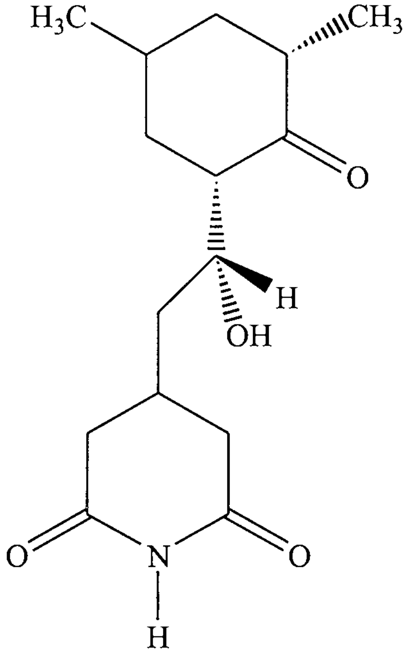
A specific and potent JAK2 protein tyrosine kinase inhibitor. Also inhibits EGF receptor autophosphorylation, with an IC_{50} of 100nM. Inhibits DNA synthesis and cell growth; induces apoptosis. Blocks growth of leukemic cells *in vitro* and *in vivo*. (information supplied by upstate biotechnology, NY).



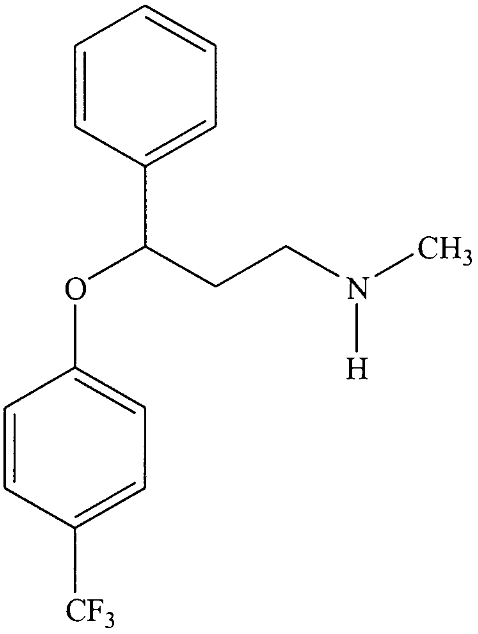
Cytochalasin B

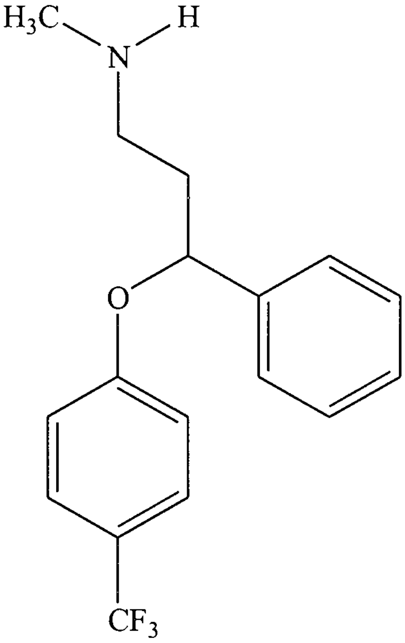


Cycloheximide

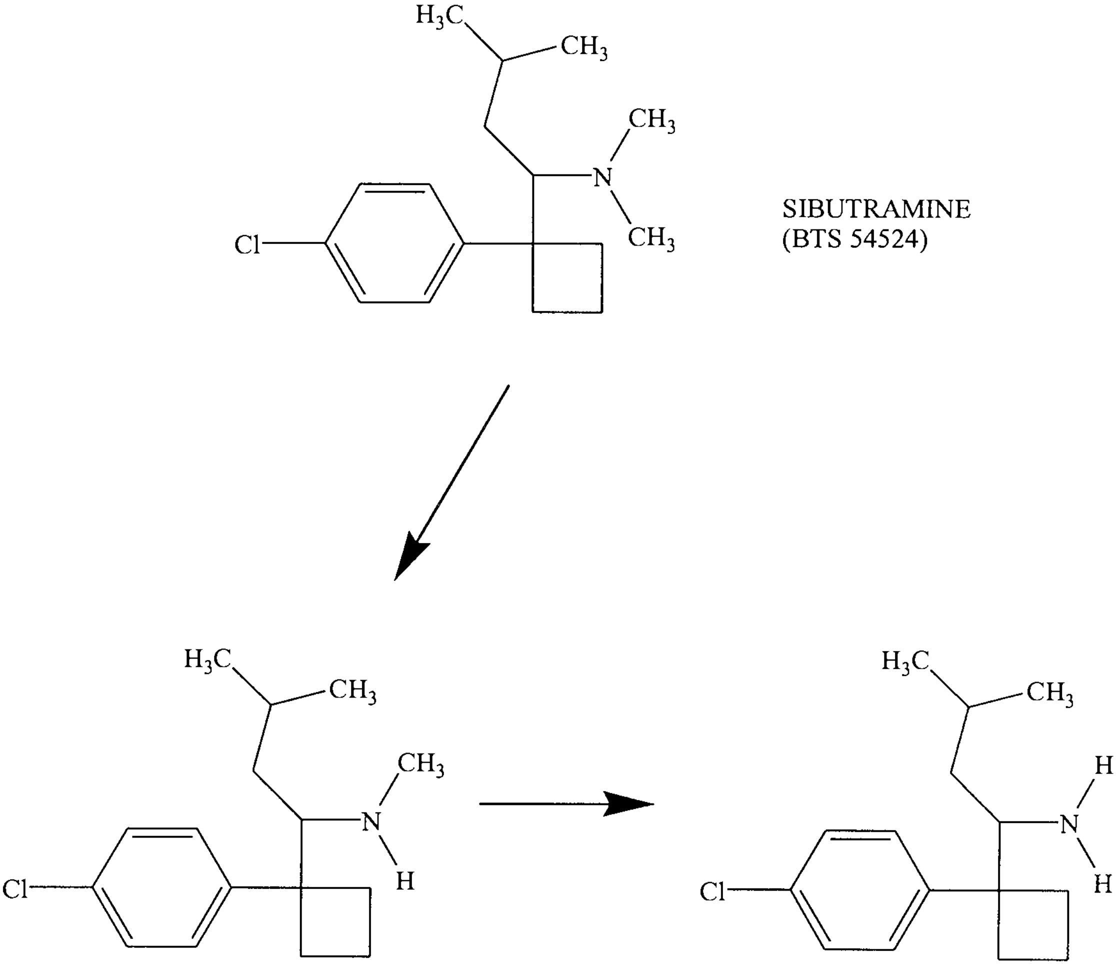


Fluoxetine





Sibutramine, M1 and M2

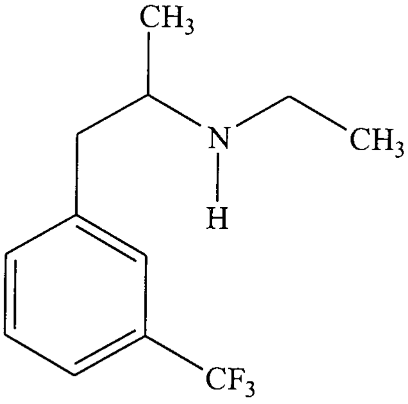


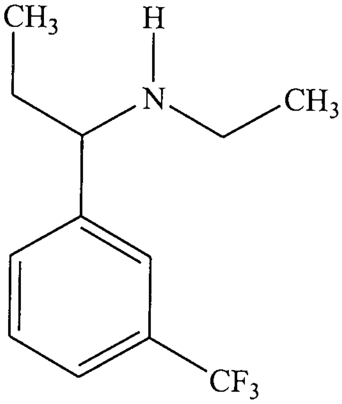
SIBUTRAMINE
(BTS 54524)

