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**INTERVENTIONS AGAINST OBESITY THROUGH
INCREASED LIPOLYSIS IN ADIPOSE TISSUE**

**Dawn Katrina Richardson
Doctor of Philosophy**

**Aston University
September 2002**

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Summary

Aston University

Interventions against obesity through increased lipolysis in adipose tissue

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Obesity is a disease of excess adiposity affecting >17% of men and >20% of women in Britain. Clinically, it is defined by a Body Mass Index (BMI, kg/m²) of ≥ 30 . Obesity is a confounding factor that promotes insulin resistance, hyperinsulinaemia and type 2 diabetes. Type 2 diabetes accounts for >90% of all cases of diabetes, with a prevalence of 2-6% of adults in most western societies, a majority of which are overweight or obese. Weight loss in obese patients reduces the risk of developing diabetes by >50%. This thesis has investigated the first part of a two-stage therapeutic intervention against obesity in which adipose tissue lipolysis will be combined with increased energy expenditure: the approach is also designed to consider agents that will benefit glycaemic control in coexistent obesity and diabetes by improving insulin sensitivity.

Rodent and human *in vitro* models of adipocyte biology and skeletal muscle have been developed, characterised and evaluated. They include isolated epididymal and parametrial adipocytes of lean and obese diabetic *ob/ob* mice, cultured 3T3-L1 preadipocytes, isolated human omental and subcutaneous adipocytes and rat L6 cultured muscle cells. Compounds investigated for anti-obesity and anti-diabetic properties include M2 (sibutramine metabolite), 3-guanidinopropionic acid and mazindol. *In vivo* studies were undertaken to investigate these compounds further in lean and *ob/ob* mice.

In vivo studies indicated that M2 and 3-guanidinopropionic acid reduced body weight gain in *ob/ob* mice. The three compounds increased lipolysis in adipocytes isolated from lean and *ob/ob* mice and human adipose depots. The direct action of these compounds was mediated via a pathway involving the β adrenoceptors and components of the lipolytic signalling pathway, including protein kinase A and p38 MAP kinase. In addition, M2 and mazindol were capable of increasing glucose uptake into insulin sensitive tissues. M2 and mazindol can act directly on adipose tissue and skeletal muscle to increase glucose uptake via a pathway involving new protein synthesis and activation of the glucose transporters. The M2-stimulated pathway is activated by the conversion of phosphatidylinositol biphosphate to phosphatidylinositol trisphosphate by phosphatidylinositol 3-kinase. Thus, M2, mazindol and 3-GPA showed pharmacodynamic properties which suggested they might be potential therapeutic treatments for obesity and diabetes.

**Type 2 Diabetes Mellitus, Insulin Resistance, Adipocytes, Glucose Uptake,
L6 Skeletal Muscle Cells**

...the whole of the
...the whole of the
...the whole of the

For Mum and Dad

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Chapter 1: Introduction

1.1 Obesity Defined

Obesity is a major health problem characterised by alterations in metabolic function, which result from an increase in body fat (Kopelman and Albon, 1997). It is a serious medical condition affecting >17% of men and >20% of women in Britain (Kopelman, 2000; Wilding, 1997) and ~20% of men and ~25% of women in the USA (Flegal *et al.*, 1998). The rise in obesity is not confined to Europe and America, but other populations susceptible include the Caribbean, Australia and South-East Asia. In one epidemiological study, the prevalence of obesity in Samoa has been estimated at 75% for adult women and 60% for adult men (Hodge *et al.*, 1995). Practical methods used in clinical studies to assess obesity are illustrated below in table 1.

Table 1: Defining Obesity

METHOD	DEFINITION
Body Mass Index (BMI)	Weight in kilograms divided by the square of the height in metres (kg/m ²)
Waist Circumference	Measured (in centimetres) at midpoint between lower border of the ribs and upper border of the pelvis
Skinfold Thickness	Measurement of skinfold thickness (in centimetres) with callipers provides a more precise assessment if taken at multiple sites
Bioimpedence	Based on the principle that lean mass conducts current

	<p>better than fat mass because it is primarily an electrolyte solution; measurement of resistance to a weak current applied across extremities provides an estimate of body fat using an empirically derived equation</p>
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(Adapted from Kopelman, Obesity as a medical problem. *Nature*. 2000, Vol. 404, page 646).

Many clinicians and epidemiologists use BMI to define overweight and obesity. Individuals with a BMI of 25 or greater are classified as overweight and those over 30 are considered obese (World Health Organisation, 1997). The main limitation of this technique is that it does not distinguish between fat mass and lean mass. Waist circumference and waist-to-hip ratio provide measures for assessing upper body fat deposition. Men with a waist circumference $\geq 94\text{cm}$ and women $\geq 88\text{cm}$ were at a greater risk of developing a metabolic complication associated with cardiovascular disease (Lean *et al.*, 1998). However neither technique provides precise estimates of intra-abdominal fat.

1.2 Clinical Implications of Obesity

Obesity is associated with premature onset and increased severity of many chronically morbid and fatal diseases (see table 2) including type 2 diabetes mellitus (T2DM), coronary heart disease, hypertension, gallbladder disease and enhanced cancer risk. In addition there are numerous detrimental endocrine and metabolic changes

associated with obesity including dyslipidaemia, insulin resistance, polycystic ovary syndrome (PCOS) and other reproductive disorders (Bray, 1998).

Table 2: Morbidity in Obesity

MORBIDITY IN OBESITY
Cardiovascular: hypertension, coronary heart disease, cerebrovascular disease, varicose veins, deep venous thrombosis.
Respiratory: breathless, sleep apnoea, hypoventilation syndrome.
Gastrointestinal: hiatus hernia, gallstones and cholelithiasis, fatty liver and cirrhosis, haemorrhoids, hernia, colorectal cancer.
Metabolic: hyperlipidaemia, insulin resistance, type 2 diabetes mellitus, polycystic ovarian syndrome, hyperandrogenisation, menstrual irregularities, dyslipidaemia.
Neurology: nerve entrapment.
Renal: proteinuria.
Breast: breast cancer, male gynaecomastia.
Uterus: endometrial cancer, cervical cancer.
Urological: prostate cancer, stress incontinence.
Skin: sweat rashes, fungal infections, lymphoedema, acanthosis nigricans
Orthopaedic: osteoarthritis, gout
Endocrine: growth hormone and IGF-1 reduced, reduced prolactin response, hyperdynamic ACTH response to CRH, increased urinary free cortisol, altered sex hormones.
Pregnancy: obstetric complications, caesarean operation, large babies, neural tube defects.

(Adapted from Jung, Obesity as a disease. *British Medical Bulletin*. 1997, Vol. 53 (No. 2): 310)

The greatest increase in risk attributable to obesity is for T2DM, where a BMI above 35kg/m² increases the risk by 93 fold in women and 42 fold in men (Jung, 1997). A 14yr prospective study of 114,281 nurses (Colditz *et al.*, 1995) and a study of 51,529 men (Chan *et al.*, 1994) showed that BMI was a predictor and an increase in risk for developing T2DM. The main predisposing factor for T2DM is insulin resistance, which is also a common feature in obese individuals. In particular android obesity, characterised by an accumulation of fat in and around the abdominal cavity is closely associated with this insulin resistance and T2DM (Cnop *et al.*, 2002; Bailey, 1999).

1.3 Insulin Resistance

Insulin resistance describes an impaired biological response to insulin (Bailey, 1999). It is a common pathological state frequently associated with a number of diseases, including chronic infection, cancer and obesity. Obesity is a confounding factor that promotes insulin resistance and hyperinsulinaemia and as such, predisposes the obese individual to serious cardiovascular complications and T2DM. There is substantial evidence to show that the development of insulin resistance is initially compensated by an increase in insulin levels, resulting in hyperinsulinaemia. The insulin resistance gives rise to T2DM when the hyperinsulinaemia is no longer sufficient to compensate. Insulin resistance in obesity gives rise to an impairment of insulin-stimulated muscle glucose uptake and glycogen synthesis predominantly by skeletal muscle, and

generally accompanied by a reduction in glucose phosphorylation (Petersen *et al.*, 1998). Moreover, the β cells are unable to compensate for the insulin resistance resulting in insulin deficiency, which can be found in T2DM (DeFronzo, 1992). Various factors conspire to cause insulin resistance and deficiency, generally reflecting a genetically determined susceptibility to the condition (due to tissue specific alterations in the levels of expression of genes encoding for regulators of nutrient metabolism and thermal control) together with acquired factors from the internal and external environment such as a high fat diet and sedentary lifestyle (Reaven, 1995; DeFronzo, 1997). Internal mediators of insulin resistance include circulating insulin antagonists, notably elevated free fatty acids (FFA) which disrupt the glucose-fatty acid cycle (Randle, 1963), aggravating insulin resistance in muscle and liver (Mingrone *et al.*, 1999). Adipose tissue turnover increases plasma FFA and may promote insulin resistance further by the release of certain cytokines, notably tumour necrosis factor- α (Hotamisligil, 1995) and interleukin-6 (Pickup *et al.*, 1997).

1.4 Diabetes Mellitus

Diabetes mellitus is a chronic endocrine disorder recognised as a syndrome: a collection of diseases and conditions that have hyperglycaemia and glucose intolerance in common, due to either impaired effectiveness of insulin action and/or insulin deficiency (Harris and Zimmet, 1992). There are two principal types of diabetes. In type 1 (juvenile onset, insulin dependent) diabetes mellitus there is progressive autoimmune-mediated destruction of pancreatic β cells and eventually total failure to secrete insulin. With T2DM, the mechanisms causing the disease are

to a great extent unknown. However in early stages of the disease, there is usually a state of insulin resistance associated with an inadequate insulin secretion relative to the raised blood glucose concentration (DeFronzo, 1992). Although the insulin secreting β cells are structurally intact in T2DM there is an impairment of function and eventually a loss of β cell mass. T2DM is the most common form of diabetes accounting for >90% of all cases of diabetes and a prevalence of 2-6% of adults in most occidental societies (Astrup and Finer, 2000; Amos *et al.*, 1997; King and Rewers, 1993). It usually develops in individuals between the ages of 40 to 70, and is strongly associated with life-style, environmental factors and an inherited predisposition to reduced insulin sensitivity and limited capability for adaptation of β cell function (Vadheim and Rotter, 1992). The most prominent of the environmental/life-style factors is obesity, particularly abdominal fat distribution. Between 80-90% of all T2DM individuals are or have been obese (Astrup and Finer, 2000; Harris and Zimmet, 1992).

1.5 Metabolic Syndrome

The metabolic syndrome (Syndrome X or Insulin Resistance Syndrome) is a cluster of abnormalities brought about by insulin resistance, sometimes through the associated hyperinsulinaemia. The conditions aggravated by insulin resistance and (initially) hyperinsulinaemia that comprise the insulin resistance syndrome include impaired glucose tolerance (IGT), obesity, T2DM, dyslipidaemia, hypertension, atherosclerosis and a procoagulant state (Pinkney *et al.*, 1997). Reaven (1988) was the first to develop a hypothesis to account for the clustering of these metabolic risk factors and

diseases together, although he did not include obesity in his original description of the metabolic syndrome. This was probably because the average person in the USA is already overweight or obese. There is no doubt that obesity, particularly fat mass localised in the central regions is a confounding factor for insulin resistance and consequently the metabolic syndrome (Maison *et al.*, 2001). In one study, the significance of abdominal visceral fat accumulation was evaluated in men with IGT. Here, it was shown that visceral fat accumulation was a major contributor to the metabolic syndrome in men with IGT (Nagaretani *et al.*, 2001). Weight gain has been shown to be a strong predictor of the metabolic syndrome, especially visceral fat accumulation where there is an increased turnover of free fatty acids, which interfere with the action of insulin (Bosello and Zamboni, 2000). Dyslipidaemia usually features an increase in very low-density lipoprotein triacylglyceride (VLDL-TAG), which is probably brought about by hyperinsulinaemia stimulating hepatic VLDL production. Dyslipidaemia also features an increase in low-density lipoprotein cholesterol (LDL-C) particularly the atherogenic small dense LDL particles, and there is often a reduction in high-density lipoprotein cholesterol (HDL-C) (Reaven, 1995). The metabolic syndrome is now accepted as a major risk factor for coronary heart disease (Suzuki *et al.*, 1996). Moreover an increased risk of cardiovascular disease is associated with obesity and body fat distribution, most notably excess accumulation of visceral fat (Morricone *et al.*, 1999). Angiotensinogen is a substrate of renin and shown to be released by rodent adipocytes and adipocyte cell lines (van Harmelen *et al.*, 2000). Angiotensinogen can be converted to angiotensin I, which is the precursor to angiotensin II, by the adipose tissue. Angiotensin II is an important regulator of blood pressure and it has been shown that the angiotensinogen gene in adipose tissue might be involved in upper body (visceral) obesity (van Harmelen *et al.*, 2000).

Obesity predisposes and contributes to the metabolic syndrome and thus represents a valuable potential opportunity for therapeutic intervention to reduce the morbidity and mortality from a wide range of associated medical conditions. Recent studies have also noted that markers of low-grade inflammation (e.g. raised level of C-reactive protein) frequently accompany the metabolic syndrome, but the potential role of low-grade infection in the development of the syndrome is unclear (Pickup *et al.*, 1997).

1.6 Insulin Action, Receptor and Signalling

Insulin's main effect is to increase glucose uptake into target tissues and promote glucose conversion to glycogen and triacylglyceride. It also inhibits triacylglyceride and glycogen breakdown. Insulin is synthesised in the β cells as preproinsulin and is converted by endoproteases in secretory vesicles to form C-peptide and insulin. Following its synthesis and secretion, insulin circulates in the blood until it reaches its target tissues. The action of insulin is initiated by its binding to the $\alpha_2\beta_2$ tetramer insulin receptor. The insulin receptor is a large glycoprotein (400KDa) consisting of two α and 2 β subunits. The β subunits of the receptor lie within the cell and possess tyrosine kinase activity (Kahn, 1994).

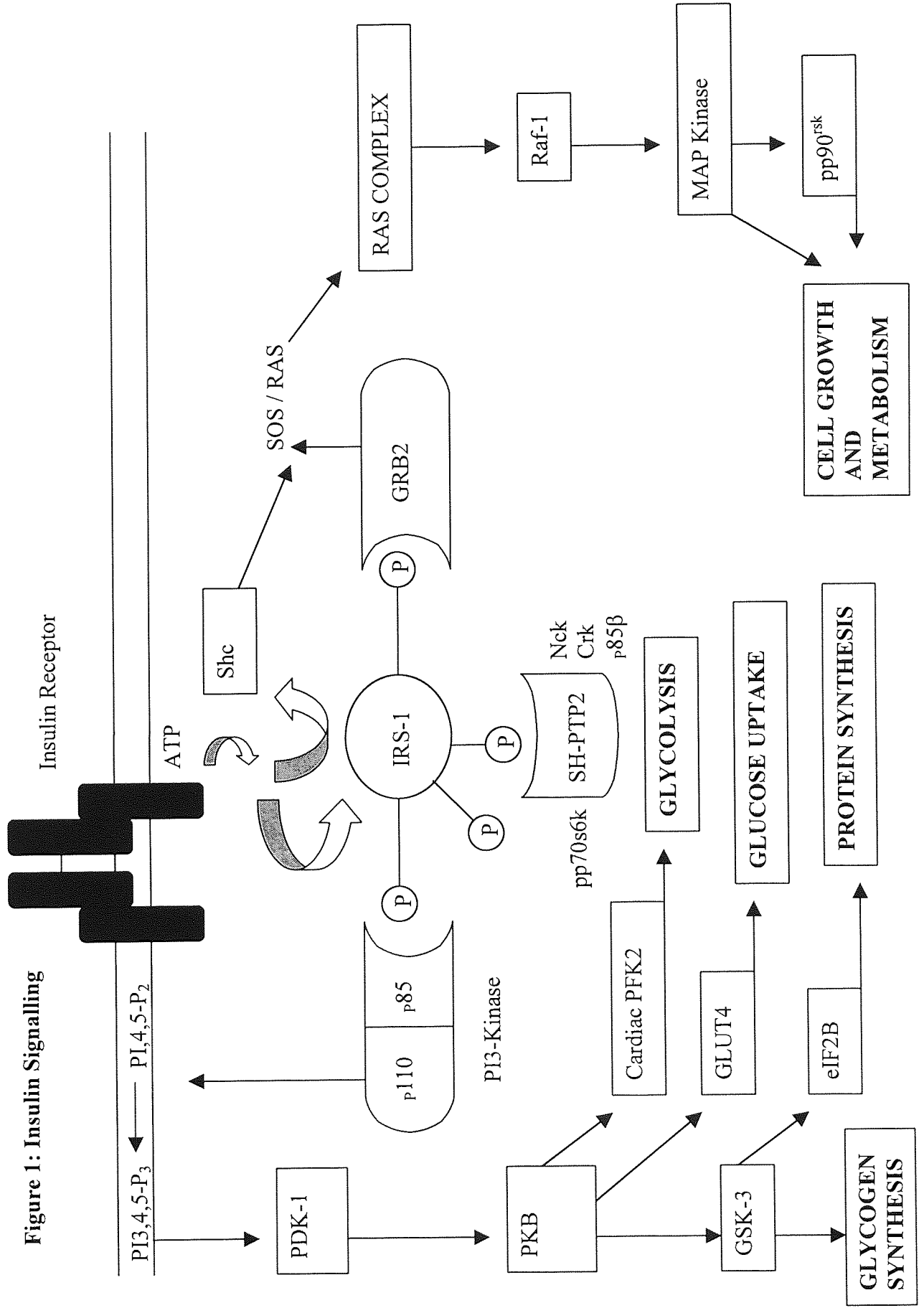
Insulin binding to the α subunits leads to the phosphorylation of the β subunits of the receptor on the tyrosine residues (Holman and Kasuga, 1997). This autophosphorylation is associated with an increased tyrosine kinase activity. Tyrosine phosphorylation sites at positions 1158, 1162 and 1163 are essential for insulin signalling transduction (Hunter and Garvey, 1998). Following insulin binding, the

insulin receptor substrate-1 (IRS-1) and an adaptor protein with homology with Src and collagen (Shc) are phosphorylated (White, 1997). Both these subunits are capable of interaction with other proteins within the target cell. Both the IRS-1 and Shc are strongly associated to the guanidine nucleotide exchange factor, 'son of sevenless' (SOS), which activates the RAS signalling pathway. This initiates a cascade leading to sequential phosphorylation and activation of the mitogen-activated protein (MAP) kinases. The MAP kinase pathway mediates the mitogenic and growth-promoting effects of insulin (Saltiel and Kahn, 2001).

The IRS-1 subunit is bound strongly to the α -p85 subunit of the phosphatidylinositol 3-kinase (PI3-Kinase). In addition, the IRS-1 forms a binding site with the growth factor receptor bound protein 2 (GRB2), a small adaptor protein closely associated with SOS (Holman and Kasuga, 1997).

Recruitment and activation of the PI3-Kinase results in the phosphorylation of phosphatidylinositol bisphosphate (PI_{4,5}-P₂) to phosphatidylinositol trisphosphate (PI_{3,4,5}-P₃) and ultimately results in the generation of enzymes important in glycolysis, glucose uptake, protein synthesis and glycogen synthesis (figure 1). Insulin signalling generates the recruitment of glucose transporters to the cell surface to allow the passage of glucose from the blood into the target tissue. Glucose transporter-4 (GLUT4) is the main insulin responsive glucose transporter and is primarily located in the muscle and adipose tissue (Shepherd and Kahn, 1999).

Figure 1: Insulin Signalling



1.7 Aetiology of Obesity

People become obese for numerous reasons. At a simple level weight is only gained when net energy intake exceeds energy expenditure over a prolonged period of time. Hence the individual is in a state of positive energy balance. Overeating, causing an imbalance of food intake relative to energy expenditure remains a major element in the origin and the maintenance of overweight and obesity (Van Gaal, 1997). However numerous factors appear to predispose an individual to obesity and complicate the removal of excess body fat. It is clear that genetic factors (particularly affecting metabolic rate, nutrient partitioning and cycling), ethnicity, socio-economic factors, psychological, social and cultural factors and endocrine disorders (e.g. abnormalities of the hypothalamus-pituitary thyroid and adrenal axes, islets of Langerhans and gastro-intestinal tract) provide clues to why some individuals become obese (Bray, 1976).

1.7a Set-Point Theory

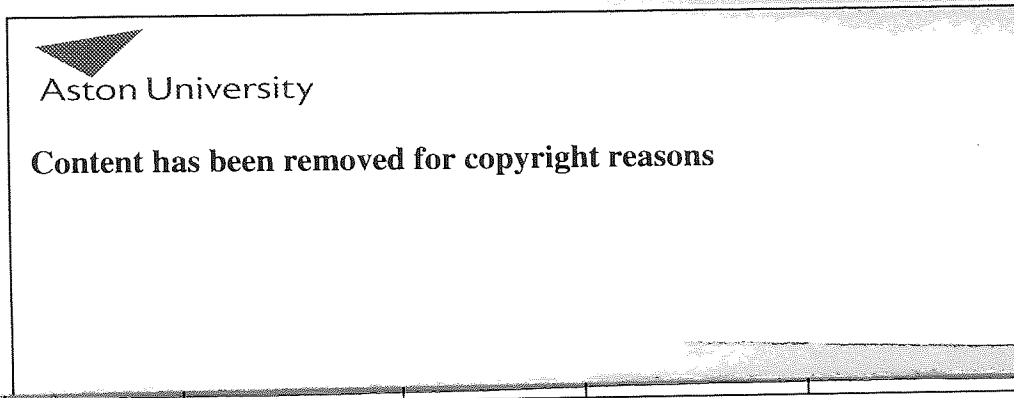
The lateral and ventromedial regions of the hypothalamus have traditionally been regarded as the feeding centre and the satiety centre, respectively. It has been proposed that each individual has a set point to which body weight and fat mass are regulated. An individual's set point range is determined principally by genetic factors and by early factors from the internal environment such as the intrauterine temperature and growth (Wilding *et al.*, 1997). Environmental factors can shift the set point within an individual, for example, physical activity is a major determinant

and in rodents exposure to highly palatable foods and prolonged overfeeding appears to cause an increase in body weight set point (Wilding *et al.*, 1997), apparently due to re-adjustment of the hypothalamic responses to long term satiety signals. Such a defect in the hypothalamic hunger-satiety mechanism causes these individuals to maintain body weight above the original set point than normal (Friedman and Leibel, 1992). Thus chronic obesity often involves the development of a new level of net balance intake of food.

1.7b Genetic Factors

There is evidence that genetic factors have a strong predisposing influence on macronutrient intake as well as energy expenditure (Bouchard *et al.*, 1998). The risks of becoming obese when one or two parents are overweight or obese are increased, although it is difficult to distinguish between inherited factors and behavioural features acquired through family life. The molecular pathogenesis of obesity is unknown. Five single gene mutations in mice (table 3) that result in an obese phenotype have been described (Friedman and Leibel, 1992). The first of the recessive obesity mutations, the obese mutation (*ob*) was identified in 1950. The *Ob* gene produces leptin, a protein secreted by adipose cells. A defect in the *Ob* gene (no leptin production) results in unregulated feeding, massive obesity, insulin resistance, hyperinsulinaemia and T2DM (Zhang *et al.*, 1994). Hence genetic defects can affect an individual's body weight, such as leptin deficiency (*ob/ob* mice) (Zhang *et al.*, 1994) and leptin resistance (*db/db* mouse) (Chua *et al.*, 1996).

Table 3: Genetically Inherited forms of Obesity



(York & Hansen (1997). *Animal Models of Obesity. Handbook of Obesity*. Edited by Bray, Bouchard and James. Chapter 11: 191-221).

1.7c Metabolic Efficiency

Metabolic efficiency is a measure of the amount of energy that is derived from nutrient fuels and channelled into biological functions other than thermogenesis. People who gain weight are usually metabolically more efficient, whereas those who maintain or easily lose weight are less efficient. Since changes in body weight result from the differences between energy intake and energy expenditure, people who gain weight, must ingest more calories than expended through basal metabolism and physical exercise. Basal metabolism can be more efficient in some individuals than others (e.g. the thrifty gene hypothesis of Neel (1962), and the somatotype differences) due largely to inherited patterns of gene expression designed to conserve

energy (Lev-Ran, 1999). So individuals carry a genetic predisposition to obesity if they have an efficient metabolic profile, and will require less energy intake to maintain body weight. Often these individuals gain weight rapidly if they overeat or under exercise. These individuals then have a positive energy balance and become overweight or obese. Once obesity is established and the set point for body weight becomes altered, obese individuals do not necessarily eat any more than thin individuals but have adapted to maintain the extra fat mass (Lean, 1998). Individuals who have been obese since childhood are very efficient at storing the excess calories they ingest and often show increased numbers of mature adipocytes.

1.7d Environmental Factors

Environmental factors such as diet and lifestyle play a critical role in the development of obesity. Environmental influences act via an increase in energy intake and/or a decrease in energy expenditure. There is evidence that high fat diets are associated with an increased risk of obesity, because fat is $2\frac{1}{4}$ more energy dense than carbohydrate and most people have a taste preference of fat-rich foods (Lissner and Heitmann, 1995). There is a well-known relationship between low levels of exercise and an increased risk of becoming obese (Lean, 1998). Obese individuals tend to lead sedentary lifestyles, and have behavioural tendencies that reduce energy expenditure - e.g. fat insulates and decreases heat loss; obese people are less likely to engage in non-specific voluntary movement, e.g. they don't fidget. Obesity is also positively correlated with car ownership, television viewing, urbanisation, smoking and alcohol

consumption. These factors together with other environmental influences play an important role in the aetiology of obesity.

1.7e Psycho-Social Factors

The eating habits of individuals are often influenced by psychological and social factors. The extent to which psychological and social factors are a cause of obesity in any given individual is difficult to quantitate but such influences can certainly modify an individual's eating habits. Many individuals eat excessively when socialising, especially during entertainment, leisure and business activities, or they eat because they follow an inflexible daily meal routine (Jebb, 1997). Other psychosocial factors contributing to weight gain include depression, stress and boredom.

1.8 Metabolic Features of Obesity

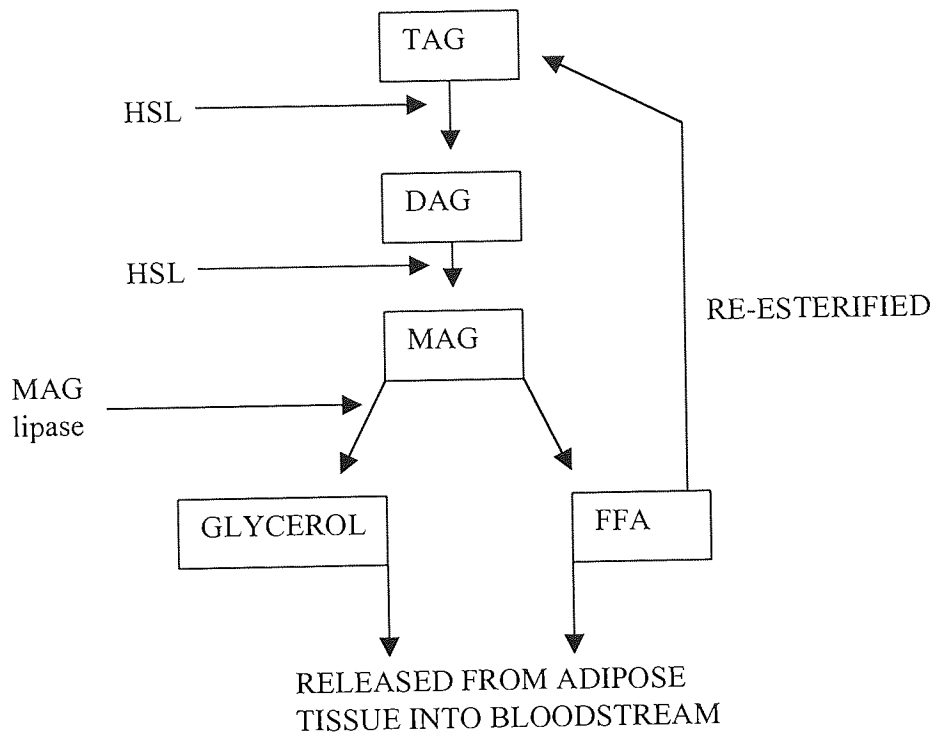
Adipose tissue is the body's largest energy reservoir, where energy is stored in adipocytes as triacylglycerol (TAG). Fat mass can range from 2-3% of body weight in some athletes to 60-70% in obese individuals. Normal fat mass values are 9-18% for males and 14-28% for females. The storage of fatty acids in the adipose tissue to form TAG is known as "lipogenesis" (Arner and Eckel, 1997). The main source for TAG is circulating chylomicrons and very low density lipoproteins from which TAG is hydrolysed by lipoprotein lipase (LPL) (Arner and Eckel, 1997). LPL is a secretory glycoprotein synthesised in a number of tissues including the adipose tissue. It is

located on the walls of capillaries within adipose tissue where it plays an important role in the provision of fatty acids for their uptake and storage as TAG. The FFA are transported into the adipose cells by membrane fatty acid carriers (Fisher *et al.*, 2001; Hamilton and Kamp, 1999; Abumrad *et al.*, 1991). TAG is formed from glucose derived glycerol 3-phosphate and FFA.

In contrast lipolysis of TAG is achieved by hormone sensitive lipase (HSL) to yield diacylglyceride (DAG) and monoacylglyceride (MAG) (Arner, 1996). HSL is the rate-limiting step during the intracellular lipolysis. Monoacylglyceride lipase is the abundant enzyme required to hydrolyse MAG to yield 3 moles of FFA and 1 mole of glycerol. Some FFA are re-esterified to yield TAG, whereas remaining FFA and glycerol are transported into the bloodstream (see figure 2). FFA are used as an energy source in muscles and liver and other tissues, and glycerol is used as a source of gluconeogenesis and esterification in other tissues.

Lipolysis is stimulated by hormones acting on the cell surface receptors coupled to adenylate cyclase via Gs proteins. This activates the adenylate cyclase, which stimulates the formation of cyclic AMP from ATP. Cyclic AMP activates the protein kinase A, which in turn stimulates the phosphorylation of HSL. This issue is addressed in detail in chapter 5.

Figure 2: Basic Pathways of Lipolysis

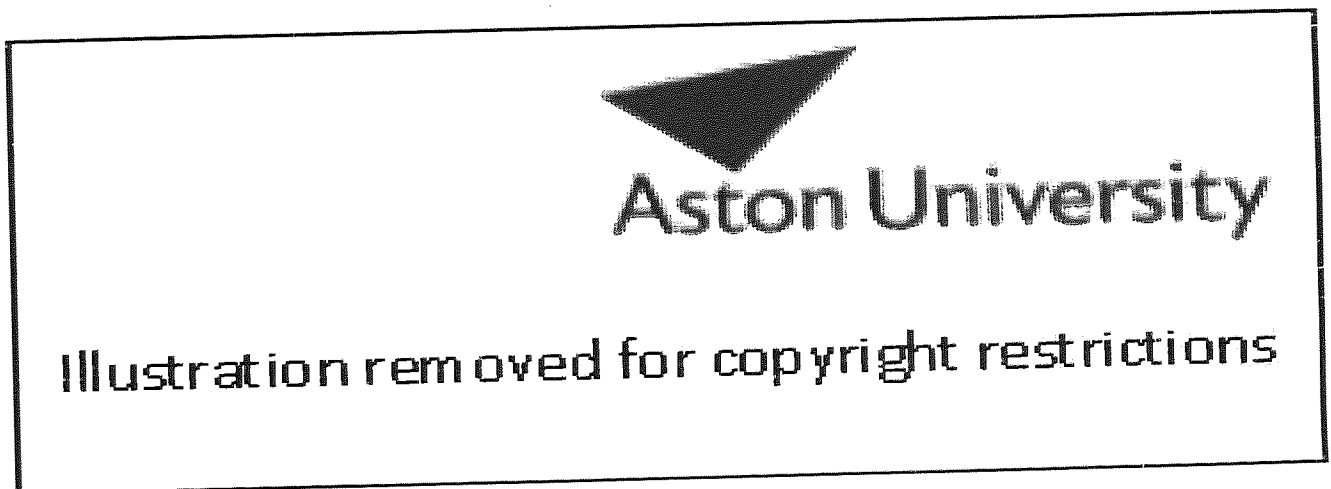


1.9 Adipose Tissue Development & Depots

White adipose tissue depots are located in three major anatomical regions (1) subcutaneous (inguinal, axillary and intercapsular fat depots), (2) dermal and (3) intraperitoneal (mesenteric, omental, perirenal, retroperitoneal, epididymal and parametrial fat depots) (Cinti, 1999). Cells which form the adipose tissue mass include mainly adipocytes, pericytes, adipose precursor cells and fibroblasts (Ailhaud and Hauner, 1997). White adipose cells proliferate most rapidly from birth to 2 years of age and during late childhood and puberty (Bray *et al.*, 1998). Normally, there are no more than 60 billion adipocytes in the body. In some types of obesity, the number of fat cells can increase 3-5 times above the normal number. The production and

turnover of white adipocytes is a continuous process throughout life as summarised in figure 3.

Figure 3: Stages of cell differentiation of the adipocytes



(Ailhaud and Hauner (1997) Development of White Adipose Tissue. *Handbook of Obesity*. Edited by Bray, Bouchard and James. Chapter 16: 360).

The major processes involved in adipogenesis are the proliferation and differentiation of the stem cell into mature adipocytes. In addition the stem cells also proliferate to form vascular precursor cells, which ultimately differentiate to form the mature capillary bed (Ailhaud and Hauner, 1997).

1.9a Hypertrophic and Hyperplastic Obesity

Hypertrophic obesity is an increase in the size of the adipose tissue brought about by the enlargement of individual cells rather than by cell multiplication. In contrast hyperplastic obesity is an increased production and differentiation of normal cells in the adipose tissue (Ailhaud and Hauner, 1997). The adipose tissue becomes larger but retains its normal form. Both forms of obesity occur in association with positive energy balance, with hypertrophy often preceding hyperplastic obesity (Bjorntorp, 1972). Obesity can result from enlarged fat cells or an increase in fat cell number or a combination of both. Childhood obesity is characterised by a combination of fat cell hyperplasia and hypertrophy, whereas in adult onset obesity fat cell hypertrophy is predominant (Salans *et al.*, 1973). Studies in lean and obese individuals have shown that obese children exhibit a more rapid increase in fat cell number and size compared to the lean children (Knittle and Ginsberg-Fellner, 1972). However studies have shown that in severe obesity, there is both an increase in cell number and an increase in cell size (Hirsch and Batchelor, 1976). When hypertrophy and hyperplasia exist together, adipose tissue becomes increasingly difficult to remove by diet, exercise and pharmacological measures.

Obese individuals can usually be classified into two categories on the basis of the distribution of the excess adiposity: android and gynoid obesity. Android (central) obesity is adipose tissue within and around the abdominal region, whereas gynoid obesity is characterised by adipose tissue distribution in the hips and thighs mainly subcutaneous (Lean, 1998). Adipose tissue excess in and around the abdomen is associated with an increased risk of the development of T2DM and vascular disease (Montague and O’Rahilly, 2000). Hence it is important that individuals with this type of obesity reduce their fat stores.

1.10 Adipose Tissue Signals

Adipose tissue can be classed as an ‘endocrine’ organ because it releases blood-borne and locally (autocrine and paracrine) acting substances which signal to the hypothalamus (Ailhaud, 2000). These ‘signals’ released by the adipose tissue play a role in food regulation and aggravate insulin resistance. Known mediators of insulin resistance include elevated free fatty acids (FFA), tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6). Leptin secreted by the adipose tissue provides a satiety signal and promotes thermogenesis (Dagogo-Jack, 1999). The adipose tissue is also a source of paracrine growth factors, such as insulin-like growth factor-1 (IGF-1), insulin-like growth factor (IGF) binding proteins, angiotensinogen and macrophage colony-stimulating factor (MCSF), which are capable of stimulating adipocyte proliferation (Rehman, 2000).

1.10a Free Fatty Acids (FFA)

FFA are stored in the body in the form of triacylglycerides (TAG), which are located in white adipose tissue depots. Lipolysis of TAG by the hormone sensitive lipase (HSL) yields the FFA and glycerol. In insulin resistance the adipocytes are insensitive to the antilipolytic action of insulin, which results in an elevation in FFA (Arner, 2001). Elevated FFA levels but not TNF- α , angiotensin II, growth hormone and insulin-like growth factor-1, are commonly seen in individuals at an early stage of insulin resistance and in obese individuals. This suggests that elevated FFA concentration is a primary metabolic abnormality leading to insulin resistance (Bluher *et al.*, 2001). Resistance to circulating insulin and elevated FFA levels incurs an imbalance in the glucose-fatty acid cycle, which contributes further to the hyperglycaemia. In addition, FFA inhibits whole body glucose utilisation and oxidation and it has been demonstrated that elevated levels of FFA inhibit insulin stimulated glucose uptake (Boden, 1996), thus suggesting that elevated levels of FFA play a major role in the development of insulin resistance. If the β cells can no longer compensate for the small increments in the glucose concentrations through increased insulin production, then glucose levels will rise more substantially and T2DM supervenes (DeFronzo, 1997 and 1992).

Higher concentrations of insulin are required to increase glucose uptake into muscle than for decreasing hepatic glucose output; and a lower insulin concentration still will decrease lipolysis. Therefore, when insulin resistance develops it is usually the glucose uptake into the muscle (which is the least sensitive of the actions of insulin) that is the first to become reduced. Hence T2DM develops and the individual can still

be obese because adipose cells are sensitive enough to insulin to prevent excess lipolysis. Insulin-stimulated glucose uptake is impaired and the suppression of lipolysis is decreased in the adipose cells. When insulin resistance becomes severe plasma FFA concentration will not be suppressed, resulting in an increase in plasma FFA concentration which will lead to increased hepatic glucose production (Reaven, 1988). Elevated circulating FFA will disrupt the glucose-fatty acid cycle (Randle *et al.*, 1963), aggravating insulin resistance in muscle and liver (Flier, 1982).

Visceral adipose tissue has the highest FFA turnover rate, with the turnover rate being lowest in the subcutaneous adipose tissue depot (Arner, 2002 and 1997). In addition, the visceral adipocytes are more lipolytically active due to their complement of adrenergic receptors (Kahn and Flier, 2000). This issue is addressed in detail in chapter 5. The high turnover rate in the visceral adipose tissue impairs hepatic insulin sensitivity because of the direct link between the visceral fat and the liver through the portal vein (Zraika *et al.*, 2002; Bjorntorp, 1991). Hence, there is an increase in glucose output and an inhibition of insulin clearance. Thus, raised plasma TAG and FFA concentrations play a role in the pathogenesis of insulin resistance.

1.10b Leptin

Leptin, a hormone produced in adipocytes, plays a vital role in the regulation of adipose tissue size. It provides a signal to the brain indicating the size of the adipose tissue mass in the body and acts on specific regions of the hypothalamus (arcuate, ventromedial, paraventricular, dorsomedial and lateral nuclei) to induce satiety and

energy expenditure (Della-Fera *et al.*, 2001). Leptin was first shown (now shown in other models) to enhance energy utilisation in *ob/ob* mice by binding to the leptin receptor (Ob-R) in the hypothalamus where it acts to selectively increase peripheral fat oxidation (Commins *et al.*, 1999). The Ob-R has been found to be most abundantly expressed in the hypothalamus (Mercer *et al.*, 1996), with the intracellular domain of the long receptor isoform containing sequence motifs suggestive of intracellular signal-transducing (Tartaglia *et al.*, 1995). Therefore the Ob-R appears to be essential for leptin signalling. In mice, leptin's metabolic effects include inhibition of food intake, stimulation of energy expenditure, reversal of obesity and amelioration of insulin resistance (Dagogo-Jack, 1999). Leptin replacement in *ob/ob* mice reduces insulin resistance in skeletal muscle, and this effect is largely attributable to reduced food intake (Bailey *et al.*, 1999). In *ob/ob* mice, leptin is absent whereas in obese humans, leptin is present, often in high concentrations suggesting leptin resistance (Ahima *et al.*, 1996). This could be caused by a mutation of leptin receptors in the brain (not found evidence from peripheral leptin receptor mRNA); which do not respond appropriately to the leptin in the circulation or due to down regulation of the receptors (similar to down regulation of insulin receptors in states of hyperinsulinaemia). Leptin goes undetected by the brain, so these individuals do not control their food intake until a higher set point is achieved (Dagogo-Jack, 1999). In one study, the administration of leptin in rats caused adipose tissue to undergo apoptosis in addition to activating lipolysis (Della-Fera *et al.*, 2001). Receptors for leptin have been identified on skeletal muscle. When leptin was incubated with muscle cells, it was found that the hormone does not exert a chronic direct influence on glucose transport by muscle cells, but it does have an acute direct but transient effect (Bates *et al.*, 2002). Increases in insulin levels with sulphonylurea

therapy were associated with increases in leptin levels despite weight loss (Williams, 1995). However, other studies provide evidence that leptin can directly inhibit both basal and glucose-stimulated insulin secretion (Fruhbeck and Salvador, 2000). These apparently contradictory studies suggest that leptin is involved in insulin production and release, however the exact relationship remains unclear and complex.

1.10c Tumour Necrosis Factor- α (TNF- α)

TNF- α is expressed as a 26kDa membrane protein that can be cleaved by the metalloprotease TNF- α -converting enzyme (TACE) to release a 17kDa soluble TNF- α form (Trifilieff *et al.*, 2002; MacEwan, 2001). TNF- α is a cytokine produced by activated macrophages and monocytes, and many other cell types including B lymphocytes and fibroblasts (MacEwan, 2001). The pleiotropic agent is over-expressed in the adipose tissue of rodents. It is thought that this cytokine plays a role in the pathogenesis of obesity and insulin resistance (Winkler *et al.*, 1998). Increased TNF- α expression in adipose tissue is also present in human obesity and correlates with the level of hyperinsulinaemia. Hence TNF- α is proposed to provide an indirect measure of insulin resistance (Zahorska-Markiewicz *et al.*, 2000; Hotamisligil *et al.*, 1995). TNF- α has many effects on adipocyte function, and these include actions to inhibit lipogenesis and to increase lipolysis (Kahn and Flier, 2000). TNF- α has also been shown to produce a decrease in both insulin-stimulated insulin receptor autophosphorylation and subsequent tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) (Hotamisligil and Spiegelman, 1994). TNF- α therefore inhibits signalling activity and interferes with insulin action in obesity and diabetes. Short-

term exposure of skeletal muscle to TNF- α does not produce insulin resistance (Nolte *et al.*, 1998), however exposure of adipocytes to TNF- α for 3-4 days makes them insulin resistant. TNF- α appears to act in a paracrine and autocrine manner rather than hormonally, acting more on adipose tissue than muscle. A study investigating the Nco I polymorphism of the TNF- α gene and its relationship with insulin resistance, percent body fat and serum leptin levels was undertaken (Fernandez-Real *et al.*, 1997). The results of the study suggest that TNF- α Nco I polymorphism may exacerbate the alterations in leptin levels normally found among insulin resistant subjects. In another study, TNF- α mRNA expression from subcutaneous adipose tissue was significantly increased in obese women during a period of weight loss with a very low calorie diet, therefore suggesting a role for this cytokine in the control of fat mass during weight loss (Bastard *et al.*, 1999).