METABOLITE 1
SECONDARY AMINE

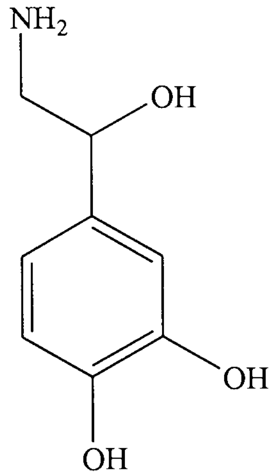
METABOLITE 2
PRIMARY AMINE

Fenfluramine

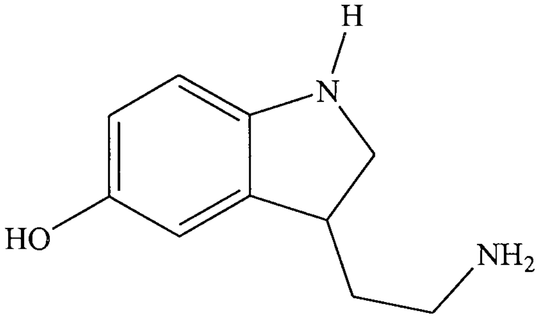




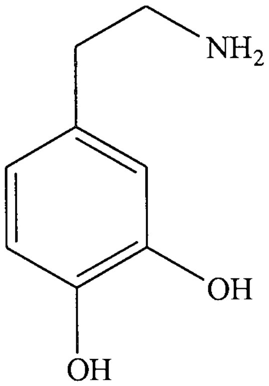
Noradrenaline



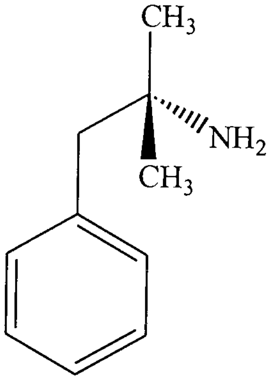
Serotonin



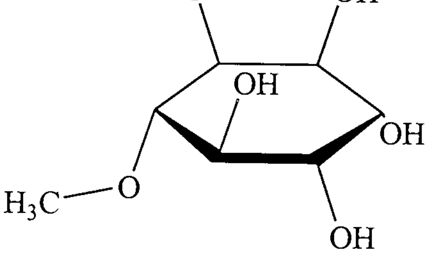
Dopamine



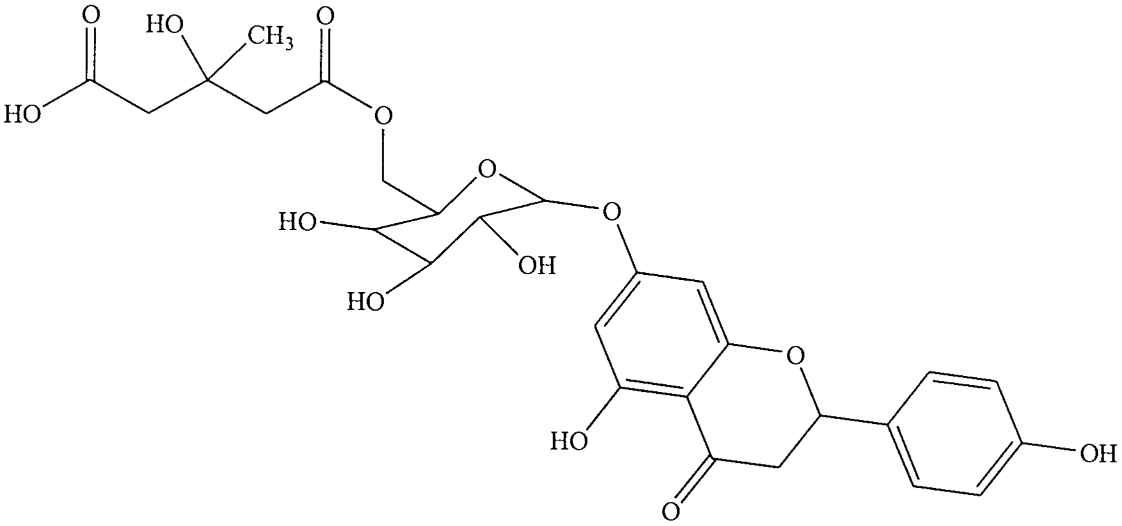
Phentermine



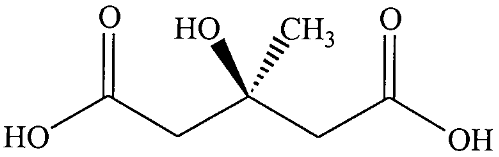
Pinitol



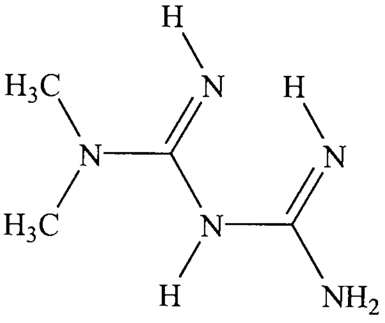
Chamaemeloside



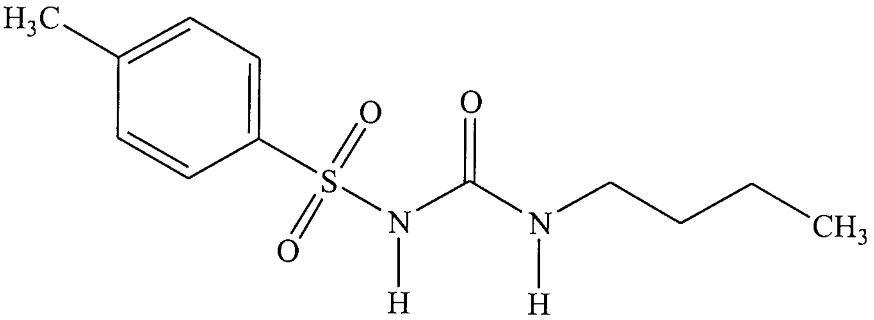
3-Hydroxy-3-mehylglutaric acid (HMG)



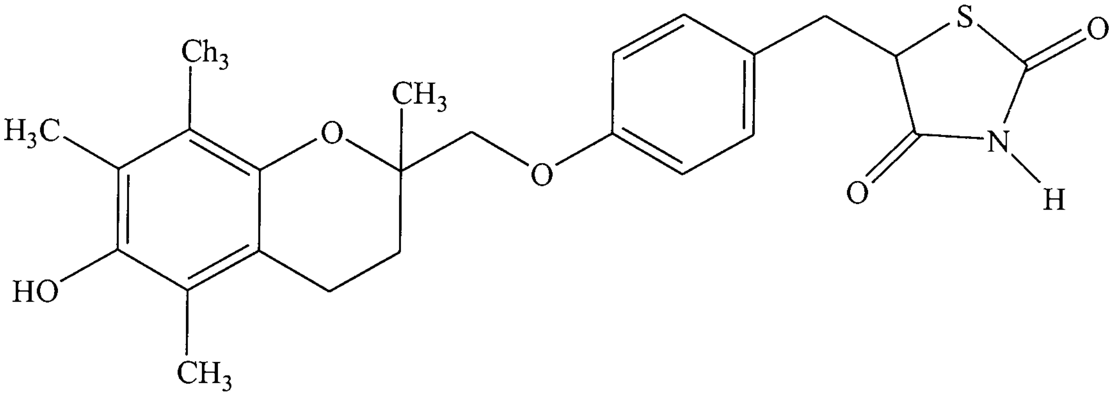
Metformin



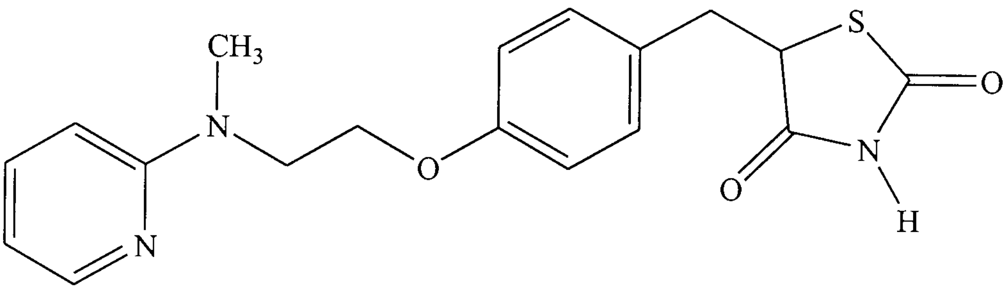
Tolbutamide



Troglitazone

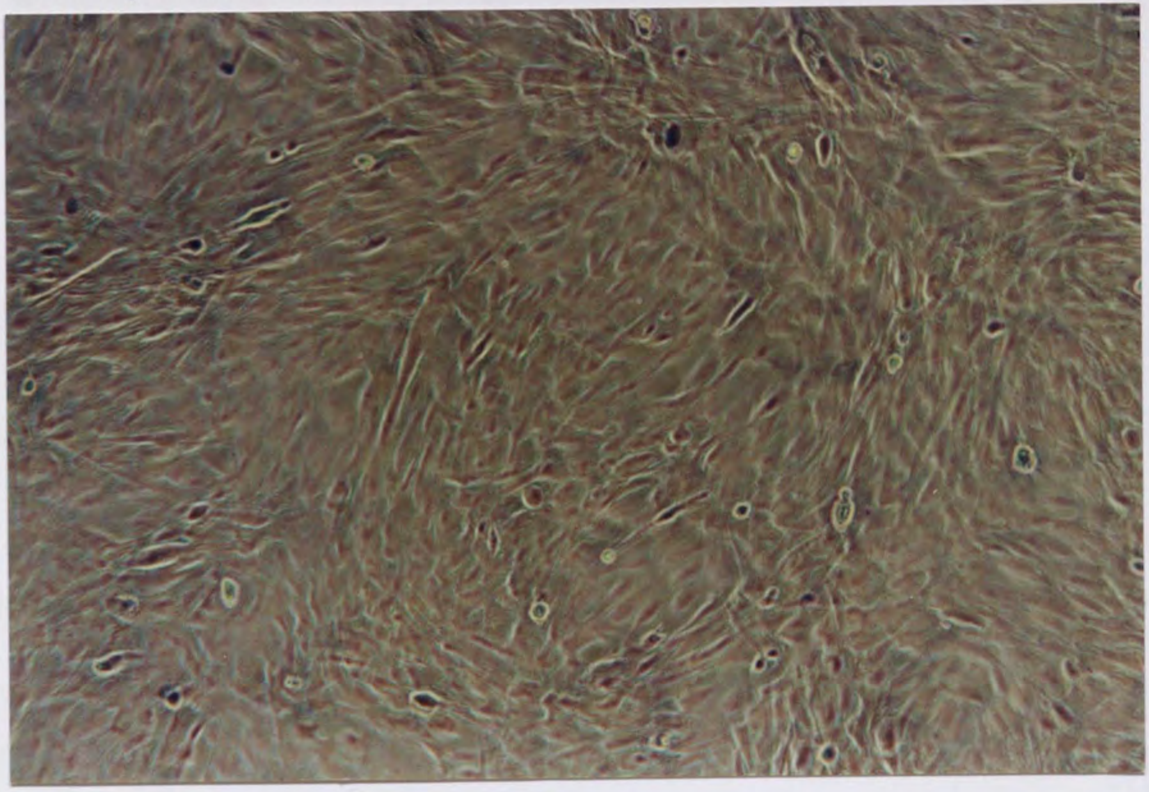


Rosiglitazone

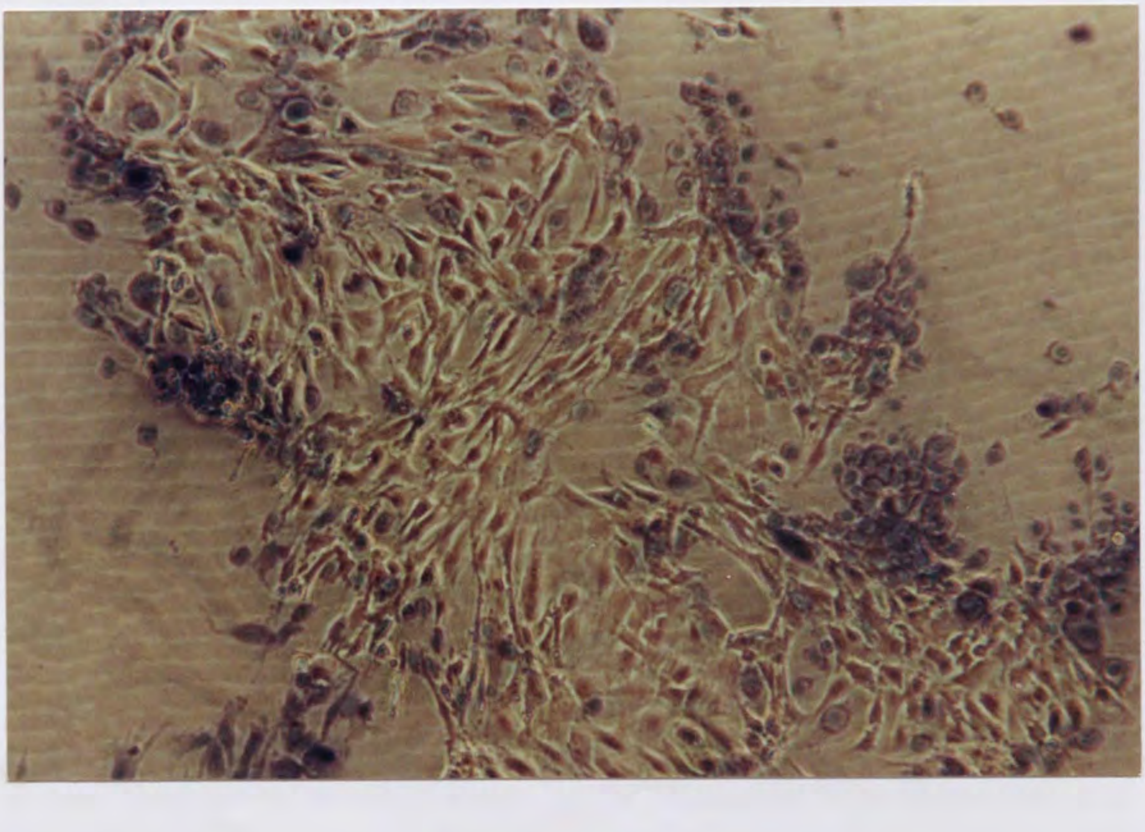


Appendix III: Photographs of Rat L6 Skeletal Muscle Cells

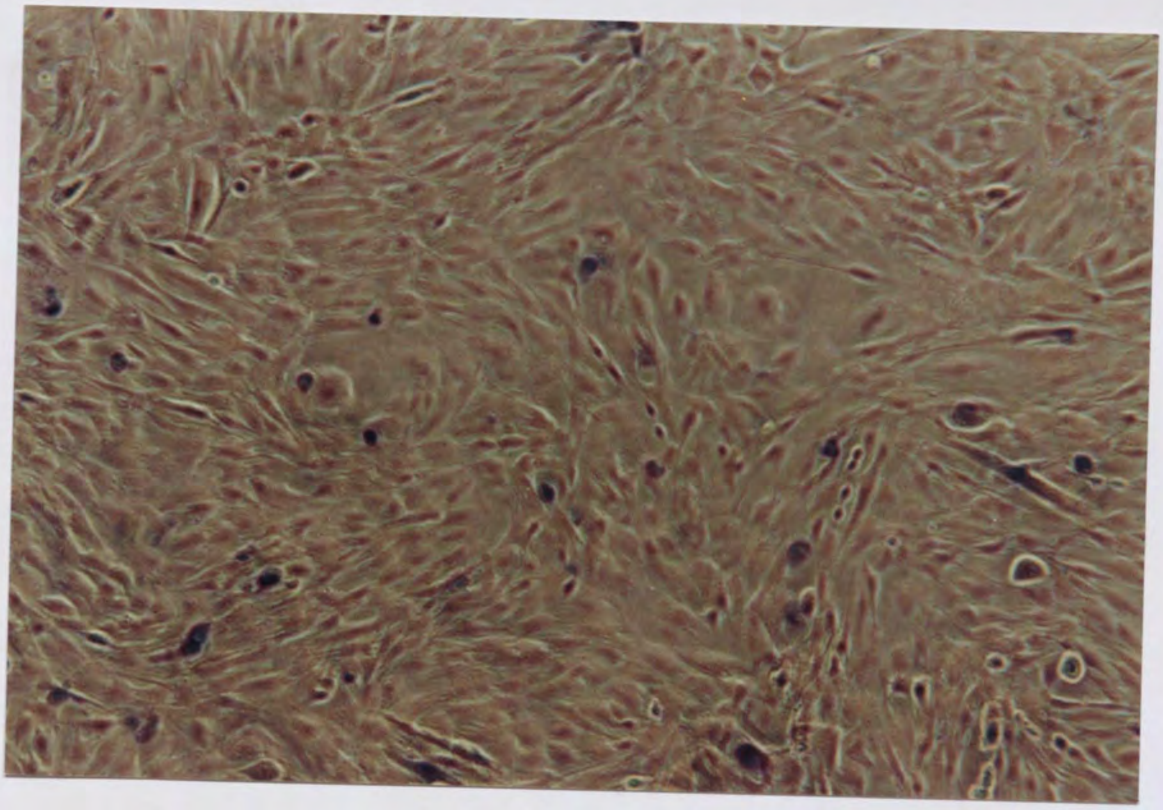