1.10d Interleukin-6 (IL-6)

IL-6 is a multifunctional cytokine produced by many different cell types including immune cells, fibroblasts, endothelial cells, myocytes and a variety of endocrine cells (Fried *et al.*, 1998). The pro-inflammatory cytokine is raised in both T2DM and obesity. IL-6 is released by the adipose tissue (Pickup *et al.*, 1997), with omental tissue releasing 2-3 times more of the cytokine than subcutaneous adipose tissue (Fried *et al.*, 1998). It is thought that this cytokine promotes insulin resistance, although its mode of action and physiological significance are as yet uncertain.

1.10e Other Adipokines

Several other adipocyte-secreted factors have been identified that have regulatory roles in metabolism, and in particular modifying insulin sensitivity. Those with the most relevance to diabetes and the metabolic syndrome are reviewed by Perry *et al.* (2001) and are listed below with their main metabolic and vascular effects.

- *Acyl stimulating protein* – component of the complement pathway, role in triglyceride storage
- *Adipsin* – co-factor in formation of acyl stimulating protein
- *Adiponectin* – insulin-sensitizing action, possibly by reducing muscle and liver triglyceride deposition
- *Agouti* – may have a role in development of insulin resistance
- *Angiotensinogen* – involved in conversion of angiotensin I to angiotensin II, thus may play a role in blood pressure regulation
- *Interleukin-8* – inflammatory cytokine
- *Plasminogen-activator inhibitor-1* – inhibits tissue plasminogen activator, so reducing fibrinolysis
- *Prostaglandins* – modify vascular flow to adipose tissue beds
- *Resistin* – negative effect on glucose uptake
- *Transforming growth factor- β* - promotes adipocyte differentiation and PAI-1 secretion

1.11 Role of Neuropeptides in Obesity

In the last few years rapid advances have been made in the understanding of the mechanisms that regulate body weight and fat content. The role of neuropeptide Y, leptin, 5-hydroxytryptamine and other neurotransmitters are being investigated for their implications in the regulation of energy balance.

1.11a Neuropeptide Y (NPY)

In the early 1980s a neurotransmitter called neuropeptide Y was shown to be an important component in appetite and food intake (Widdowson, 1997). It is a 36 amino acid peptide synthesised in the hypothalamus and is released in the 'satiety centre' of the hypothalamus. When injected directly into the brain of laboratory animals, the animals begin to feed within ten minutes and consume a whole day's amount of food within four hours (Frankish *et al.*, 1995). Repeated injections of NPY over a few days result in obesity, which is due to increased food intake and reduced thermogenesis (Stanley *et al.*, 1986). NPY inhibits thermogenesis by reducing the firing rate of sympathetic nerves that innervate brown adipose tissue in rodents (Jeanrenaud, 1994). Restricting food increases the activity of NPY and restoring food to hungry rats reduces NPY hypothalamic activity, suggesting that NPY is responsible for feelings of hunger.

1.11b 5-Hydroxytryptamine (5-HT)

5-HT (serotonin) suppresses food intake in freely feeding or food deprived rats. Microinjections of 5-HT in various regions of the brain have shown that the inhibitory actions on food intake are localised in the paraventricular nucleus (PVN) and the ventromedial regions of the hypothalamus (Wilding *et al.*, 1997). There may be additional effects outside of the hypothalamus, which have yet to be established. Serotonergic agents and 5-HT reuptake inhibitors are effective in reducing food intake (Bray and Ryan, 1997). Elevations within the synaptic cleft in both 5-HT and noradrenaline are required for the effective reduction in food intake in humans, as is the case with sibutramine, a satiety-inducing agent.

1.11c Corticotrophin-Releasing Factor (CRF)

CRF is expressed in the PVN of the hypothalamus and regulates the secretion of adrenocorticotrophic hormone (ACTH). The CRF is an important mediator of weight control, because when injected into the PVN, weight loss is observed (Wilding *et al.*, 1997). The weight loss is accomplished by reducing appetite and stimulating thermogenesis. CRF and NPY act in an opposing fashion on the regulation of energy balance and there is evidence of synaptic connections between CRF and NPY neurones in the PVN (Mercer *et al.*, 1996). CRF may also interact with serotonin, as serotonergic drugs decrease hypothalamic CRF concentrations (Appel *et al.*, 1991).

1.11d Melanin Concentrating Hormone (MCH)

Increasing evidence suggests that the neuropeptide, melanin-concentrating hormone (MCH), plays an important role in feeding regulation (Gee, 1998). Neurons containing MCH are located in the zona incerta and in the lateral hypothalamus, which are both involved in the regulation of ingestive behaviour. When MCH was injected into the lateral ventricles of rats, food consumption was increased (Qu *et al.*, 1996). Hence MCH participates in hypothalamic regulation of body weight. MCH is a cyclic, 19 amino acid neuropeptide that antagonises the action of α -melanin stimulating hormone (MSH) in the skin of teleost fish. However at high concentrations, MCH in reptiles and amphibians has MSH-like actions (Qu *et al.*, 1996). MCH-deficient mice have a reduced body weight, leanness due to hypophagia and an increase in metabolic rate; therefore antagonists of MCH may be effective treatments for obesity (Shimada *et al.*, 1998). The physiological function of MCH in mammals is unknown, but from recent data it appears that MCH is involved in the regulation of feeding.

1.11e Glucagon-like peptide-1 (GLP-1)

GLP-1 is a hormone synthesised within the brain as well as the intestine. The sequence of GLP-1 (7-36) amide is completely conserved in all mammalian species studied, implying that it plays a critical physiological role (Turton *et al.*, 1996). It is effective in reducing food intake when injected into the third ventricle of rat brain. Receptors for GLP-1 are present in appetite regulating areas of the CNS, such as the

PVN of the hypothalamus and the central nucleus of the amygdala (Wilding *et al.*, 1997). To confirm its actions a selective GLP-1 antagonist was synthesised and it was found to increase food intake in the rat (Turton *et al.*, 1996).

1.11f Other Neuropeptides

Several other neuropeptides that are involved in the regulation of appetite and energy homeostasis have been identified and reviewed extensively by Wilding *et al.* (2002) and are listed below.

- Orexin - found to stimulate food intake
- Galanin - transiently increases food intake when administered centrally
- Ghrelin - potent stimulus to food intake
- Nitric oxide - has been proposed as a neurotransmitter or neuromodulator involved in appetite regulation
- Bombesin/gastrin-releasing peptide - reduces food intake in experimental animals and humans
- Calcitonin gene-related peptide
- Cholecystinin – is released from the duodenum in response to the presence of digested food and acts as a satiety signal
- Glucagon - reduces food intake after a meal
- Neurotensin - reduces food intake

PVN of the hypothalamus and the central nucleus of the amygdala (Wilding *et al.*, 1997). To confirm its actions a selective GLP-1 antagonist was synthesised and it was found to increase food intake in the rat (Turton *et al.*, 1996).

1.12 Treatment of obesity

Thyroid extract was first used for the treatment of obesity in 1893 (Bray, 1976). Dinitrophenol uncouples oxidative phosphorylation and was the second drug used for treating obesity. In 1937 amphetamines were introduced and from here many combinations of the drugs were introduced (Lesses and Myerson, 1938). Current treatments for obesity (see table 4) include diet, exercise, surgery, behavioural therapy and drug therapy.

Table 4: Treatment of obesity

Treatment of obesity	
Diet	<p>Restriction of total caloric intake.</p> <p>Diet composition, e.g. restrict fats.</p> <p>Meal pattern, e.g. have frequent smaller meals.</p> <p>Temporary starvation: but include vital nutrients in diet.</p>
Exercise	<p>Relatively ineffective unless strenuous and/or prolonged.</p> <p>Not usually compatible with general health.</p>
Drugs	<p>(1) drugs that reduce food intake, (2) drugs that increase energy expenditure and (3) drugs that exert other effects on metabolism (Bray, 1998).</p> <p>Generally effective but effect may not be maintained.</p>
Behavioural	<p>Modification of eating behaviour associated with aspects of feeding other than food consumption,</p> <p>E.g. group therapy programmes.</p>

Lipectomy	Removal of adipose tissue, relatively successful, but only short-term.
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Centrally-Acting Anti-Obesity Agents

Amphetamines, which enhance central noradrenergic activity were early centrally acting anti-obesity drugs. Despite the efficacy on weight loss the use of amphetamine and its derivatives in obesity was not justified as any possible benefits outweighed the risks involved. Other centrally acting anti-obesity agents include noradrenergic agents (diethylpropion, phentermine, phenylpropanolamine and mazindol) that release norepinephrine or block its reuptake into neurons, and serotonergic drugs that release serotonin or block its reuptake into neurons. These treatments act on the central nervous system to decrease food intake, hence acting as anorectic drugs. Dopamine and noradrenaline have been linked with food intake, whilst serotonin has been linked with the sensation of satiety. The effects observed depends on the receptors they fire and where (Bray and Tartaglia, 2000).

Serotonergic agents include fluoxetine (serotonin reuptake inhibitor). Recently withdrawn agents include dexfenfluramine and fenfluramine (serotonin releasers). Serotonergic agents have been shown to improve insulin sensitivity independently of weight reduction (Scheen and Lefebvre, 1999). Serotonin agents act by suppressing food intake in the paraventricular nucleus (PVN) and the ventromedial regions of the hypothalamus. To date, 14 subtypes of 5-HT receptor have been described (Bickerdike *et al.*, 1999). Of these the 5-HT_{2C} and 5-HT_{1A} receptors are

perhaps the most promising target for the development of a novel drug to treat obesity, because they are the most important mediators of satiety effects (Dourish, 1995).

Sibutramine is a novel 5-hydroxytryptamine (5-HT) and noradrenaline (NA) reuptake inhibitor. It decreases food intake by enhancing satiety (McNeely and Goa, 1998) and it has also been demonstrated to produce weight loss in obese patients (Weintraub *et al.*, 1991, Bickerdale *et al.*, 1999). The hypophagic effects of sibutramine occur due to its ability to inhibit 5-HT and NA reuptake (Jackson *et al.*, 1997).

Drugs Blocking Fat Digestion and Absorption

Orlistat (tetrahydrolipostatin), a semisynthetic derivative of lipostatin is a potent and selective inhibitor of gastric and pancreatic lipases (Scheen and Lefebvre, 1999). The drug inhibits intestinal hydrolysis of triacylglycerides, thus reduces subsequent absorption of monoglycerides and free fatty acids. It has predictable side effects of malabsorption including liquid stools and steatorrhoea, particularly after a meal rich in fat (McNulty and Williams, 1999).

1.13 Benefits of Weight Loss

Weight gain has usually taken place over many years, and when it is accompanied by a new set point for body weight, and the metabolic efficiency has readapted after its initial increase, then it becomes a problem to remove the excess mass (McNulty and Williams, 1999). Numerous methods have been employed to treat obesity and different methods are better suited to different types and degrees of overweight and obesity. Obese individuals find it difficult to lose weight (Bray, 1998) because (1) they are unable to undertake physical activity, (2) they want to lose weight immediately, however find it difficult due to an efficient metabolic profile, which is seen in overweight individuals (less energy intake to maintain body weight). The benefits of weight loss are significant health benefits (see table 5). Weight loss of 0.5kg (1lb) - 1kg (2lb) per week is considered a good rate of weight loss but is difficult to sustain beyond about 3 months because any weight loss is often accompanied by an increase in metabolic efficiency, therefore a new plateau is soon reached (Lean, 1998). Weight loss of more than 1kg (2lb) per week can potentially be dangerous due to mobilisation of lean body mass.

Table 5: Benefits of a 10kg weight loss over 3-4 months

Weight loss of 0.5kg - 1kg per week.

Mortality	20-25% fall in total mortality 30-40% fall in diabetes related deaths 40-50% fall in obesity related cancer deaths
Blood Pressure	Fall of 10 mmHg systolic pressure

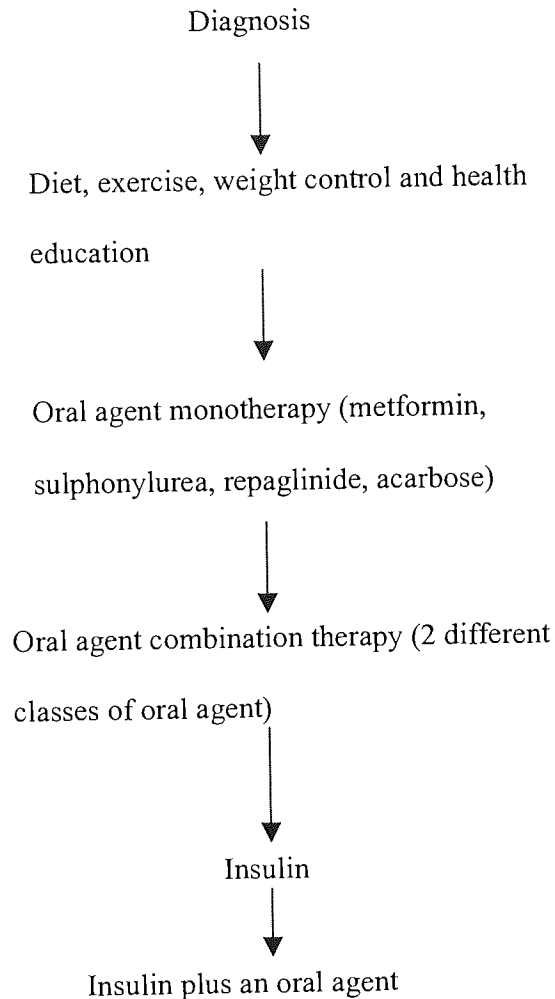
Angina	Fall of 20 mmHg diastolic pressure Reduced symptoms by 91% 33% increase in exercise tolerance
Lipids	Fall by 10% in total cholesterol Fall by 15% in LDL cholesterol Fall by 30% in triacylglycerides Increase by 8% in HDL cholesterol
Diabetes	Reduces risk of developing diabetes by > 50% Fall of 30-50% in fasting blood glucose Fall of 15% in HbA1c

(Adapted from Jung, Obesity as a disease. *British Medical Bulletin*. 1997, Vol. 53 (No. 2): 311)

1.14 Treatment of Type 2 Diabetes Mellitus

To treat type 2 diabetes mellitus (T2DM), it is essential to control the hyperglycaemia. First line treatment begins with diet, exercise, weight control and health education to facilitate weight loss and reduce the obesity. If this proves to be ineffective then second line treatment with an oral agent and failing this a combination therapy with two different classes of oral agents. Insulin therapy will only be introduced when the oral agents are inadequate at improving glycaemia (see figure 4).

Figure 4: Treatment of Type 2 Diabetes Mellitus



(Adapted from Bailey, Antidiabetic drugs. *The British Journal of Cardiology*. 2000, Volume 7, Issue 5).

Sulphonylureas

Sulphonylureas reduce hyperglycaemia by directly stimulating insulin release from β cells. Insulin release occurs when the oral agent binds to the sulphonylurea receptor

to close K⁺ ATP channels, thus depolarising the cell and opening Ca²⁺ channels. This generates an influx of Ca²⁺ and an increase in intracellular Ca²⁺ resulting in a stimulation of insulin release (Panten *et al.*, 1996).

Table 5: Sulphonylureas

	Duration of action
Chlorpropamide	Long-acting
Glibenclamide	Intermediate
Gliclazide	Intermediate
Glimepride	Intermediate
Glipizide	Short-intermediate
Gliquidone	Short-intermediate
Tolazamide	Short-intermediate
Tolbutamine	Short

The insulin release from the β cell into the portal vein suppresses hepatic glucose output and the insulin acts on the peripheral tissues to utilise glucose in the blood. Hence blood glucose concentrations are reduced by initially 2-3mmol/L (Bailey, 2000). Side effects with sulphonylureas include hypoglycaemia, weight gain and hyperinsulinaemia contributing further to insulin resistance (Scheen and Lefebvre, 1999). In the UK Prospective Diabetes Study, treatment with the chlorpropamide or glibenclamide resulted in weight gain in obese diabetic patients prescribed the sulphonylurea therapy for 6 years (UKPDS, 1998).

Repaglinide is a very short-acting insulin releasing agent modified from the non-sulphonylurea portion of glibenclamide. It is quickly absorbed and thus must be taken with meals to stimulate post-prandial insulin release from the β cells. Hence, insulin release will coincide with the increase in glucose concentration seen immediately after eating.

Metformin

One of the most widely used anti-diabetic drugs is a biguanide metformin (Bailey, 1992). Metformin was first introduced in 1957 as an oral glucose-lowering agent to treat T2DM (Sterne, 1969). The effects of metformin include increasing glucose utilisation in the body without stimulating insulin secretion, without weight gain and without causing overt hypoglycaemia. Due to the latter property, metformin is regarded as an anti-hyperglycaemic agent rather than a hypoglycaemic agent. Metformin reduces hepatic gluconeogenesis and enhances insulin stimulated glucose uptake by skeletal muscle (Bailey, 2000). Metformin often promotes weight loss especially in patients who follow a low energy diet (UKPDS, 1998; Bailey, 1992). A decrease in blood glucose concentration by 2-3mmol/L can be achieved with metformin treatment. Side effects of metformin treatment include gastrointestinal symptoms and rarely lactic acidosis (Bailey and Turner, 1996).

Acarbose

Acarbose is an α -glucosidase inhibitor which reduces postprandial elevations of blood glucose and plasma insulin concentrations. It acts at the intestinal brush border to inhibit the activity of the intestinal α -glucosidases (amylase, dextrinase, glucoamylase, isomaltase, maltase and sucrase). Thus, α -glucosidase inhibitors slow the rate of digestion of the dietary carbohydrates and lowering basal glucose concentrations, along with reducing triacylglyceride levels. Side effects of acarbose treatment include abdominal discomfort and diarrhoea.

Thiazolidinediones (TZDs)

Thiazolidinediones (troglitazone, pioglitazone and rosiglitazone) are a relatively new class of treatments for T2DM. They act as agonists of the nuclear peroxisome proliferator-activated receptor-gamma (PPAR γ). Stimulation of the PPAR γ results in an increase in the transcription of insulin sensitive genes. These drugs enhance insulin action by stimulating non-oxidative glucose metabolism in muscle and by suppressing gluconeogenesis in the liver (Scheen and Lefebvre, 1999). Thiazolidinediones improve insulin resistance, glucose tolerance and triacylglyceride levels in obese type 2 diabetic individuals (Bailey and Day, 2001; Day 1999; Kaneko, 1997; Spencer and Markham, 1997; Kumar *et al.*, 1996). However, troglitazone has recently been withdrawn in the US due its toxic effects on the liver. Other side effects of the thiazolidinediones include fluid retention and haemodilution (Bailey, 1999).

Insulin

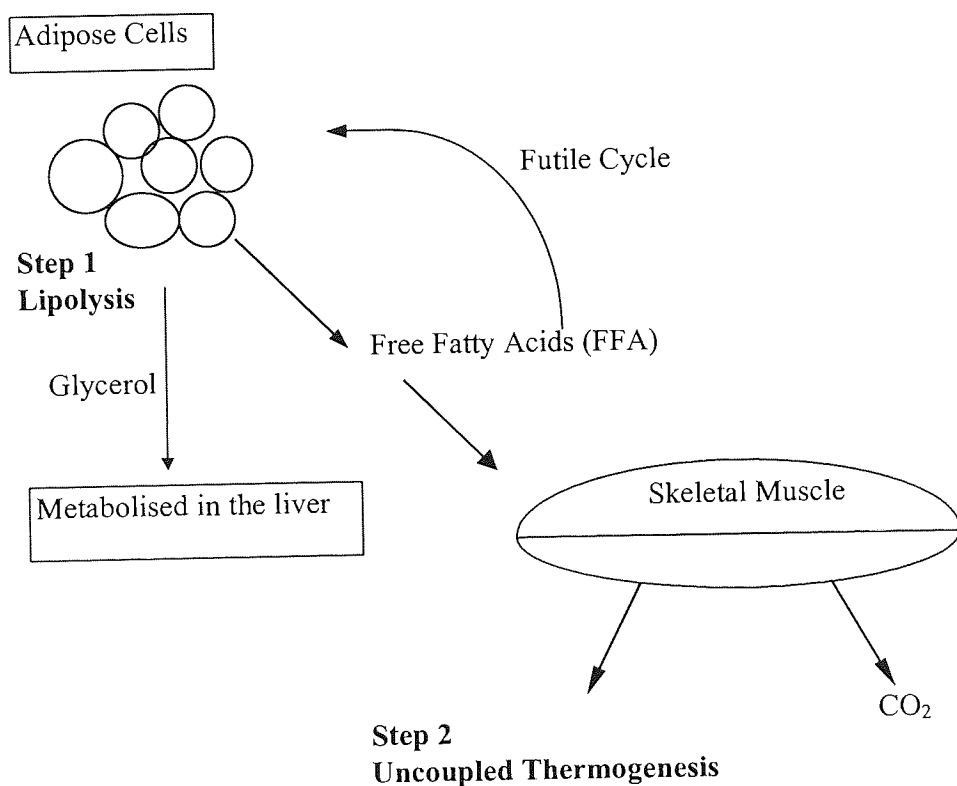
Insulin is required when diet and oral drugs, alone or in combination fail to reduce the hyperglycaemia associated with type 2 diabetes. Usually insulin is only required when there is an impairment of function and loss of β cell mass. Approximately 5-10% of patients on oral treatment for type 2 diabetes will require to start insulin each year (Bailey, 2000). Injecting the insulin results in a reduction in hepatic glucose output and an increase in glucose uptake and metabolism into the peripheral tissues, notably skeletal muscle.

1.15 Aims

The main focus of this research is to treat the obesity by increasing weight loss, which will improve insulin sensitivity and ultimately have benefits for type 2 diabetes. Therefore, the aim of the present study is to investigate new interventions against obesity through increased lipolysis of adipose tissue and increased energy expenditure (such as thermogenesis) in adipose tissue or in other tissues (see figure 5). To decrease adiposity in obese individuals, it is essential to increase lipolysis and as such models of adipocyte biology have been developed as part of this research. Thus, the present research includes the development and characterisation of methods for isolation and evaluation of metabolic activity (especially lipolysis) by human and animal adipose tissue and cultured 3T3-L1 adipocytes. In addition, this research will use novel and established anti-obesity and anti-diabetic agents, and investigate the mechanism of action of these agents in cultured L6 muscle cells, cultured 3T3-L1

fibroblast cells, rodent adipose tissue, human adipose tissue and in animal models of diabetes, obesity and insulin resistance. Known inhibitors of the lipolytic and insulin signalling pathways will be used to define and understand the mechanism of action of these agents.

Figure 5: Aims of Study



1.16 Objectives

The specific objectives of the study are detailed herein. Suppliers of materials and the methods used to conduct the studies in this thesis will be described in chapter 2. Characterisation of the 3T3-L1 cell line will be discussed in chapter 3, along with the characterisation of lean and obese adipocytes isolated from rodent adipose tissue (chapter 5). In addition the adipose tissue is one of the sites responsible for insulin-stimulated glucose uptake, thus the 3T3-L1 cells will be characterised as a model for determining the effects of pharmacological agents on glucose uptake (chapter 3).

Although discussed in detail elsewhere (Bates, 1999), the L6 muscle cell line will be investigated. Preliminary experiments will be undertaken in chapter 4 to identify the L6 cells as a model for skeletal muscle.

In chapter 6, the mechanism of action of M2 (sibutramine metabolite) on adipose tissue will be investigated. The action of M2 on the adipose tissue (rodent and human tissue) will be investigated *in vivo* and *in vitro*. The research will focus on its ability as an anti-obesity agent and/or an anti-diabetic agent. Its effects will be compared with other anti-obesity and anti-diabetic agents.

A well-known anti-diabetic agent is metformin, and in chapter 7 the main area of research will focus on another guanidine derivative, 3-guanidinopropionic acid (3-GPA). The mechanism of action of 3-GPA on the adipose tissue and skeletal muscle will be investigated *in vivo* and *in vitro*. The ability of 3-GPA to have either anti-diabetic and/or anti-obesity effects will be compared with metformin.

Mazindol, a centrally acting appetite suppressant used as an anti-obesity agent will be investigated in chapter 8. The effect of mazindol on glucose transport into L6 muscle cells and its mechanism of action will be determined. In addition, the lipolytic effect of mazindol on isolated adipocytes from human and rodent tissue and 3T3-L1 preadipocytes will be investigated.

Chapter 9 will focus on the *in vitro* clinical work using human adipose tissue extracted from subcutaneous and visceral sites. The action of novel and established anti-obesity agents on the adipose tissue will be investigated here.

Chapter 2: Materials and Methods

2.1 Materials

All chemicals were standard laboratory reagents and of analytical grade, unless otherwise stated. Knoll Pharmaceuticals kindly supplied Sibutramine, its metabolites, BRL 37344, dexfenfluramine, fenfluramine, phentermine, nisoxetine and fluoxetine. Non-esterified fatty acid kits (NEFA) were supplied by Wako Chemicals, USA. The mouse insulin ELISA kits was obtained from Mercodia, Sweden. Plasticware was from Scientific Laboratories Supplies, Nottingham, UK and Sarstedt, Leicester, UK.

Suppliers of chemicals were:

Amersham International Ltd, Bucks, UK

2-Deoxy-D-[1-³H]glucose

Calbiochem-Novabiochem Ltd, Beeston, Nottingham, UK.

H-89

LY 294 002

SB 202190

Wortmannin

Fisher Scientific, Loughborough, UK

Ethanol

Glacial acetic acid

D-Glucose anhydrous

Hydrochloric acid

Magnesium sulphate

Methanol

Methylated spirit

Optiphase Hisafe 3

Perchloric acid

Potassium dihydrogen orthophosphate

Potassium hydroxide

Sodium carbonate

Sodium chloride

Sodium dihydrogen orthophosphate

Sodium hydrogen carbonate

Sodium hydroxide

Sucrose

ICN Biomedicals, Hampshire, UK

Atenolol

Clonidine

Dobutamine

Fenoterol

Phenylephrine

Phosphate buffered saline tablets (PBS)

Invitrogen, Paisley, Scotland, UK

Bovine foetal calf serum (FCS)

Dulbecco's Modified Eagle Medium (DMEM)

Penicillin-Streptomycin

Trypsin/EDTA

Sigma-Aldrich Co. Ltd, Dorset, UK

Adenosine 5'-triphosphate (ATP)

Bovine serum albumin (BSA)

Calcium chloride

Clonidine

Collagenase type II

Cycloheximide

Cytochalasin B

Dexamethsone

2-Deoxy-D-Glucose

1,1-Dimethylbiguanide

Dimethyl sulphoxide (DMSO)

Ephinephrine (adrenaline)

Forskolin

L-Glutamine
Glycerokinase
Glycerol
Glycine
3-Guanidinopropionic acid (3-GPA)
3-Hydroxytyramine
5- Hydroxytryptamine (5-HT)
ICI 118551
Insulin
3-Isobutyl-1-methylxanthine (IBMX)
Isoproterenol
Lactate dehydrogenase
Magnesium chloride
Mazindol
 α -Nicotinamide adenine dinucleotide-reduced form (NADH)
Norepinephrine (noradrenaline)
Oil red O
Phosphoenolpyruvate (PEP)
Potassium chloride
Prazosin
Propranolol
Pyruvate kinase
Sodium bicarbonate
SR 59230A
Triethanolamine

Trypan blue

Yohimbine

2.2 Methods

2.2.1 Animal *in vivo* studies

2.2.2 Animals

The mice used in the present studies were originally derived from the Jackson Laboratory, Maine USA. They were out-crossed to other strains at the University of Edinburgh before a colony was established at Aston University. Details of the history of the Aston colony are given elsewhere by Flatt and Bailey (1981). Homozygous lean *+/+* and obese *ob/ob* mice were used. Characteristics of the *ob/ob* mice, notably absence of leptin, hyperphagia, obesity, hyperinsulinaemia, insulin resistance and hyperglycaemia have been described fully elsewhere (Bailey and Flatt, 1994). The nature of the *ob* mutation has been identified namely a premature stop codon in the leptin gene that renders the leptin inactive (Zhang *et al.*, 1994). Mice were caged in separate groups and maintained at 21°C with a 12h light-dark cycle. Fresh water and rodent pellet (SDS Economy Rodent Breeder, Special Diet Services, Witham, Essex) were available at all times, unless otherwise stated. Food and water consumption were monitored before, during and after the study. Body weights of the mice were measured daily starting 7 days prior to, during and for 14 days after drug administration, unless otherwise stated.

2.2.3 Drug dosing

Administration of the drug to the animals was via oral gavage (po), intra-peritoneal injection (IP) or in the drinking water.

2.2.4 Determination of Glucose, Insulin, Non-Esterified Fatty Acid (NEFA) Concentration

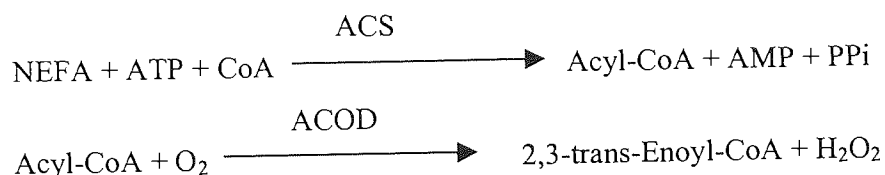
Blood samples taken from the tail tip of the mice were collected in heparinised (500U/ml) microfuge tubes and stored on ice. Blood glucose levels in all mice were measured using a glucose-oxidase based portable glucose monitor (Glucotrend®, Boehringer Mannheim). 3-5µl of blood was required for the glucose determination. To ensure absolute accuracy the blood was pipetted onto the solid phase reagent patch. Glucose oxidase in the reagent patch oxidises the glucose to glucuronic acid, and hydrogen peroxide is liberated. Consequently there is a change in redox potential, which is recorded by the glucose monitor. The analyser automatically corrects the read out value to give the plasma equivalent value. The method has been compared against the Beckman plasma glucose analyser in our laboratory and shows identical results.

The plasma was separated from the blood by centrifugation (13000rpm (11600g) /1min (MSE Microcentaur Centrifuge, Fisher Scientific, UK)) and stored at -40°C until analysed for insulin (25µl plasma required) and free fatty acid (10µl plasma

required) concentration. Typically 80-100 μ l of blood would be required for the determination of glucose, insulin and NEFA concentration.

The principle of the insulin ELISA kit is based on a direct sandwich technique in which two monoclonal antibodies are directed against different antigenic determinants on the insulin molecule. Here the insulin in the plasma sample (25 μ l) was incubated with 100 μ l peroxidase-conjugated anti-insulin antibodies and the anti-insulin antibodies bound to the wells in the plate for 1h at room temperature on a shaker. Any unbound enzyme antibodies were removed by a washing buffer, which was repeated 4 times. The bound insulin was then detected using 200 μ l 3,3',5,5'-tetramethylbenzidine. The reaction was stopped using 50 μ l 1M H₂SO₄. Absorbance of samples was read at 450nm on the spectrophotometer (Ultraspec®K LKB Biochrom, UK) and the concentration determined from the standard curve. A standard curve was prepared using the rat insulin standards supplied with the Mercodia insulin ELISA kit. The sensitivity of the assay was 0.07 μ g/ml (lowest concentration distinguishable from zero) and the intra-assay co-efficient of variations was typically 2-3%. All samples that were directly compared were analysed in the same assay run to avoid inter-assay variability.

To determine NEFA concentrations the Wako NEFA test kit was used. This enzymatic method relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase. The acyl-CoA thus produced is oxidised by added acyl-CoA oxidase with the generation of hydrogen peroxide.



The hydrogen peroxide in the presence of the added peroxidase allows the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxy-ethyl)-aniline with 4-aminoantipyrine to form a purple coloured adduct, which can be measured colourimetrically at 550nm. 10µl plasma was added to 200µl colour reagent A and incubated for 10min at 37°C. 400µl of colour reagent B was then mixed with the solution and incubated at the same temperature for 10min.

Constituents of Colour Reagent A:

Acyl-coenzyme A synthetase (ACS)

Ascorbate oxidase (AOD)

Coenzyme A (CoA)

Adenosine triphosphate (ATP)

Phosphate buffer, pH 6.9

Magnesium chloride

Surfactant

Stabilizers

Constituents of Colour Reagent B:

Acyl-coenzyme A oxidase

Peroxidase (POD)

3-methyl-N-ethyl-N-(β-hydroxy-ethyl)-aniline (MEHA)

Surfactant

The samples were then allowed to cool to form the purple colour as described above, and the absorbance read at 550nm on the spectrophotometer (Ultraspec®K LKB Biochrom, UK). The concentration in the plasma was then determined by reading the absorbance from the standard curve. A standard curve was prepared using the NEFA standards supplied with the kit and following the protocol as outline above. The test is linear over the range 0.0 to 2.0 mEq/L. All samples that were directly compared were analysed in the same assay run to avoid inter-assay variability.

2.2.5 Intraperitoneal Glucose Tolerance Test (IPGTT)

To assess improvements in glucose homeostasis an IPGTT was performed on the mice at the end of the study. A basal glucose concentration was measured for each mouse (fed) using the glucose analyser described in 2.2.4. This was followed by IP administration of 2g glucose/kg of body weight in 40% (w/v) solution. The blood glucose levels were then recorded each hour for 3h.

2.3 Isolated Animal and Human Tissue

2.3.1 Animal Tissues

Mice were killed by cervical dislocation and the adipose tissue was immediately removed from either the lean or obese (*ob/ob*) mice. The lean mice used in the

experiments were 20-30 weeks old and had a weight range of 35g-50g. The obese mice used in the experiments were age-matched (20-30 weeks) and weighed 80g-100g. The animal experiments were performed using the minimum numbers consistent with good scientific practice for the experiments concerned and conformed to the UK Home Office Scientific procedures licence and the Aston University Bioethical committee requirements. The adipocytes were isolated from the adipose tissue using the method described below, which was introduced by Rodbell in 1964.

2.3.2 Human Tissue

Abdominal subcutaneous and omental adipose tissue biopsies were obtained from adult patients undergoing elective surgery at Birmingham City Hospital, Dudley Road, Birmingham. All patients were fasted preoperatively, and all underwent general anaesthesia. City Hospital Research Ethics Committee approval was obtained, and all patients involved gave their informed consent. A summary of the patient consent procedure and a sample copy of consent form are given in appendix II.

2.3.3 Isolation and Incubation of Mouse and Human Adipocytes to determine Lipolysis

The same experimental procedure was used to measure lipolytic activity of putative agents with mouse and human adipose tissue. For mouse adipose tissue this was accomplished by removing the parametrial and epididymal fat pads, total weight

about 2g, from the mouse and placing each pad (minced into small pieces) in a 5ml bijou containing 1.5mg collagenase/ml (total volume 2ml) of Krebs Ringer bicarbonate buffer (KRB) containing 3% bovine serum albumin (BSA) for the lean pads and 5% BSA for the obese pads. Similarly, about 2g of human adipose tissue was minced into small pieces and placed in a 5ml bijou containing 1.5mg collagenase/ml of KRB buffer supplemented with 5% BSA. A high albumin concentration is required for the more fragile human fat cells and also to stabilise the buffer (Pederson *et al.*, 1981). After incubating the tissue in a slow shaking water bath at 37°C for 1h, the fat layer floating on the top of the medium was removed by a plastic pipette and transferred into a universal with fresh KRB buffer (37°C) and centrifuged at room temperature at 1000rpm (500g) for 1min (MSE Mistral 2,000, Fisher Scientific, UK). The fat layer was reaspirated, washed with fresh KRB buffer (37°C) and centrifuged (1000rpm for 1min) four times in total. After the final centrifugation the adipocytes were removed and added to a series of samples, including controls for the experiment. The controls in the experiment consisted of a negative control (KRB buffer only) and the positive control (isoproterenol), which determined that the whole experimental procedure was working reproducibly. Adipocytes (10^5 fat cells counted using a haemocytometer (see characterisation of murine lean and obese adipocytes)) were incubated in a working solution diluted in KRB to give 10^5 cells/ml. Test compounds were included in the pre-gassed KRB buffer and incubations were performed for 2h at 37°C in the slow shaking water bath. The samples were gassed at the beginning of the incubation period with 5% CO₂ 95% O₂. Control samples containing adipocytes alone were analysed to determine the spontaneous basal glycerol release. After incubation, 0.5ml of the medium was added to 0.5ml of 10% perchloric acid (1.7M) to stop the reaction. After centrifuging at

13000rpm (11600g) for 5min (MSE Microcentaur Centrifuge, Fisher Scientific, UK), the infranatant was collected and the pH for each sample was adjusted to pH 7.0 by the addition of 40% potassium hydroxide (7.1M). The amount of glycerol released by the adipocytes was then measured using the method described in 2.3.4.

2.3.4 Measuring Lipolytic Activity

The concentration of glycerol released was determined enzymatically by the method of Wieland, 1974. A 200 μ l sample of medium was added to 830 μ l spectrophotometer buffer

Constituents of spectrophotometer buffer - to 100ml distilled water add:

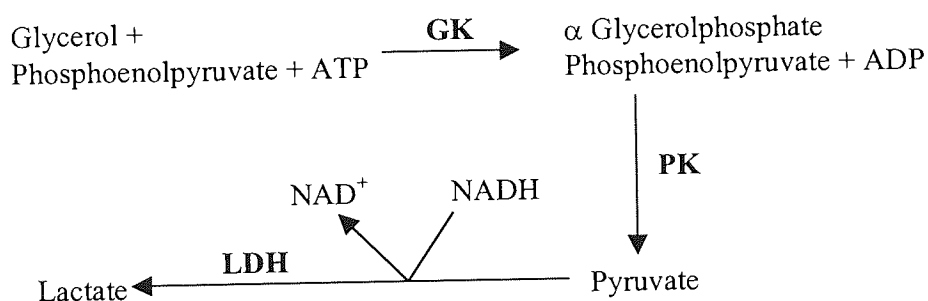
Magnesium Sulphate	0.0493g
Triethanolamine	1.5g
PEP	0.0183g
ATP	0.0609g
NADH	0.0177g

pH 7.4 (adjusted with hydrochloric acid or potassium hydroxide)

73 μ l lactate dehydrogenase (LDH) and 53 μ l pyruvate kinase (PK) was added to the reaction mixture.

Glycerokinase was diluted 100µl to 400µl distilled water and 10µl was added to the assay mixture. The samples were shaken and immediately absorbance read at 340nm using a spectrophotometer (Ultraspec®K LKB Biochrom, UK). Samples were allowed to stand at 37°C and then a second reading was taken after 15min. The difference between the two values was calculated. Glycerol is converted to glycerophosphate by ATP and glycerokinase (GK). The oxidation of glycerophosphate to the NAD-dependent lactate serves as the indicator reaction.

Principle of Method



The formation of NAD⁺, as measured by the changes of extinction at 340nm is proportional to the amount of glycerol present. To calculate the concentration of glycerol (mM) in each sample, the following equation was used.

$$(\text{change in absorbance}) \times 1.61^* = \text{mM [Glycerol]}$$

Using the equation, the lipolytic activity of putative agents was expressed as mM glycerol released/10⁵ adipocytes/2h.

* 1.61 is obtained for the extinction co-efficient of NADH (6.22)

2.4 3T3-L1 Fibroblast Cells

2.4.1 Cell Culture - growth and passaging

3T3-L1 preadipocytes were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine, 100U/ml penicillin G, 10mg/ml streptomycin and 25µg/ml amphotericin B. The cells were seeded at 10^7 cells/ml, grown in 200ml flasks and maintained in a fresh 20ml volume of 10% FCS/DMEM every 2 days. Once confluence was reached 5ml of trypsin/EDTA solution was added and the flask agitated until the cells detached. The cells and trypsin were transferred to a universal containing 5ml of the 10% FCS/DMEM and centrifuged at 800rpm (400g) for 5min (MSE Mistral 2,000, Fisher Scientific, UK). The pellet containing the cells was then resuspended in 10ml of fresh medium. 1ml of cell suspension (10^7 cells/ml) was added to fresh flasks for further passaging and the remaining cells were grown in 100mm culture dishes for differentiation or in 24 well plates for lipolysis experiments (seeded at 10^6 cells/ml). The cells were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

2.4.2 3T3-L1 cell growth curves

Characterisation of the cells and growth curves were undertaken for the 3T3-L1 cell line and can be found in chapter 3.

2.4.3 Differentiation of 3T3-L1 cells

1ml of the cell suspension after passaging was added to 100mm culture dishes together with 7ml of medium. Cultures were replaced with fresh 8ml medium every 2 days during exponential growth. Once the preadipocytes were grown to confluence, the cells were either differentiated into adipocytes (Rubin *et al.*, 1978; Frost and Lane, 1985) or used as preadipocytes. Two days post confluence (day 0) differentiation was induced with 0.5mM IBMX, 0.25 μ M dexamethasone and 1 μ g/ml insulin in DMEM containing 10% FCS, 2mM L-glutamine, 100U/ml penicillin G, 10mg/ml streptomycin and 25 μ g/ml amphotericin B. After two days the IBMX and dexamethasone were removed and insulin was maintained for 2 additional days. On day 4 the insulin was removed from the medium and the 10% FCS/DMEM was replaced every 2 days. Oil Red O, a fat-soluble dye was used to assess the extent of fat accumulation post differentiation using a small aliquot of cells. Cells were used for experimentation between 8-14 days and incubated in low serum (0.5% FCS) DMEM for 2h prior to incubation with test compounds.

2.4.4 ³H2DG Uptake into Differentiated 3T3-L1 cells

At the end of the incubation period, the effect of the test compound on glucose transport into the cells was measured. Cell monolayers were washed with glucose-free KRB buffer at 22°C, then incubated in 8ml of this buffer supplemented with 0.1mM 2-deoxy-D-glucose (0.0162mg/ml) and 7.4Kbq/ml 2-deoxy-D-[³H]-glucose (0.2 μ Ci/ml) for 10min at 22°C. The buffer was then aspirated and cells were

washed twice with ice cold KRB buffer. 4ml of 1M NaOH was then pipetted into the culture dishes and left for 1h. The NaOH cell digest (1ml) was placed into vials containing 8ml of scintillant (Optiphase HiSafe 3) and the ^3H radioactivity was counted using the packard 1900TR liquid scintillation counter.

2.4.5 Lipolysis experiments

4ml of the cell suspension after passaging was added to 46ml of fresh DMEM supplemented with 10% FCS. The cells and medium were then transferred into 24 well (16mm) plates at 1ml/well at a concentration of 10^6 cells/ml. Once confluence was reached (3 days), the medium was removed and replaced with 1ml of 0.5% FCS DMEM in each well. The cells were serum starved (0.5% FCS) for 24h and then incubated with test agents for 10min to 72h. After incubation with the test compounds, the amount of glycerol released from the 3T3-L1 preadipocytes was measured using the method described in 2.3.4.