a. Healthy Control Cells



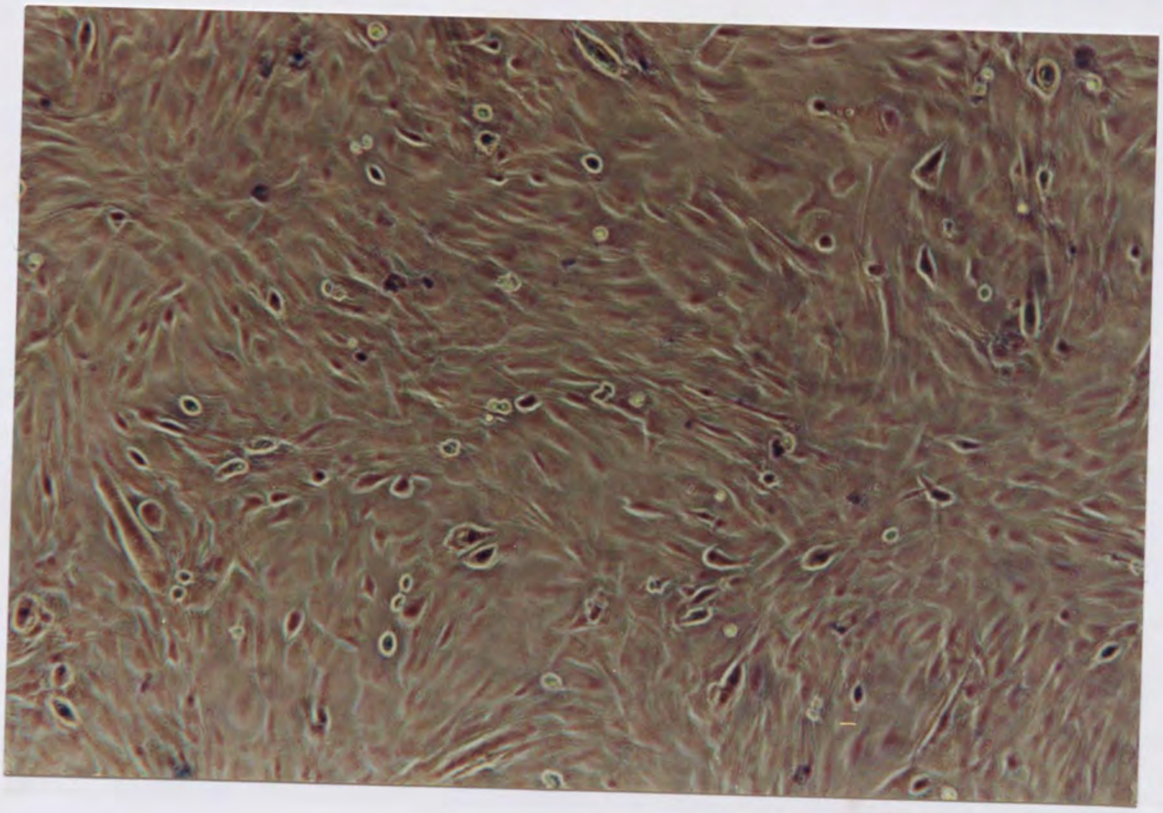
b. Cells Exposed to 10^{-4}M Wortmannin for 50 minutes



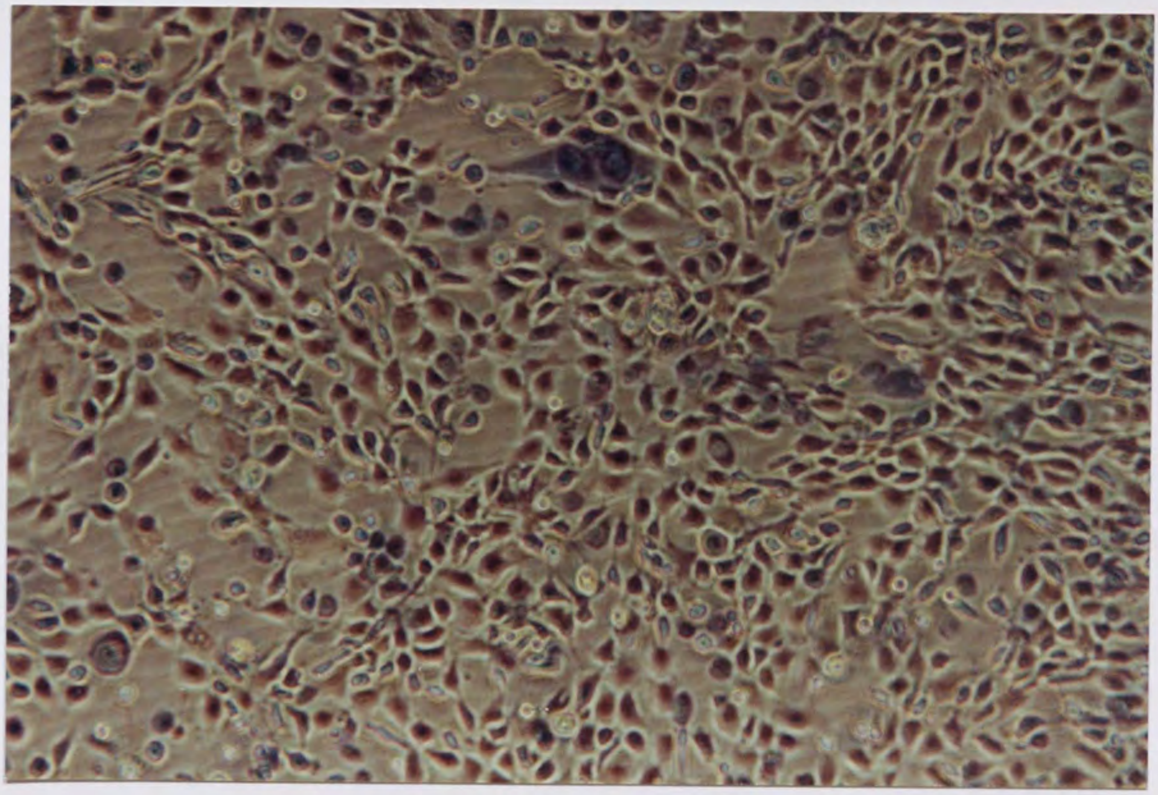
c. Cells Exposed to 10^{-6}M Wortmannin for 50 minutes



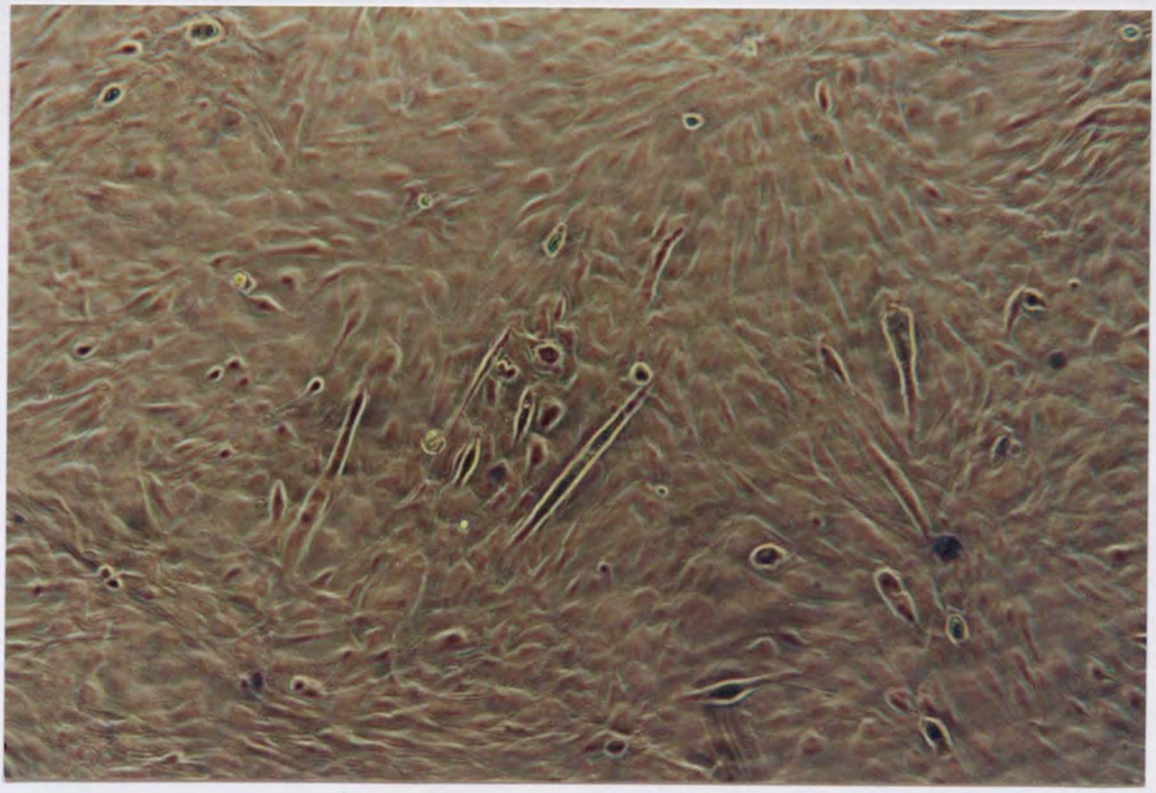
d. Cells Exposed to 10^{-8}M Wortmannin for 50 minutes