2.5 L6 Muscle Cells

2.5.1 Cell Culture - growth and passaging

L6 muscle cells (obtained from the European Culture Collection, Porton Down) were grown in DMEM supplemented with 5% FCS, 2mM L-glutamine, 100U/ml penicillin G, 10mg/ml streptomycin and 25 μg /ml amphotericin B. The cells were seeded at 1 x

10^7 cells/ml, grown in 200ml flasks and when confluent the 20ml medium in which they were maintained was removed. 5ml of trypsin/EDTA solution was added and the flask agitated until the cells detached. The cells and trypsin were transferred to a universal containing 5ml of the 5% FCS/DMEM and centrifuged at 800rpm (400g) for 5min (MSE Mistral 2,000, Fisher Scientific, UK). The supernatant was decanted off and 10ml of fresh medium was added to the cells (in the pellet) and resuspended. 1ml of cells (1×10^7 cells/ml) was added to fresh flasks for further passaging and 4ml of the cells was added to 46ml of fresh 5% FCS/DMEM, which was plated out in a 24 well (16mm) plate at 1ml/well. The cells were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Once the cells reached confluence (3 days), the medium was removed and replaced with 1ml of 0.5% FCS/DMEM in each well. The cells were serum starved (0.5% FCS) for 24h to induce differentiation and fusion of myoblasts into myotubes. The myotubes were then incubated with test compounds for periods of 10min to 72h, after which ³H2DG uptake was determined during a further 10min incubation.

2.5.2 L6 muscle cell growth curves

Preliminary experiments and growth curves have been obtained for the L6 muscle cell line and can be found in chapter 4.

2.5.3 ³H2DG Uptake into L6 Muscle Cells

At the end of the incubation period, the effect of the test compound on glucose transport into the cells was measured. Cell monolayers were washed with glucose-free KRB buffer at 22°C, then incubated in 1ml of this buffer supplemented with 0.1mM 2-deoxy-D-glucose (0.0162mg/ml) and 7.4Kbq/ml 2-deoxy-D-[³H]-glucose (0.2μCi/ml) for 10min at 22°C. The buffer was then aspirated and cells were washed twice with ice cold KRB buffer. 0.5ml of 1M NaOH was then pipetted into the wells and left for 1h. The cell digest was placed into vials containing 4ml of scintillant (HiSafe 3) and the ³H radioactivity was counted using the packard 1900TR liquid scintillation counter.

2.6 Cell counting

Trypan blue and the haemocytometer were used to determine total cell counts and viable cell number. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Normal cells are able to exclude the dye, but trypan blue diffuses into cells in which membrane integrity has been lost. It is a quick technique facilitating the visualisation of cell morphology. The technique is particularly important for visualising the effects of unknown compounds on cell viability and morphology. The following method was used for the L6 cells, 3T3-L1 cells and lean and obese isolated adipocytes. A cell suspension was prepared by placing the cells in 1ml PBS. 500μl of 0.4% trypan blue solution was added to 300μl of PBS and 200μl of the cell suspension. The solution was mixed

thoroughly and incubated for 10min at room temperature. The trypan blue-cell suspension was transferred to a haemocytometer using a Pasteur pipette and the number of viable and non-viable cells was determined. The number of cells in the 1mm centre square and four 1mm corner squares were counted in 2 separate chambers and the average cells/ml was determined using the following equation.

$$\text{Cells per ml} = \text{Cell count per square} \times \text{Dilution factor} \times 10^4 \text{ (count 10 squares)}$$

This procedure was repeated 3 times to ensure accuracy of cell number and cell viability. Viability was expressed as % cells that were clear of dye.

2.7 Statistical analyses

All data was expressed as mean values \pm standard error. For 2-deoxyglucose uptake experiments, the uptake of 2DG was expressed as % change compared with the control (100%). The amounts of glycerol produced in the lipolysis experiments were expressed as mM glycerol. For the *in vivo* studies, glucose and NEFA concentrations were expressed as mmol/L, and insulin concentrations were expressed as pmol/L.

A Student's t-test was used to compare the difference between two means. A paired t-test was used when the results formed pairs from the same animal or same tissue sample. An unpaired t-test was used when the treatments were applied to separate animals and tissues. Where repeated t-tests were used a Bonferroni correction was included to avoid type 2 error.

An analysis of variance (ANOVA) was used when the experiment had more than two treatments and the differences between the groups were to be compared. A post Dunnett's test was used to compare the control mean with the other means and a Tukey's test was used to measure the significance between all means with each other. Probability levels of $p < 0.05$ were accepted as significant.

Chapter 3: Characterisation of the murine 3T3-L1 cell line

3.1 Introduction

Green and Kehinde (1974, 1975 and 1976) established and cloned several lines of murine 3T3-L1 preadipocytes. These cultured 3T3-L1 cells spontaneously generate foci of adipocytes when maintained at confluence. The rounded cells contain large droplets of triglyceride when kept at confluence for 2-4 weeks in fresh medium (Rubin *et al.*, 1978).

Conversion of the 3T3-L1 preadipocytes into adipocytes can be accelerated by the treatment of confluent cells with isobutylmethylxanthine (IBMX), dexamethasone and insulin (Frost and Lane, 1985). Other stimulators of the adipocyte conversion process include 20-30% serum (Green and Meuth, 1974), prostaglandin F₂ α (Russell and Ho, 1976), biotin (Mackall *et al.*, 1976) and more recently thiazolidinediones, which act by stimulating the nuclear peroxisome proliferator-activated receptor-gamma (Spiegelman *et al.*, 1998; Brun *et al.*, 1996; Tontonoz *et al.*, 1994).

The murine 3T3-L1 cell line has been used extensively to study the action of insulin. Cells that have undergone adipocyte differentiation exhibit a 20-fold increase in the number of insulin receptors and an increase in glucose transporters (Reed *et al.*, 1981). To this end, the 3T3-L1 adipocytes have been characterised as a model for glucose uptake studies in adipose tissue. In addition, the cells have been used as a model for the study of adipocyte lipolysis. 3T3-L1 preadipocytes express a higher percentage of β 1 adrenoceptors than any other adrenoceptor subtype and have been

shown to be responsive to catecholamines. Preliminary studies are included here to characterise the present batch of cultured 3T3-L1 preadipocytes as a model for studying the lipolytic action of putative agents.

3.2 3T3-L1 cell growth

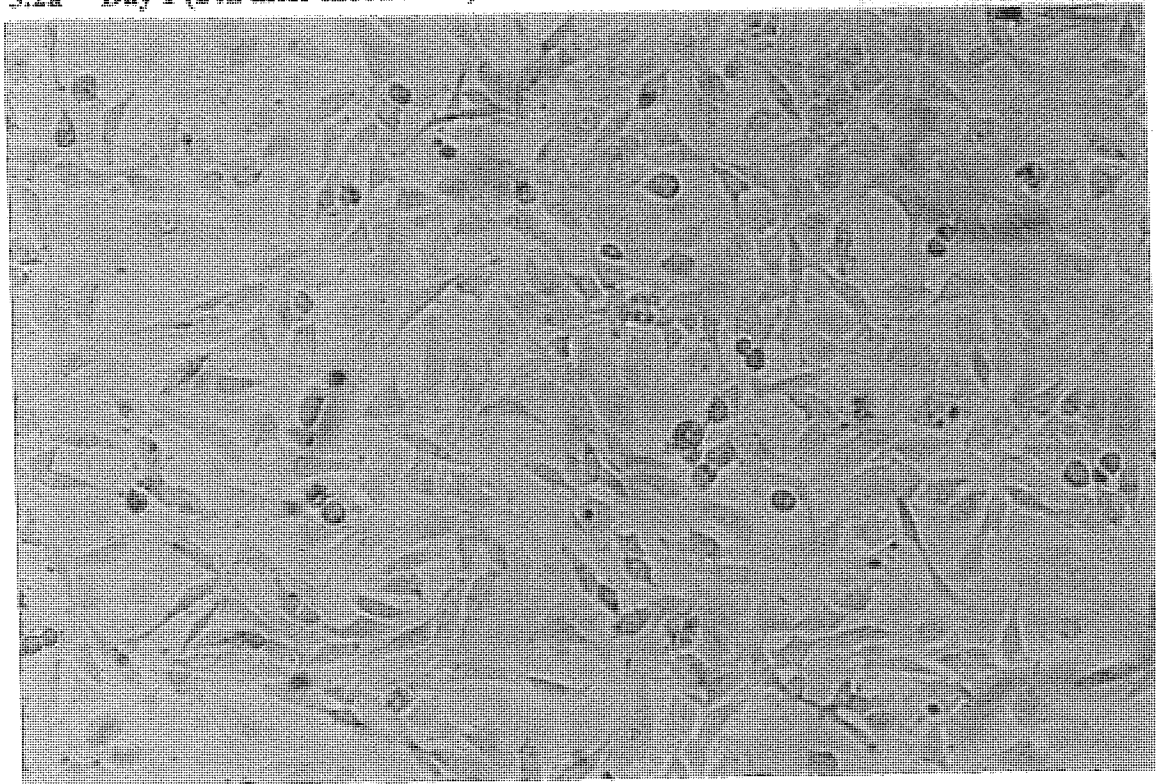
3T3-L1 cells were grown as described in section 2.4.1, and their rate of growth was monitored daily. Cell growth rate was determined over a 5-day period. The 3T3-L1 fibroblasts were seeded at 10^7 cells and grown in 75cm^3 flasks containing 20ml 10% FCS DMEM. Each day the number of cells in each flask was calculated using a haemocytometer. Trypsin (5ml) was added to the flask to remove the cells and centrifuged at 800rpm for 5min. The cells were then resuspended in fresh medium (5ml) and the number of cells counted. Dilution factors were accounted for and the data were expressed as number of cells per ml. The cell counts were carried out at noon each day and photographs were taken daily.

3.2.1 3T3-L1 cell growth results

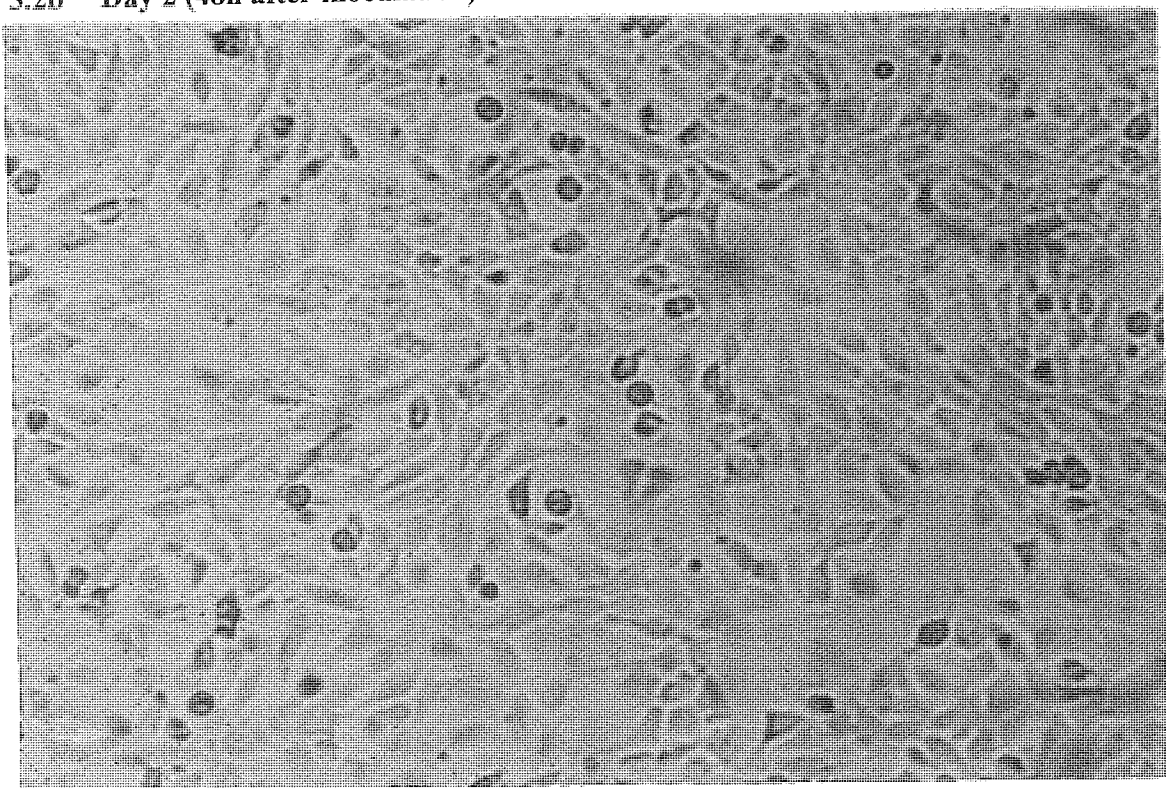
The 3T3-L1 fibroblasts grow at a slow rate for the first 48h (see figure 3.1). However, at 72h post-seeding there is a substantial increase in the number of 3T3-L1 fibroblasts (3×10^7 to 8×10^7 cells/ml, $p < 0.05$, 48h versus 72h). The number of cells at 96h and 120h does not increase significantly further, thus confluence is reached at 72h

(day 4 of seeding). Photographs of 3T3-L1 cells were taken 24h, 48h and 72h after inoculation of 1×10^7 cells (see figure 3.2a, b, and c).

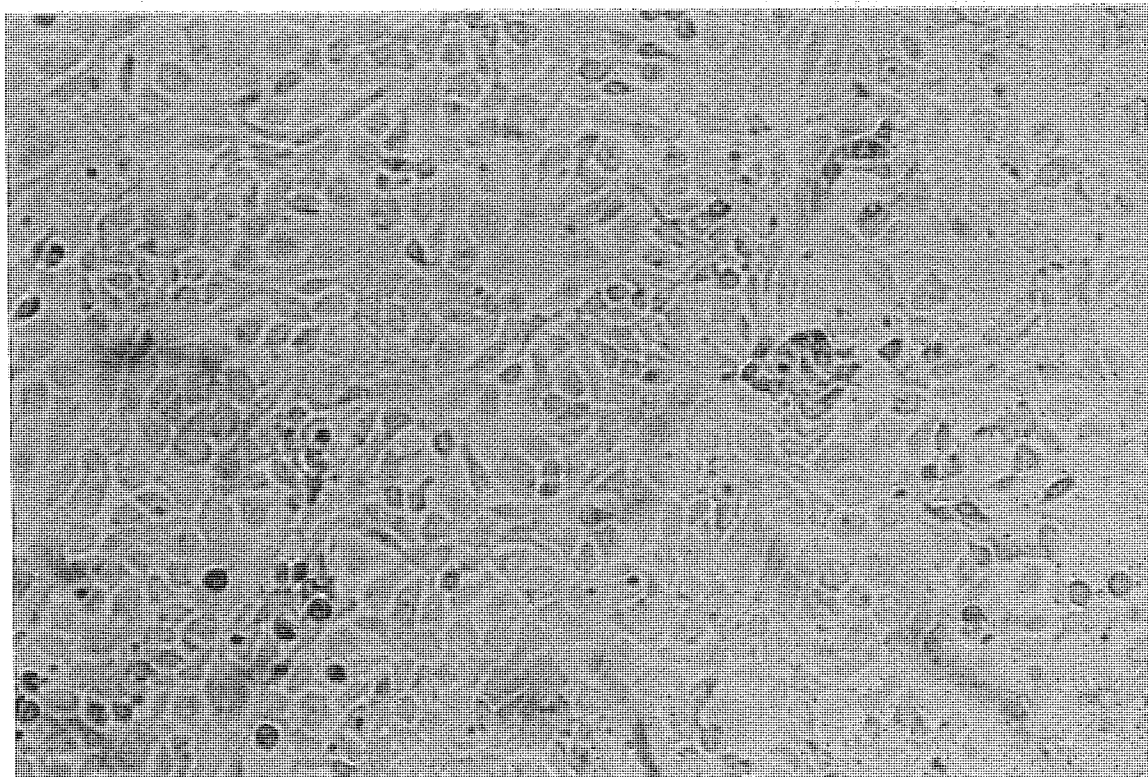
3.2a Day 1 (24h after inoculation)



3.2b Day 2 (48h after inoculation)

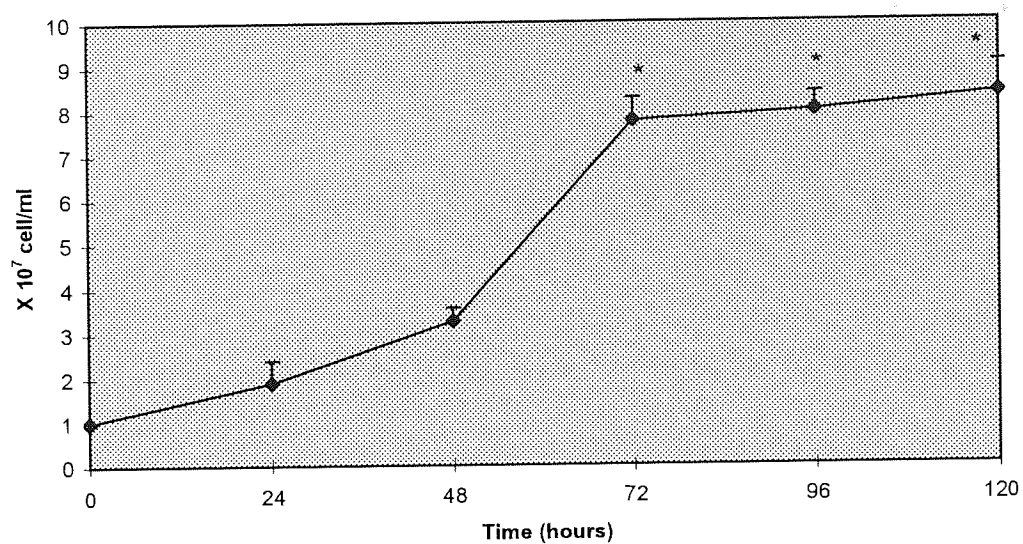


3.2c Day 3 (72h after inoculation)



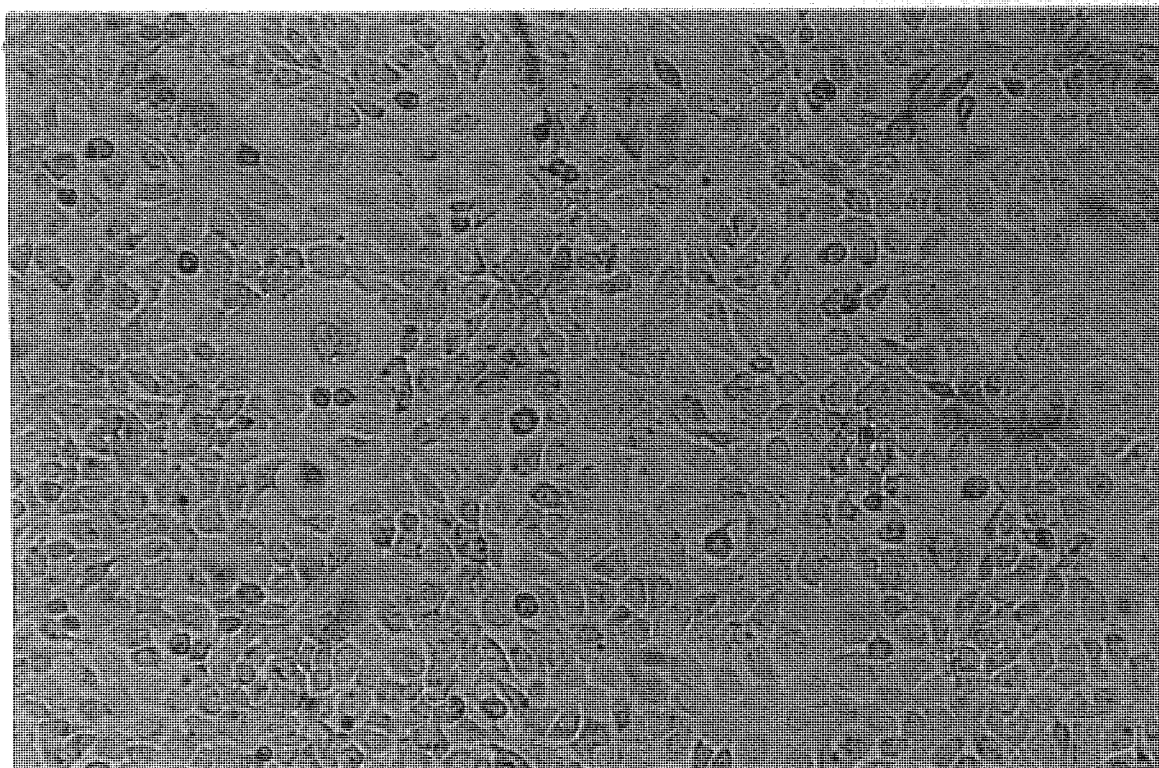
Once the cells reached confluence they were used directly as fibroblasts in the lipolysis experiments or were differentiated into adipocytes using the method described, and used for $^3\text{H}2\text{DG}$ uptake experiments. 3T3-L1 fibroblasts were differentiated as described in chapter 2 (section 2.4.3) and at each stage a photograph was taken for morphological analysis (see figure 3.3a, b, and c).

Figure 3.1 3T3-L1 cell growth curve. Cells were inoculated at 1×10^7 cells/ml. Growth was determined by counting cells using a haemocytometer. Results expressed as mean values \pm SEM (n= 3). * p<0.05 versus control.

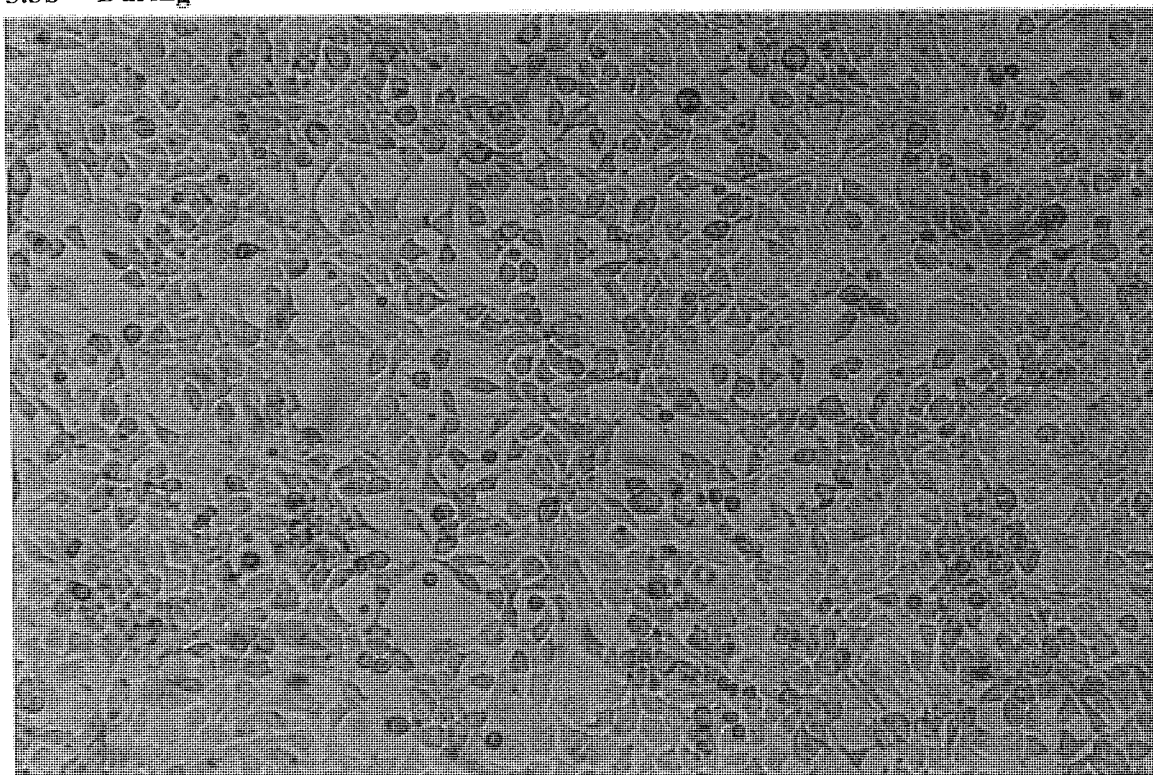


3.3 Differentiated 3T3-L1 cell growth

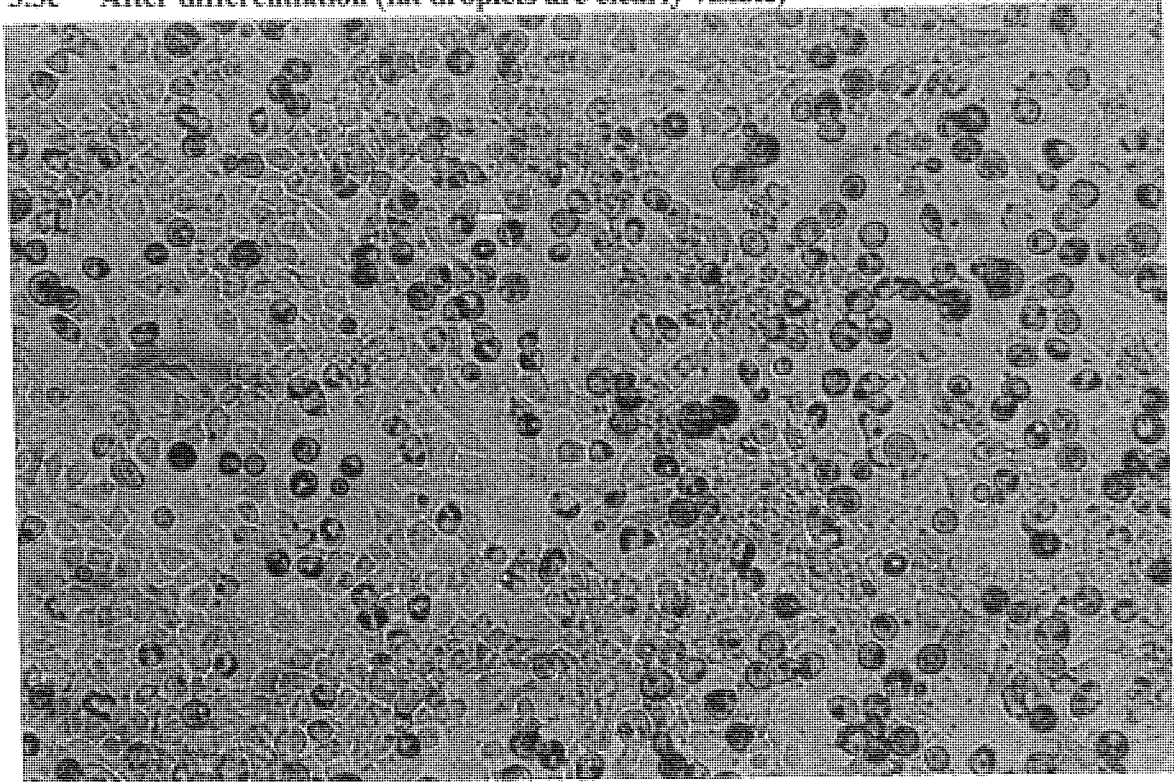
3.3a Before differentiation



3.3b During differentiation



3.3c After differentiation (fat droplets are clearly visible) (see Fig. 3.3a)



3.4 Preliminary studies on differentiated 3T3-L1 cells: Insulin-stimulated 2-deoxy-[³H]-glucose uptake

3.4.1 Introduction

Insulin stimulates glucose transport in adipose tissue by the translocation of intracellular GLUT4-containing vesicles and to a lesser extent GLUT1 from microsomes located throughout the cytoplasm to the cell surface. There is approximately a 2-fold increase in GLUT1 translocation to the membrane compared with a 15 to 20-fold increase in GLUT4 upon insulin stimulation (Tanner and Lienhard, 1987; Calderhead *et al.*, 1990). Hence, glucose uptake into the differentiated 3T3-L1 cells is via the insulin-responsive GLUT1 and GLUT4 transporters.

Differentiated 3T3-L1 cells have a considerable capacity to metabolise glucose, thus in order to measure glucose transport activity directly, it is necessary to use a non-metabolizable glucose analogue. 2-deoxyglucose (2DG) is a glucose analogue that is taken into the 3T3-L1 differentiated cells by glucose transporters in an identical manner to glucose. Glucose is phosphorylated by hexokinase to yield glucose 6-phosphate. The glucose-6-phosphate produced from glucose is isomerised to yield fructose 6-phosphate by phosphoglucosomerase. Because the 2DG molecule lacks the keto group on the C-2, this molecule cannot be metabolised further and hence an intracellular pool of 2-deoxyglucose phosphate can be detected within the cells. The 2DG molecule labelled with a ³H radionuclide allows accurate measurement of the glucose levels in the cells.

Under the conditions used in the present study involving incubation of 3T3-L1 adipocytes with 0.1mM 2-deoxy-D-glucose (0.0162mg/ml) and 7.4Kbq/ml 2-deoxy-D-[³H]-glucose (0.2μCi/ml) for 10min at 22°C, the cellular accumulation of phosphorylated ³H2DG has been shown to be approximately linear and highly reproducible, indicating that it is a representative measure of glucose uptake (Frost and Lane, 1985).

3.4.2 Method

3T3-L1 preadipocytes were grown in 100mm culture dishes until confluence was reached. Post confluence, the cells were differentiated as described previously and serum starved 2h prior to use. Bovine insulin was freshly prepared in PBS and filter sterilised. The cells were incubated with insulin (10^{-9} - 10^{-5} M) for 10min, 1h, 4h, 8h and 24h and its effect on glucose transport into the differentiated 3T3-L1 cells was measured by ³H2DG uptake during an additional period of 10min at the end of the main incubation. Experiments were performed in duplicate and undertaken on at least 3 occasions. All data were expressed as mean values ± standard error. Uptake of 2DG was expressed as % change compared with the control (100%). Significance was assessed by ANOVA (post Dunnett's test) and probability levels of $p < 0.05$ were considered to be significant.

3.4.3 Results

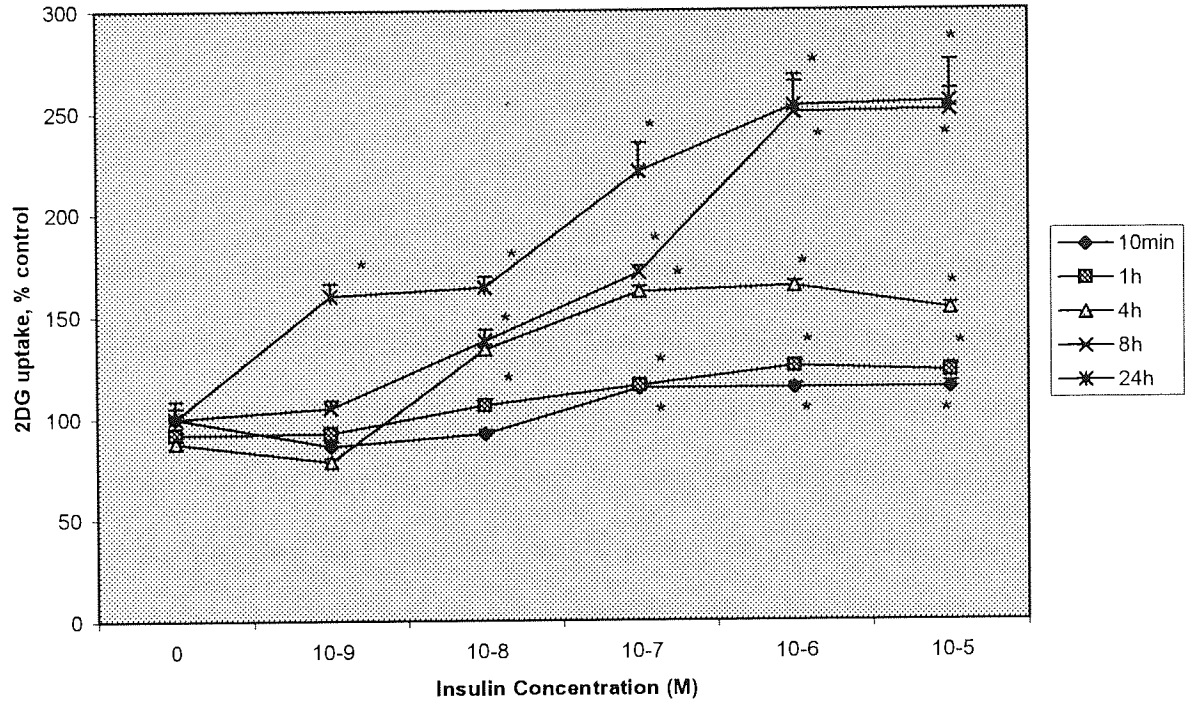
Insulin significantly increased glucose uptake into the differentiated 3T3-L1 cells in a concentration and time dependent manner (see figure 3.4). Maximal insulin (10^{-6} M)-stimulated glucose uptake was observed at 24h where there was an increase by 155% into the differentiated 3T3-L1 cells. Insulin (24h incubation) significantly increased glucose uptake into the cells at 10^{-9} - 10^{-5} M, by 60-155%, $p<0.05$.

A similar result was observed with the 8h incubation where insulin significantly increased 2DG into the cells at 10^{-8} - 10^{-5} M, by 38-151%, $p<0.05$. Maximal insulin-stimulated glucose uptake into the cells at 8h was similar to the 24h incubation, suggesting that a maximal response can be achieved by 8h insulin exposure.

2DG uptake was also significantly increased into the differentiated 3T3-L1 cells after a 4h incubation with insulin at 10^{-8} - 10^{-5} M, by 33-53%, $p<0.05$. However, the effect of insulin was less than with the longer incubation periods.

At 1h incubation, insulin (10^{-8} - 10^{-5} M) significantly increased glucose uptake into the 3T3-L1 cells by 15-31%, $p<0.05$. Uptake of 2DG by insulin was also observed at 10min, where significance was achieved at 10^{-7} - 10^{-5} M by an increase of 14%, $p<0.05$. Thus, the effect of insulin on 2DG uptake into the differentiated 3T3-L1 cells diminishes considerably at the 10min and 1h incubations.

Figure 3.4 Effect of insulin 10^{-9} - 10^{-5} M on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to insulin for 10min, 1h, 4h, 8h and 24h. Results expressed as mean values \pm SEM (n= 6). * $p < 0.05$ versus control.



3.5 Mechanism of Insulin Action in Differentiated 3T3-L1 Cells

3.5.1 Effect of LY 294 002, Wortmannin, Cytochalasin B and Cycloheximide on Basal and Insulin-Stimulated Glucose Uptake in 3T3-L1 differentiated cells

3.5.2 Introduction

In adipose tissue and skeletal muscle, insulin stimulates the uptake of glucose. Phosphatidylinositol 3-Kinase (PI3-Kinase) activation is necessary to generate the acute metabolic actions of insulin. PI3-Kinase is an enzyme that phosphorylates phosphatidylinositol bisphosphate (PI,4,5-P₂) to phosphatidylinositol trisphosphate (PI3,4,5-P₃). Activation of PI3-Kinase and the generation of PI3,4,5-P₃ results in signalling cascade that activates enzymes important in glycolysis, glucose uptake, protein synthesis and glycogen synthesis (Saltiel and Kahn, 2001), (see section 1.6).

Inhibitors of the PI3-Kinase include the microbial metabolite wortmannin (*Penicillium wortmanni*) and LY 294 002 also known as 2-(4-morpholinyl)-8-phenyl-[4H]-1-benzopyran-4-one. Wortmannin is a selective and irreversible inhibitor of PI3-Kinase and has been demonstrated to inhibit the insulin stimulation of 2DG uptake in rat adipocytes (Okada *et al.*, 1994). Moreover, in isolated rat and cultured 3T3-L1 adipocytes, wortmannin inhibited insulin-stimulated glucose transport without a significant effect on basal transport (Evans *et al.*, 1995; Lam *et al.*, 1994). Wortmannin specifically binds to the catalytic subunit of PI3-Kinase, p110 α to inhibit insulin-stimulated glucose transport (Wymann *et al.*, 1996).

LY 294 002 is a competitive, reversible inhibitor of PI3-Kinase and has been shown to abolish PI3-Kinase activity in human neutrophils, smooth muscle cells and L6 muscle cells (Bates, 1999; Vlahos *et al.*, 1994). In addition, LY 294 002 inhibited insulin-stimulated glucose uptake, amino acid uptake and protein synthesis in 3T3-L1 fibroblasts transfected with human insulin receptors (Sanchez-Margalet *et al.*, 1994). Cheatham *et al.* (1994) analysed the effects of LY 294 002 on insulin receptor signalling in 3T3-L1 cells. They demonstrated that LY 294 002 interfered with insulin-mediated translocation of GLUT4 and also the inhibition p70 S6 kinase. The findings with LY 294 002 and wortmannin indicate that insulin-stimulated PI3-Kinase activity is required for maximum insulin-stimulated glucose transport and GLUT4 translocation.

The insulin-stimulated translocation of glucose transporters (GLUT1 and GLUT4) to the cell surface, which mediates increased glucose uptake by fat cells has been confirmed using membrane fractions of adipocytes, which have shown that insulin increases the number of plasma membrane glucose transporters 2-3 fold (James *et al.*, 1988). In addition, 3T3-L1 cells that have undergone adipocyte differentiation exhibit a 20-fold increase in the number of insulin receptors and glucose transporters (Reed *et al.*, 1981). In contrast, insulin causes only a 5-10 fold increase in plasma membrane cytochalasin B binding sites (James *et al.*, 1988). Cytochalasin B is a fungal alkaloid, which acts as an inhibitor of glucose transporters. Glucose and cytochalasin B compete for binding on the transporter. If cytochalasin B attaches, glucose uptake into the cell is inhibited. The inhibitor also acts on microfilaments and interferes with the assembly of actin filaments by capping them at one ends (Bates, 1999).

Cycloheximide (antibiotic derived from *Streptomyces griseus*) inhibits cytosolic protein synthesis but does not inhibit organelle protein synthesis in eukaryotic cells. It blocks the translation of messenger RNA by inhibiting the peptidyl transferase activity of the 60s ribosomal subunit. Cycloheximide has been widely used in studies to determine the specific action of pharmacological agents (Klip *et al.*, 1992), and it has been demonstrated that cycloheximide inhibits the stimulation of glucose uptake (Walker *et al.*, 1990). Hence, cycloheximide can be used to block protein synthesis to determine glucose transporter action.

These inhibitors were used in the present study to determine the role of PI3-Kinase, GLUT transporters and protein synthesis on basal and insulin-stimulated glucose uptake into differentiated 3T3-L1 cells. The inhibitors were assessed for toxicity using a cell viability test (see section 3.5.3).

3.5.3 Method

LY 294 002, wortmannin, cytochalasin B and cycloheximide were dissolved in 10mM dimethyl sulphoxide (DMSO) and prepared as 10^{-2} M stock solutions. These compounds were all light sensitive and were prepared in the dark. Prior to use the LY 294 002, wortmannin and cycloheximide were diluted in sterile PBS to give final working concentrations of 10^{-6} - 10^{-3} M. Cytochalasin B was diluted in PBS to give 10^{-7} - 10^{-4} M.

The 3T3-L1 cells were grown in 100mm culture dishes (see 2.4.1) and when confluent they were differentiated into adipocytes (see 2.4.3). These cells were serum-starved 2h prior to incubation with the inhibitors. LY 294 002, wortmannin and cycloheximide were added to the incubation medium to give a concentration of 10^{-8} - 10^{-5} M. In addition, cytochalasin B was added to the cells to give a final concentration of 10^{-9} - 10^{-6} M. To determine the effect of these inhibitors on insulin action (see figure 3.4), 3T3-L1 differentiated cells were incubated with the LY 294 002 (10^{-8} - 10^{-5} M), wortmannin (10^{-8} - 10^{-5} M), cycloheximide (10^{-8} - 10^{-5} M) and cytochalsin B (10^{-9} - 10^{-6} M) \pm insulin 10^{-8} - 10^{-6} M. Insulin (bovine) was prepared as described in 3.4.2.

All incubations on the cells were for 24h and performed in duplicate on at least 3 separate occasions. At the end of the incubation period, the effect of these compounds on basal and insulin-stimulated glucose transport was measured as described above in section 3.4.2. If required a cell viability test was used to measure toxicity of the inhibitors on the 3T3-L1 differentiated cells. Here, the cells were incubated with trypan blue diluted in PBS (1:1) for approximately 10min. Live (viable) cells do not take up the trypan blue, whereas dead (non-viable) cells do. Therefore, by counting the viable and non-viable cells, the % cell viability could be determined (see section 2.6). All data were expressed as mean values \pm standard error. Uptake of 2DG was expressed as % change compared with the control (100%). Significance was assessed by ANOVA (post Dunnett's test) and probability levels of $p < 0.05$ were considered to be significant.

3.5.4 Results

Insulin significantly increased 2DG uptake into the differentiated 3T3-L1 cells in a concentration dependent manner as previously described in 3.4.3. Incubations with LY 294 002 significantly abolished insulin-stimulated glucose uptake into the 3T3-L1 cells at all concentrations used (see figure 3.5a). Insulin (10^{-8} - 10^{-6} M)-stimulated 2DG uptake was inhibited by the LY 294 002 compound (10^{-8} - 10^{-5} M) by 35-55%, $p < 0.05$. However, basal glucose uptake into the 3T3-L1 differentiated cells was not reduced with the specific PI3-Kinase inhibitor. A cell viability test was performed to determine if the potent effects observed by LY 294 002 were due to a decrease in cell number (caused by cell death). The cells incubated with LY 294 002 (10^{-5} M) were no different morphologically to the control cells (incubated with PBS). Moreover, there were no differences observed in cell number.

Similarly, wortmannin completely inhibited the insulin-stimulated 2DG uptake into the differentiated 3T3-L1 cells (see figure 3.5b). Wortmannin (10^{-8} - 10^{-5} M) significantly inhibited insulin (10^{-8} - 10^{-6} M) action by about 40-50%. Wortmannin (10^{-5} M only) reduced basal glucose uptake into the 3T3-L1 differentiated cells by approximately 25%, $p < 0.05$. As before, a cell viability test was performed to determine any differences between the control cells vs wortmannin treated cells. Wortmannin (10^{-5} M only) was clearly toxic on the 3T3-L1 differentiated cells (see figure 3.6a and 3.6b). There was a decrease in cell number (visualised by 'patchy' regions) and an increase in non-viable cells, determined by the trypan blue method. However, the other concentrations of wortmannin tested (10^{-8} - 10^{-6} M) did not affect the number or morphology of the differentiated 3T3-L1 cells.

Both cytochalasin B and cycloheximide significantly reduced insulin-stimulated glucose uptake in a concentration dependent manner (see figures 3.5c and 3.5d). Cytochalasin B (10^{-8} - 10^{-6} M) significantly reduced the insulin (10^{-6} M)-stimulated glucose uptake in the 3T3-L1 differentiated cells by 29-57%. Similarly, cycloheximide (10^{-7} - 10^{-5} M) inhibited maximal insulin (10^{-6} M)-stimulated 2DG uptake in the same cells by approximately 15-78%, $p < 0.05$. Both cytochalasin B and cycloheximide (10^{-5} M only), significantly decreased basal 2DG uptake into the differentiated 3T3-L1 cells by 16% and 26%, respectively. The differentiated 3T3-L1 cells were incubated with cytochalasin B (10^{-6} M) and cycloheximide (10^{-5} M) for approximately 24h and a cell viability test was performed at the end of the incubation period. There were no differences in cell number and morphology of the treated cells versus the control cells (incubated with PBS).

Figure 3.5a Effect of insulin (10^{-8} - 10^{-6} M) \pm LY 294 002 (10^{-8} - 10^{-5} M) on 2DG uptake in differentiated 3T3-L1 cells. **Figure 3.5b** Effect of insulin (10^{-8} - 10^{-6} M) \pm wortmannin (10^{-8} - 10^{-5} M) on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to insulin \pm LY 294 002 and wortmannin for 24h. Results expressed as mean values \pm SEM (n= 6). LY 294 002 and wortmannin significantly inhibited insulin action at all concentrations.

Figure 3.5a

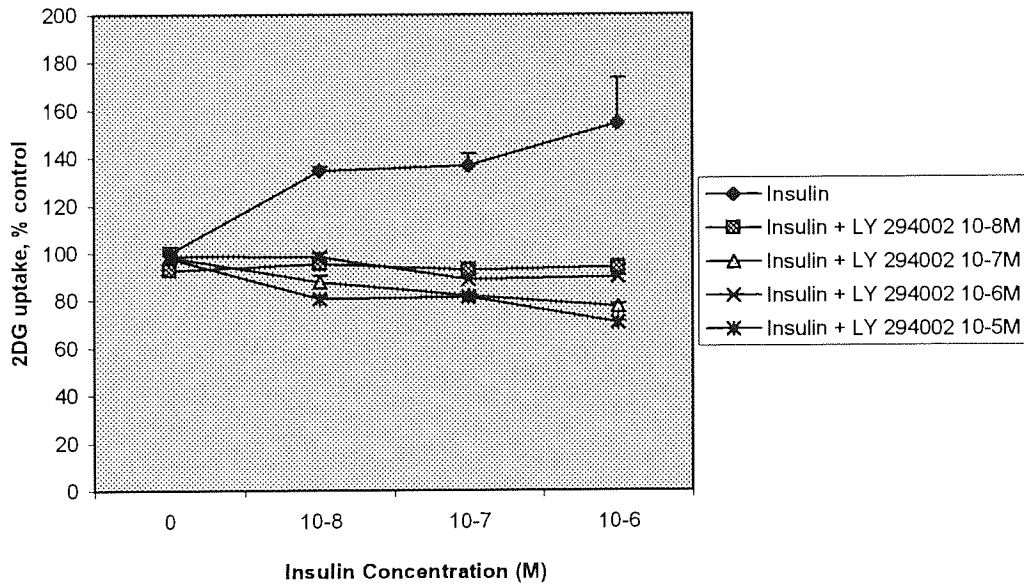


Figure 3.5b

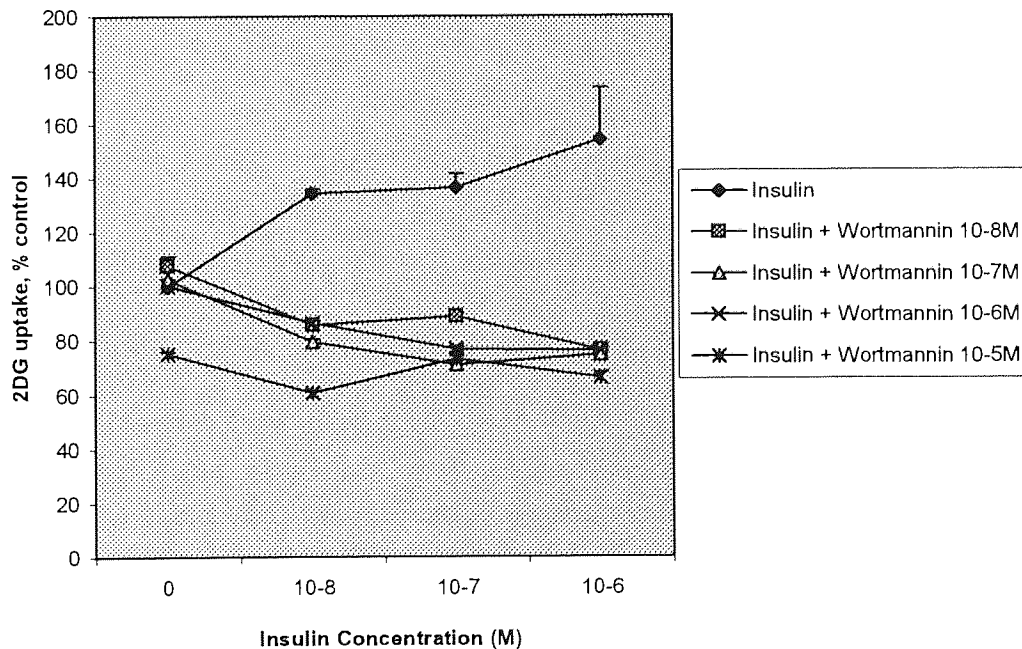


Figure 3.5c Effect of insulin (10^{-8} - 10^{-6} M) \pm cytochalasin B (10^{-9} - 10^{-6} M) on 2DG uptake in differentiated 3T3-L1 cells. **Figure 3.5d** Effect of insulin (10^{-8} - 10^{-6} M) \pm cycloheximide (10^{-8} - 10^{-5} M) on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to insulin \pm cytochalasin B and cycloheximide for 24h. Results expressed as mean values \pm SEM (n= 6). + p<0.05 versus insulin alone

Figure 3.5c

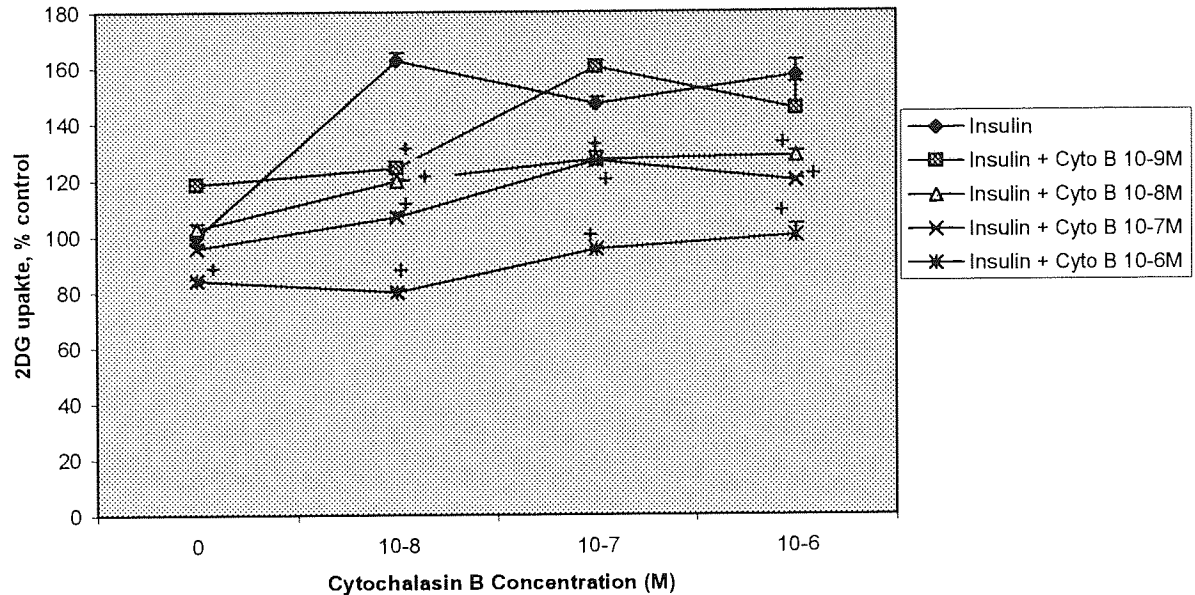
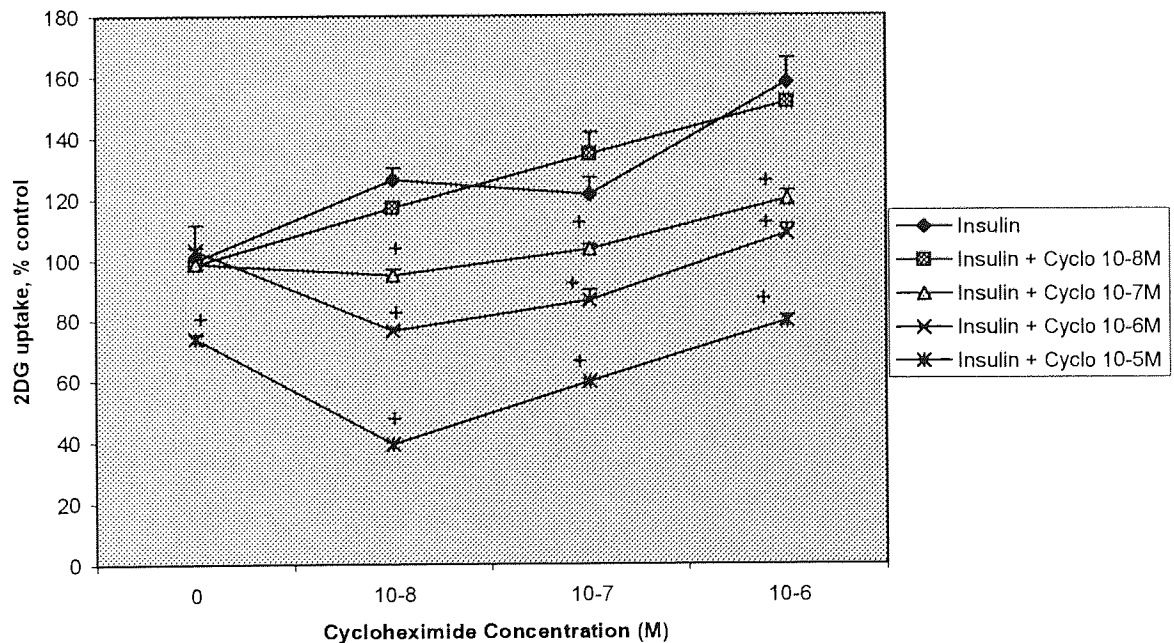
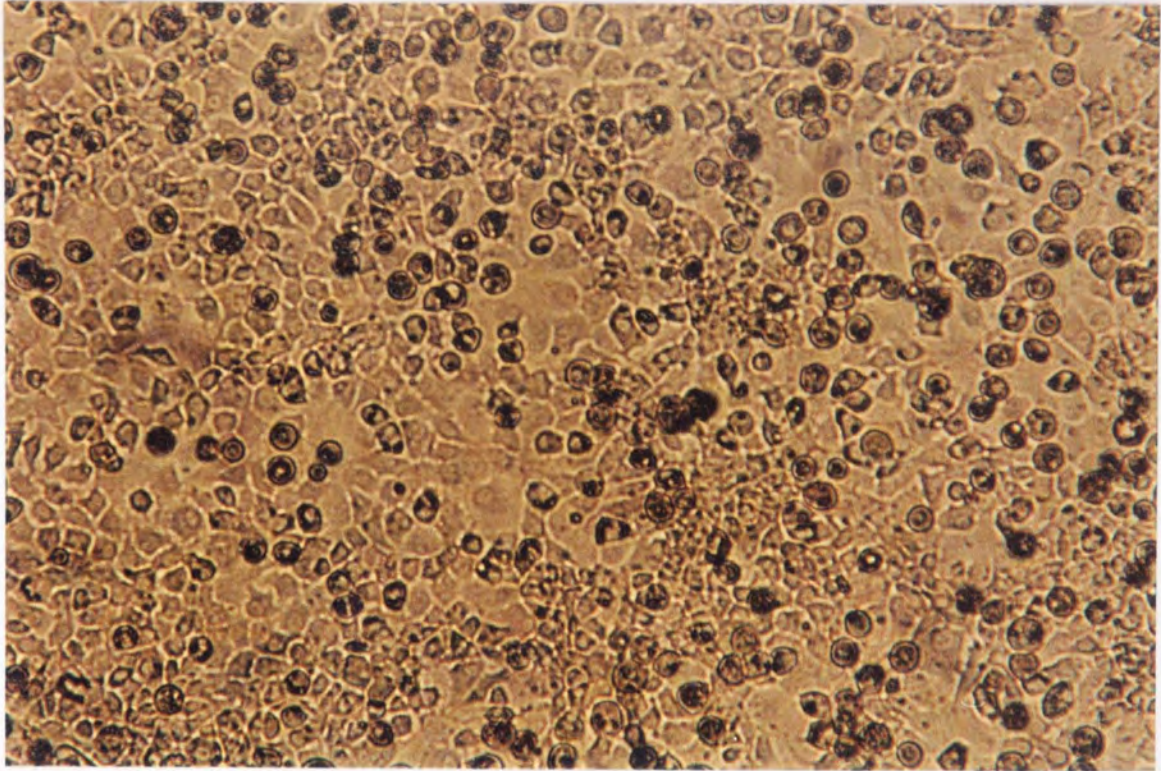


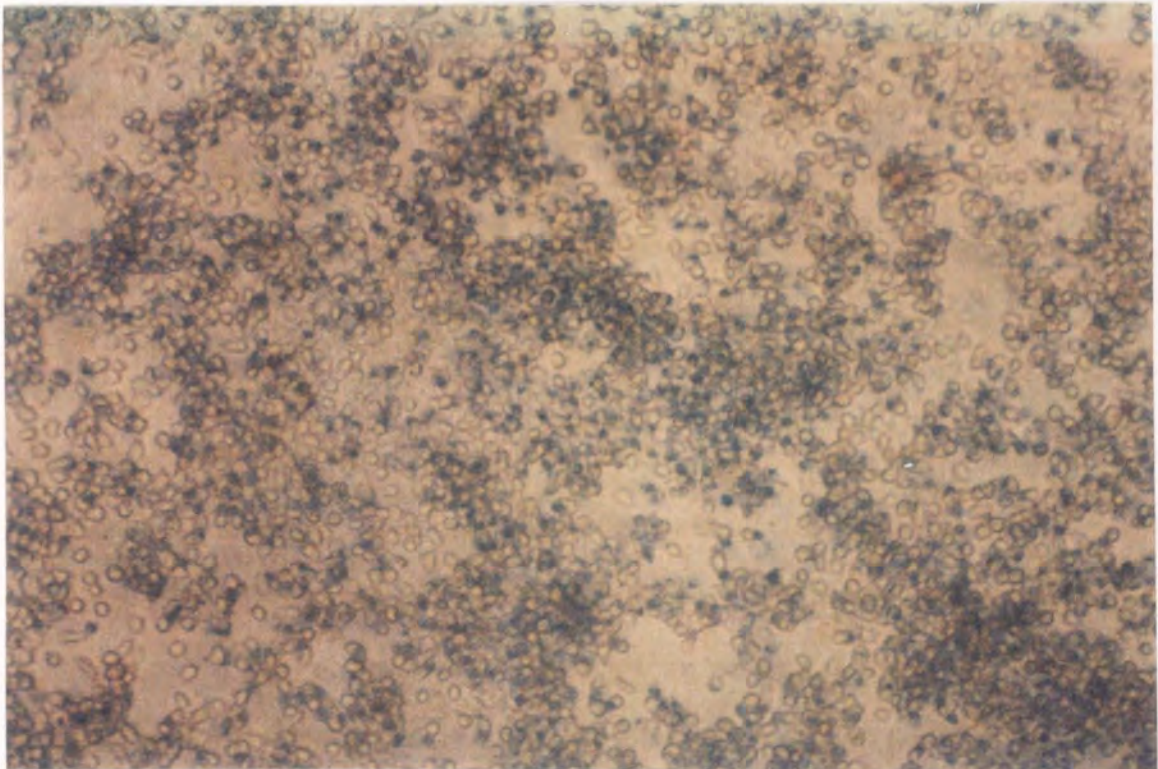
Figure 3.5d



3.6a Control cells



3.6b Differentiated 3T3-L1 cells treated with wortmannin (10^{-5} M) for 24h



3.5.5 Discussion

LY 294 002 and wortmannin proved to be potent inhibitors of the insulin-stimulated 2DG uptake in differentiated 3T3-L1 cells. Both PI3-Kinase inhibitors abolished insulin-stimulated glucose uptake into these cells at 10nM concentrations. This suggests that the majority of insulin signalling to glucose transport requires PI3-Kinase. Basal glucose transport was unaffected by wortmannin and LY 294 002 at concentrations up to 10nM. Thus it is unlikely that the inhibition of insulin-stimulated glucose uptake can be attributed to a cytotoxic effect of these inhibitors.

These results are consistent with previous studies that have demonstrated an essential role of PI3-Kinase in insulin receptor signalling (Evans *et al.*, 1995; Smith *et al.*, 1995; Okada *et al.*, 1994; Rahn *et al.*, 1994; Sanchez-Margalet *et al.*, 1994). Cheatham *et al.* (1994) and Sanchez-Margalet *et al.* (1994) have published the effects of LY 294 002 on basal and insulin-stimulated 2DG uptake into differentiated 3T3-L1 cells. Both groups found that LY 294 002 interfered with the insulin-stimulated uptake of glucose and completely inhibited p70 S6 kinase activation, indicating that this serine/threonine kinase lies downstream to PI3-Kinase.

Evans *et al.* (1995) have demonstrated that in differentiated 3T3-L1 cells wortmannin inhibited insulin-stimulated, but was without effect on basal 2DG transport. The same study reported that the effects of insulin on GLUT4 redistribution were completely abolished when the adipocytes were exposed to wortmannin prior to insulin stimulation. This inhibitory effect on transport was associated with a reduction in cell surface transporter, but not the total cellular transporters, of both the GLUT1 and

GLUT4 isoforms. Taken together, these studies indicate that PI3-Kinase plays a major role in generating the acute metabolic actions of insulin observed in the 3T3-L1 differentiated cell line.

Uptake of 2DG was dose dependently inhibited by cytochalasin B in the 3T3-L1 differentiated cells. Inhibition of glucose uptake by 40% was achieved with a 10nM cytochalasin B concentration. This result suggests that insulin stimulates glucose transport in adipose tissue by increasing the number of glucose transporters (GLUT1 and GLUT4) translocated to the cell surface. It has been demonstrated elsewhere that exposure of cells to a maximally stimulatory concentration of insulin results in an approximately 6-fold increase in the concentration of glucose transporters in the cell membrane (Simpson and Cushman, 1986). Suzuki and Kano (1980) have demonstrated that insulin facilitates translocation of these transporters from an intracellular storage site to the cell surface membrane. Further evidence for a unique insulin regulatable glucose transporter in adipose tissue was demonstrated by using the selective inhibitor cytochalasin B (James *et al.*, 1988). In another study, the uptake of 2DG into L6 skeletal muscle cells was non-competitively inhibited by cytochalasin B, and the identification of the glucose transporters involved in that 2DG uptake study was achieved using the high affinity (³H) cytochalasin B (Klip *et al.*, 1983 and 1982). Hence, these results demonstrate that activation of glucose transporter translocation to the cell membrane in the differentiated 3T3-L1 cells requires insulin action.

Cycloheximide, the inhibitor of protein synthesis reduced insulin-stimulated 2DG uptake in a dose dependent manner. A 50% inhibition of the translation of glucose

transporters was achieved by 10nM cycloheximide. Basal 2DG uptake was inhibited at concentrations above 10 μ M, however this inhibition on glucose transport was not attributed to cytotoxic effects. The results suggest that glucose transporters and the translocation of these cell membrane proteins are essential for basal (high concentrations only) and insulin-stimulated glucose transport into the differentiated 3T3-L1 cells.

In summary, insulin-stimulated 2DG uptake in differentiated 3T3-L1 cells requires the activation of PI3-Kinase. Moreover, insulin stimulation via PI3-Kinase is required for the activation of GLUT1 and GLUT4 transcription and translation in these cells.

The differentiated 3T3-L1 cell line is an alternative model to the isolated adipocytes extracted from lean and obese mice (chapter 5), particularly for studies of insulin signalling and glucose uptake. 3T3-L1 differentiated cells are particularly responsive to insulin (Frost and Lane, 1985) and maintain cell viability for extended periods of time. Thus, these cells have been used in the following chapters for the screening and mechanistic evaluation of anti-diabetic and anti-obesity drugs.

3.6 Preliminary studies on 3T3-L1 preadipocytes: Noradrenaline-stimulated lipolysis

3.6.1 Introduction

Stimulators of lipolysis include catecholamines, thyroid stimulating hormones, glucagon and parathyroid hormone (Arner, 1996). Binding of catecholamines to the β adrenoceptors on the adipocyte cell membrane activates adenylate cyclase via a stimulatory G protein (Gs), which results in the generation of the cAMP (see chapter 5). Hormone sensitive lipase (HSL) and less importantly, monoacylglyceride lipase are the main enzymes required for the hydrolysis pathway. HSL is rate limiting and is activated by protein kinase A, an enzyme which is activated by cAMP. During lipolysis, triacylglycerides (TAG) are broken down to yield diacylglycerides (DAG) and monoacylglycerides (MAG), to produce FFA and glycerol. Glycerol is water-soluble and can be used in *in vitro* assays to measure the lipolytic effect of compounds. The fate of FFA includes re-esterification and binding to circulating albumin. Glycerol release by the cells is proportional to the hydrolysis of TAG, hence this is the chosen parameter used to measure the rate of lipolysis.

The 3T3-L1 cell line is well established and has been shown to possess the three functional β -adrenoceptors (Shimizu *et al.*, 1996). This cell line is suitable for lipolysis experiments because it acquires morphologic and metabolic characteristics of adipocytes (Green and Kehinde, 1974).

3.6.2 Method

The 3T3-L1 preadipocytes were seeded (10^6 cells/ml) in 24 well plates until confluence was reached (see section 2.4.1). The cells were used at higher passages (>15) because they displayed morphological features of adipocytes and therefore did not need to be differentiated further. Post confluence, the cells were serum-starved 2h prior to incubation with noradrenaline. Noradrenaline was freshly prepared in PBS and filter sterilised. The cells were incubated with noradrenaline (10^{-10} - 10^{-6} M) for 2h, 24h and 48h at 37°C in an atmosphere of 5% CO₂ and 95% air. The concentration of glycerol released into the culture medium, which is indicative of triacylglyceride breakdown, was measured enzymatically (see section 2.3.4). Experiments were performed in duplicate and undertaken on at least 3 occasions. All data were expressed as mean values \pm standard error. Significance was assessed by ANOVA (post Dunnett's test) and probability levels of $p < 0.05$ were considered to be significant.

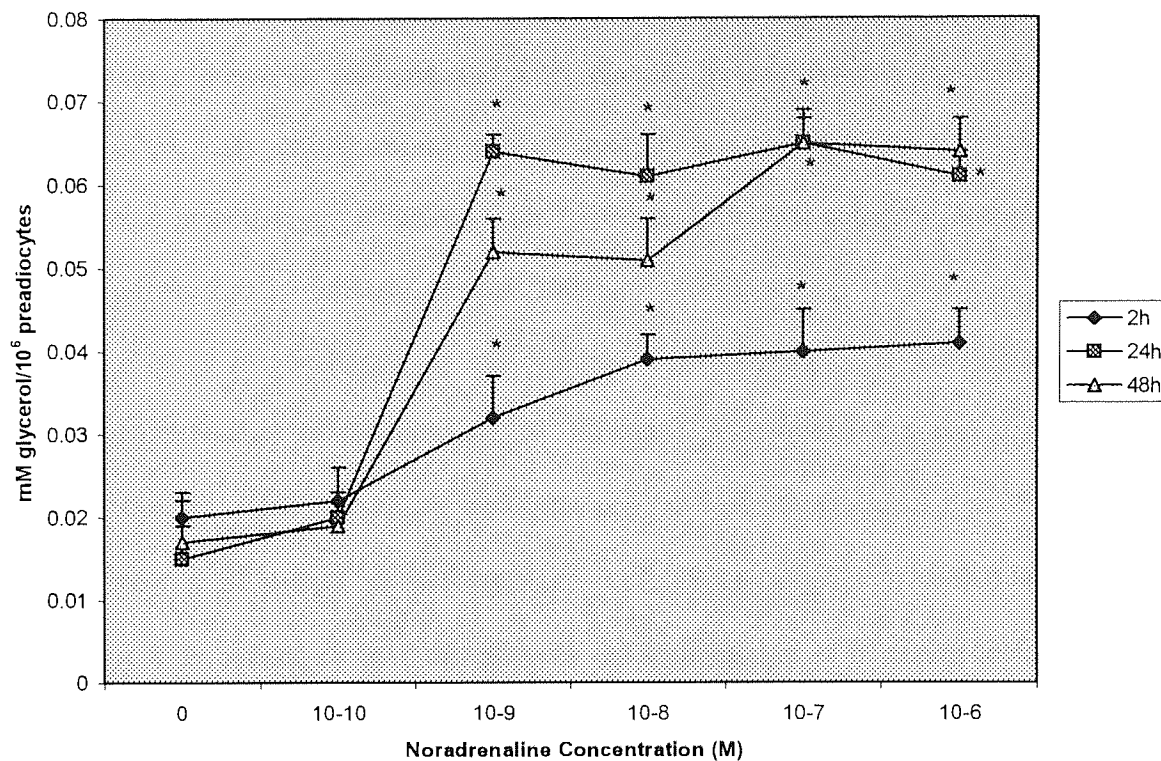
3.6.3 Results

Noradrenaline significantly stimulated lipolysis by the 3T3-L1 preadipocytes in a concentration and time dependent manner (see figure 3.7). Maximal noradrenaline (10^{-6} M)-stimulated lipolysis was observed at 24h. Noradrenaline (24h incubation) increased glycerol release by the preadipocytes at 10^{-9} - 10^{-6} M, by 200-250%, $p < 0.05$.

A similar result was observed with the 48h incubation where noradrenaline (10^{-9} - 10^{-6} M) significantly increased lipolysis of preadipocytes by 200-250%. Maximal noradrenaline-stimulated lipolysis by these cells at 48h was similar to the 24h incubation, suggesting that a maximal response can be achieved after 24h noradrenaline exposure.

In addition, noradrenaline (10^{-9} - 10^{-6} M) simulated lipolysis in the 3T3-L1 preadipocytes at the 2h incubation. Glycerol release by the preadipocytes was increased by about 50-100%, $p < 0.05$. The effect of noradrenaline on adipocyte lipolysis had diminished by approximately 100% at the 2h incubation (0.061 ± 0.003 noradrenaline (10^{-6} M) on the preadipocytes for 24h vs 0.041 ± 0.004 noradrenaline (10^{-6} M) on the preadipocytes for 2h, $p < 0.05$). Results are expressed as mM glycerol/ 10^6 preadipocytes).

Figure 3.7 Lipolytic activity of noradrenaline on 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were exposed to noradrenaline (10^{-10} - 10^{-6} M) for 2h, 24h and 48h at 37°C. Results expressed as mM glycerol/ 10^6 preadipocytes. Mean values \pm SEM (n=6). *p<0.05 versus control.



3.6.4 Discussion

Noradrenaline increased lipolysis in the 3T3-L1 preadipocytes at all incubation times. The effects of noradrenaline are near maximal at 24h incubation, which suggests that noradrenaline exerts its full potential after chronic incubation with the 3T3-L1 preadipocytes. The catecholamine-induced lipolysis is consistent with a previous study where preadipocytes incubated with 2.5 μ M isoproterenol enhanced adenylate cyclase activity 2 to 2.5-fold (Rubin *et al.*, 1977).

Noradrenaline is unselective and affects β 1, β 2 and β 3 adrenoceptors, in addition to α 2 adrenoceptors. The 3T3-L1 preadipocytes possess a significant population of β 1 adrenoceptor vs β 2 and β 3 adrenoceptor (Lai *et al.*, 1981). Preadipocytes that have undergone differentiation induce the synthesis and accumulation of β 2 adrenoceptors, which replace the β 1 adrenoceptors (Lai *et al.*, 1982). 3T3-L1 preadipocytes are most suitable for comparison with similar studies performed in isolated lean and obese mouse adipocytes, since these cells exhibit a higher number of β 1 and β 3 adrenoceptors vs β 2 adrenoceptors (see chapter 5).

These preliminary results confirm that adrenoceptors under catecholamine (noradrenaline) stimulation accelerate the lipolysis rate. These 3T3-L1 preadipocytes are responsive to catecholamines and hence, were used in the forthcoming studies as a screening tool for putative anti-obesity agents.

Chapter 4: Characterisation of the rat skeletal L6 cell line

4.1 Introduction

Skeletal muscle is the major tissue responsible for glucose disposal from the circulation (DeFronzo, 1988). It is also a major site of insulin resistance in type 2 diabetes and therefore an important tissue to study with respect to glucose uptake. Yaffe (1968) originally derived myoblasts of the L6 cell line from rat skeletal thigh muscle. Experiments showed that, under appropriate culture conditions, it is possible to maintain myogenic cell lines for many months. These spindle shaped myoblasts, which are the mononuclear precursor cells of muscle fibres, retained their capacity to fuse and differentiate into post-mitotic multinucleated muscle fibres.

L6 cell culture systems offer unique advantages over intact tissue preparations for the study of insulin signalling, because they grow in monolayers and maintain cell viability for extended periods of time. Freeze storing (-80°C) makes the use of this L6 cell system convenient for investigations. The L6 cell line has been used extensively, since these cells have been shown to possess several characteristics of skeletal muscle *in vivo* (Klip *et al.*, 1982).

Preliminary studies are included herein to characterise the present batch of cultured L6 skeletal muscle cells as a model for studying the basal and insulin-stimulated glucose uptake of putative anti-diabetic and anti-obesity agents. Previous studies in this laboratory have characterised other batches of these cells (Eason, 2002; Bates

1999) and there is a consistency of the growth morphology and metabolic function of the different batches.

4.2 L6 cell growth

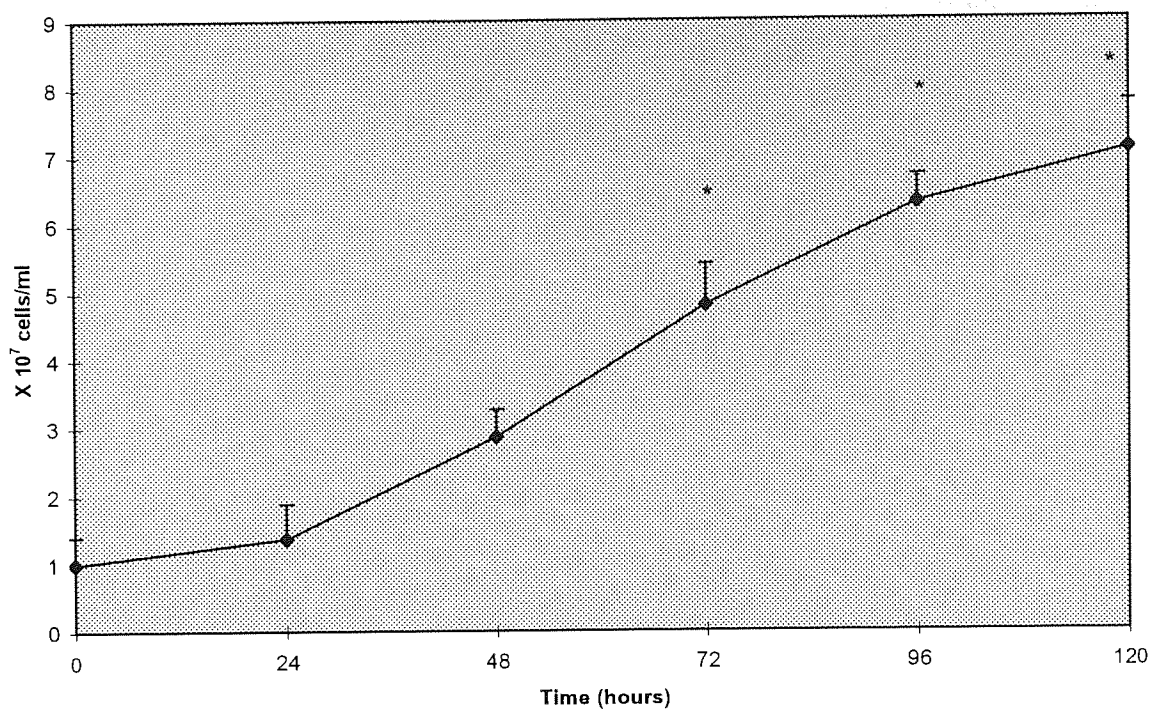
L6 cells were grown as described (see section 2.5.1), and their growth rate was monitored daily. Cell growth rate was determined over a 5-day period. The L6 muscle cells were seeded at 1×10^7 cells per ml and grown in 75cm^3 flasks containing 20ml 5% FCS/DMEM. Cells were seeded at this initial density for ease of accurate counting of growth of these cells. Each day the number of cells in each flask was calculated using a haemocytometer. Trypsin (5ml) was added to the flask to remove the cells and centrifuged at 800rpm. The cells were then resuspended in fresh medium (5ml) and the number of cells counted. Dilution factors were accounted for and the data were expressed as number of cell per ml. The cell counts were carried out at noon each day and photographs were taken daily.

4.2.1 L6 cell growth results

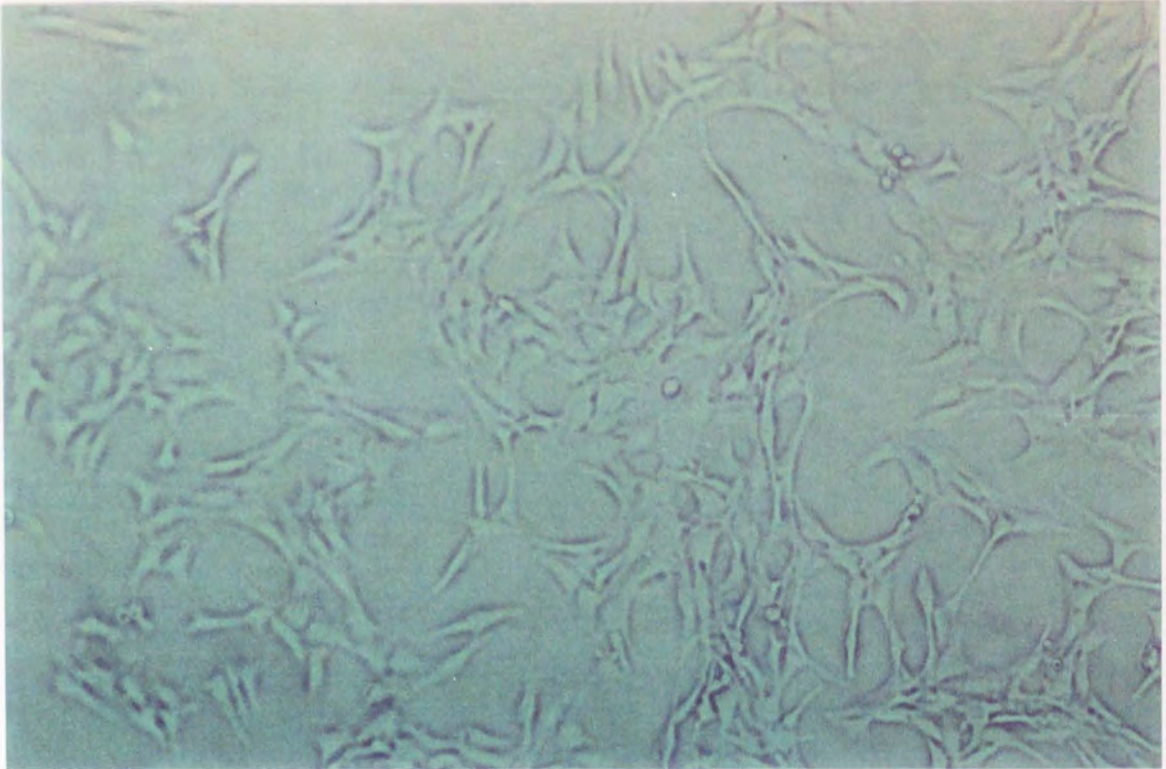
The L6 cells grow slowly for the first 48h (see figure 4.1). However, at 72h post seeding there is a substantial increase in the number of L6 muscle cells (3.27×10^7 to 8.01×10^7 cells/ml, $p < 0.05$, 48h versus 72h). The number of cells at 96h and 120h does not increase significantly further, thus confluence is reached at day 4 of seeding.

Photographs of L6 muscle cells were taken 24h, 48h and 72h after initial inoculation of 1×10^7 cells per ml (see figure 4.2a, b, and c).

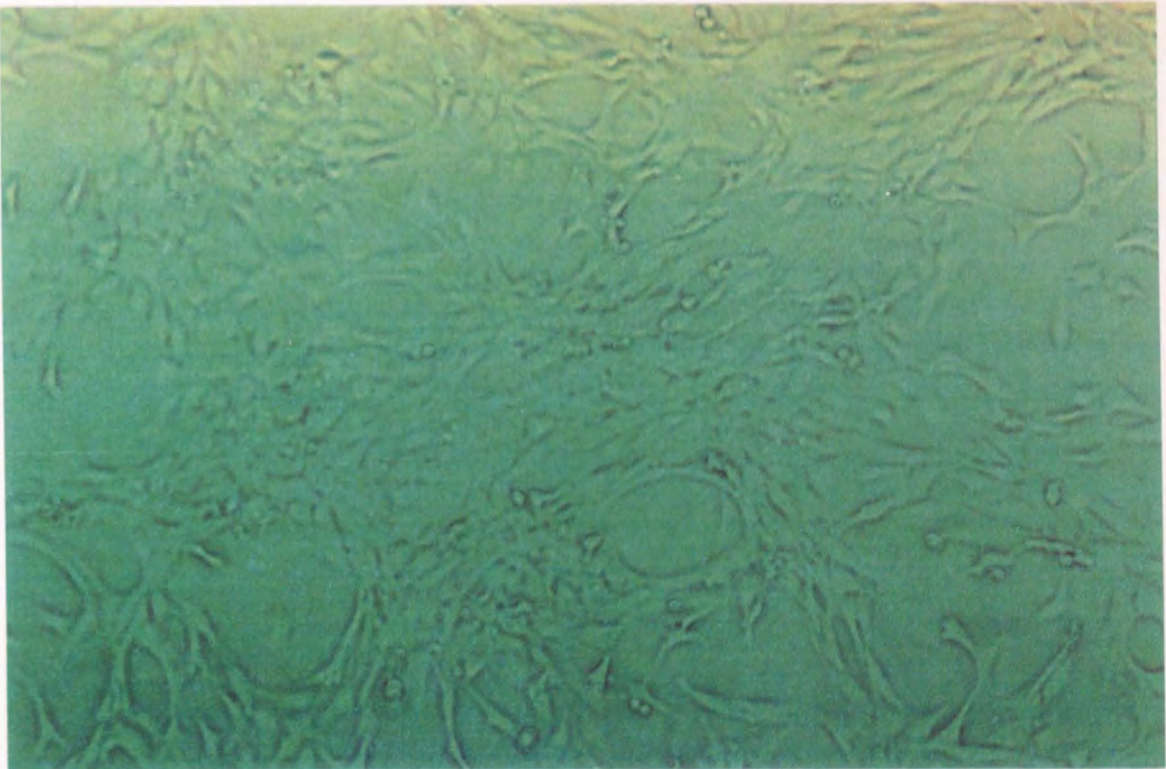
Figure 4.1 L6 muscle cell growth curve. Cells were inoculated at 1×10^7 cells/ml. Growth was determined by counting cells using a haemocytometer. Results expressed as mean values \pm SEM (n= 3). * $p < 0.05$ versus control.



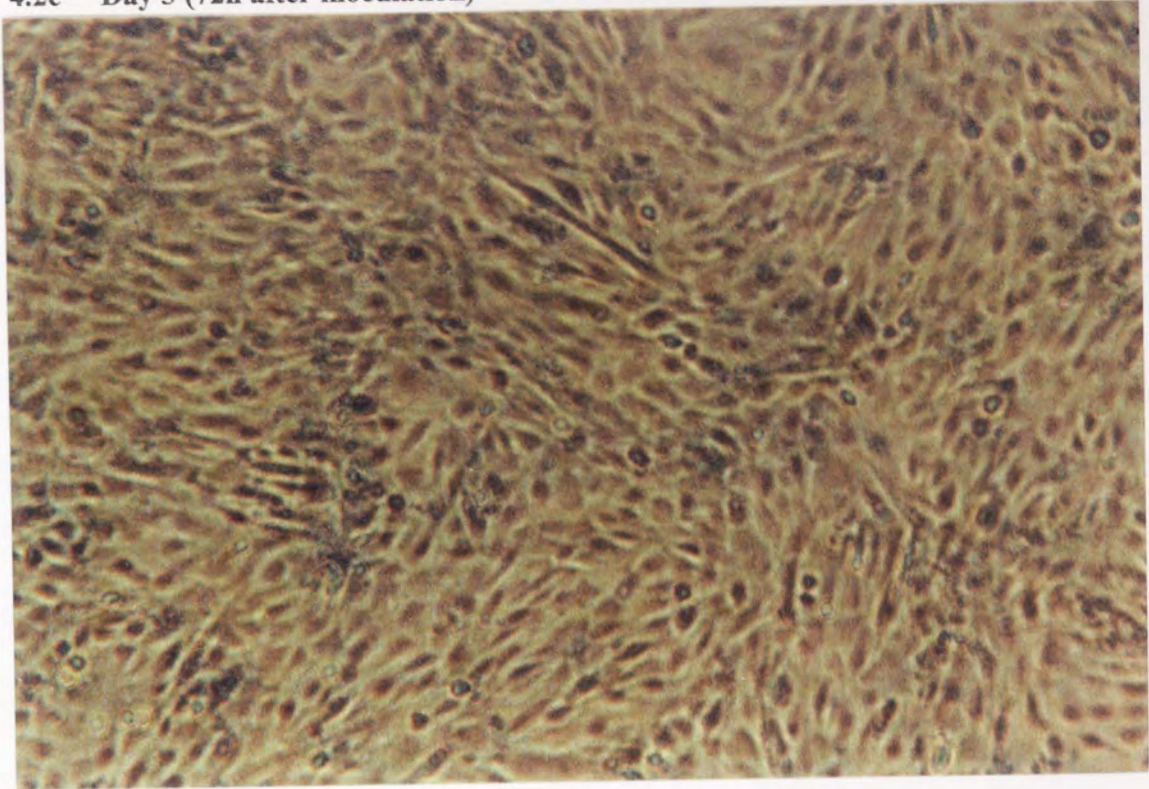
4.2a Day 1 (24h after inoculation)



4.2b Day 2 (48h after inoculation)



4.2c Day 3 (72h after inoculation)



4.3 Preliminary studies on L6 skeletal muscle cells: Insulin-stimulated 2-deoxy-[³H]-glucose uptake

4.3.1 Introduction

Glucose transporters are responsible for the movement of glucose across the cell surface membrane. Insulin has been demonstrated to acutely regulate glucose transport activity in responsive L6 muscle cells via the recruitment of preformed glucose transporters to the cell membrane. Chronic exposure to insulin *in vivo* or *in vitro* has been found to increase glucose transport activity in skeletal muscle cells (Young *et al.*, 1986). GLUT4 protein and mRNA are uniquely expressed in skeletal and adipose tissues, which suggest that this transporter is the major insulin responsive form in these tissues (Birbaum, 1989). GLUT1 mRNA is also found in skeletal muscle (Walker *et al.*, 1990). Hence, glucose uptake into L6 skeletal muscle cells is via the insulin-responsive GLUT1 and GLUT4 transporters.