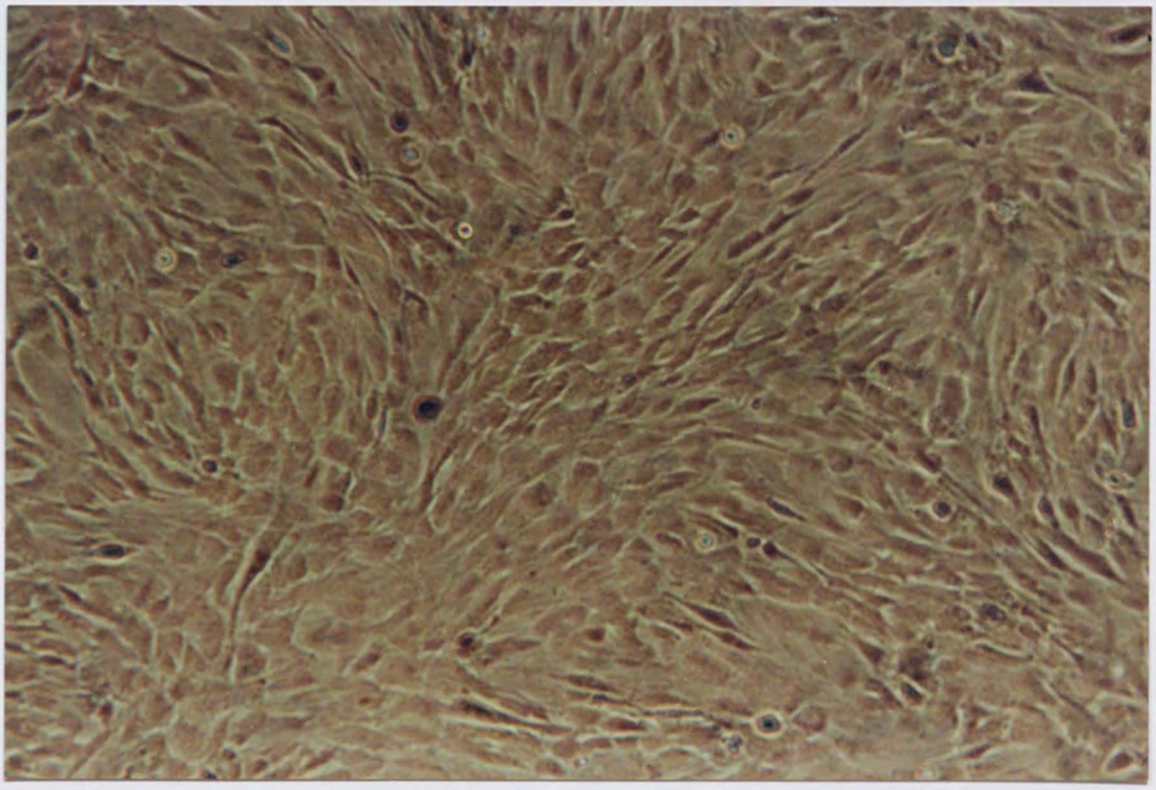
e. Cells Exposed to 10^{-4}M LY-294,002 for 24 hours



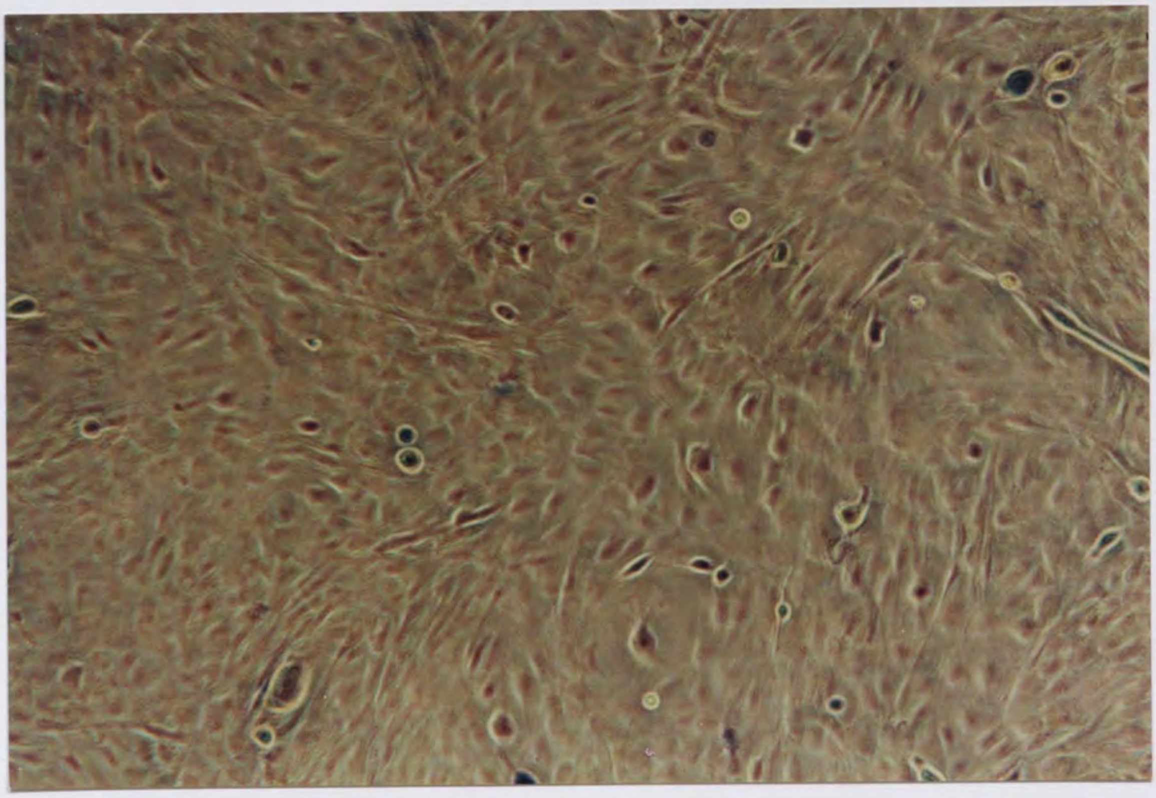
f. Cells Exposed to 10^{-6}M LY-294,002 for 24 hours



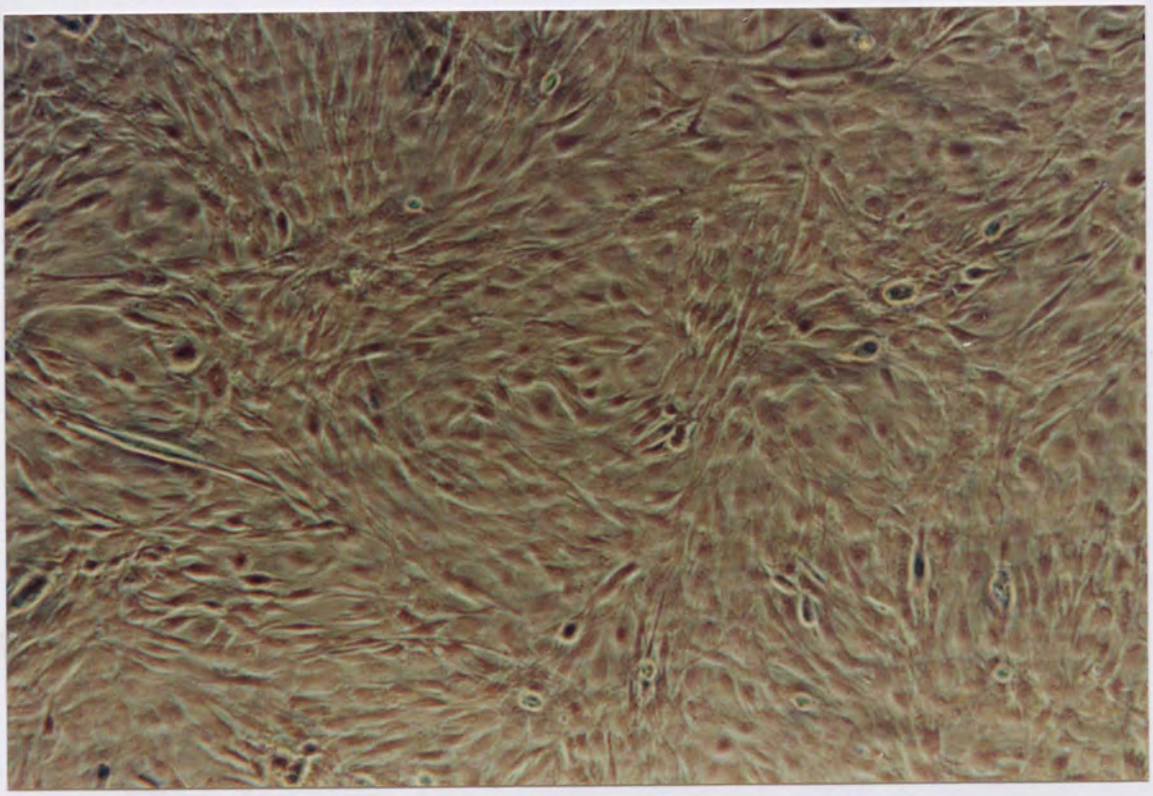
g. Cells Exposed to 10^{-8}M LY-294,002 for 24 hours