In skeletal muscle, uptake of glucose across the cell membrane is the rate-limiting step to its utilisation. In order to measure basal and insulin-stimulated glucose transport activity directly in the L6 muscle cells, it is necessary to use a non-metabolizable glucose analogue. Two glucose analogues widely used to study the kinetics of glucose transport include, 2-deoxyglucose (2DG) and 3-*O*-methyl glucose (3OMG). These analogues are taken into the L6 muscle cells by glucose transporters in an identical manner to glucose, but are not metabolised further (see section 3.4.1). 2DG is phosphorylated, therefore there may be some dependence of hexokinase

activity. 3OMG is not phosphorylated but uptake can only be measured over very short periods for linear uptake.

4.3.2 Method

L6 skeletal muscle cells were grown in 24 well (16mm) plates at 1ml/well until confluence was reached. The cells were maintained in an atmosphere of 5% CO₂ 95% O₂ at 37°C. Once 90% confluence was reached the L6 muscle cells were serum starved for 24h to induce differentiation and fusion of myoblasts into myotubes. (see section 2.5.1). These cells were then incubated with insulin prior to use for the ³H2DG uptake experiments (see section 2.5.3). Bovine insulin was freshly prepared in PBS and filter sterilised. The cells were incubated with insulin (10⁻¹⁰-10⁻⁶M) for 1h, 8h and 24h and its effect on glucose transport into the L6 muscle cells were measured. Experiments were performed in duplicate and undertaken on at least 3 occasions. All data were expressed as mean values ± standard error. Uptake of 2DG was expressed as % change compared with the control (100%). Significance was assessed by ANOVA (post Dunnett's test) and probability levels of p<0.05 were considered to be significant.

4.3.3 Results

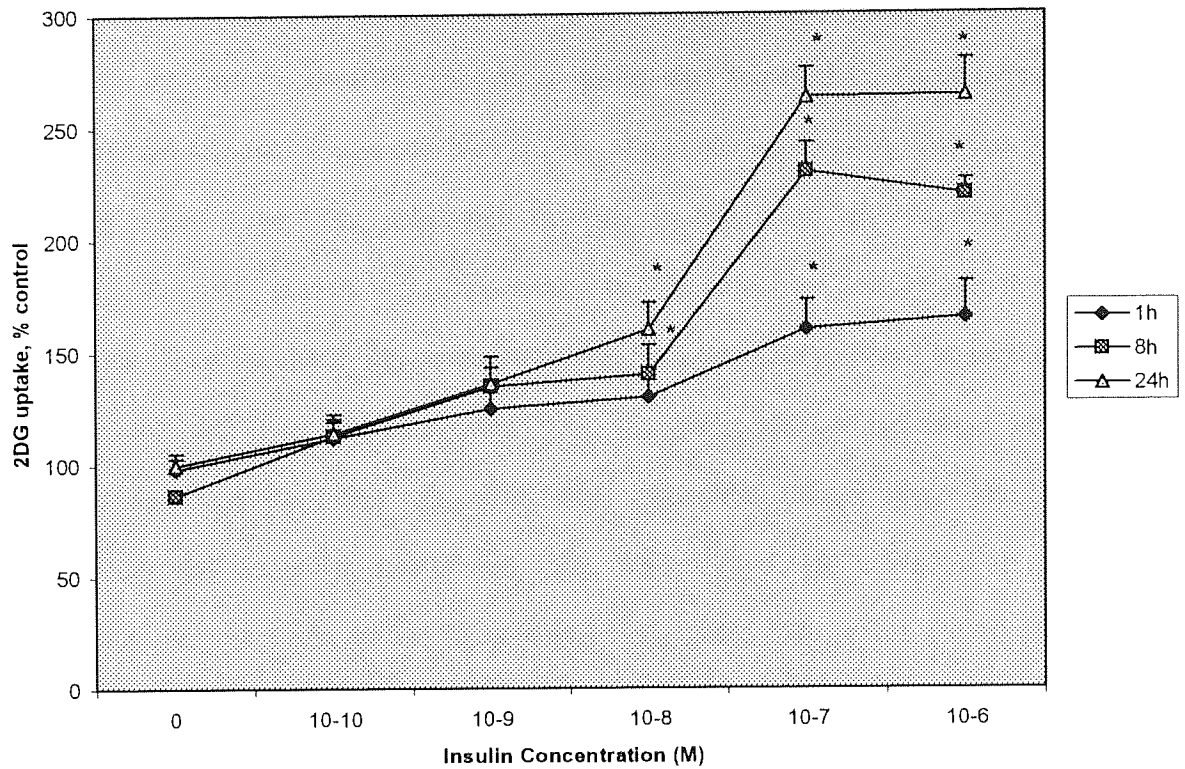
Insulin significantly increased glucose uptake into the L6 skeletal muscle cells in a concentration-dependent and time-dependent manner (see figure 4.3). Maximal

insulin (10^{-6} M)-stimulated glucose uptake was observed at 24h where there was an increase by 164% into the L6 muscle cells. Insulin (24h incubation) significantly increased 2DG uptake into the cells at 10^{-8} - 10^{-6} M, by 60-164%, respectively. 10^{-10} and 10^{-9} M insulin had no measurable effect.

A concentration dependent curve was also observed after 8h insulin incubation on the L6 muscle cells. Insulin increased 2DG into the cells at 10^{-8} - 10^{-6} M, by 35-120%, ($p < 0.05$ versus control). 10^{-10} and 10^{-9} M insulin had no effect on stimulating 2DG uptake into the L6 cells.

In addition, 2DG uptake was significantly increased into the L6 muscle cells at 1h incubation by insulin at 10^{-7} and 10^{-6} M, by 30 and 65%, ($p < 0.05$ versus control). 10^{-10} - 10^{-8} M insulin had no effect when exposed to the cells for 1h. 2DG uptake was significantly diminished with 1h insulin exposure vs the 24h exposure (65 ± 16.85 2DG uptake, % control at 1h vs 164 ± 16.01 2DG uptake, % control at 24h, $p < 0.05$).

Figure 4.3 Effect of insulin 10^{-10} - 10^{-6} M on 2DG uptake in L6 skeletal muscle cells. Cells were exposed to insulin for 1h, 8h and 24h. Results expressed as mean values \pm SEM (n= 6). * $p < 0.05$ versus control.



4.3.4 Discussion

The L6 cell culture system is an ideal model to study insulin signalling. The cells were responsive to the insulin in a concentration and time dependent manner. Uptake of glucose into these cells after exposure to insulin was achieved for 1h or longer, until maximal stimulation was achieved at 24h. It must be appreciated here that the L6 cells would have been previously exposed to insulin in prior culture (in DMEM/FCS) during growth and differentiation to achieve normal healthy growth of these cells. These results clearly demonstrate that the L6 muscle cells can be used as an alternative model to skeletal muscle *in vitro*. The results are consistent with previous studies that have demonstrated an insulin-stimulated glucose uptake into L6 muscle cells and skeletal muscle (Bates, 1999; Walker *et al.*, 1990; Walker *et al.*, 1989; Simpson and Cushman, 1986; Young *et al.*, 1986; Klip *et al.*, 1984). In addition, previous studies have indicated that the L6 cell line possesses several characteristics of skeletal muscle *in vivo* (Bates, 1999; Klip *et al.*, 1982).

Acute insulin stimulation on the L6 muscle cells results in an increase in 2DG uptake. The major action of acute insulin treatment in these cells is due to a rapid recruitment of preformed GLUT transporters to the cell membrane (Walker *et al.*, 1989). The uptake of chronic insulin-stimulated glucose transport into the L6 muscle cells is probably achieved by the translocation of the GLUT transporters to the cell surface membrane (4.3.1) and an increase in glucose transporter mRNA expression. Previous studies have shown that the long-term effects of insulin on glucose transport activity correlate with a time dependent increase in both the glucose transporter and mRNA (Walker *et al.*, 1989).

Bates (1999) demonstrated that insulin-stimulated glucose uptake was inhibited by both cytochalasin B (which binds to and competitively inhibits glucose transporters) and cycloheximide (which blocks protein synthesis and is used to determine glucose transporter action). In this study, basal and insulin-stimulated glucose transport via the activation of GLUT1 and GLUT4 transporters appear to be regulated by both protein synthesis and translocation of the transporters to the membrane. In addition, LY 294 002 (inhibitor of PI3-Kinase) decreased both basal and insulin-stimulated glucose uptake in a concentration time dependent manner. The effects were observed at 24h, and therefore indicate that PI3-Kinase is pivotal to both acute and chronic insulin exposure to L6 cells (Bates, 1999). The kinetics of glucose transport in L6 cells and the importance of PI3-Kinase in the activation of glucose transport by insulin have been described previously (Klip *et al.*, 1982; Cheatham *et al.*, 1994; Clarke *et al.*, 1994). Separate trypan blue exclusion studies have confirmed almost 100% cell viability in the studies performed by Bates (1999).

In summary, the L6 muscle cells are responsive to insulin and the insulin-stimulated 2DG uptake in differentiated 3T3-L1 cells requires the activation of PI3-Kinase (Bates, 1999; Cheatham *et al.*, 1994; Clarke *et al.*, 1994). In addition, insulin stimulation via the PI3-Kinase subunit is required for the activation of GLUT1 and GLUT4 transcription and translation in these cells (Bates, 1999; Klip *et al.*, 1982).

The L6 cell culture system is an alternative model to isolated skeletal muscle, particularly for studies of insulin signalling. L6 muscle cells offer many advantages to using intact skeletal muscle tissue. These cells are particularly responsive to insulin, maintain cell viability for extended periods of time and the technique allows an even and rapid accessibility of cells to substrates. Thus, these cells have been used

in the following chapters for the screening and mechanistic evaluation of anti-diabetic drugs.

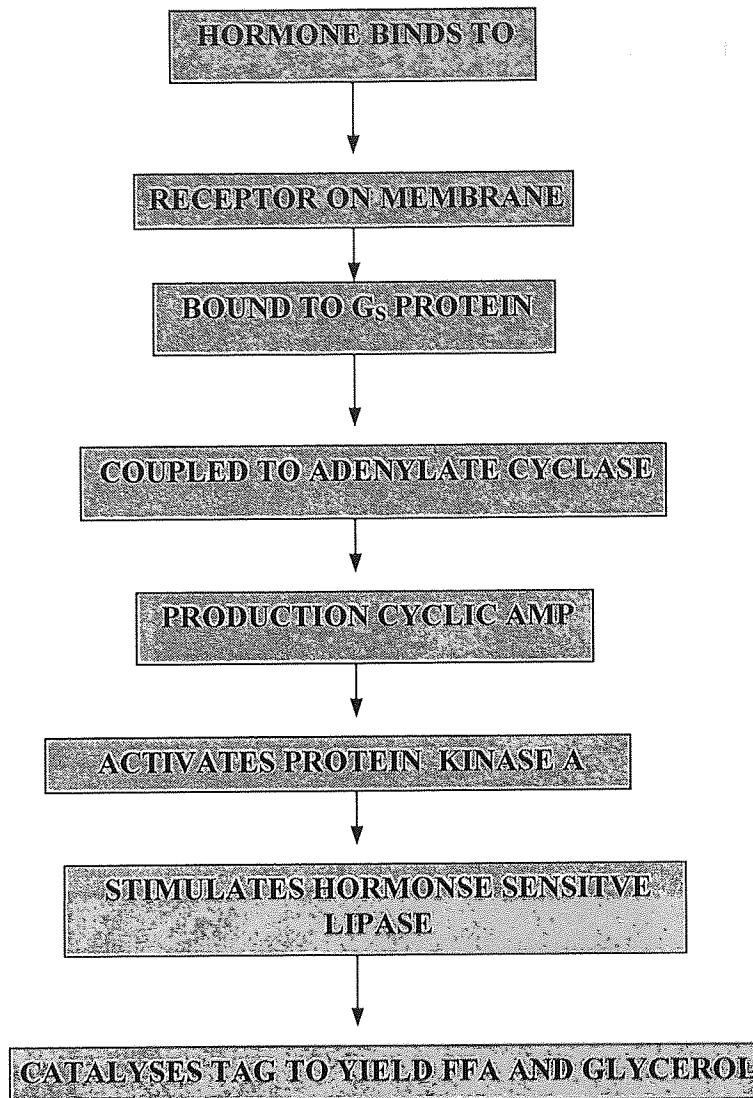
Chapter 5: Characterisation of lean and obese mouse adipocytes

5.1 Introduction

To decrease adiposity in obese individuals, it is essential that excess stored triglyceride is broken down and metabolised to yield energy, rather than be re-esterified and re-stored. Adipose tissue is the body's main energy storage site, where the energy is stored as triacylglycerols (TAG). When lipolysis occurs, either through hormonal or chemical means, TAG is broken down by the rate limiting enzyme hormone sensitive lipase to yield diacylglycerol (DAG), and it also catalyses the breakdown of DAG to yield monoacylglycerol (MAG). The MAG is then hydrolysed by the enzyme monoacylglycerol lipase to yield the end products of lipolysis, free fatty acids (FFA) and glycerol. The free fatty acids are either re-esterified to yield TAG or are used as an energy source in muscles and liver. Excess glycerol is used for gluconeogenesis in the liver (Bjorntorp, 2000).

The cell surface receptors involved in the control of lipolysis are coupled to the G protein complex. During lipolysis, hormones bind to the appropriate receptor, which activates adenylate cyclase via G_s proteins (stimulatory GTP sensitive coupling proteins), which increases cyclic AMP. Cyclic AMP activates protein kinase A, which in turn phosphorylates hormone sensitive lipase (HSL). HSL then catalyses TAG to yield FFA and glycerol (Arner and Eckel, 1997) (see figure 1). Hutchinson *et al.* (2002) have described the β 3a and β 3b receptor subtypes. They have demonstrated that the β 3a adrenoceptor couples solely to the G_s proteins, whereas the β 3b adrenoceptor is coupled to G_s and G_i proteins (Hutchinson *et al.*, 2002).

Figure 1:

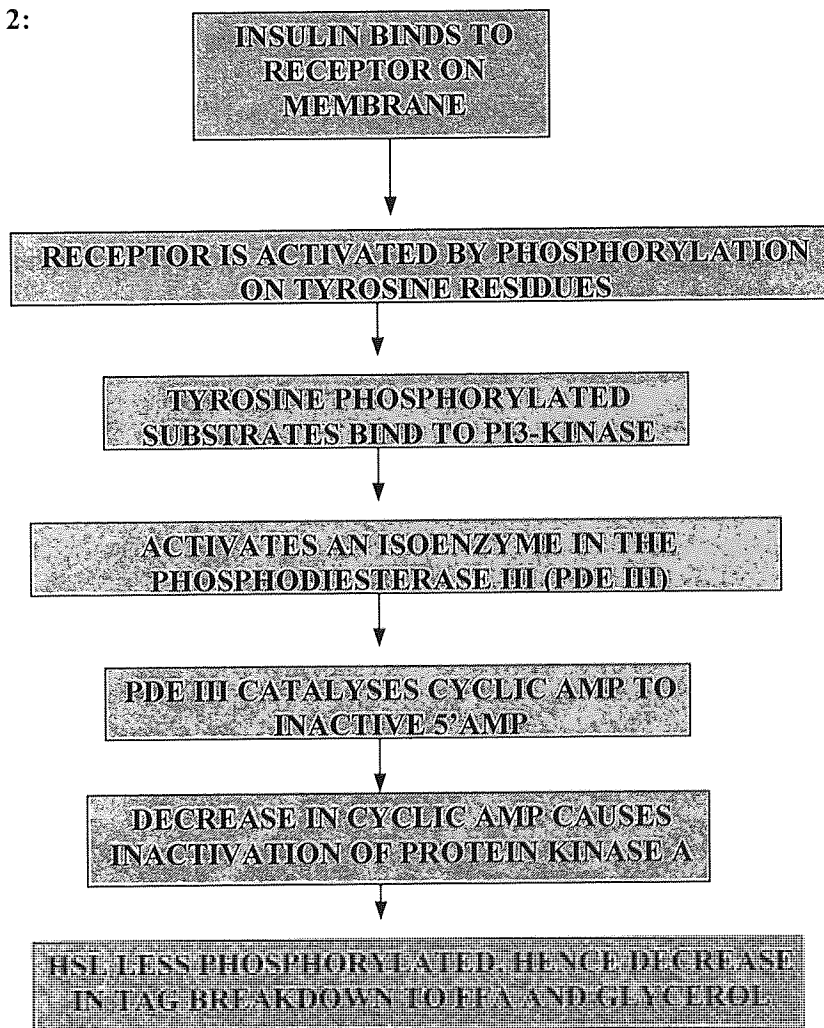


(Adapted from Arner and Eckel (1997). *Adipose Tissue as a Storage Organ. Handbook of Obesity*. Edited by Bray, Bouchard, James. Chapter 17: 381).

Catecholamines play a key role in the regulation of lipolysis in white adipose tissue. It is well known that the lipolytic properties of catecholamines are mediated through β adrenoceptor subtypes, including β_1 , β_2 and β_3 , and possibly β_4 (Galitzky *et al.*, 1997). In contrast, activation of the α adrenoceptors inhibits lipolysis (Guo *et al.*, 1997).

Lipolysis can be inhibited by the activation of the α adrenoceptors coupled to adenylate cyclase via Gi protein. The Gi proteins are inhibitory and as such, inhibit the production of cyclic AMP and the activation of protein kinase A. Inhibition of the lipolytic pathway can also occur via hormones binding to the specific receptors that are linked to the phosphatidylinositol 3-kinase (PI3-Kinase) (Hutchinson *et al.*, 2002; Arner, 1996). An example of inhibition via this route is by the hormone insulin (see figure 2).

Figure 2:



(Adapted from Arner and Eckel (1997). *Adipose Tissue as a Storage Organ. Handbook of Obesity*. Edited by Bray, Bouchard, James. Chapter 17: 381).

5.2.1 β adrenoceptors subtypes in mouse adipose tissue

Lands *et al.* (1967) first described the β_1 and β_2 -adrenoceptor subclasses. Since then, many studies have investigated the role of adrenoceptors, and it became apparent soon after this initial discovery that the degree of expression of these receptors by brown adipose tissue (BAT) and white adipose tissue (WAT) and the potency of stimulation by specific catecholamine agonists were far too low to explain the magnitude of the lipolytic effects that they mediate (Furchgott, 1972; Harms *et al.*, 1974 and 1977). Over a decade later β_3 adrenoceptors were identified (Arch and Ainsworth, 1983) in rodent adipose tissue. Use of β agonists with different subclass specifications, in BAT and WAT indicated that lipolysis by these tissues was predominantly mediated by β_3 receptors (Hollenga and Zaagsma, 1989). Most recently β_3 receptors have been shown to comprise two distinct categories, β_3a and β_3b based on their G protein associations (Hutchinson *et al.*, 2002).

β_1 , β_2 and β_3 adrenoceptor subtypes all participate in physiological binding of the catecholamines, norepinephrine (noradrenaline) and epinephrine (adrenaline) (Herrera *et al.*, 2000). Although the β_3 adrenoceptors play a dominant role in lipolysis (Arner and Eckel, 1997), a minor role exists for β_1 adrenoceptor in this respect (Germack *et al.*, 1997). Indeed many studies have confirmed that only the β_1 and β_3 subtypes can stimulate lipolysis in rat (Hollenga and Zaagsma, 1989; Van Liefde *et al.*, 1992, Murphy *et al.*, 1993): a role of β_2 adrenoceptors in the lipolysis of rodent white adipocytes is uncertain and still needs to be investigated further.

5.2.2 β adrenoceptors subtypes in human adipose tissue

Before the β_3 adrenoceptor was identified, it was reported that human adipose tissue expressed mainly the β_2 adrenoceptor (Lai *et al.*, 1982). Several studies suggested a stimulatory role of β_2 adrenoceptors and inhibitory α_2 adrenoceptors as the dominant mediators of lipolysis by human adipocytes (Enocksson *et al.*, 1995; Galitzky *et al.*, 1993). Recently, level of expression and role of β_3 adrenoceptors in human adipocytes has been a matter of debate. Langin *et al.* (1991) reported β_3 adrenoceptor mRNA expression in human adipose tissue, however the level of expression was lower than in rodents. Another study investigated the respective degree of expression of β_3 adrenoceptors in human omental white adipose tissue. Deng *et al.* (1996) reported that no β_3 adrenoceptor mRNA could be detected in human white adipocytes and it was noted that 9% and 91% of the total mRNA was β_1 and β_2 adrenoceptor, respectively. A more recent study established that β_3 adrenoceptors mediate some lipolysis in human white adipose tissue, and there is possibly also a β_4 adrenoceptor (van Harmelen, 1997; Galitzky *et al.*, 1997; Sennitt *et al.*, 1998). Identification and characterisation of the β_3a and β_3b adrenoceptor subtype by Hutchinson *et al.* (2002) has revealed that both adrenoceptor subtypes increase cyclic AMP by signalling through the Gs protein. In addition, β_3b adrenoceptor exerts its effects by signalling through the Gi protein. These receptor subtypes may play a role in human white adipose tissue mobilisation. Sequence identities between β_1 and β_2 adrenoceptors are 40-50% in both human and rat, whereas the rat and human β_3 adrenoceptors are 79% identical (Arch and Wilson,

1996). Hence, synthetic β_3 agonists developed in rodents are not necessarily effective in humans and may have some cross reactivity with other β adrenoceptor subtypes.

It is pertinent to note that BAT, which is evident throughout life in distinctive depots in rodents, is mostly lost in neonatal life in humans. Adult humans have small numbers of BAT and some poorly differentiated BAT-like adipocytes in the spinal and infra-renal WAT depots. Thus, BAT does not make a significant contribution to the overall adipocyte number in adult humans. However the small numbers of BAT cells in adult humans are metabolically active and capable of very high rates of lipolysis if appropriately stimulated (Chaudhry and Granneman, 1999; Jockers *et al.*, 1998). Hence, BAT in humans could still represent a relevant target to enhance lipolysis and thermogenesis.

5.3 Adipocyte biology

In the 1930's, adipose tissue was viewed as a 'storage of lipid derived from excess calories ingested' (DiGirolamo and Fried, 1987). Almost three decades later, in 1956 Dole demonstrated that the adipose tissue was a source of metabolic fuels that could be mobilised during fasting and release free fatty acids and glycerol into the bloodstream. From this observation, interest in adipocyte biology arose and many metabolic and hormonal regulators of the adipose tissue have now been established (Perry *et al.*, 2001).

Adipose tissue is regulated by a number of physiological and hormonal factors, such as fasting, feeding, catecholamines and insulin. These factors play an important role in regulating the rate of lipolysis in adipocytes. Martin Rodbell (1964), first described a method for extracting and isolating adipocytes from adipose tissue using collagenase. With this method the isolated adipocytes are separated from the dense stromal vascular cells by floatation. Rodbell (1964) clearly showed that the adipose cells maintained intrinsic metabolic characteristics of the intact adipose tissue.

Isolated adipocytes are now the preferred preparation to use when studying adipocyte biology. Intact tissue that is cut into small pieces can be used, however it is important to take into account the influence of the stromal vascular cells on the adipocyte function. Other disadvantages to this method include tissue thickness, reduced perfusion and diffusion, mixed cellular composition and variability associated with the region of the pad whence the tissue is cut. The main advantage of using tissue fragments is the limited manipulation of the tissue and the immediate incubation of

the tissue with the test agent after the animal is killed. Another *in vitro* method used for studying the metabolism of adipose tissue, is the 'perifused' adipocyte preparation developed by Allen *et al.* (1973). Here, the adipocytes are placed in a perfusion chamber containing buffer that passes through the chamber, around and through the tissue, and the perfusate is collected for analysis. The method is more complex versus the Rodbell (1964) isolation method. However the flowing system provides a constant supply of fresh buffer to the cells and prevents the accumulation of metabolites, such as free fatty acids that could alter the cell metabolic activities of the cell. The perifused system also offers a simple, reproducible method by which minute to minute dynamic changes in the rate of lipolysis of isolated adipocytes may be monitored.

5.3.1 Studies using mouse lean and obese adipocytes

5.3.2 Adipocyte cell size and number

Epididymal and parametrial fat pads were removed from lean and *ob/ob* mice (figure 3) and adipocytes were isolated from the adipose tissue using the Rodbell (1964) method. Several modifications of the original method have been employed here, including the use of 5% albumin (3% for lean adipocyte) in the incubation medium to minimise obese adipocyte breakage (Pederson *et al.*, 1981). Many differences in adipose tissue from different sites have been observed in rodents and the response of the adipose tissue to different lipogenic and lipolytic hormones. Hence, the epididymal and parametrial fat pads have been used for all the adipocyte experiments,

to avoid variability in the experiments. Once isolated, the adipocyte cell number was determined by use of a haemocytometer, which permitted the calculation of cell number and hence an equal number of adipocytes in the cell suspension with reproducible accuracy (10^5 cells per ml). Cell concentration is a critical variable in the lipolysis experiments. It has been shown that high cell concentrations markedly reduce the rate of lipolysis per cell, possibly due to the accumulation of free fatty acids in the buffer (DiGirolamo *et al.*, 1993), which are then partially taken up by the cells and re-esterified. Cell size of the adipocytes was also calculated using photomicrographs to determine any difference between the lean and obese adipocytes (figure 4a and 4b).

Table 1: Cell number from isolated epididymal and parametrial preparations

Mouse	Number of cells per ml	Number mice
Lean	$6.57 \times 10^5 \pm 0.42$	50
Obese	$6.42 \times 10^5 \pm 0.40$	50

Values are mean \pm SEM

There are no significant differences between the number of cells isolated from the adipose tissue of the lean and obese mouse. Cell size is an important determinant and from the photomicrographs (see below) it can clearly be seen that adipocytes from the lean mouse are much smaller versus those isolated from the obese mouse. Moreover, there were no significant differences between adipocyte cell size and the sex of the mouse when comparing epididymal with parametrial fat depots.

Table 2: Cell size from isolated epididymal and parametrial preparations

Mouse	Size of cells	Number mice
Lean	75 ± 6.9µm	25
Obese	160 ± 14.0µm *	25

Values are mean ± SEM *p<0.001 versus lean adipocytes

There was a significant difference in the size of the adipocyte isolated from the obese adipose tissue. The mean size of the obese adipocyte was 160µm versus 75µm for the lean adipocyte, p<0.001.

Figure 3: Lean and obese mouse



Figure 4a: Lean adipocytes

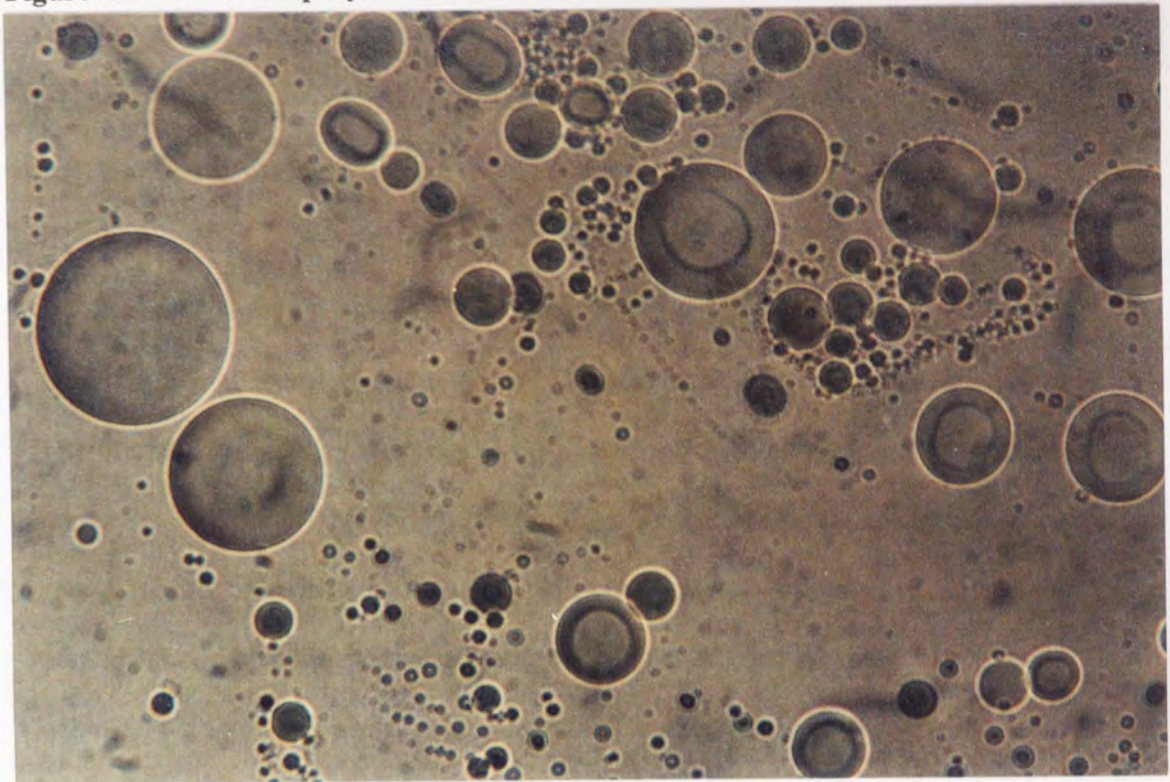
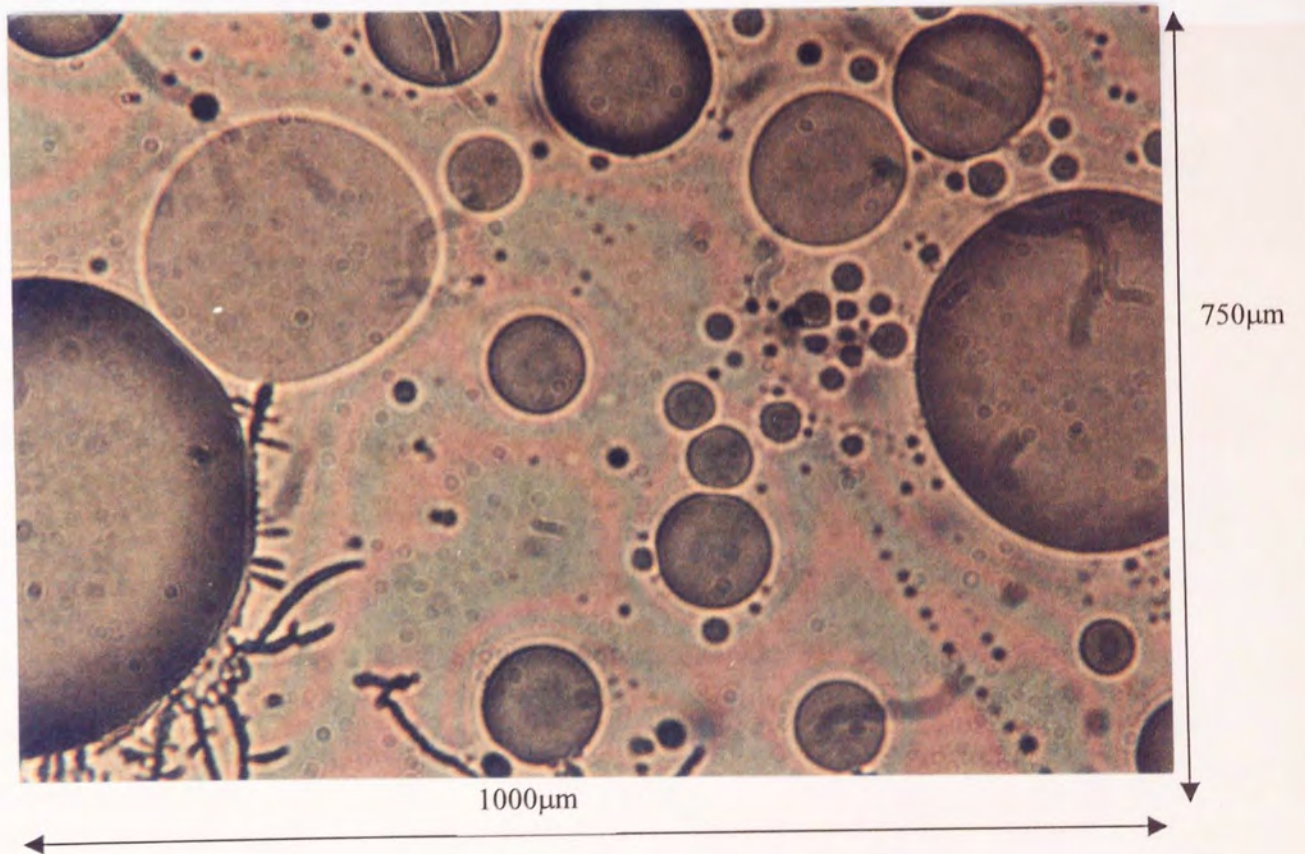


Figure 4b: Obese adipocytes



The isolated adipocytes are spherical and contain a store of triacylglyceride surrounded by a thin cytoplasm. The nucleus is seldom visible on the photomicrographs; it is typically offset to the rim of the cytoplasm. Along with the triacylglyceride storage, there are small amounts of water.

A prominent feature of the adipocytes isolated from the obese mouse is that they maintain a high basal lipolytic rate when incubated *in vitro* in the absence of a lipolytic stimulating agent (expressed per number of cells) compared with lean mouse adipocytes. When a lipolytic agent stimulates lipolysis, a much higher incremental rate is usually obtained in the lean mouse adipocytes compared with the obese adipocytes. Alternatively the maximum lipolytic rate may be the same but it is achieved with a lower concentration of agonist in lean mouse adipocytes. A possible explanation could be the fat cell size, where a number of studies have shown a positive correlation between the rate of basal lipolysis and fat cell size (Arner, 1988). Another possible explanation is that the obese mouse has a diminished response to lipolytic hormones due to a decrease in β adrenoceptors on the cell surface membrane. Although this is a partial explanation for reduced responsiveness to β agonists, it is not a confluent explanation as agents such as luteinizing hormone (LH), which act independently of adrenoceptors show a reduced lipolytic effect in *ob/ob* adipocytes. Hence, the reduced responsiveness in obese adipocytes may possibly be due to reduced Gs expression or activity. Other hormone-induced cellular responses mediated by G-protein linked receptors and the generation of cyclic AMP include thyroid stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH) and glucagon.

Changes in insulin binding with obesity exist, and it is known that obese rodents have a decreased responsiveness to insulin associated with the larger adipocytes (Olefsky, 1976; Gliemann and Sonne, 1978). Older rodents have also shown a decrease response to lipolytic hormones and a greater rate of fatty acid re-esterification (DiGirolamo and Fried, 1987). Hence, the adipocytes used here were isolated from 20-30 week old mice to reduce variability between the lean and obese studies. This age period also corresponds to maximum weight achievement and stability of body weight in *ob/ob* mice. To this end, the lean and obese mouse adipocytes have been characterised as a tool for analysing putative and novel lipolytic agents.

5.3.3 Preliminary experiments on the lean and obese adipocytes

In the present study the role of the α and β adrenoceptors in the lipolysis of adult mouse epididymal and perimetrial white adipocytes was investigated using selective α and β agonists and antagonists. Adipocytes of lean and obese (*ob/ob*) mice were studied. The aim of the current work was to establish whether there were differences between the lipolytic effect of the agonists on the lean and obese adipose tissue. The main agents used to characterise adrenoceptor status are shown in table 3.

Table 3: Agents used to characterise lean and obese adipocytes

ADRENOCEPTOR TYPE	AGONISTS	ANTAGONISTS
Non-selective β	Isoproterenol	Propranolol
β_1	Dobutamine	Atenolol
β_2	Fenoterol	ICI 118 551
β_3	BRL 37344	SR 59230A
α_1	Phenylephrine	Prazosin
α_2	Clonidine	Yohimbine

A broad range of concentrations of each agonist (10^{-9} - 10^{-5} M) was used but typically one concentration of the antagonist (10^{-6} M), which was towards the lower end of the inhibitory concentration range, was chosen. The effectiveness to decrease lipolysis by the antagonist reflects its potency. The adipocytes were incubated for 2h at 37°C and the concentration of glycerol released into the medium, which is indicative of triacylglyceride breakdown, was measured enzymatically as described in detail in chapter 2.

5.3.4 Data analysis

All concentration-response curves were expressed in mM glycerol/ 10^5 adipocytes (determined by use of a haemocytometer). Data are given as means \pm SEM, n=6 (duplicates analyses of tissues from three mice for each agonist with or without the

antagonist, unless otherwise stated). Significance was assessed by ANOVA and Student's paired 't'-test, * $p < 0.05$ was considered to indicate a significant difference.

5.3.5 Results

Non-selective β agonist (Isoproterenol) and antagonist (Propranolol)

Isoproterenol 10^{-9} to 10^{-5} M significantly increased lipolysis in lean adipocytes, and propranolol (10^{-6} M) significantly inhibited lipolysis at 10^{-9} to 10^{-7} M (figure 5.1a).

Likewise, isoproterenol 10^{-6} and 10^{-5} M significantly increased lipolysis in obese adipocytes and propranolol (10^{-6} M) significantly inhibited lipolysis at 10^{-5} M. In addition, propranolol reduced lipolysis at a non-stimulatory concentration of isoproterenol (10^{-9} M) (figure 5.1b).

Selective β_1 agonist (Dobutamine) and antagonist (Atenolol)

Dobutamine (10^{-5} M) at a maximal concentration significantly increased lipolysis in lean adipocytes and atenolol 10^{-6} M significantly inhibited dobutamine action at 10^{-7} to 10^{-5} M (figure 5.2a). Dobutamine 10^{-6} to 10^{-5} M significantly increased lipolysis in obese adipocytes, and atenolol at 10^{-6} M significantly inhibited dobutamine action at 10^{-9} to 10^{-5} M (figure 5.2b).

Selective β_2 agonist (Fenoterol) and antagonist (ICI 118 551)

Fenoterol 10^{-5} M significantly increased lipolysis in lean adipocytes, however ICI 118 551 10^{-6} M did not significantly inhibit fenoterol action (figure 5.3a).

Since ICI 118 551 10^{-6} M did not inhibit fenoterol action in the lean white adipocytes, this indicates that although the antagonist is very selective for the β_2 receptor, it has a low potency. Thus ICI 118 551 (10^{-6} - 10^{-4} M) was incubated with the fenoterol 10^{-5} M

only (figure 5.3b). ICI 118 551 10^{-5} and 10^{-4} M significantly inhibited the action of fenoterol in the lean murine adipocytes. Fenoterol (10^{-6} and 10^{-5} M) significantly increased lipolysis in obese adipocytes. In contrast to the lean adipocytes, ICI 118 551 (10^{-6} M) significantly inhibited fenoterol action at 10^{-8} to 10^{-5} M (figure 5.3c and 5.3d).

Selective β_3 agonist (BRL 37344) and antagonist (SR 59230A)

BRL 37344 (10^{-9} - 10^{-5} M) significantly increased lipolysis in lean adipocytes, and SR 59230A (10^{-6} M) significantly antagonised the action of the selective β_3 agonist (figure 5.4a). Similarly, BRL 37344 (10^{-9} - 10^{-5} M) increased lipolysis in the obese adipocytes and the antagonist SR 59230A inhibited this lipolysis at all concentrations (5.4b).

Selective α_1 agonist (Phenylephrine) and antagonist (Prazosin)

Phenylephrine had no lipolytic effect in lean white adipocytes (figure 5.5a) and equally had no effect in adipocytes isolated from obese mice (figure 5.5b).

Selective α_2 agonist (Clonidine) and antagonist (Yohimbine)

Clonidine is an α_2 agonist and binds to the inhibitory α_2 adrenoceptor of the adipocytes. The results show that clonidine has no lipolytic effect in lean or obese white adipocytes (figure 5.6a and 5.6b): because clonidine is an inhibitory agonist this was expected.

Figure 5.1a Lipolytic activity of isoproterenol ± propranolol (10^{-6} M) on lean mouse fat. **Figure 5.1b** Lipolytic activity of isoproterenol ± propranolol (10^{-6} M) on obese mouse fat. Adipocytes were exposed to isoproterenol (10^{-9} - 10^{-5} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values ± SEM (n=6). *p<0.05 versus control, +p<0.05 versus isoproterenol only.

Figure 5.1a

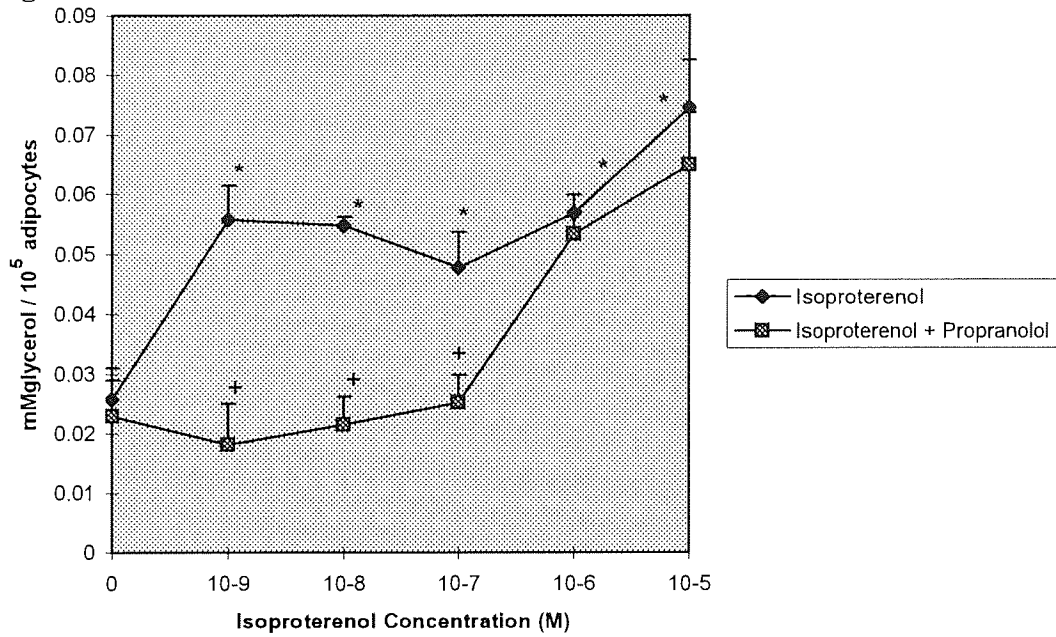


Figure 5.1b

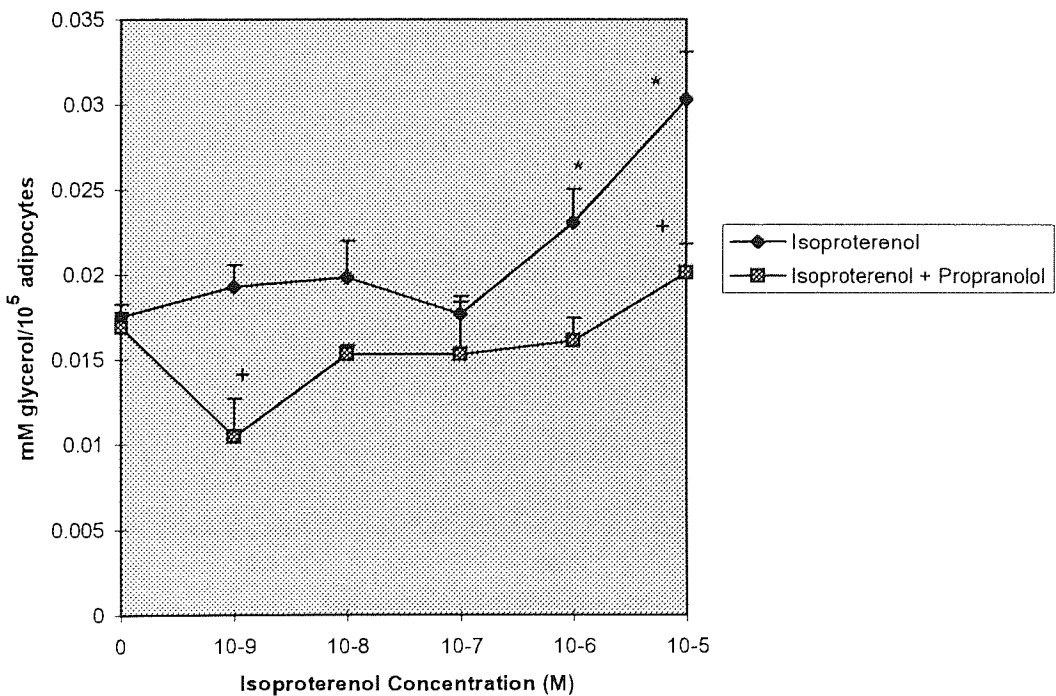


Figure 5.2a Lipolytic activity of dobutamine ± atenolol (10^{-6} M) on lean mouse fat.

Figure 5.2b Lipolytic activity of dobutamine ± atenolol (10^{-6} M) on obese mouse fat.

Adipocytes were exposed to dobutamine (10^{-9} - 10^{-5} M) for 2h at 37°C . Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (n=6). *p<0.05 versus control, +p<0.05 versus dobutamine only.

Figure 5.2a

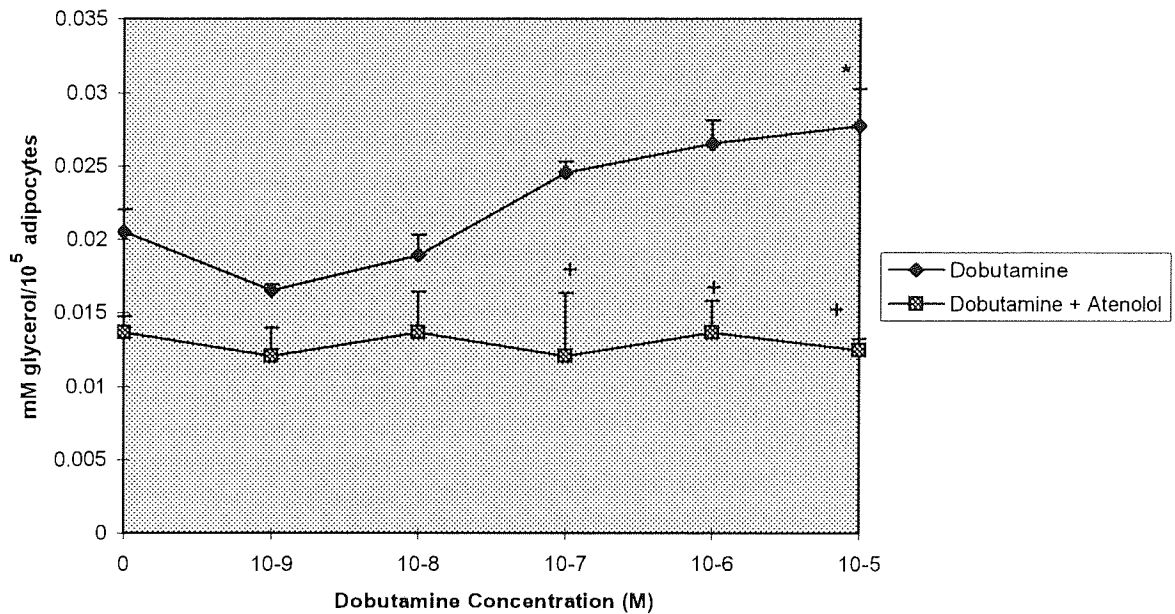


Figure 5.2b

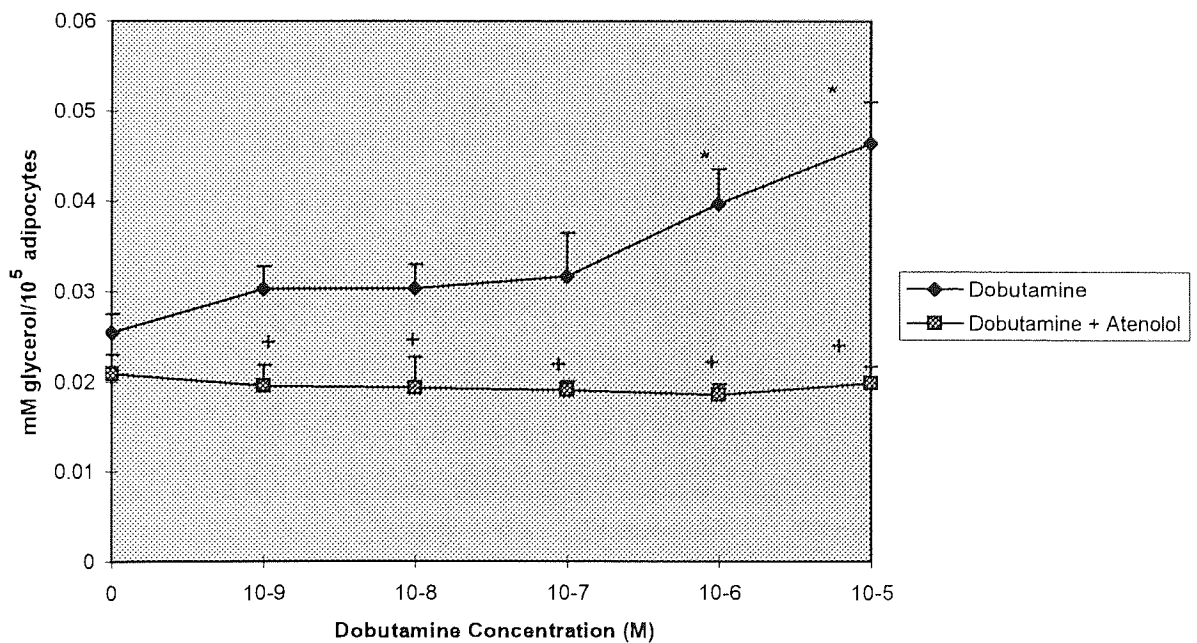


Figure 5.3a Lipolytic activity of fenoterol ± ICI 118-551 (10^{-6} M) on lean mouse fat.

Figure 5.3b Lipolytic activity of fenoterol ± ICI 118-551 (10^{-6} to 10^{-4} M) on lean mouse fat. Adipocytes were exposed to fenoterol (10^{-9} - 10^{-5} M) for 2h at 37°C . Results expressed as mM glycerol/ 10^5 adipocytes. Mean values ± SEM (n=6). *p<0.05 versus control, +p<0.05 versus fenoterol only.

Figure 5.3a

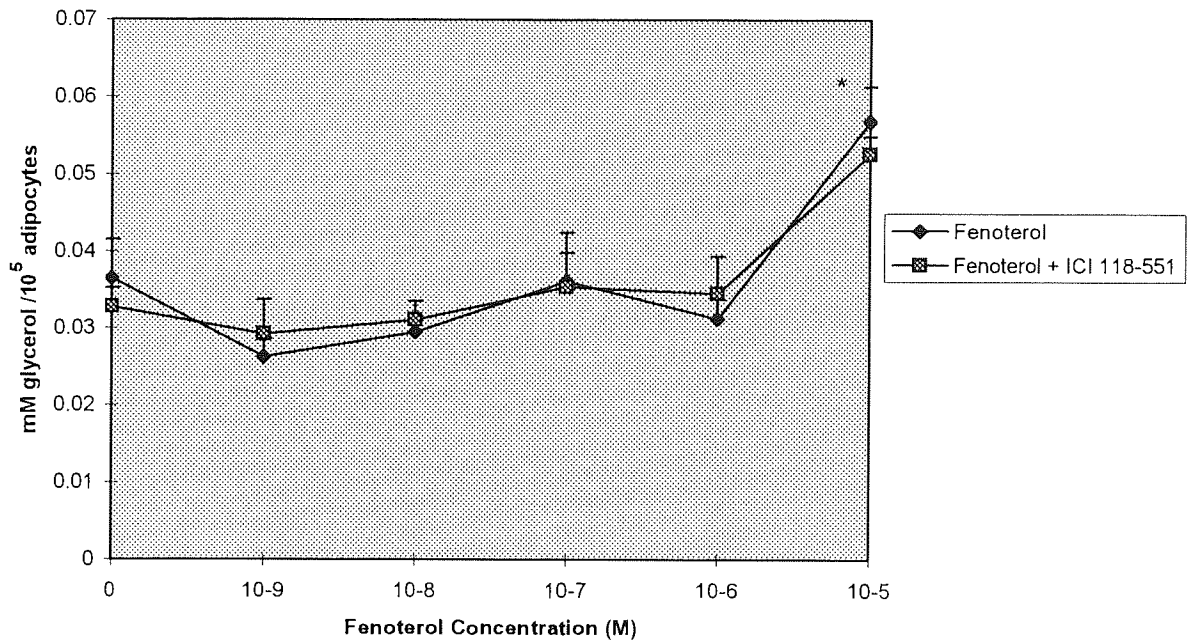


Figure 5.3b

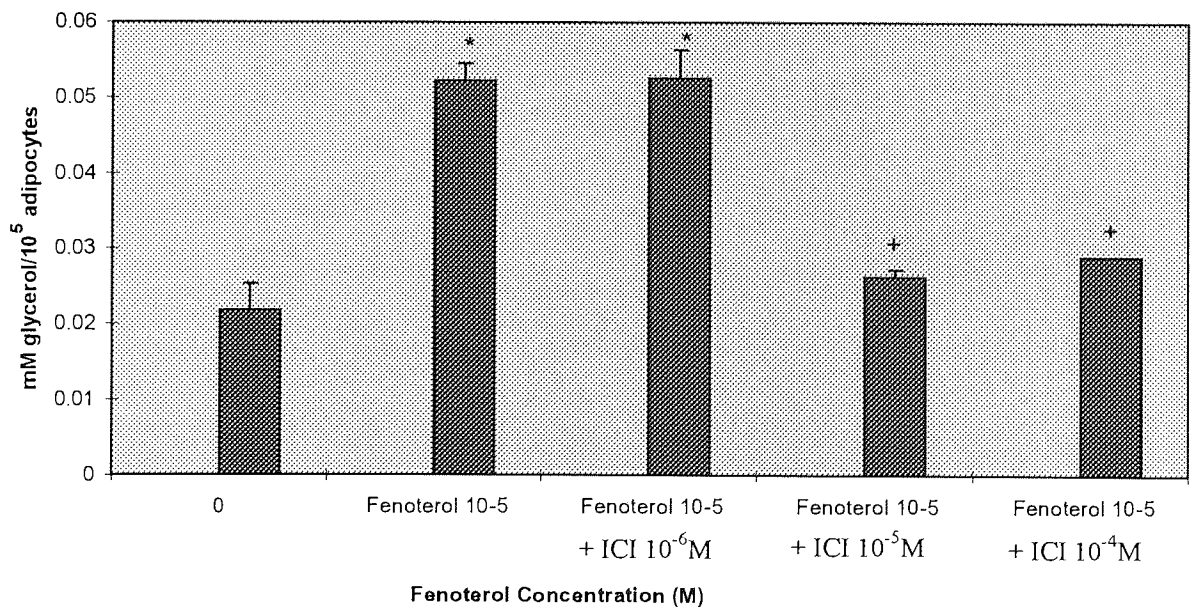


Figure 5.3c Lipolytic activity of fenoterol ± ICI 118-551 (10^{-6} M) on obese mouse fat.

Figure 5.3d Lipolytic activity of fenoterol ± ICI 118-551 (10^{-6} to 10^{-4} M) on obese mouse fat. Adipocytes were exposed to fenoterol (10^{-9} - 10^{-5} M) for 2h at 37°C . Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (n=6). *p<0.05 versus control, + p<0.05 versus fenoterol only.

Figure 5.3c

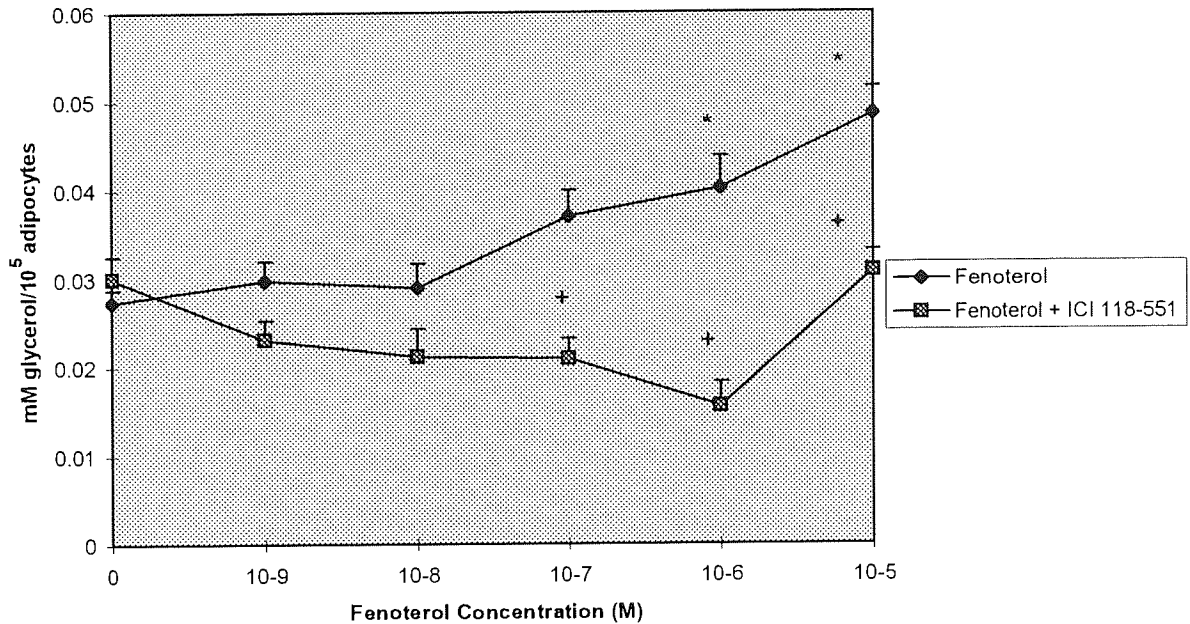


Figure 5.3d

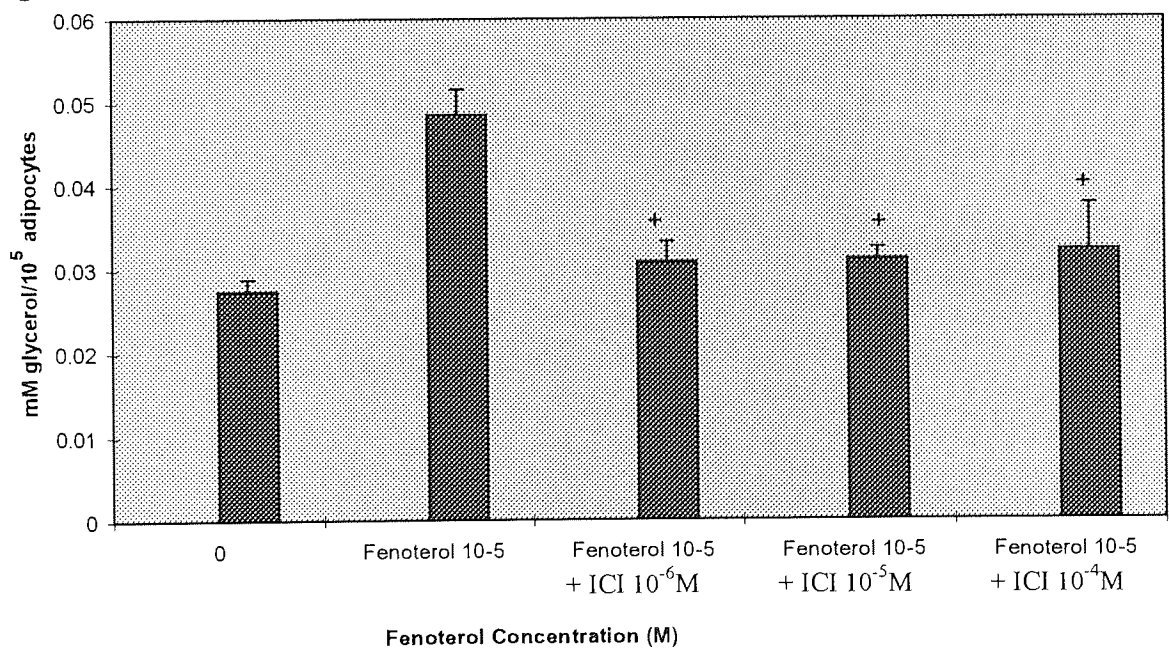


Figure 5.4a Lipolytic activity of BRL 37344 ± SR 59230A (10^{-6} M) on lean mouse
Figure 5.4b Lipolytic activity of BRL 37344 ± SR 59230A (10^{-6} M) on obese mouse fat. Adipocytes were exposed to BRL 373 44 (10^{-9} - 10^{-5} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values ± SEM (n=6). *p<0.05 versus control, + p<0.05 versus BRL 37344 only.

Figure 5.4a

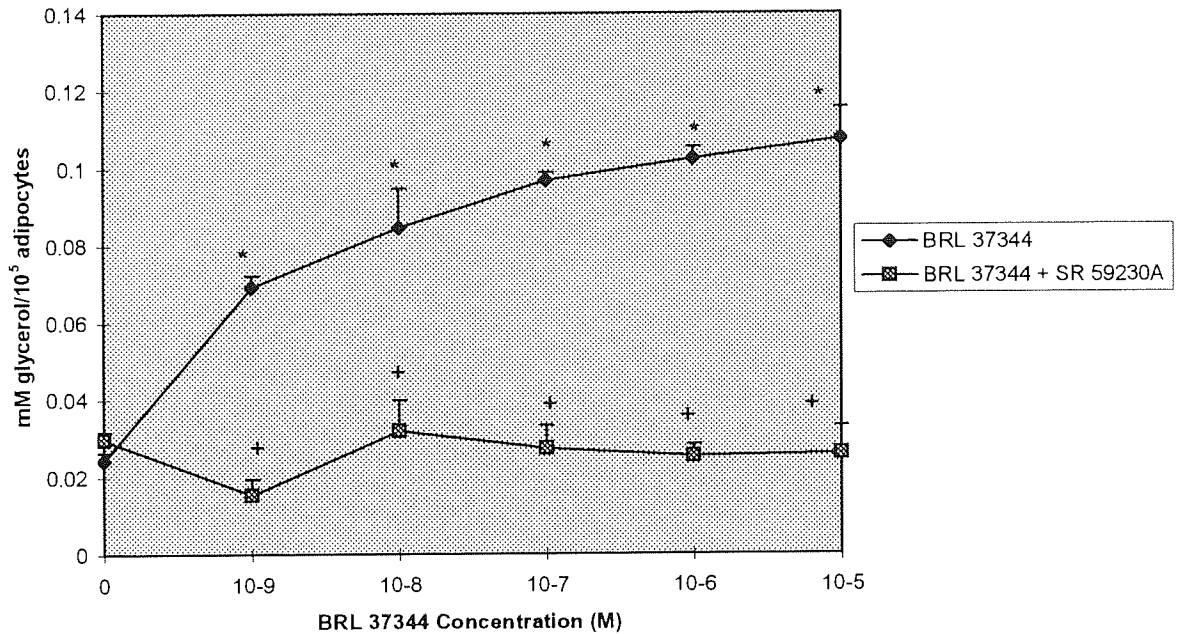


Figure 5.4b

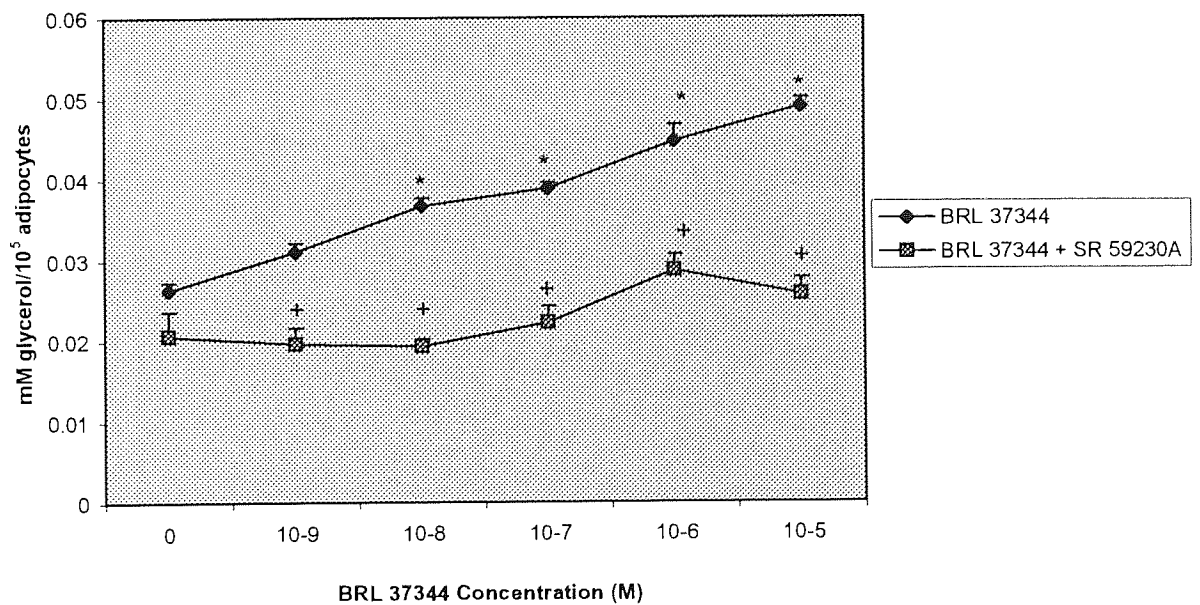


Figure 5.5a Lipolytic activity of phenylephrine ± prazosin (10^{-6} M) on lean mouse fat.

Figure 5.5b Lipolytic activity of phenylephrine ± prazosin (10^{-6} M) on obese mouse fat.

Adipocytes were exposed to phenylephrine (10^{-9} - 10^{-5} M) for 2h at 37°C . Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (n=6).

Figure 5.5a

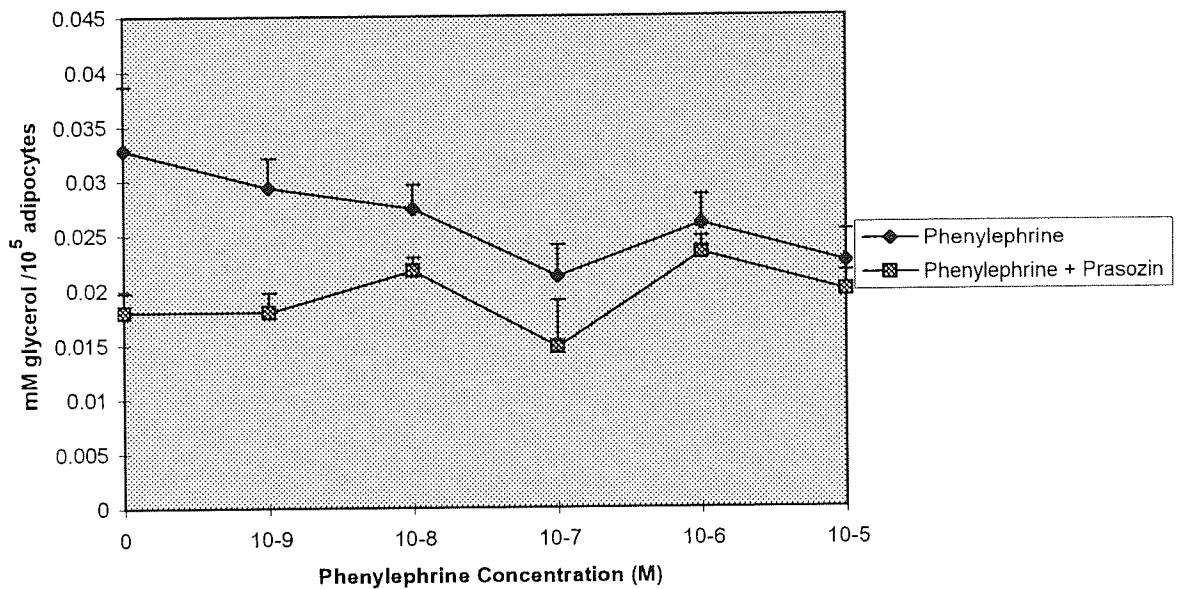


Figure 5.5b

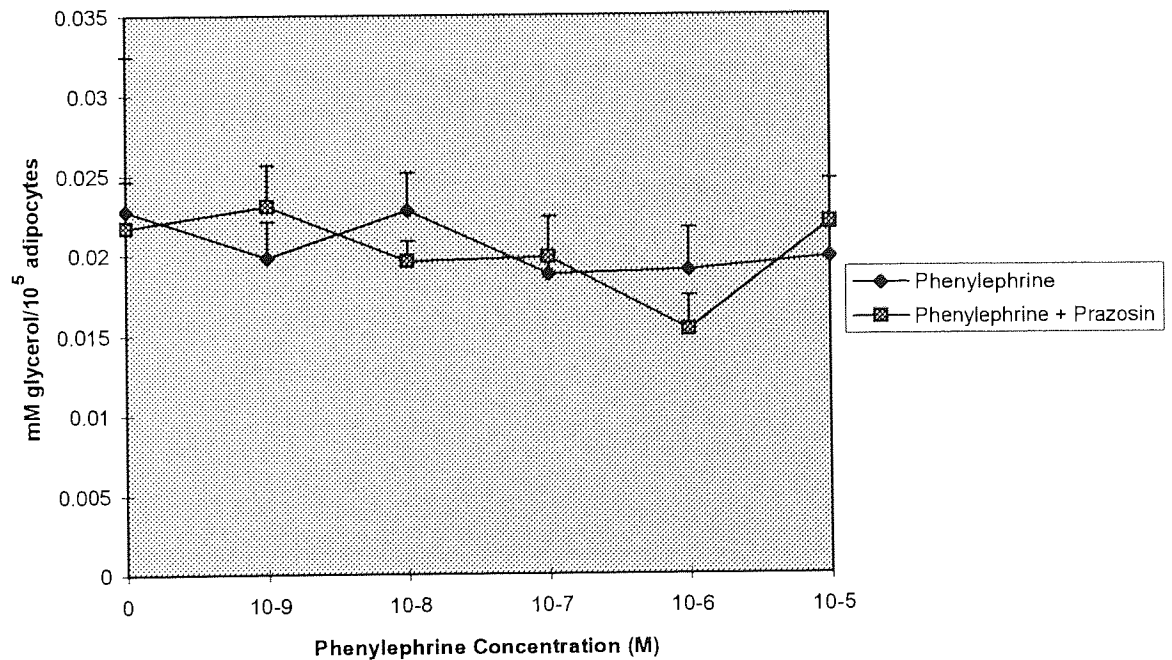


Figure 5.6a Lipolytic activity of clonidine ± yohimbine (10^{-6} M) on lean mouse fat.

Figure 5.6b Lipolytic activity of clonidine ± yohimbine (10^{-6} M) on obese mouse fat.

Adipocytes were exposed to clonidine (10^{-9} - 10^{-5} M) for 2h at 37°C . Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (n=6).

Figure 5.6a

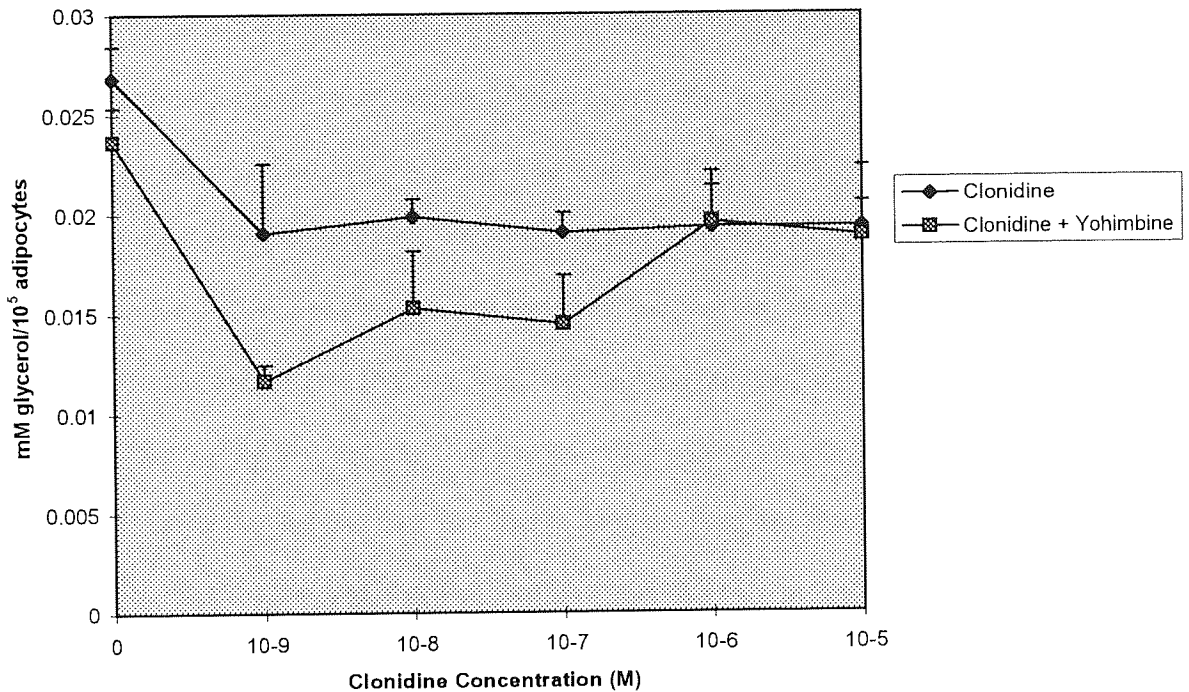
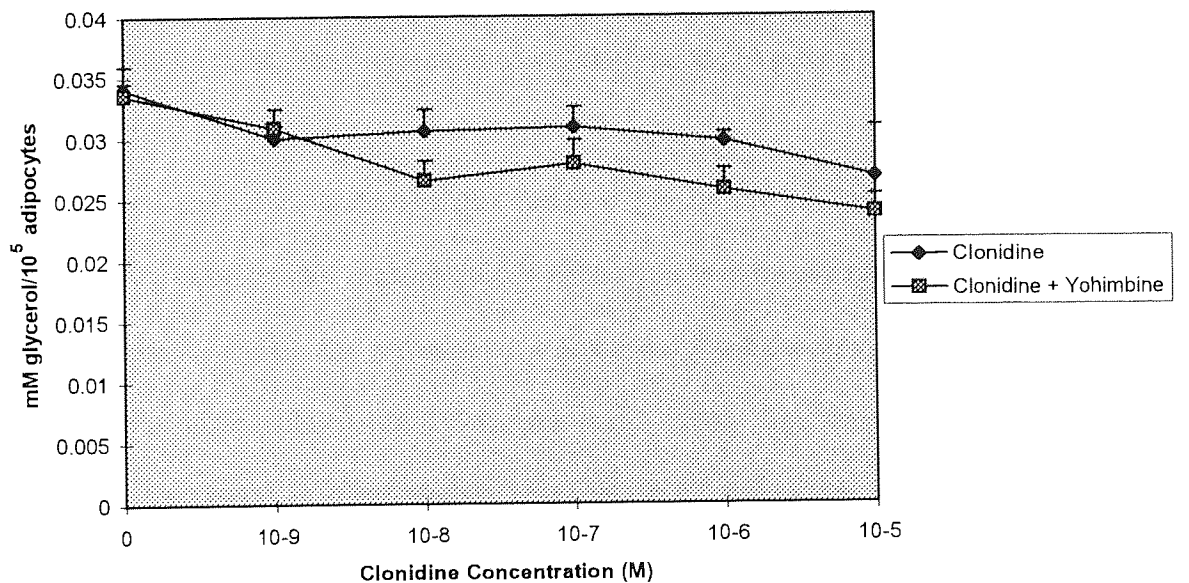


Figure 5.6b



5.3.6 Discussion

The lean and obese adipocytes have been characterised and the findings indicate that the obese adipocytes were larger in size in comparison to the lean adipocytes. Although there was no significant difference in adipocyte cell number isolated from the epididymal and perimetrial depots of lean mouse versus the obese mouse ($6.57 \times 10^5 \pm 0.42$ cells/ml versus $6.42 \times 10^5 \pm 0.40$ cells/ml, respectively), the basal rate of lipolysis (expressed per number of cells) was generally higher in adipocytes of obese than lean mice. This is possibly due to the larger adipocytes isolated from the obese mice, which are well known to show increased lipolysis per cell but decreased relative to triacylglyceride (TAG) mass (i.e. decreased when expressed per volume or weight of cell). It has become customary to express lipolysis as glycerol production per cell number, but the difference between cell number and cell size in lean and obese mice must be considered when interpreting data. All incubations in the adipocytes were conducted for 2h to ensure cell viability, which was evident by usual inspection of cells at the end of the incubation. This was further confirmed by trypan blue exclusion studies when the preparation was initially tested (results not shown).

The marked and concentration-related increase (up to 150 to 250%) in lipolysis produced by isoproterenol (non-specific β agonist) in lean mouse adipocytes (figure 5.1a) is consistent with the presence of a functional β adrenoceptor present in the membrane of these cells (Arner, 1996; Arch *et al.*, 1984). The stimulatory effect is similar to that noted in other studies using the WAT adipocytes preparation and provides further validation of the viability and functional integrity of the preparation. The much-reduced lipolytic stimulus of isoproterenol in obese mouse adipocytes,

which showed 25 and 75% increase in lipolysis at only the higher concentrations (10^{-6} - 10^{-5} M) reaffirms the impaired lipolytic responses of these adipocytes (Lemonnier, 1972). This reduced effect of isoproterenol raises the possibility that obese adipocytes express either a reduced number of β adrenoceptors or a defect post-receptor signalling mechanism (DiGirolamo and Fried, 1987). Addition of propranolol at a concentration (10^{-6} M) which is sufficient to block β adrenoceptors significantly blocked the stimulation of lipolysis by isoproterenol. Propranolol, the non-selective β antagonist reduced the stimulatory effect of isoproterenol by 100-150% in the lean adipocytes and 40-75% in the obese adipocytes. Together these results confirm that functional β adrenoceptors are present on the lean and obese white adipocytes and that the lipolytic response demonstrated by isoproterenol is abolished by the non-selective β antagonist, propranolol.

Dobutamine induced lipolysis in lean and obese white adipocytes. The marked increase (up to 100%) in lipolysis produced by dobutamine (specific β_1 agonist) in lean and obese mouse adipocytes indicate that both the lean and obese adipocytes possess functional β_1 adrenoceptors. Atenolol (specific β_1 antagonist) significantly reduced dobutamine action on the adipocytes. In lean adipocytes, atenolol (10^{-6} M) reduced dobutamine action (10^{-7} , 10^{-6} and 10^{-5} M) by approximately 50%. Obese adipocytes incubated with 10^{-9} to 10^{-5} M dobutamine and exposed to atenolol 10^{-6} M, were significantly inhibited at all dobutamine concentrations by 50-100%. The obese adipocytes were more responsive to dobutamine suggesting a higher number of β_1 adrenoceptors on the obese adipocytes cell surface membrane or an impaired post receptor signalling in the lean adipocytes. Taken together, these results indicate a

minor role for the β_1 adrenoceptor in lipolysis activation similar to previous conclusions (Germack *et al.*, 1997; Hollenga and Zaagsma, 1989).

The functional studies suggest that β_2 adrenoceptors could play a minor role in mediating lipolysis since fenoterol (β_2 agonist) was able to induce the lipolytic response with a potency (1nM) close to that observed by Moss *et al.* (1999). It has been demonstrated elsewhere that procaterol, a β_2 agonist stimulated lipolysis in rat white adipocytes, which further suggests a minor role for these receptors (Germack *et al.*, 1997). In this study, fenoterol in the lean adipocytes was only active at 10^{-5} M, increasing lipolysis by 100%. Obese adipocytes incubated with fenoterol, increased lipolysis at 10^{-6} and 10^{-5} M by 50-100%. It appears from these results that both lean and obese adipocytes appear to express the β_2 adrenoceptors. The modest effect observed by fenoterol at such a high concentration is likely to reflect cross-reactivity with other β adrenoceptors. These results suggest either very low expression of β_2 adrenoceptors, possibly no β_2 adrenoceptor expression, which is doubtful given the level of effect or an impaired signal generation intracellularly (Van Liefde *et al.*, 1992; Langin *et al.*, 1991; Hollenga and Zaagsma, 1989).

The selective β_2 antagonist, ICI 118 551 (10^{-6} M) on the lean adipocytes did not inhibit fenoterol, however at the same concentration in the obese adipocytes the β_2 agonist was inhibited at 10^{-8} - 10^{-5} M, by 20-100%. When ICI 118 551 concentration was increased to 10^{-5} and 10^{-4} M, and incubated with fenoterol 10^{-5} M using the lean adipocytes, the antagonist significantly reduced fenoterol action by 100%. This indicates that the obese adipocytes were more responsive to ICI 118 551, than the lean adipocytes suggesting a higher number of β_2 adrenoceptors on the obese adipocytes.

This could reflect the greater expanse of plasma membrane associated with the much larger size of the obese adipocytes. However, the number of β_2 adrenoceptors per obese adipose cell has been reported to be lower than lean adipocytes.

Arch and co-workers (1984) reported BRL 37344 (specific β_3 agonist) to be 400 and 20 fold selective for rat brown adipocyte lipolysis as compared to atrial β_1 and tracheal β_2 adrenoceptor responses. The lipolytic potency was demonstrated 5-6 times higher than isoproterenol. Similarly, Hollenga and Zaagsma (1989) demonstrated BRL 37344 to be over 10 times more lipolytically potent than isoproterenol. In this study BRL 37344 (10^{-9} - 10^{-5} M) significantly increased lipolysis in the lean adipocytes by 200-400%, suggesting that the β_3 adrenoceptor is most abundant on the lean adipocytes versus the β_1 and the β_2 adrenoceptor. The lipolytic effect of the BRL 37344 on the lean adipocytes was completely abolished by the β_3 antagonist (SR 59230A). An increase in lipolysis was also observed with the BRL 37344 (10^{-8} - 10^{-5} M) in the obese adipocytes by 50-100%, however the effect was lower versus the lean adipocytes suggesting a reduction in adrenoceptor number on the obese cell surface membrane. Similarly, the SR 59230A antagonist inhibited the BRL 37344-stimulated lipolysis in the obese adipocytes. In a recent study, the β_3a and β_3b receptor subtypes were identified and characterisation of these adrenoceptors demonstrated that BRL 37344 had almost identical binding affinities for the two distinct receptors (Hutchinson *et al.*, 2002).

Below is a summary table (table 3) indicating the % increase in lipolysis in the lean and obese adipocytes with the various agonists (range of concentrations). The table also shows the % inhibition of the agonists by the respective antagonists (at 10^{-6} M

concentration). For each agonist the appropriate antagonist produced a proportionally similar effect with the apparent exception of propranolol in lean mouse adipocytes, which were strongly responsive to isoproterenol. This indicates the existence of other β receptors in the adipocytes that are responsive to isoproterenol but not proportionally inhibited by a non-specific antagonist.

Table 4: Antagonistic effects of β antagonists on rat white adipocyte lipolysis induced by various agents

<u>Adipocytes - % increase in lipolysis</u>		
<u>(% inhibition by antagonist)</u>		
Agonist (Antagonist)	Lean	Obese
Isoproterenol (Propranolol)	150-250% (100-150%)	25-75% (40-75%)
Dobutamine (Atenolol)	50% (50%)	50-100% (50-100%)
Fenoterol (ICI 118 551)	100% (100%)	50-100% (20-100%)
BRL 37344 (SR 59230A)	200-400% (200-400%)	50-100% (50-100%)

Lipolysis was not stimulated with either of the α adrenoceptor agonists tested, namely the α_1 specific (phenylephrine) and α_2 specific (clonidine) adrenoceptor agonists. It is well known that activation of the α adrenoceptors inhibits lipolysis, which is consistent with the lack of stimulation of lipolysis or a small reduction in mean values seen (Lafontan and Berlan, 1993).

The stimulation of lipolysis in the lean and obese adipocytes is predominantly mediated by the β_1 , β_2 and β_3 adrenoceptors. From the results of the present characterisation it is confirmed that β_1 and β_2 adrenoceptors play a minor role in lipolysis of WAT, with the β_1 and β_2 selective agonists exerting a stimulatory effect only at high concentrations. Hence, the β_3 adrenoceptor appears to be the most important receptor involved in adipocyte lipolysis.

Chapter 6: Sibutramine and its Metabolites

6.1 Introduction

Monoamine reuptake inhibitors, which act by increasing levels of serotonin (5-HT) and noradrenaline (NA) in specific areas of the brain, are the most promising pharmacological agents used in the treatment of obesity (chapter 1.12). One such treatment is sibutramine (BTS 54524; N-[1-(4-chlorophenyl)-cyclobutyl]-3-methylbutyl]-N,N-dimethylamine hydrochloride monohydrate), a centrally-acting agent that dose-dependently inhibits serotonin and noradrenaline reuptake (table 1). It is a member of a new class of drugs called serotonin and noradrenaline reuptake inhibitors (SNRIs). Sibutramine is a tertiary amine and when administered to humans and animals it is demethylated to form the secondary (M1; BTS 54 354) amine and then the primary (M2; BTS 54 505) amine.