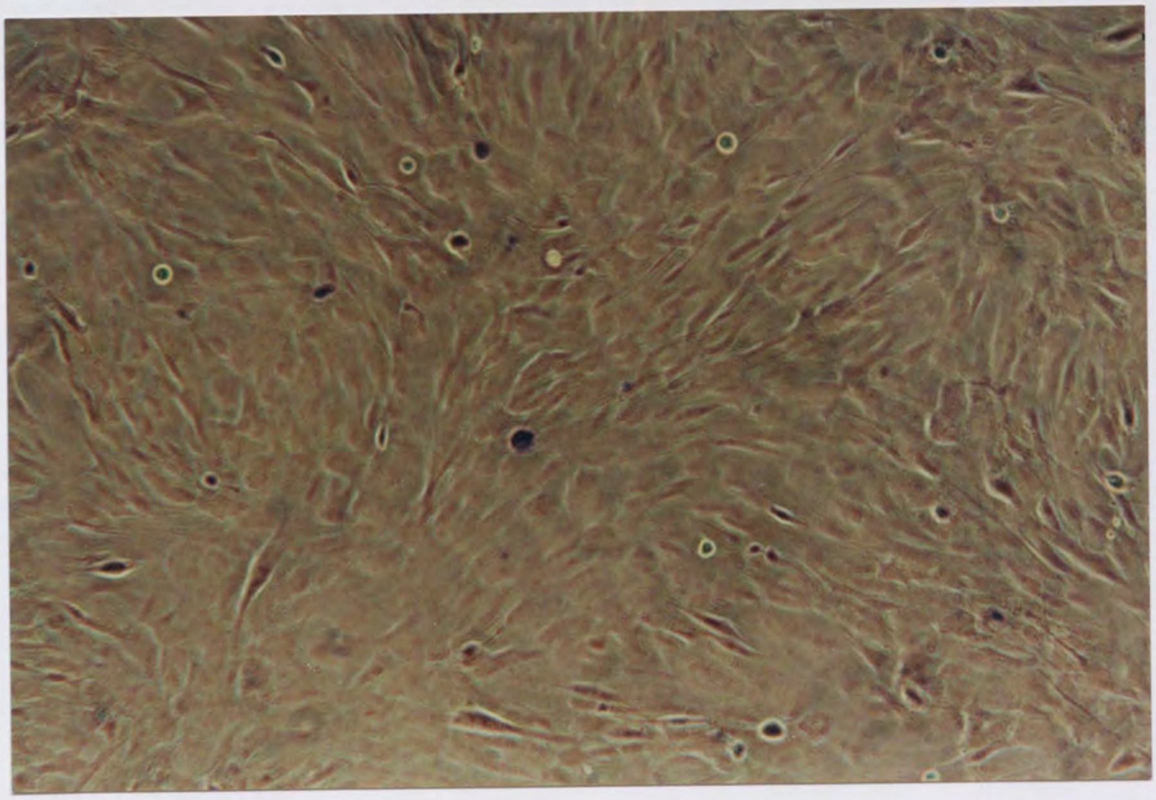
h. Cells Exposed to 10^{-6}M Cytochalasin B for 24 hours



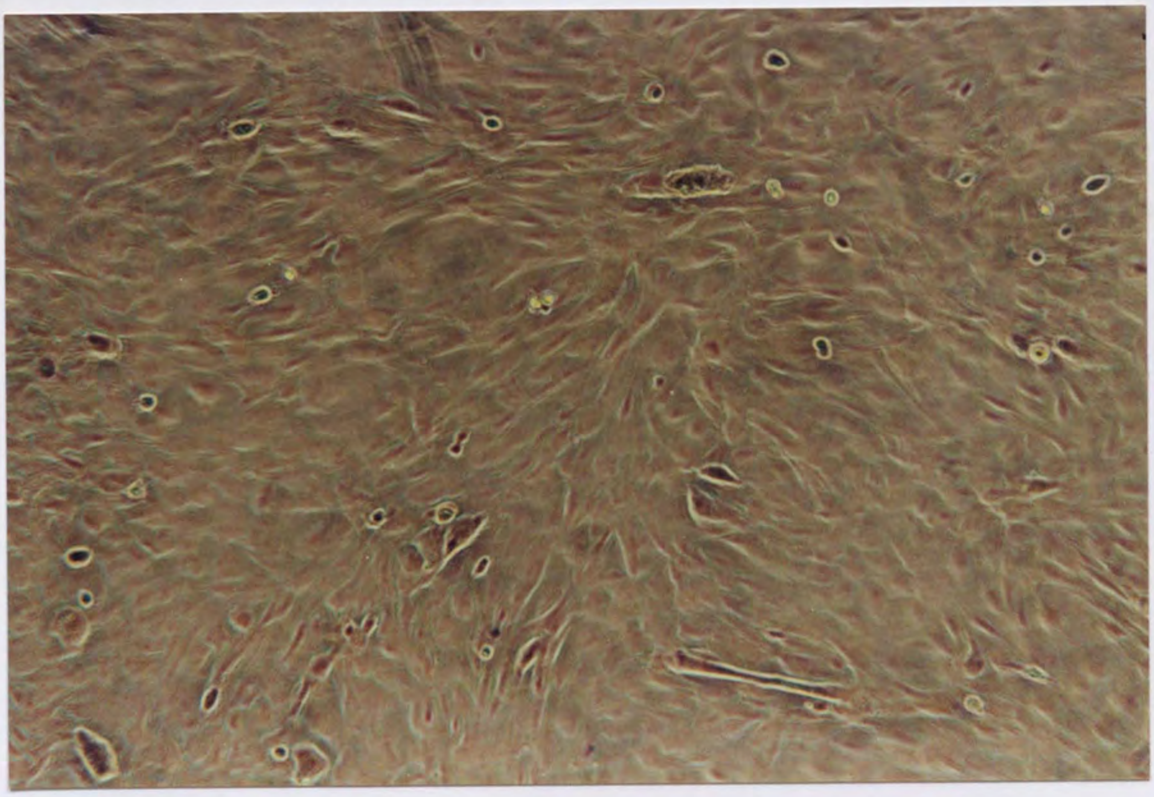
i. Cells Exposed to 10^{-7}M Cytochalasin B for 24 hours



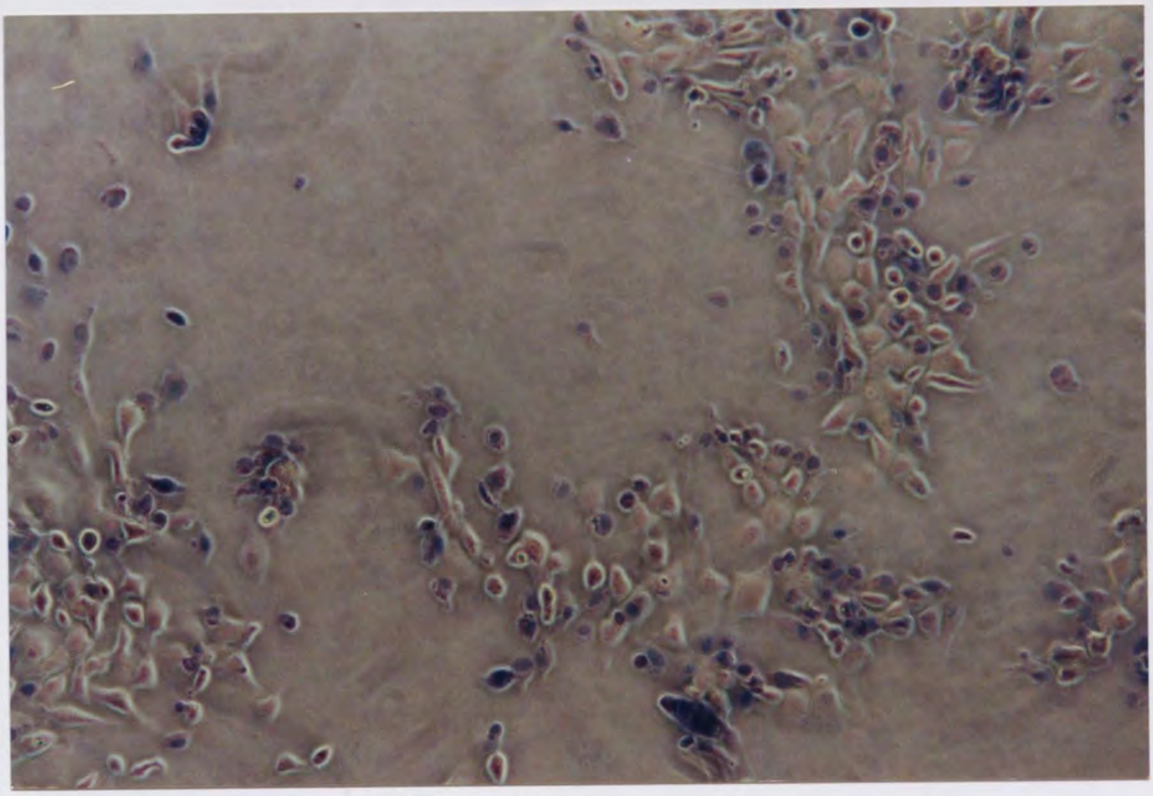
j. Cells Exposed to 10^{-5}M Cycloheximide for 24 hours



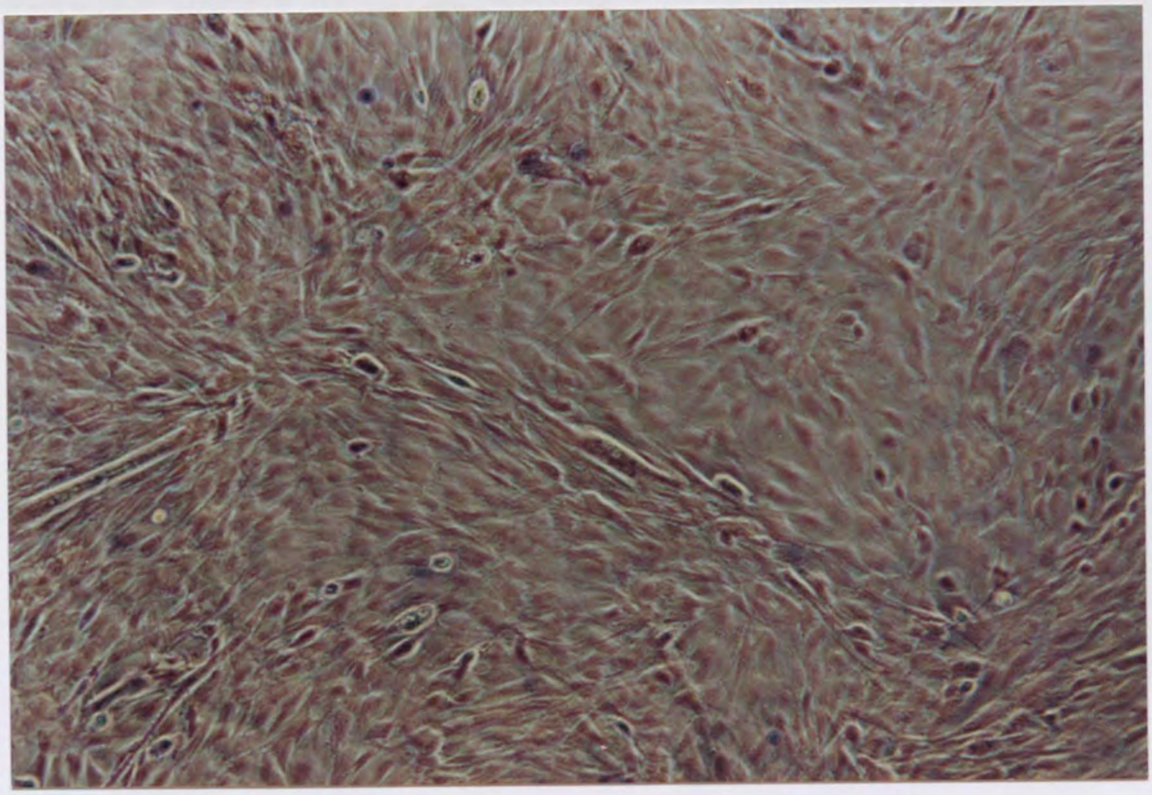
k. Cells Exposed to 10^{-6}M Cycloheximide for 24 hours



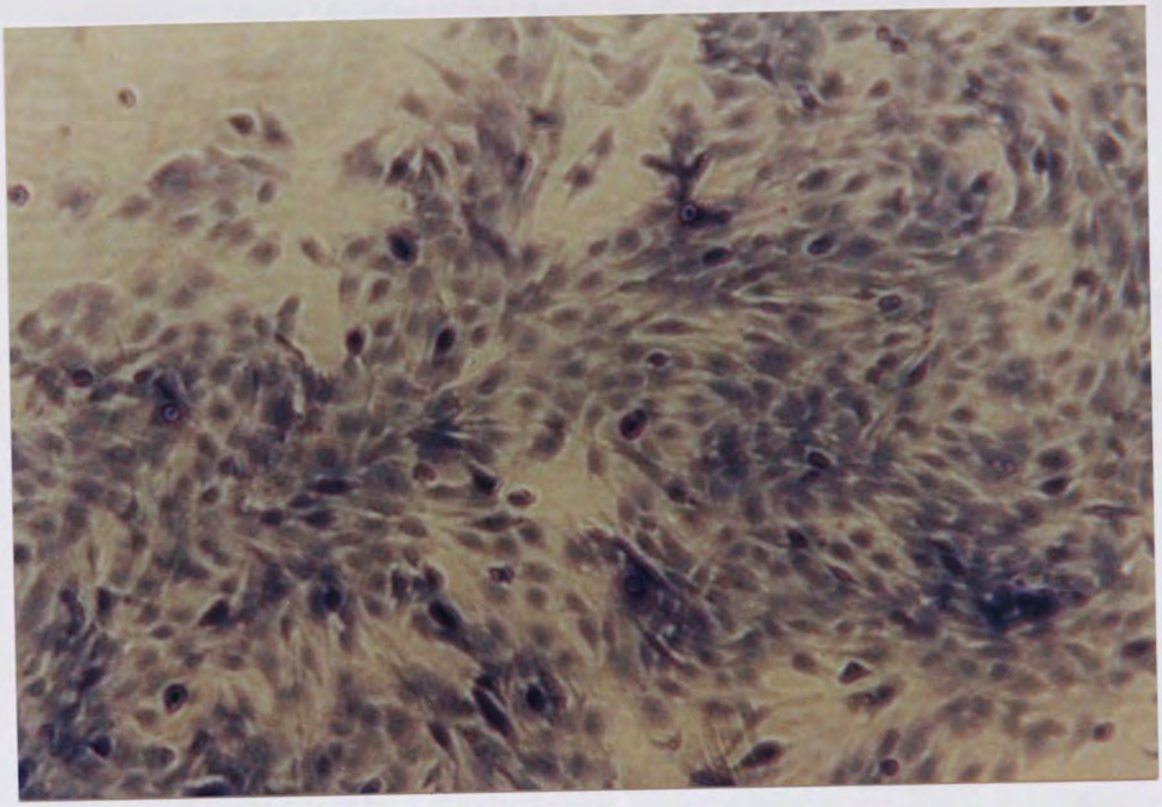
l. Cells Exposed to 10^{-4}M Vanadyl Sulphate for 24 hours