Table 1: Pharmacological Properties of Sibutramine and its Metabolites

Pharmacological properties of sibutramine and its metabolites
M1 and M2 metabolites are 100-fold more potent reuptake inhibitors than sibutramine
Sibutramine decreases food intake in a dose-dependent manner
Sibutramine decreases size and duration of meals and decreases body weight
Low abuse potential in rats and humans

Some thermogenic activity evident at doses of 10mg/kg
sibutramine

(Adapted from McNeely and Goa, Sibutramine: A Review of its Contribution to the Management of Obesity. *Drugs*. 1998, Vol. 56, page 1101).

In clinical studies sibutramine has been shown to promote a mean weight loss of 8kg in obese patients (Lean, 1997). Sibutramine produced statistically and clinically significant weight loss in patients treated with the drug for 24 weeks (Fujioka *et al.*, 2000). In addition these patients also showed improvements in fasting insulin, triacylglycerides, HDL cholesterol and quality of life assessments. In humans and rats, food intake is significantly reduced after the administration of sibutramine (Rolls *et al.*, 1998; Fantino *et al.*, 1995). The hypophagic effects of sibutramine are due to the enhancement of satiety in the ventromedial hypothalamus. The effects are mediated at least in part through the activation of 5-HT_{2C} receptor, where agonists for this receptor subtype have demonstrated a marked decrease in food intake in food-deprived rats (Bickerdike *et al.*, 1999). Sibutramine has also been shown to improve glycaemic control in overweight type 2 diabetic patients (McNeely and Goa, 1998). In *ob/ob* mice, sibutramine has been shown to reduce weight gain, lower NEFA concentrations, decrease hyperinsulinaemia and ameliorate insulin resistance (Day and Bailey, 1998). Side effects of sibutramine include an increase in blood pressure and heart rate along with minor adverse effects such as headache, dry mouth, anorexia, insomnia and constipation.

Studies in rodents and humans indicate that the metabolites are largely responsible for the action of sibutramine by inducing satiety (Halford *et al.*, 1995), stimulating thermogenesis (Connoley *et al.*, 1999) and reducing body weight gain (Fantino *et al.*, 1995; Weintraub *et al.*, 1991). In *ob/ob* mice, acute administration of M2 lowered blood glucose concentration (Day and Bailey, 1998). *In vitro*, the M1 and M2 metabolites were more active than sibutramine at inhibiting the reuptake of serotonin into rat cortical slices (Cheetham *et al.*, 1990; Luscombe *et al.*, 1989).

Sibutramine metabolites have been shown to increase glucose uptake *in vitro* using cultured L6 muscle cells. M2 has been shown to increase glucose uptake at 24h and M1 at 72h (Bailey *et al.*, 2001). The glucose uptake has been shown to be independent of the SNRI properties of sibutramine. Therefore sibutramine and its metabolites appear to have provided a means of reducing insulin resistance, which is separate from that associated with decreased adiposity (Bailey *et al.*, 2001).

6.2 Section One: *In vitro* lipolysis experiments

Since the active metabolites of sibutramine have a peripheral effect on skeletal muscle, there is a possibility that M1 and M2 could also act on the adipose tissue. Sibutramine and its metabolites may increase weight loss and/or reduce weight gain by acting directly on the adipose tissue, to increase lipolysis. To investigate this possibility, sibutramine, M1 and M2 were incubated with lean and obese (*ob/ob*) adipocytes. The 3T3-L1 preadipocytes were also used as an additional model to study the lipolytic action of sibutramine and its metabolites.

6.2.1 Methods

6.2.2 Effect of Sibutramine, M1 and M2 on Lipolysis

Adipose tissue from lean and *ob/ob* mice was removed (as described 2.3.1), characterised (chapter 5) and the isolated adipocytes were incubated with M1, M2 and sibutramine (10^{-12} to 10^{-5} M). Sibutramine, M1 and M2 (supplied by Knoll Pharmaceuticals) were dissolved in PBS by sonication for approximately 1h prior to use. Experiments on the isolated adipocytes were at 37°C for a 2h incubation period, to ensure cell viability. The 3T3-L1 preadipocytes were grown to confluence in 24 well plates (2.4.1) and incubated with M2 (10^{-11} to 10^{-5} M) for 2h, 8h and 24h at 37°C. Similarly, sibutramine and M1 (10^{-10} to 10^{-6} M) were incubated with the preadipocytes for 2h and 24h at 37°C. The concentration of glycerol released into the medium, which is indicative of triacylglyceride breakdown, was measured enzymatically (see 2.3.4).

Concentration-response curves for sibutramine, M1 and M2 were expressed in mM glycerol/ 10^5 adipocytes for the isolated adipocytes and mM glycerol/ 10^6 preadipocytes per ml for the 3T3-L1 preadipocyte work. Data are given as means \pm SEM, n=6 (duplicates analyses from three separate experiments). Significance was assessed by ANOVA post Dunnett's test. Probability levels of $p < 0.05$ were considered to indicate a significant difference.

6.2.3 Mechanism of Action of M2

To determine the mechanism of action of the active M2 on the adipose tissue the following experiments were performed on the isolated adipocytes and the cultured 3T3-L1 preadipocytes.

6.2.3a Isolated adipocytes

To determine the effect of the β_3 adrenoceptor agonist (BRL 37344) on a typical M2 concentration-response curve (10^{-10} - 10^{-5} M), adipocytes were isolated from the adipose tissue and incubated with BRL 37344 (10^{-8} M) for 2h.

In addition selective α and β adrenoceptor antagonists (10^{-6} M) were incubated with M2 (10^{-10} to 10^{-5} M) for approximately 2h at 37°C to characterise the effects of M2 on *in vitro* lipolysis in isolated lean and obese adipocytes. The selective antagonists (table 2) were as follows:

Table 2: Selective α and β Adrenoceptor Antagonists

ADRENOCEPTOR TYPE	ANTAGONISTS
Non-selective β	Propranolol
β_1	Atenolol

β 2	ICI 118 551
β 3	SR 59230A
α 1	Prazosin
α 2	Yohimbine

Other compounds were also used to further characterise and determine the mechanism of action of M2 (10^{-10} to 10^{-5} M) (table 3).

Table 3: Other Compounds

Compound	Site of Action
H-89 (10^{-6} M)	Inhibitor of cAMP dependent A kinase
SB 202190 (10^{-6} M)	MAP kinase inhibitor

The effects of the antagonists (as described in chapter 5) and known inhibitors were used to investigate the lipolytic pathway of M2 on the adipocytes.

6.2.3b 3T3-L1 preadipocytes

Noradrenaline (10^{-8} M) was incubated with a typical M2 concentration-response curve (10^{-8} - 10^{-6} M) on the preadipocytes to determine any additional lipolytic effect using the non-selective α/β adrenoceptor agonist.

Similarly, incubations on the 3T3-L1 preadipocytes for 24h with serotonin, noradrenaline and agents (table 4) known to stimulate release or inhibit reuptake of these substances in nervous tissues were performed. Concentrations of these agents were 10^{-8} - 10^{-6} M and were incubated with or without noradrenaline (10^{-6} M) to establish any additional lipolytic effect. The effects of these agents on the preadipocytes were used to compare with sibutramine, M1 and M2.

Table 4: Serotonin and Noradrenaline Releasing and Inhibiting Agents

Agent	Description
Fluoxetine	Selectively inhibits the reuptake of serotonin
Nisoxetine	Selectively inhibits the reuptake of noradrenaline
Phentermine	Noradrenaline releasing anorectic agent
Fenfluramine	Serotonin releasing anorectic agent
Nordexfenfluramine	Active metabolite of fenfluramine

6.2.4 Results

6.2.4a Effect of Sibutramine, M1 and M2 on Glycerol Release by Lean and Obese Mouse Adipocytes and 3T3-L1 Preadipocytes

M2 (10^{-10} - 10^{-5} M) significantly increased lipolysis by white adipocytes isolated from lean mouse adipose tissue by 100-125%, respectively. Similarly, in adipocytes isolated from *ob/ob* mice, M2 (10^{-9} - 10^{-5} M) increased lipolysis by 125-200% ($p < 0.05$). The lipolytic rate of M2 (10^{-5} M), measured by glycerol release was significantly higher in the obese adipocytes versus the lean adipocytes (0.064 ± 0.007 versus 0.042 ± 0.003 mM glycerol/ 10^5 adipocytes, $p < 0.05$) (see figure 6.2a). In contrast, M1 and sibutramine failed to stimulate lipolysis in the adipocytes isolated from lean and obese mice (see figure 6.2b and 6.2c).

The 3T3-L1 cell line was used to investigate the lipolytic effect of M2 at longer incubation times. In 3T3-L1 preadipocytes, incubation with M2 (10^{-11} - 10^{-5} M) for 24h increased lipolysis (measured by glycerol release) by 100-150% ($p < 0.05$) in a concentration-related manner up to 10^{-5} M. A similar result was observed at 2h and 8h after M2 (10^{-5} M) incubation with an increase in lipolysis by 50% and 100%, respectively (see figure 6.2d). In comparison, the secondary amine M1 was ineffective at increasing lipolysis in the 3T3-L1 preadipocytes at 2h and 24h incubation (figure 6.2e). Similarly, sibutramine had no effect on the 3T3-L1 preadipocytes at 2h and 24h incubation (figure 6.2f).

Figure 6.2a Lipolytic activity of M2 on lean and obese mouse fat. **Figure 6.2b** Lipolytic activity of M1 on lean and obese mouse fat. **Figure 6.2c** Lipolytic activity of sibutramine on lean and obese mouse fat. Adipocytes were exposed to sibutramine, M1 and M2 (10^{-12} - 10^{-5} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (n=6). *p<0.05 versus control, + p<0.05 versus lean adipocytes.

Figure 6.2a

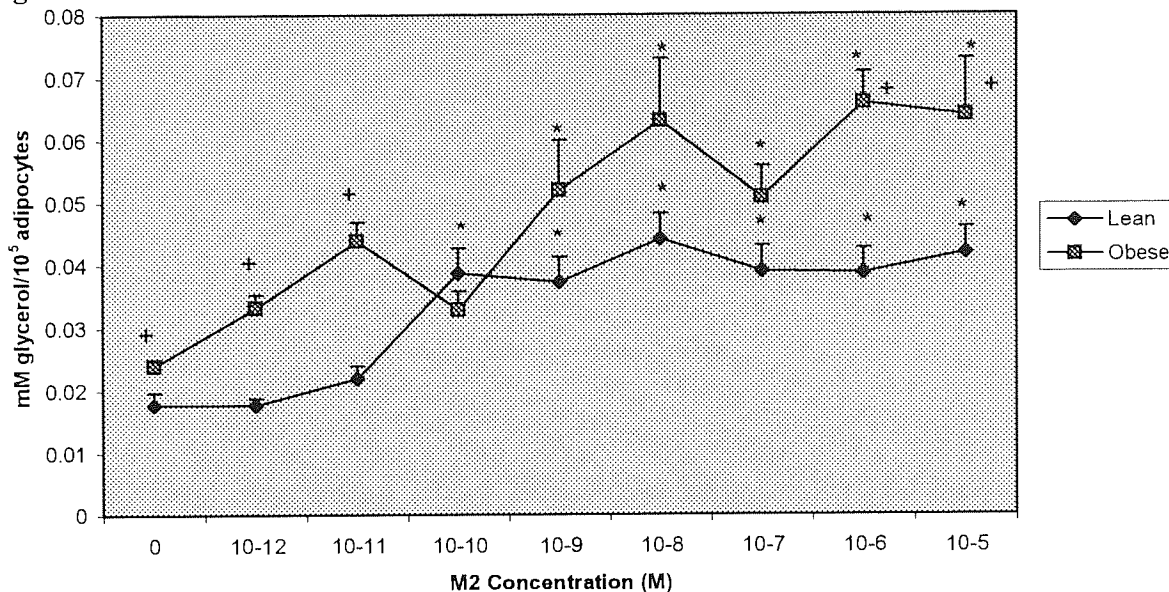


Figure 6.2b

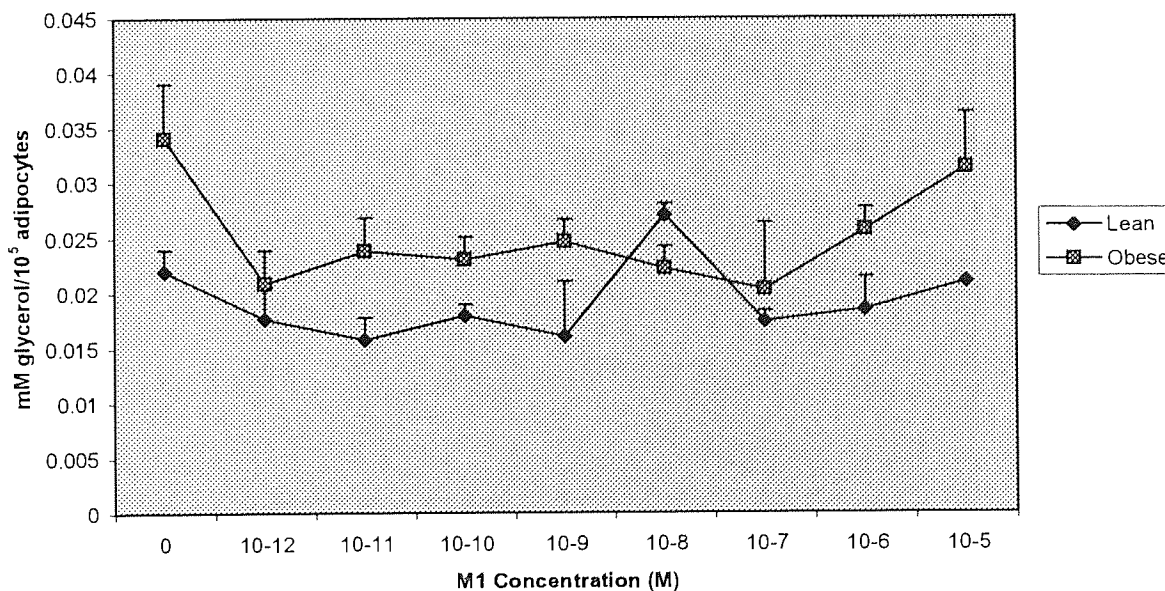


Figure 6.2c

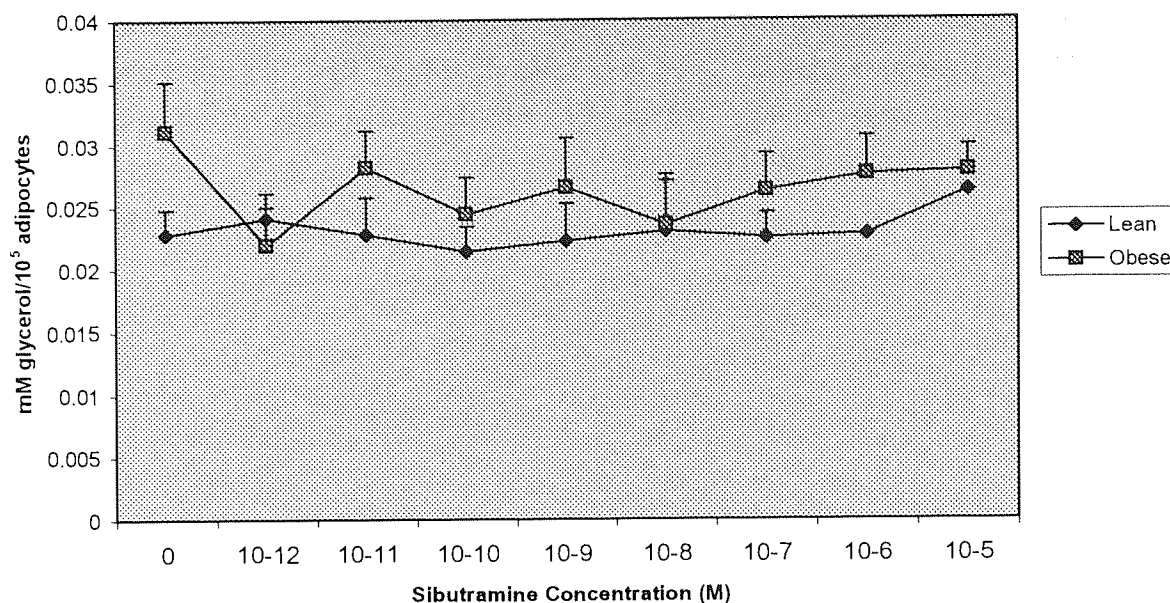


Figure 6.2d Lipolytic activity of M2 on 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were exposed to M2 (10⁻¹¹-10⁻⁵M) for 2h, 8h and 24h at 37°C. Results expressed as mM glycerol/10⁶ preadipocytes. Mean values ± SEM (n=6). *p<0.05 versus control.

Figure 6.2d

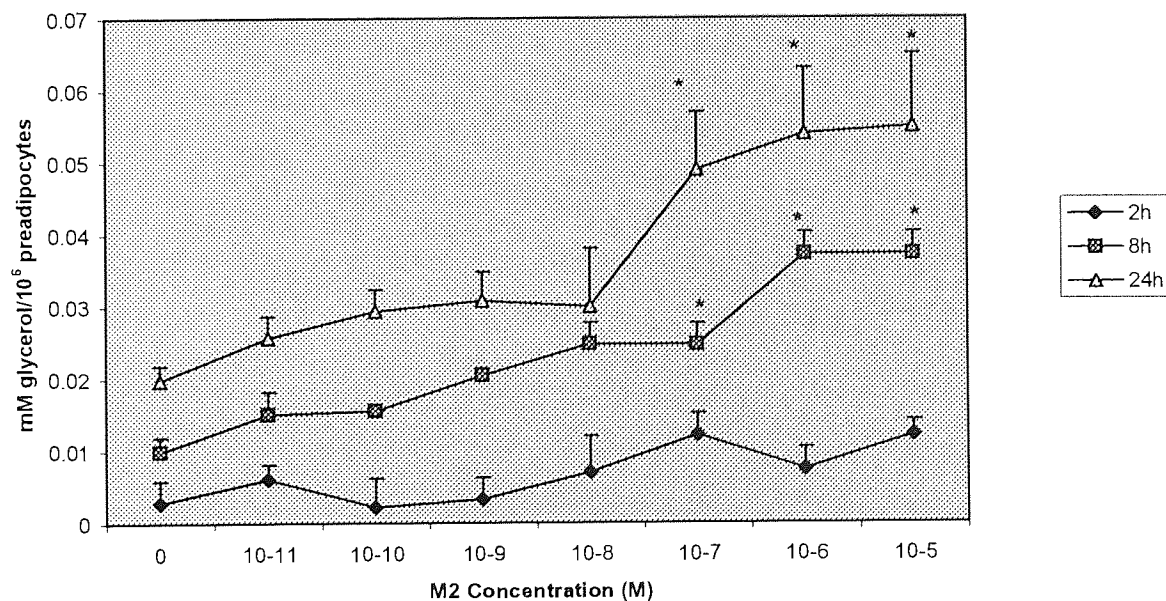


Figure 6.2e Lipolytic activity of M1 on 3T3-L1 preadipocytes. **Figure 6.2f** Lipolytic activity of sibutramine on 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were exposed to M1 and sibutramine (10^{-10} - 10^{-6} M) for 2h and 24h at 37°C. Results expressed as mM glycerol/ 10^6 preadipocytes. Mean values \pm SEM (n=6). *p<0.05 versus control.

Figure 6.2e

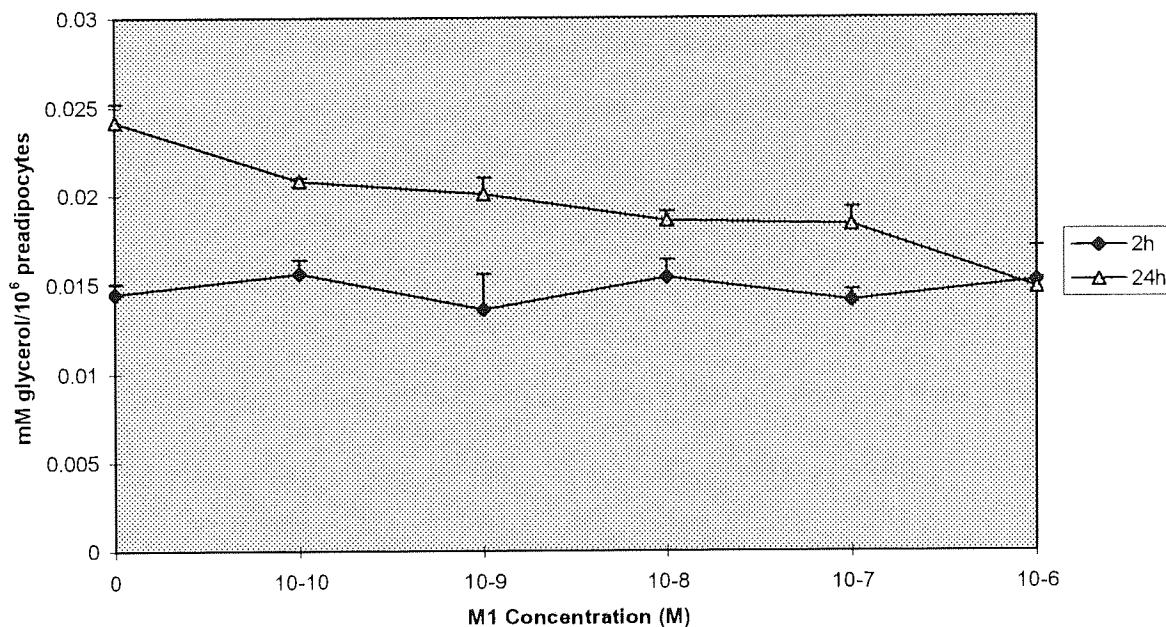
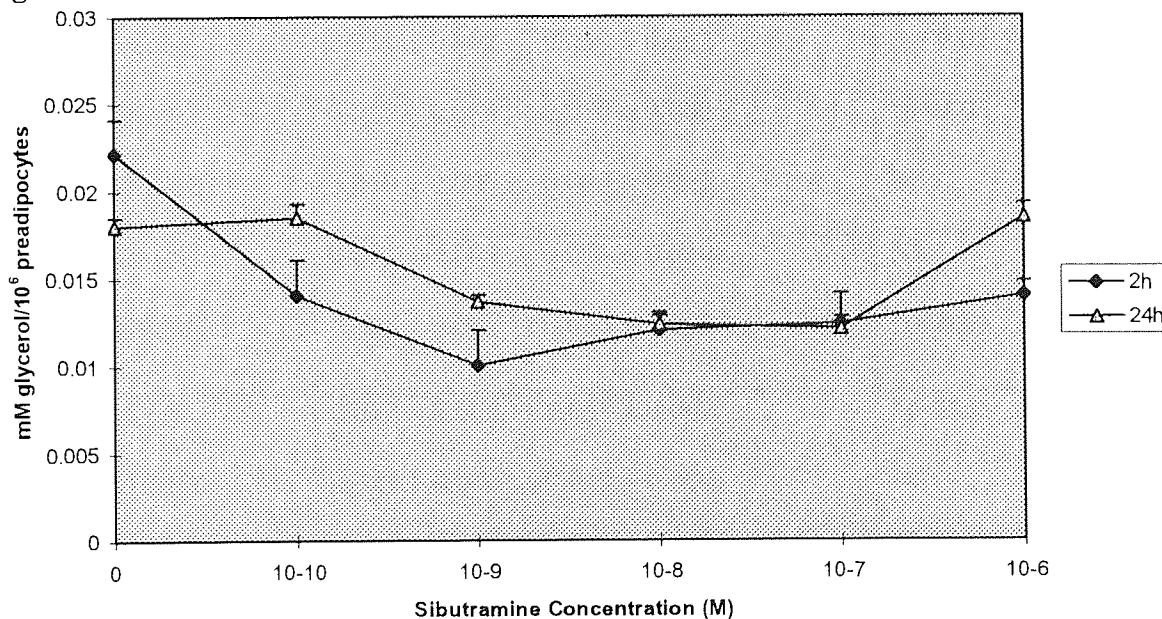


Figure 6.2f



6.2.4b Mechanism of Action of M2

During 2h incubations on isolated lean and obese adipocytes, BRL 37344 (10^{-8}M) significantly shifted a M2 concentration-response curve (10^{-10} - 10^{-5}M) to the left, but did not alter the maximum lipolytic effect of M2 (table 5). In addition, noradrenaline (10^{-8}M) on the 3T3-L1 preadipocytes significantly increased lipolysis. However, noradrenaline had no additional effect when incubated with M2 at 10^{-8} to 10^{-6}M for 24h (see figure 6.3).

Table 5: Lipolytic Activity of M2 \pm BRL 37344 (10^{-8}M) on Lean and Obese Mouse Fat.

Concentration M2	M2 alone	M2 + BRL 37344 10^{-8}M	Significance	
0	LEAN	0.021 \pm 0.003	0.046 \pm 0.004	*p<0.05
	OBESE	0.031 \pm 0.002	0.038 \pm 0.002	*p<0.05
10^{-10}	LEAN	0.022 \pm 0.005	0.050 \pm 0.002	*p<0.05
	OBESE	0.040 \pm 0.005	0.048 \pm 0.005	NS
10^{-9}	LEAN	0.042 \pm 0.004	0.061 \pm 0.010	NS
	OBESE	0.036 \pm 0.008	0.055 \pm 0.001	*p<0.05
10^{-8}	LEAN	0.042 \pm 0.005	0.057 \pm 0.002	*p<0.05
	OBESE	0.042 \pm 0.006	0.049 \pm 0.006	NS
10^{-7}	LEAN	0.057 \pm 0.009	0.074 \pm 0.004	NS
	OBESE	0.049 \pm 0.003	0.061 \pm 0.006	NS

10^{-6}	LEAN	0.065 ± 0.007	0.077 ± 0.003	NS
	OBESE	0.053 ± 0.004	0.069 ± 0.003	* $p < 0.05$
10^{-5}	LEAN	0.064 ± 0.007	0.066 ± 0.007	NS
	OBESE	0.060 ± 0.003	0.069 ± 0.006	NS

Table 5. Lipolytic activity of M2 \pm BRL 37344 (10^{-8} M) on lean and obese mouse fat. Adipocytes were exposed to M2 (10^{-10} - 10^{-5} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (n=6). * $p < 0.05$ versus M2 alone.

M2 (10^{-10} and 10^{-8} - 10^{-5} M) significantly increased lipolysis in lean adipocytes and propranolol (10^{-6} M) significantly inhibited lipolysis by 100-150% at 10^{-10} , 10^{-8} , 10^{-6} and 10^{-5} M (see figure 6.4a). Similarly, M2 (10^{-10} - 10^{-5} M) incubated with obese adipocytes significantly increased lipolysis by 75-200% and its lipolytic effects were inhibited by propranolol (10^{-6} M), at 10^{-9} - 10^{-5} M (see figure 6.4b).

Atenolol (10^{-6} M), a selective β_1 antagonist significantly inhibited M2 (10^{-5} M)-stimulated lipolysis in lean and obese adipocytes by 100% and 150%, respectively. A significant inhibition was also achieved with the selective β_3 antagonist, SR 59230A (10^{-6} M). In the obese adipocytes, a complete inhibition was observed at all concentrations of M2 using the SR 59230A. Similarly, the SR 59230A reduced the M2-stimulated lipolysis by lean adipocytes and a right-hand shift in the M2 curve can clearly be seen in figure 6.4a. In contrast, ICI 118 551 (10^{-6} M) (selective β_2 antagonist) did not inhibit the M2-stimulated lipolysis by the lean adipocytes. However, a slight inhibition was observed by the obese adipocytes at 10^{-6} and 10^{-5} M M2 concentrations (see figure 6.4b). The α adrenoceptor antagonists, prazosin (α_1)

and yohimbine (α_2) did not significantly inhibit M2-stimulated lipolysis by both lean and obese isolated adipocytes (data not shown).

In lean adipocytes, H-89 (a selective inhibitor of cAMP dependent A kinase) significantly inhibited M2 (10^{-8} and 10^{-6} M)-stimulated lipolysis. Although there was a reduction in mean values for M2-stimulated glycerol release in obese adipocytes incubated with the H-89 inhibitor, the effect proved to be statistically insignificant (see table 6).

Table 6: Lipolytic Activity of M2 \pm H-89 (10^{-6} M) on Lean and Obese Mouse Fat.

Concentration M2	M2 alone	M2 + H-89 10^{-6} M	Significance
0			
LEAN	0.027 \pm 0.008	0.023 \pm 0.002	NS
OBESE	0.019 \pm 0.006	0.023 \pm 0.004	NS
10^{-10}			
LEAN	0.043 \pm 0.006	0.042 \pm 0.009	NS
OBESE	0.032 \pm 0.002	0.048 \pm 0.027	NS
10^{-9}			
LEAN	0.053 \pm 0.009	0.033 \pm 0.004	NS
OBESE	0.058 \pm 0.016	0.029 \pm 0.010	NS
10^{-8}			
LEAN	0.078 \pm 0.006	0.043 \pm 0.013	*p<0.05
OBESE	0.053 \pm 0.008	0.041 \pm 0.019	NS
10^{-7}			
LEAN	0.069 \pm 0.007	0.052 \pm 0.012	NS
OBESE	0.065 \pm 0.011	0.052 \pm 0.013	NS
10^{-6}			
LEAN	0.081 \pm 0.011	0.044 \pm 0.006	*p<0.05
OBESE	0.066 \pm 0.011	0.049 \pm 0.029	NS

10^{-5}	LEAN	0.089 ± 0.012	0.110 ± 0.030	NS
	OBESE	0.0113 ± 0.025	0.073 ± 0.021	NS

Table 6. Lipolytic activity of M2 ± H-89 (10^{-6} M) on lean and obese mouse fat. Adipocytes were exposed to M2 (10^{-10} - 10^{-5} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values ± SEM (n=6). *p<0.05 versus M2 alone.

The potent inhibitor of p38 MAP kinase, SB 202190 (10^{-6} M), caused a significant inhibition of M2-induced lipolysis in lean and obese adipocytes (see figure 6.5a and 6.5b, respectively).

Figure 6.3 Lipolytic activity of M2 ± noradrenaline (10^{-8} M) on 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were exposed to M2 (10^{-8} - 10^{-6} M) for 24h at 37°C. Results expressed as mM glycerol/ 10^6 preadipocytes. Mean values ± SEM (n=6). *p<0.05 versus control. + p<0.05 versus M2 alone.

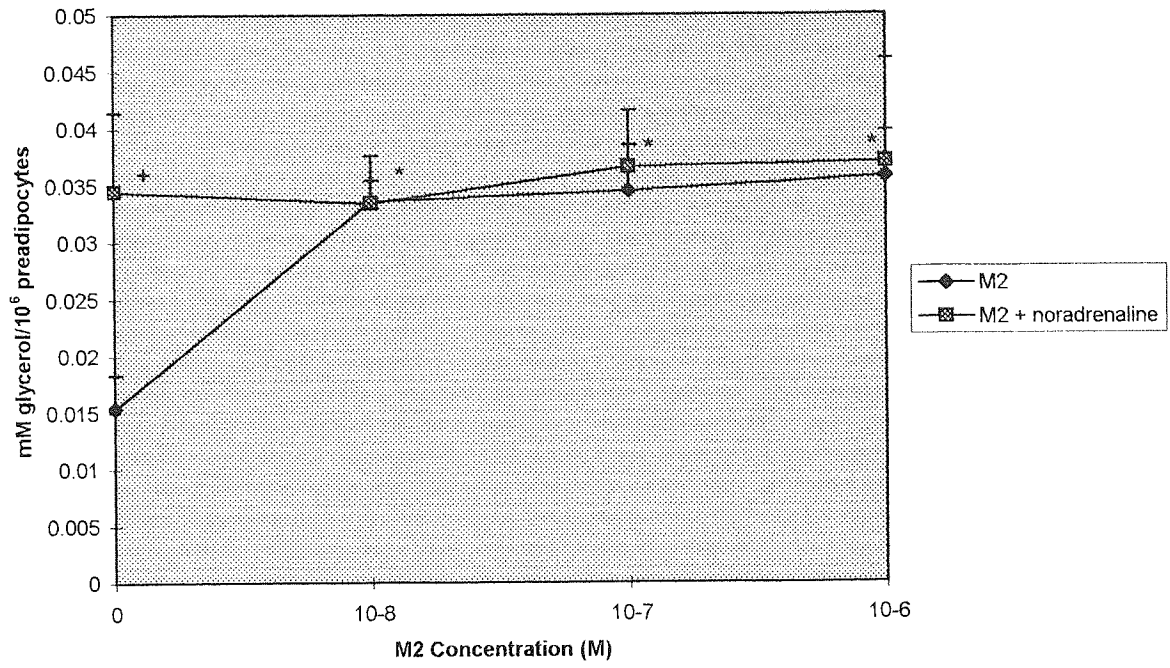


Figure 6.4a Lipolytic activity of M2 ± β antagonists (10^{-6} M) on lean mouse fat. Adipocytes were exposed to M2 (10^{-10} - 10^{-5} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (n=6). + p<0.05 versus M2 alone.

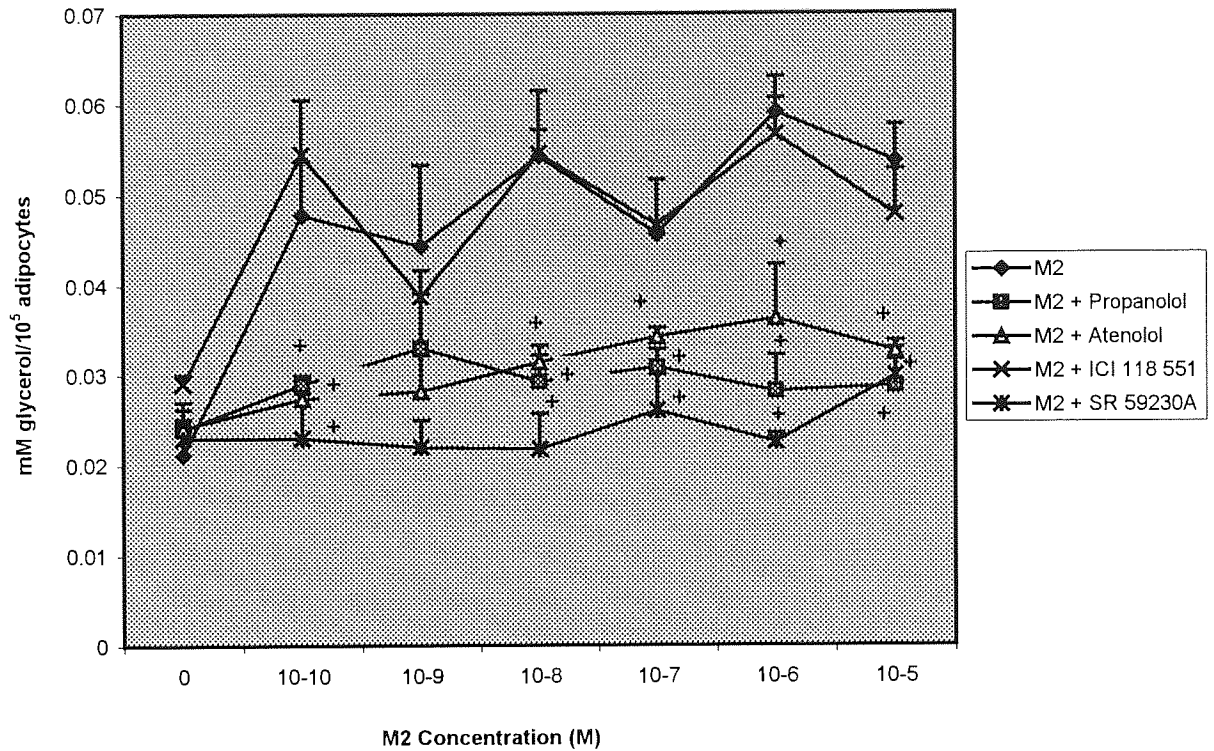


Figure 6.4b Lipolytic activity of M2 \pm β antagonists (10^{-6} M) on obese mouse fat. Adipocytes were exposed to M2 (10^{-10} - 10^{-5} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (n=6). + p<0.05 versus M2 alone.

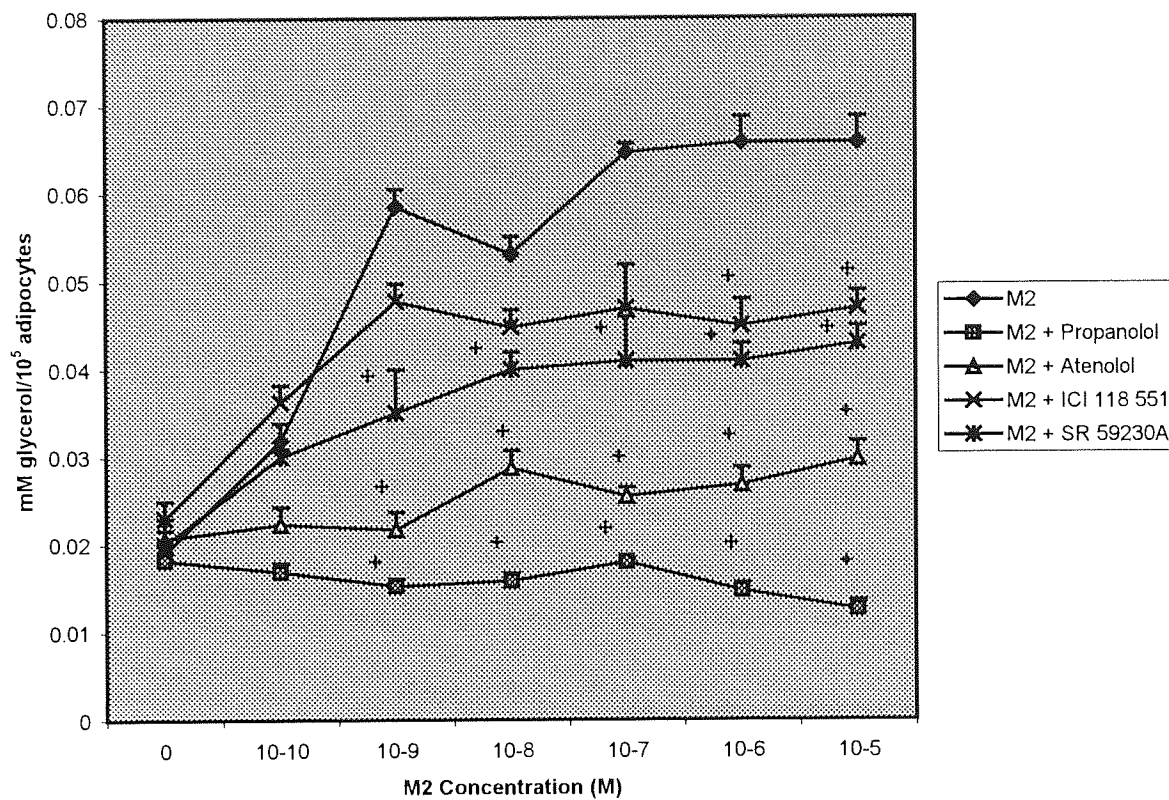


Figure 6.5a Lipolytic activity of M2 ± SB 202190 (10^{-6} M) on lean mouse fat. **Figure 6.5b** Lipolytic activity of M2 ± SB 202190 (10^{-6} M) on obese mouse fat. Adipocytes were exposed to M2 (10^{-10} - 10^{-5} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values ± SEM (n=6). + p<0.05 versus M2 alone.