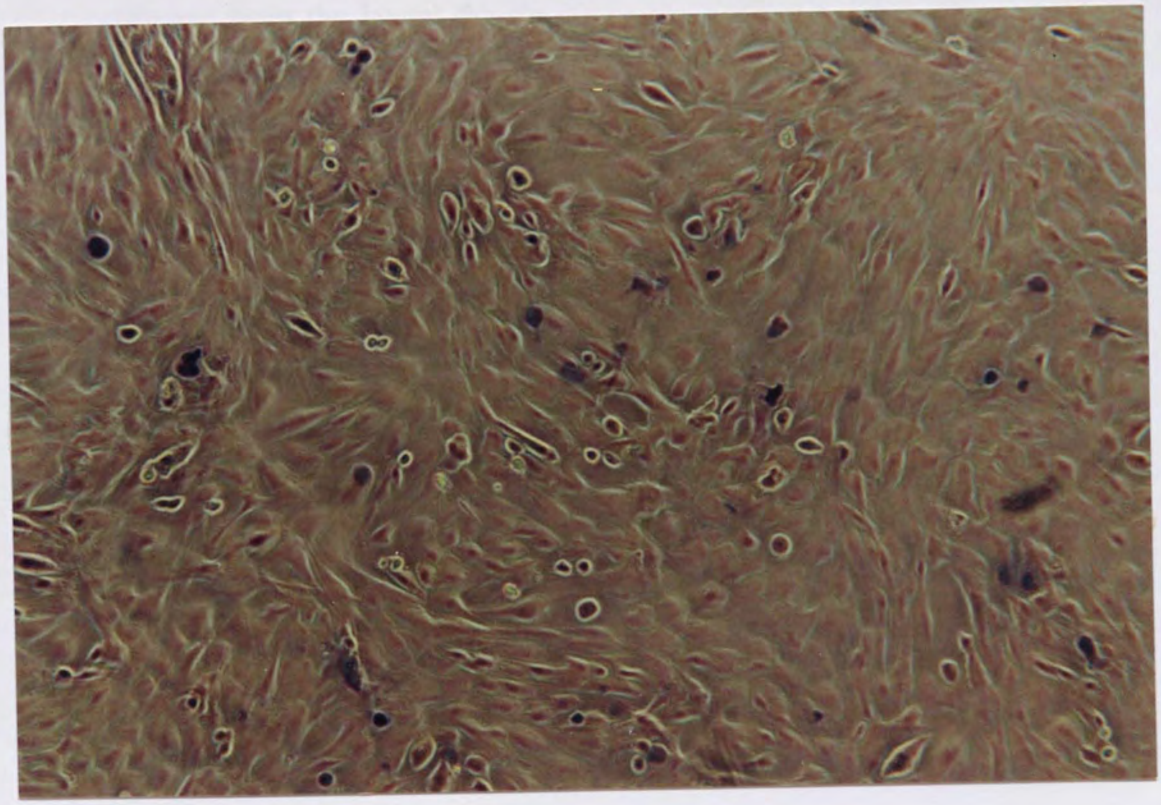
m. Cells Exposed to 10^{-6}M Vanadyl Sulphate for 24 hours



n. Cells Exposed to 10^{-3}M Thioctic Acid for 24 hours

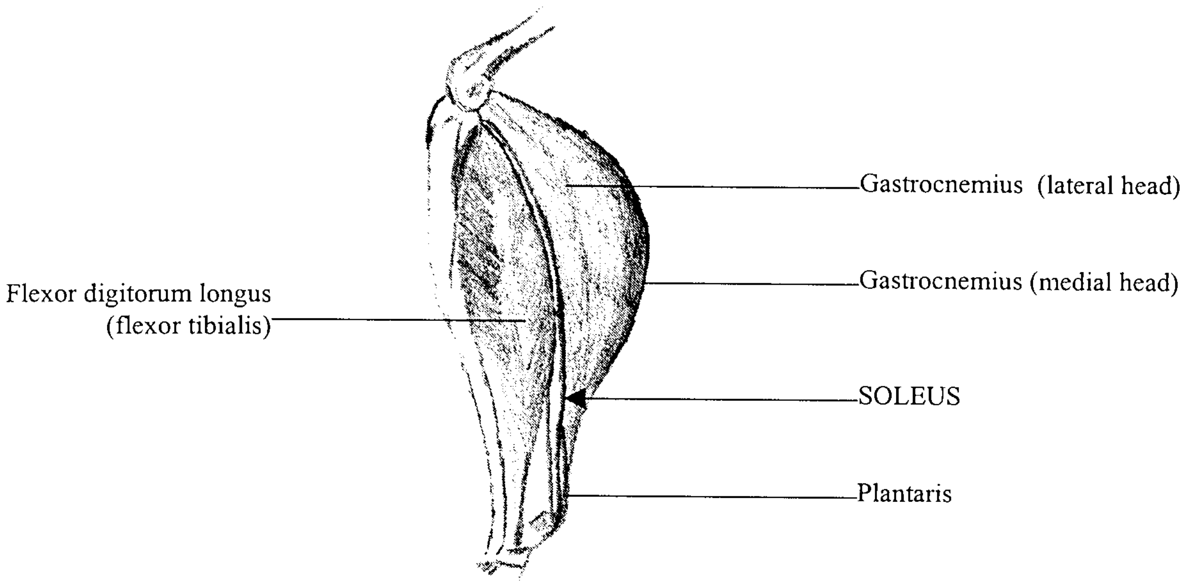


o. Cells Exposed to 10^{-4}M Thioctic Acid for 24 hours



Appendix IV: Anatomy and Dissection

Illustration I: Major Muscles of the Medial Surface of the Lower Leg.

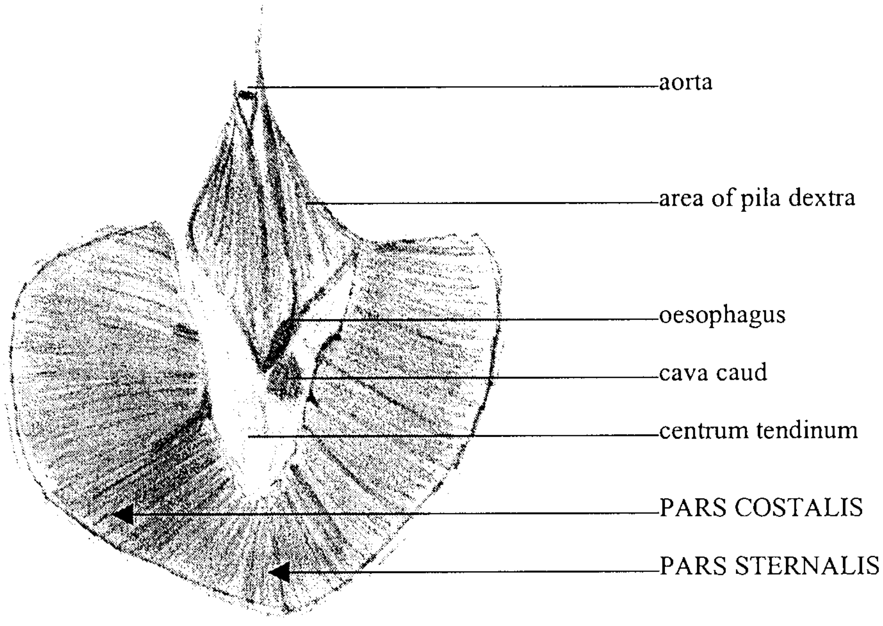

Gastrocnemius (lateral head)
Gastrocnemius (medial head)
Flexor digitorum longus (flexor tibialis)
SOLEUS
Plantaris

The soleus muscle arises by a slender tendon from the head of the fibula. It eventually unites with the gastrocnemius aponeurosis to form the achilles tendon, inserting on the middle portion of the posterior calcaneal surface. The gastrocnemius is made up of two heads, medial and lateral. The medial head arises from the medial epicondyle of the femur and from the medial fabella. The lateral head arises from the lateral epicondyle and from the lateral fabella. Their tendons are twisted with that of the plantaris. The plantaris arises from the lateral epicondyle of the femur, enwrapped by the gastrocnemius, from the lateral fabella and medial border of the head of the fibula. Its tendon passes over the tuber calcanei superficial to the triceps surae and becomes continuous with the flexor digitorum brevis (Greene, 1955).

To remove the soleus muscle for 2-deoxy-[^{3}H]-glucose uptake studies, the hind limb was removed and stripped of skin. The soleus muscle, which is located behind the gastrocnemius, was exposed and gently removed as it has a tendency to tear. The achilles tendon was held firmly by forceps and cut distally to the forceps. The combined plantaris, gastrocnemius and soleus muscles were pealed-back by gently lifting the achilles tendon. The anterior tendon to the soleus muscle was cut as close to the knee joint as possible, and the soleus was separated from the gastrocnemius by gently pulling back the soleus tendon so that the muscle itself was not damaged.

Once removed the soleus had the tendons stripped from each end and any adipose tissue was removed. It was briefly washed in room temperature physiological buffer (Krebs or PBS) and dried by blotting on tissue paper. It was weighed and immediately transferred to the experimental media.

Illustration II: The Diaphragm (abdominal surface).



To dissect the diaphragm, the upper abdomen and thorax were opened by a cross-shaped longitudinal and lateral incision. The diaphragm was cut around the pars costalis and pars sternalis, which were held with forceps to prevent the tissue from curling. The tissue around the aorta, the pila dextra, the oesophagus and the centrum tendinum were removed by cutting along the inner edges of the pars costalis and pars sternalis (Greene, 1955).

For 2-deoxy-[^{3}H]-glucose uptake studies, the tissue was rinsed briefly in physiological buffer (Krebs or PBS) and cut into two separate pieces 'hemi-diaphragms'. The hemidiaphragms were trimmed of any remaining tendons (centrum tendinum), then were weighed and transferred directly to the experimental media.

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Publications

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