Figure 6.5a

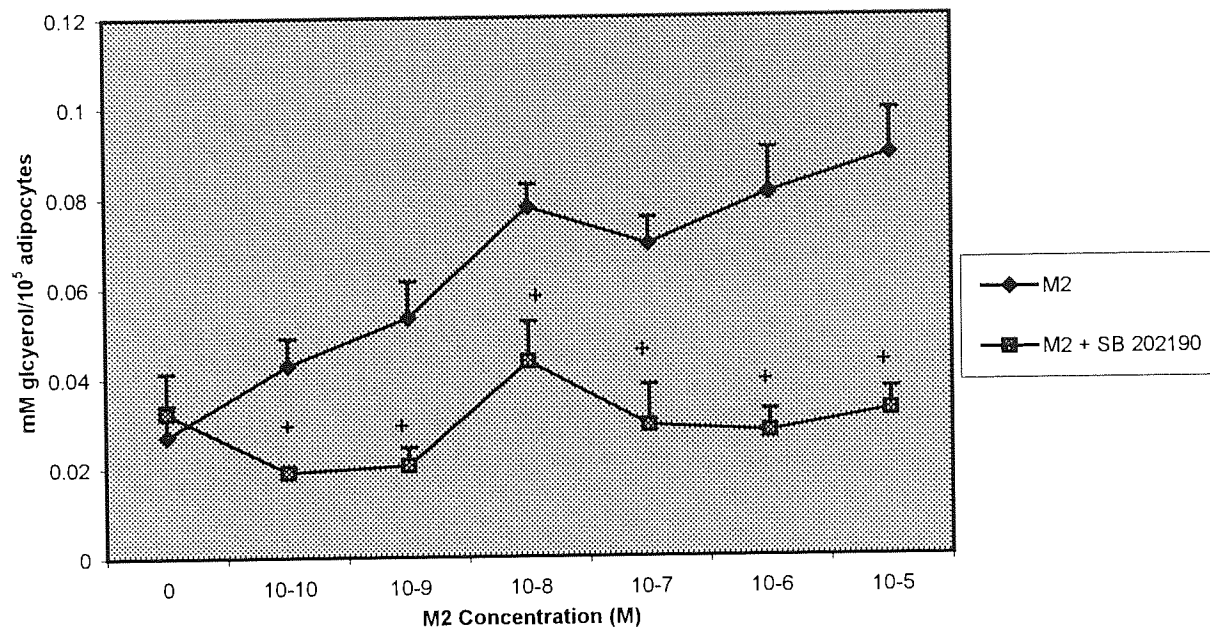
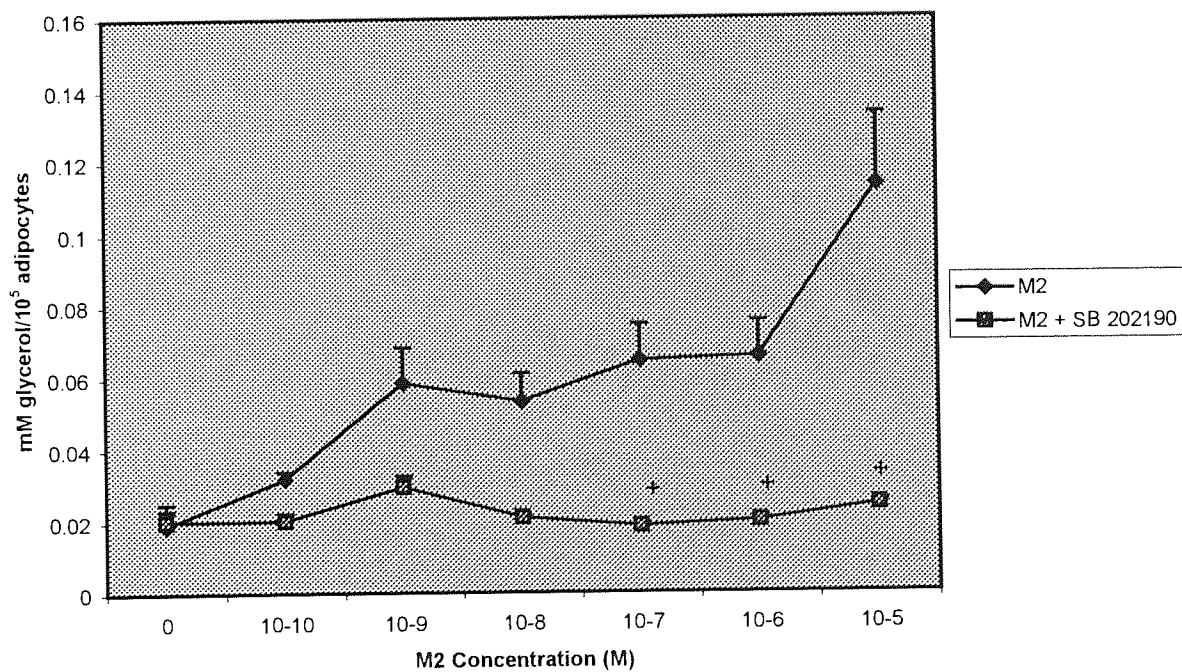


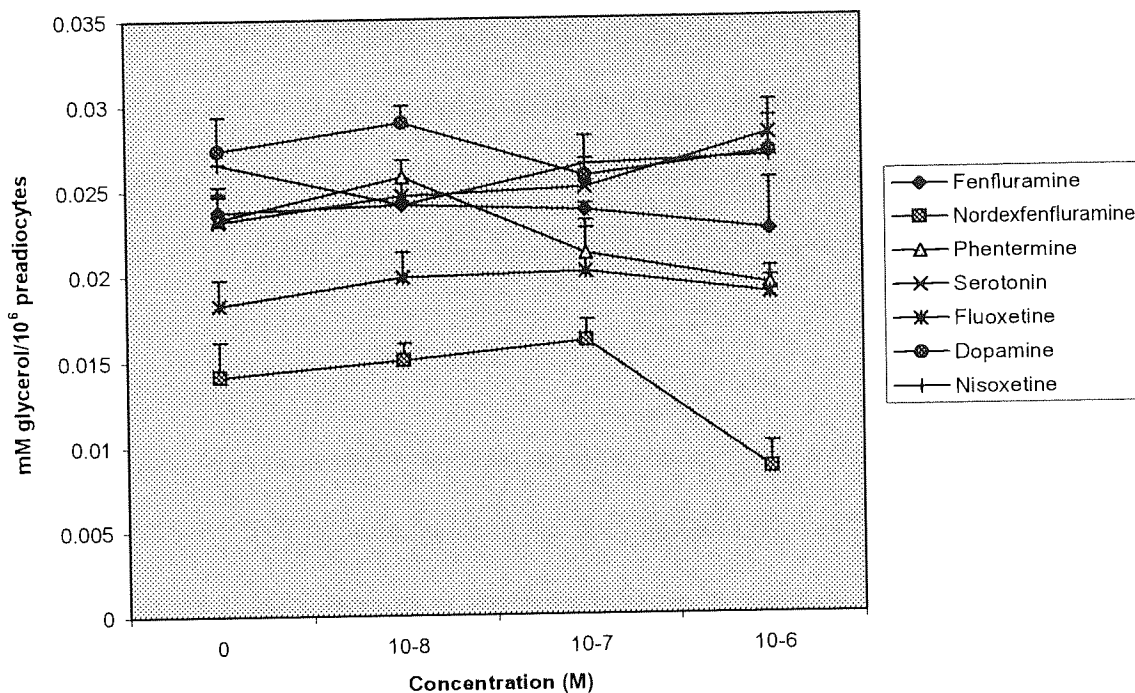
Figure 6.5b



6.2.4c Effect of Pharmacologically Related Compounds on Glycerol Release by 3T3-L1 Preadipocytes

Whereas M2 increases lipolysis, fenfluramine, nordexfenfluramine and phentermine did not significantly alter lipolysis in 3T3-L1 preadipocytes. Also, serotonin, fluoxetine, dopamine and nisoxetine were without a significant effect on lipolysis in these cells (see figure 6.6).

Figure 6.6 Lipolytic activity of fenfluramine, nordexfenfluramine, phentermine, serotonin, fluoxetine, dopamine and nisoxetine on 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were exposed to all agents at 10^{-8} - 10^{-6} M for 24h at 37°C. Results expressed as mM glycerol/ 10^6 preadipocytes. Mean values \pm SEM (n=6).



6.2.5 Discussion

M2 increased lipolysis in both lean and obese adipocytes. However, the lipolytic effect of M2 was less pronounced in adipocytes isolated from the lean mice. In addition, M2-stimulated lipolysis was achieved in the 3T3-L1 preadipocytes. Sibutramine and M1 were ineffective at increasing basal-stimulated lipolysis by the isolated adipocytes and cultured 3T3-L1 preadipocytes at all incubation times. This suggests that M2 is largely responsible for the action of sibutramine on the adipose tissue.

Propranolol, the non-selective β antagonist inhibited the effect of M2 in both lean and obese adipocytes. These results indicate that the lipolytic effect of M2 is mediated through either the β adrenoceptors or through a pathway similar to that described for lipolytic agonists (see chapter 5).

The β_1 (atenolol) and β_3 (SR 59230A) antagonist inhibited M2-stimulated lipolysis by the lean adipocytes, however the β_2 (ICI 118 551) antagonist was without effect. Similarly, atenolol and SR 59230A inhibited the lipolytic activity of M2 on the obese adipocytes. In addition, ICI 118 551 reduced the M2-stimulated glycerol release in obese adipocytes. Therefore, the responsive element of M2-stimulated lipolysis in the lean and obese adipocytes appears to be predominantly mediated through the β_1 and β_3 adrenoceptors. These results are consistent with the literature where the β_3 adrenoceptor plays a dominant role in lipolysis with a minor role for β_1 adrenoceptor being identified (Arner and Eckel, 1997; Germack *et al.*, 1997).

From the results, it appears that the β_2 adrenoceptors appear to play a role in M2-stimulated lipolysis in the obese adipocytes. During adipocyte characterisation (chapter 5), the obese adipocytes were more responsive to ICI 118 551 versus the lean adipocytes, which suggest a higher number of β_2 adrenoceptors on the obese adipocytes. However, the role of the β_2 adrenoceptor on rodent adipose tissue is uncertain and still needs to be investigated further.

The α_1 (prazosin) and α_2 (yohimbine) antagonist did not inhibit the lipolytic effect of M2 by the lean and obese adipocytes. The M2-stimulated lipolysis was not increased further with the addition of the α adrenoceptor antagonists, which suggests that M2 effects on the adipose tissue are mediated through the β adrenoceptors and are independent of the α adrenoceptors. Activation of the α adrenoceptors inhibits lipolysis (Guo *et al.*, 1997), as shown in chapter 5 where the α_1 specific (phenylephrine) and α_2 specific (clonidine) adrenoceptor agonists were ineffective at stimulating lipolysis. These agonists bind to the α adrenoceptor, which results in the coupling of the receptor to the G_i protein via adenylate cyclase. The α adrenoceptor antagonists inhibit the effect of the α agonists on the adipose tissue.

H-89, a selective inhibitor of cAMP dependent A kinase reduced M2-stimulated lipolytic response in both the lean and obese mouse adipocytes. This result further confirms that M2 is acting on the adipose tissue through the lipolytic pathway described in chapter 5.

With the addition of a sub-maximally stimulating BRL 37344 and noradrenaline concentration the basal-stimulated lipolytic effect was not increased further than the effect observed with M2 alone. This suggests that M2 directly acts on the adipose tissue to increase lipolysis independently of a sub-maximally stimulating adrenoceptor agonist concentration. However, β adrenoceptor antagonists inhibit the lipolytic effect of M2, which suggests that both M2 and BRL 37344/noradrenaline are mediating lipolysis through the same β adrenoceptor(s). This raises the possibility that the M2 and β agonists are competing for the same receptor. Hence, once the lipolytic agonist has bound to the receptor, the remaining agonist cannot generate a lipolytic response since receptor activation is required to mediate the response.

The β_3 adrenoceptor stimulates p38 MAP kinase PKA in white and brown adipocytes. The β_3 agonist CL 316 243 has been shown to stimulate the phosphorylation of p38 MAP kinase (Cao *et al*, 2001; Soeder *et al.*, 1999). To understand the mechanism of action of M2 on the adipocytes, SB 202190 (p38 MAP kinase inhibitor) was incubated with the metabolite. SB 202190 reduced the M2-stimulated lipolysis in both lean and obese adipocytes. This result illustrates that M2 activates PKA, which stimulates the phosphorylation of p38 MAP kinase and hormone sensitive lipase (HSL). It has been shown elsewhere that p38 MAP kinase activity is required for the β adrenoceptor dependent increase in UCP1 expression in brown adipocytes (Chiesi *et al* 2001; Cao *et al*, 2001). M2 acts on the adipose tissue to increase lipolysis and also activates the p38 MAP kinase signalling pathway, which plays a role in increasing UCP expression. Thermogenic activity was significantly increased in humans and rodents following administration of sibutramine (Stock *et al.*, 1998; Connoley *et al.*, 1995). These results suggest that M2 possibly has

thermogenic activity by increasing UCP expression in the adipocytes through its activation of the p38 MAP kinase pathway.

Fenfluramine, nordexfenfluramine and phentermine did not significantly alter lipolysis in 3T3-L1 preadipocytes. Also, serotonin, fluoxetine and nisoxetine failed to increase basal-stimulated lipolysis in these cells. Although it is unlikely that sibutramine and its metabolites could affect dopamine receptors or dopamine release, there was no evidence that dopamine could stimulate lipolysis in 3T3-L1 preadipocytes. This suggests that M2 can act directly on the adipocyte to increase lipolysis via a mechanism that appears to be independent of serotonin, dopamine and noradrenaline reuptake inhibition.

In conclusion M2 can act directly on adipose tissue to increase lipolysis via a pathway involving the β adrenoceptors and components of the lipolytic signalling pathway, notably PKA. M2 also activates the p38 MAP kinase pathway, which is an important downstream target of the β adrenergic/cAMP/PKA signalling pathway in adipocytes. The M2-stimulated lipolysis is independent of the serotonin and noradrenaline properties of sibutramine.

6.3 Section Two: *In vitro* glucose uptake experiments

Sibutramine acts via its metabolites to increase insulin-sensitive glucose uptake by cultured L6 muscle cells. This was shown to be independent of the SNRI properties of sibutramine (Bailey *et al.*, 2001). It is possible that sibutramine and its active metabolites may also act directly on the adipose tissue to increase glucose uptake. Thus, to investigate this possibility, sibutramine and its metabolites were incubated with 3T3-L1 differentiated cells (2.4.3). Glucose uptake was assessed using the 2-deoxy-D-[³H]-glucose analogue to measure basal and insulin-stimulated glucose uptake (2.4.4).

6.3.1 Method

6.3.2 Effect of Sibutramine, M1 and M2 on Basal and Insulin-Stimulated Glucose Uptake in 3T3-L1 differentiated cells

3T3-L1 cells were grown in 100mm culture dishes (2.4.1) characterised (chapter 3) and when confluent differentiated into adipocytes (2.4.3). Culture dishes were seeded at 10^7 cells/ml with a final concentration of 8×10^7 cells/ml (chapter 3). The cells were incubated with sibutramine, M1 and M2 with and without insulin (10^{-8} M) for 24h. At the end of the incubation period, the effect of the compounds on glucose transport into the cells was measured by ³H 2DG uptake (as described 2.4.4).

Concentration-response curves for sibutramine, M1 and M2 were expressed as % of control values. Data were given as means \pm SEM, n=6 for each concentration and test

compound (duplicates analyses from three separate experiments). Significance was assessed by ANOVA, post Dunnett's test and Student's paired t-test. Probability levels of $p < 0.05$ were considered to be significant.

6.3.3 Mechanism of Action of M2

To determine the mechanism of action of M2 on *in vitro* glucose uptake into 3T3-L1 differentiated cells the following experiments were performed.

6.3.3a 3T3-L1 differentiated cells

To determine the effect of M2 on the insulin concentration-response curve (10^{-9} - 10^{-5} M) (as described in chapter 3), 3T3-L1 differentiated cells were incubated with a maximal stimulating concentration of M2 (10^{-6} M) for 24h. A sub-maximally stimulating concentration of insulin (10^{-8} M) was also incubated with the M2 concentration-response curve (10^{-10} - 10^{-5} M) on the 3T3-L1 differentiated cells to determine additional increases on *in vitro* glucose uptake.

A selection of known inhibitors (table 7) of the intracellular signalling pathway of glucose transport in 3T3-L1 differentiated cells was used to determine the possible mechanisms by which M2 increases glucose transport. The inhibitors used have been described in chapter 3 to understand the mechanism of action in this cell line. In the

experiments, a maximal concentration of M2 (10^{-6} M) was used and incubated with the inhibitors for 24h at 37°C.

Table 7: Inhibitors of the Insulin-Stimulated Glucose Signalling Pathway

Compound	Site of Action
Cycloheximide (10^{-8} - 10^{-5} M)	Inhibits protein synthesis and therefore blocks the synthesis of glucose transporters
Cytochalasin B (10^{-9} - 10^{-6} M)	Inhibitor of glucose transporters
LY-294 002 (10^{-8} - 10^{-5} M)	Inhibitor of PI3-Kinase
Wortmannin (10^{-8} - 10^{-5} M)	Potent and selective inhibitor of PI3-Kinase

The concentration-response curves for the inhibitors on the 3T3-L1 differentiated cells are shown in brackets above.

In addition, 3T3-L1 differentiated cells were exposed to serotonin, noradrenaline and agents known to stimulate release or inhibit reuptake of these substances in nervous tissues (fluoxetine, nisoxetine, phentermine, fenfluramine and nordexfenfluramine; see chapter 6, table 4). The effects of these pharmacologically related compounds on glucose uptake in 3T3-L1 differentiated cells were used to compare with sibutramine, M1 and M2.

6.3.4 Results

6.3.4a Effect of Sibutramine, M1 and M2 on Basal and Insulin-Stimulated Glucose Uptake in 3T3-L1 differentiated cells

In the absence of added insulin, M2 (10^{-10} to 10^{-5} M) significantly increased glucose uptake into the 3T3-L1 cells (by 46%, 29%, 44%, 34%, 51% and 58% at each magnitude of concentration) (6.7a). Addition of a sub-maximally stimulating insulin concentration (10^{-8} M) to the M2 concentration-response curve did not significantly increase the uptake of 2DG in the 3T3-L1 differentiated cells. Therefore the stimulatory effect of M2 was not exceeded. M1 and sibutramine did not significantly increase glucose uptake into the 3T3-L1 differentiated cells (6.7b and 6.7c). Addition of insulin (10^{-8} M) to the M1 and sibutramine concentration-response curve for 24h significantly increased 2DG uptake into the 3T3-L1 differentiated cells at all concentrations 10^{-10} - 10^{-5} M. Addition of insulin (10^{-8} M) to the M1 (10^{-5} M) and sibutramine (10^{-5} M) curve significantly increased 2DG uptake by 18% and 26%, respectively. The uptake of 2DG in the 3T3-L1 differentiated cells is primarily due to insulin action.

Figure 6.7a Effect of M2 \pm insulin 10^{-8} M on 2DG uptake in differentiated 3T3-L1 cells.

Figure 6.7b Effect of M1 \pm insulin 10^{-8} M on 2DG uptake in differentiated 3T3-L1 cells.

Figure 6.7c Effect of sibutramine \pm insulin 10^{-8} M on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to sibutramine, M1 and M2 for 24h at concentrations 10^{-10} - 10^{-5} M. Results expressed as mean values \pm SEM (n= 6). * p<0.05 versus control, + p<0.05 versus sibutramine, M1 or M2 alone.

Figure 6.7a

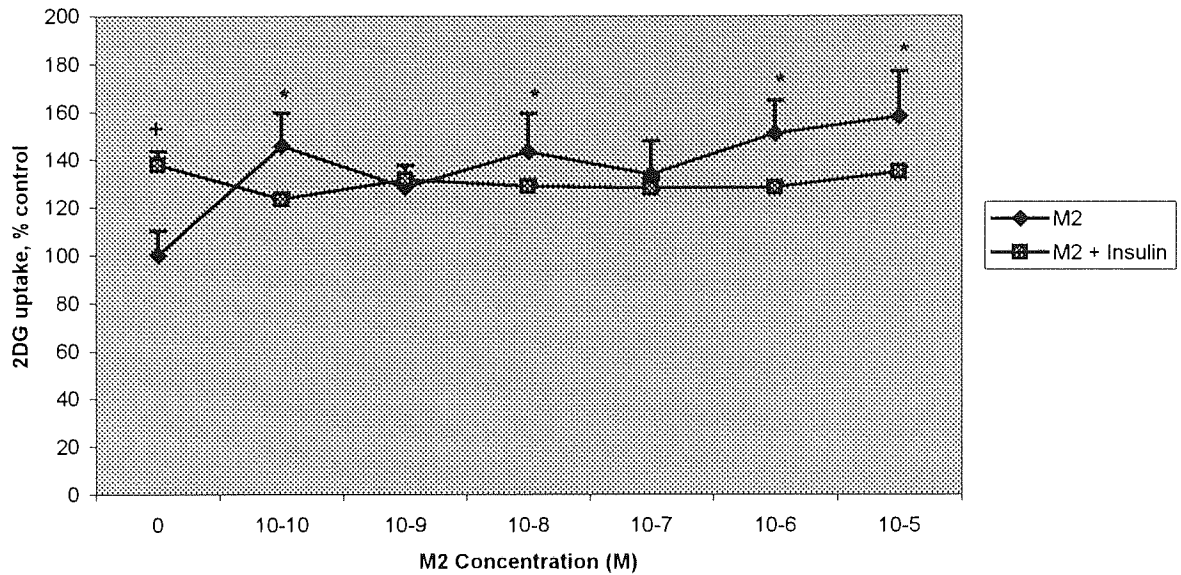


Figure 6.7b

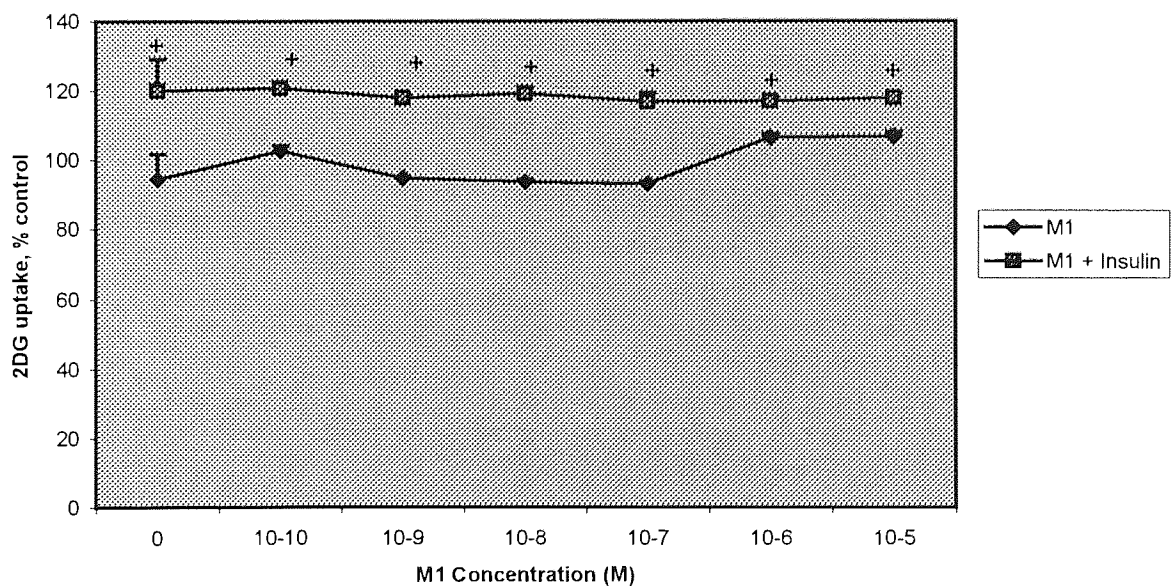
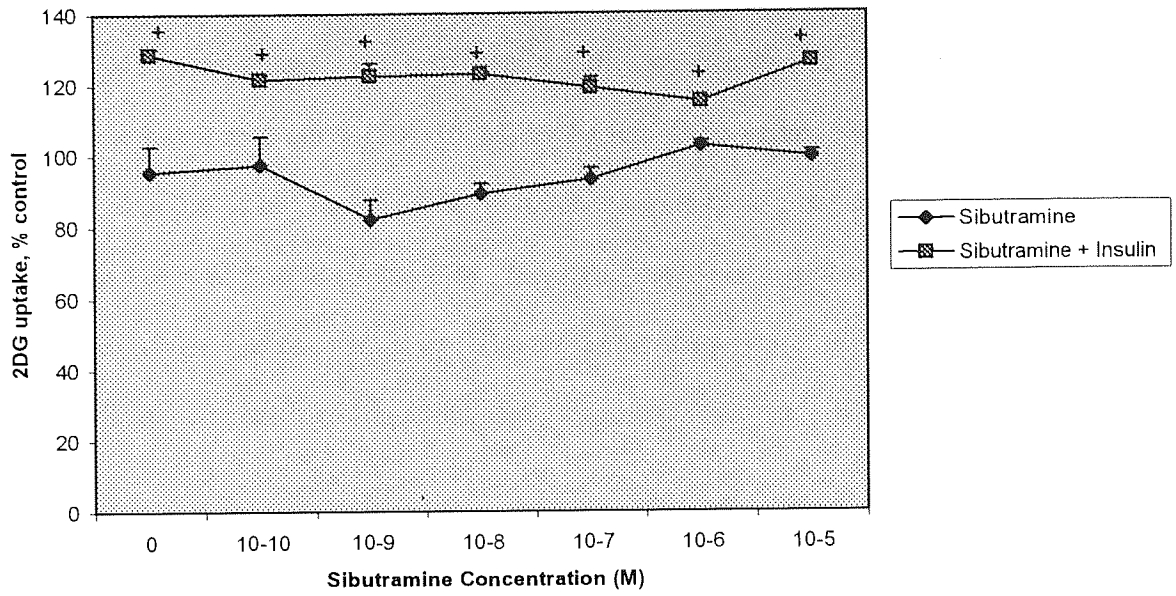


Figure 6.7c



6.3.4b Mechanism of Action of M2

During 24h incubations, M2 (10^{-6} M) significantly shifted a typical insulin concentration-response curve (10^{-9} - 10^{-5} M) to the left (ED_{50} values changed from 5×10^{-7} M in absence of M2 to 1×10^{-8} M in its presence, $p < 0.05$), but did not alter the maximum effect of insulin (figure 6.8). M2 significantly increased insulin (10^{-9} and 10^{-8} M)-stimulated glucose uptake by 31% and 38%, respectively. However, M2 did not significantly increase insulin-stimulated glucose uptake at 10^{-7} - 10^{-5} M.

The inhibitor of protein synthesis cycloheximide (10^{-7} - 10^{-5} M) caused a significant concentration-dependent inhibition of M2 (10^{-6} M)-induced 2DG uptake by 23%, 63% and 68% ($p < 0.05$). In addition, cycloheximide (10^{-6} and 10^{-5} M) significantly inhibited basal 2DG uptake by 20% (figure 6.9).

The glucose transport inhibitor, cytochalasin B significantly inhibited basal and M2-stimulated glucose transport (figure 6.10). Cytochalasin B (10^{-9} - 10^{-5} M) significantly inhibited M2 action at all concentrations by 128%-263%, respectively. Basal 2DG uptake was inhibited by cytochalasin B at 10^{-5} M only by approximately 68% ($p < 0.05$).

Inhibition of phosphatidylinositol 3-kinase (PI3-Kinase) activity using LY 294 002 (10^{-9} - 10^{-5} M) did significantly inhibit M2 action (figure 6.11). LY 294 002 at all concentrations significantly inhibited M2-stimulated 2DG uptake in a concentration-dependent manner by 79%-99% ($p < 0.05$). Similarly, LY 294 002 (10^{-8} - 10^{-5} M) inhibited basal stimulated 2DG uptake by 45%-35%, respectively.

Figure 6.8 Effect of insulin \pm M2 10^{-6} M on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to insulin for 24h at concentrations 10^{-9} - 10^{-5} M. Results expressed as mean values \pm SEM (n= 6). + p<0.05 versus insulin alone.

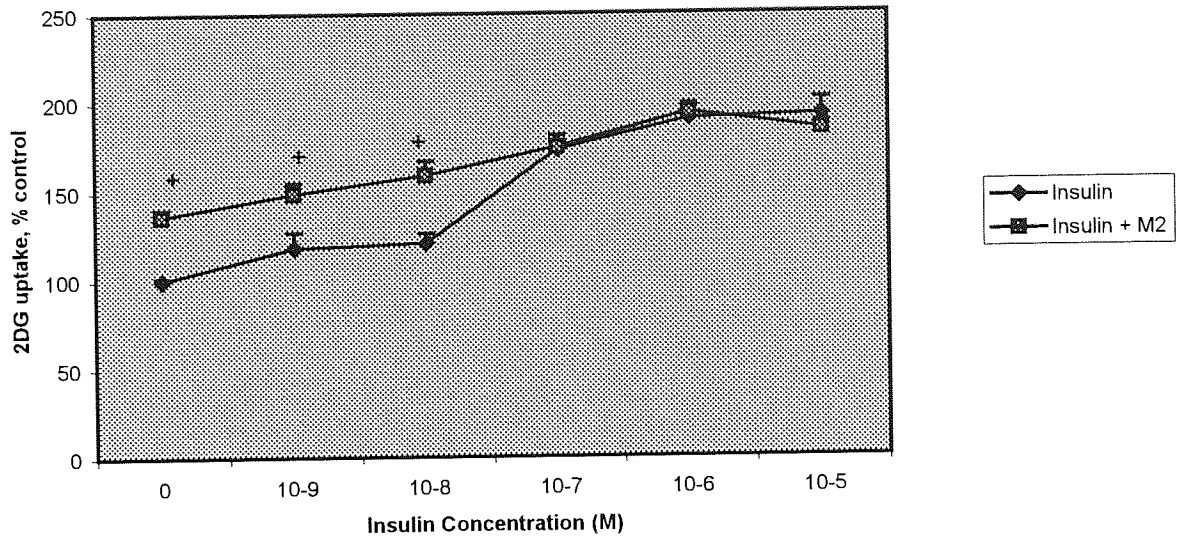


Figure 6.9 Effect of cycloheximide \pm M2 10^{-6} M on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to cycloheximide for 24h at concentrations 10^{-8} - 10^{-5} M. Results expressed as mean values \pm SEM (n= 6). * p<0.05 versus control.

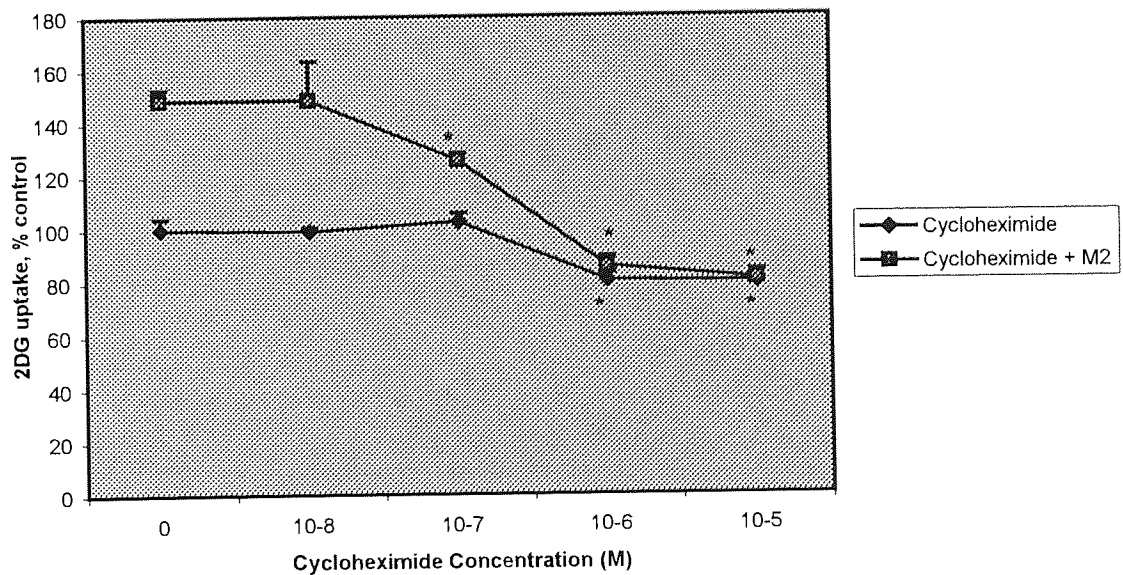


Figure 6.10 Effect of cytochalasin B \pm M2 10^{-6} M on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to cytochalasin B for 24h at concentrations 10^{-9} - 10^{-5} M. Results expressed as mean values \pm SEM (n= 6). * p<0.05 versus control.

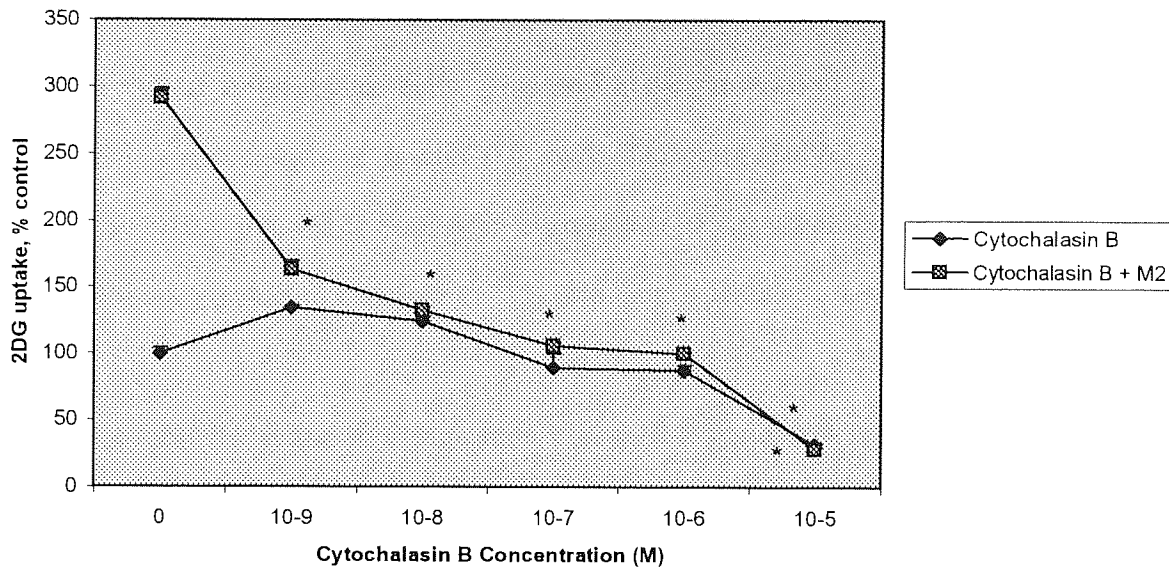
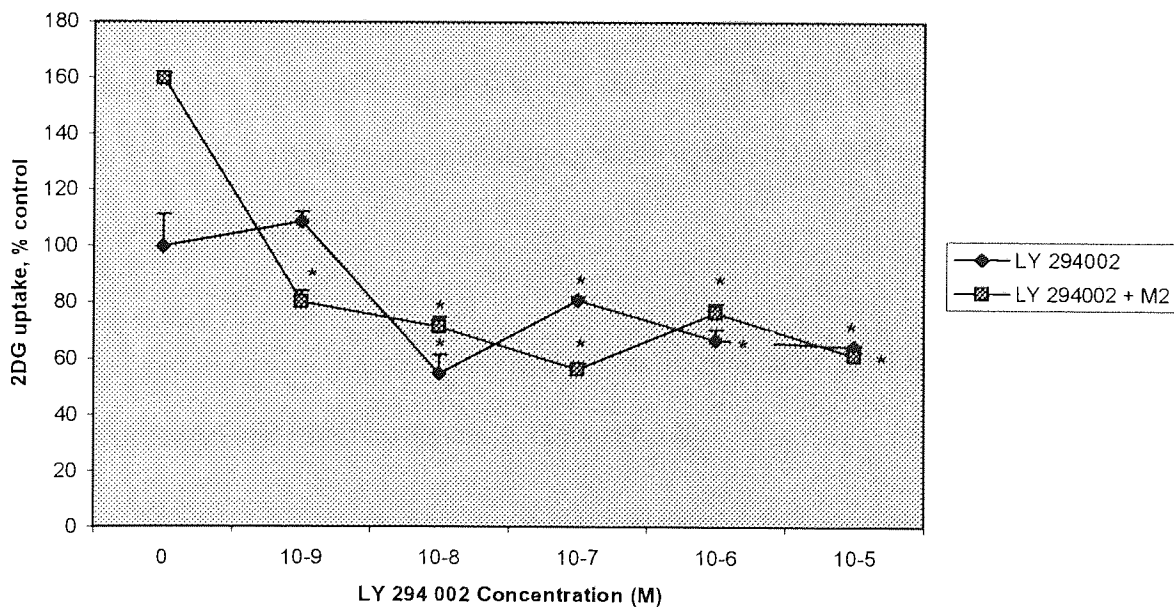


Figure 6.11 Effect of LY 294 002 \pm M2 10^{-6} M on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to LY 294 002 for 24h at concentrations 10^{-9} - 10^{-5} M. Results expressed as mean values \pm SEM (n= 6). * p<0.05 versus control.



6.3.4c Effect of Pharmacologically Related Compounds on Basal and Insulin-Stimulated Glucose Uptake in 3T3-L1 differentiated cells

Nisoxetine and fluoxetine (10^{-8} - 10^{-6} M) did not significantly alter the basal uptake of 2DG by 3T3-L1 differentiated cells. However in the presence of a sub-maximally stimulating concentration of insulin (10^{-8} M), fluoxetine (10^{-6} M) increased 2DG uptake by 41% ($p<0.05$). Similarly, nisoxetine (10^{-7} M) significantly increased insulin-stimulated glucose transport by 18% (see figures 6.12a and 6.12b).

Nisoxetine and fluoxetine in combination produced a significant increase in basal 2DG uptake at 10^{-6} M by approximately 75%. This effect was not increased further by the addition of insulin (10^{-8} M) (see figure 6.12c).

Fenfluramine and its active metabolite nordexfenfluramine did not significantly alter basal and insulin-stimulated 2DG uptake in differentiated 3T3-L1 cells (see figures 6.12d and 6.12e). Phentermine (the noradrenaline releasing anorectic agent) did significantly alter basal 2DG uptake in the cells at 10^{-6} M by 26%. However, phentermine did not affect insulin-stimulated glucose uptake in the differentiated 3T3-L1 cells (see figure 6.12f).

Serotonin and dopamine did not alter basal or insulin-stimulated 2DG uptake in the 3T3-L1 differentiated cells (see figures 6.12g and 6.12h).

However, noradrenaline (10^{-6}M) increased basal glucose uptake by 49% ($p < 0.05$).

Noradrenaline at all concentrations did not alter insulin-stimulated 2DG uptake (see figure 6.12i).

Figure 6.12a Effect of nisoxetine \pm insulin 10^{-8} M on 2DG uptake in differentiated 3T3-L1 cells. **Figure 6.12b** Effect of fluoxetine \pm insulin 10^{-8} M on 2DG uptake in differentiated 3T3 L1 cells. Cells were exposed to nisoxetine and fluoxetine for 24h at concentrations 10^{-8} - 10^{-6} M. Results expressed as mean values \pm SEM (n= 6). * $p < 0.05$ versus control, + $p < 0.05$ versus nisoxetine or fluoxetine alone.

Figure 6.12a

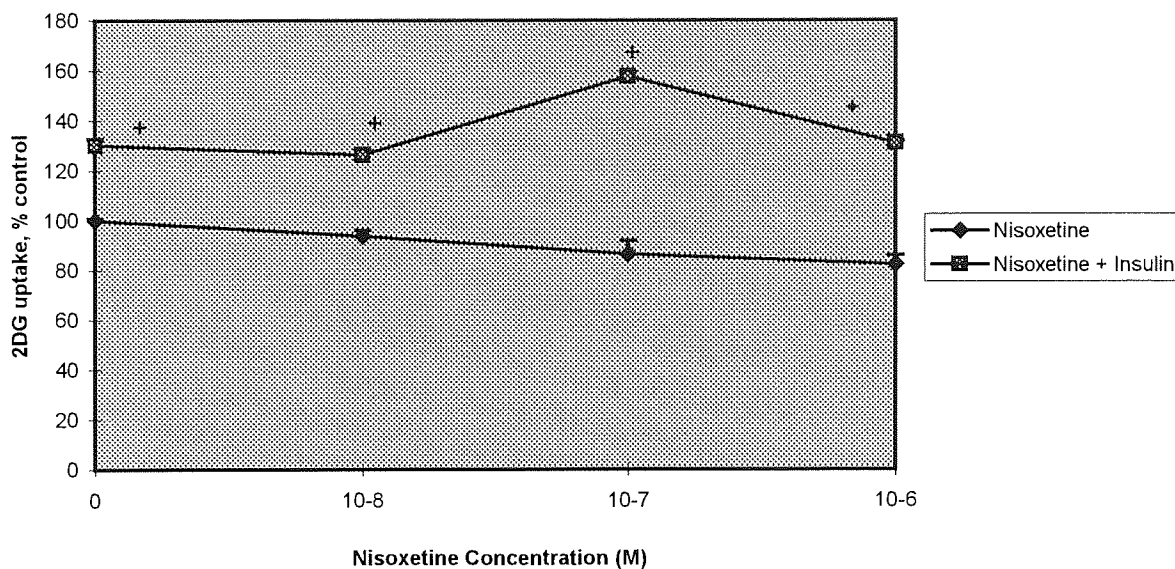


Figure 6.12b

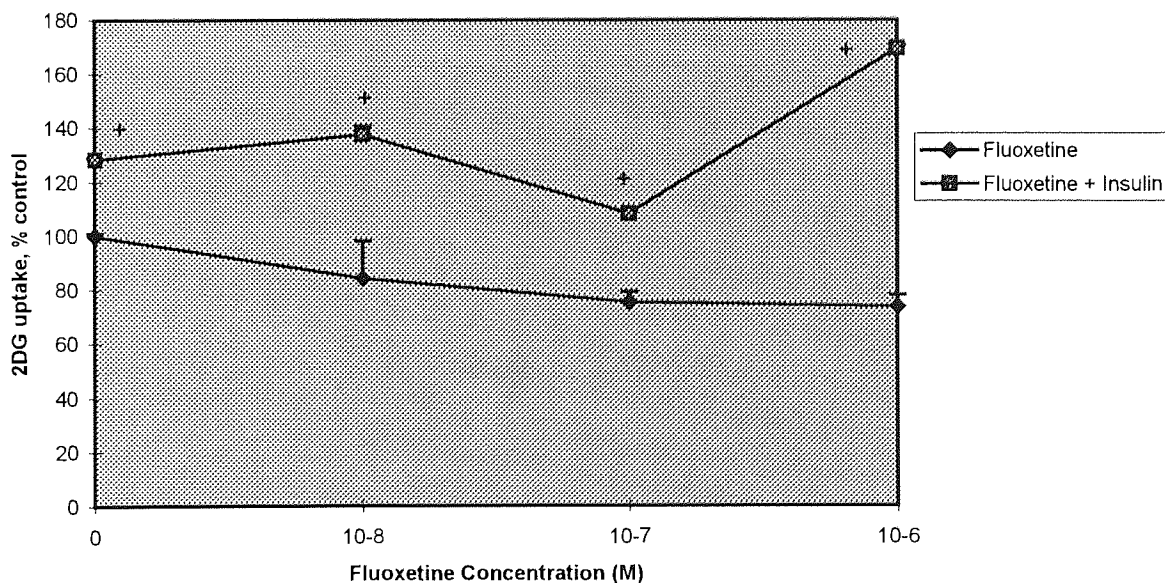


Figure 6.12c Effect of nisoxetine + fluoxetine \pm insulin 10^{-8} M on 2DG uptake in differentiated 3T3-L1 cells. **Figure 6.12d** Effect of fenfluramine \pm insulin 10^{-8} M on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to nisoxetine + fluoxetine and fenfluramine for 24h at concentrations 10^{-8} - 10^{-6} M. Results expressed as mean values \pm SEM (n= 6). * p<0.05 versus control, + p<0.05 versus nisoxetine + fluoxetine and fenfluramine alone.

Figure 6.12c

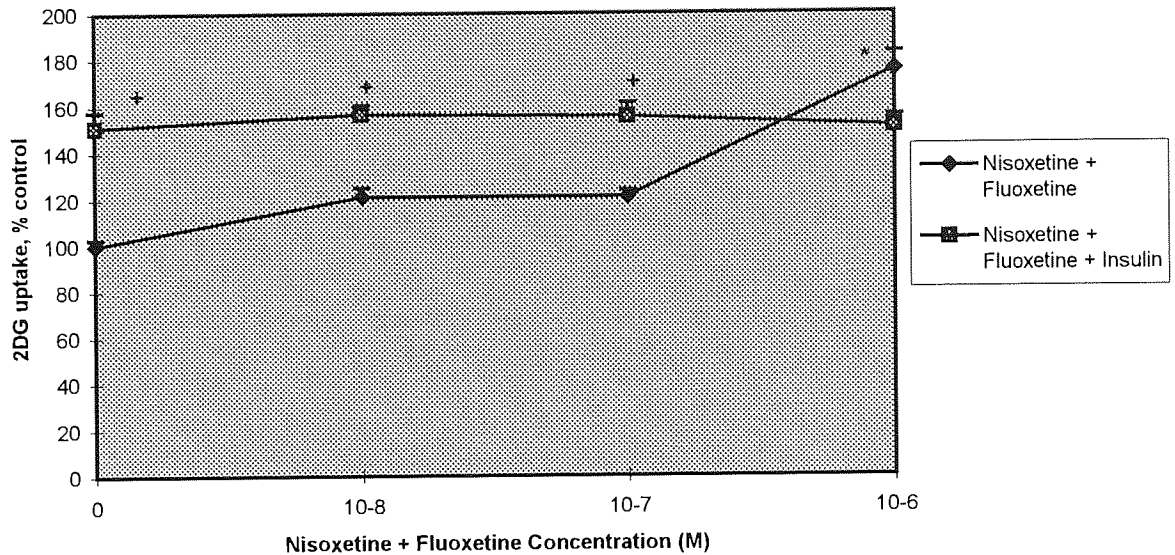


Figure 6.12d

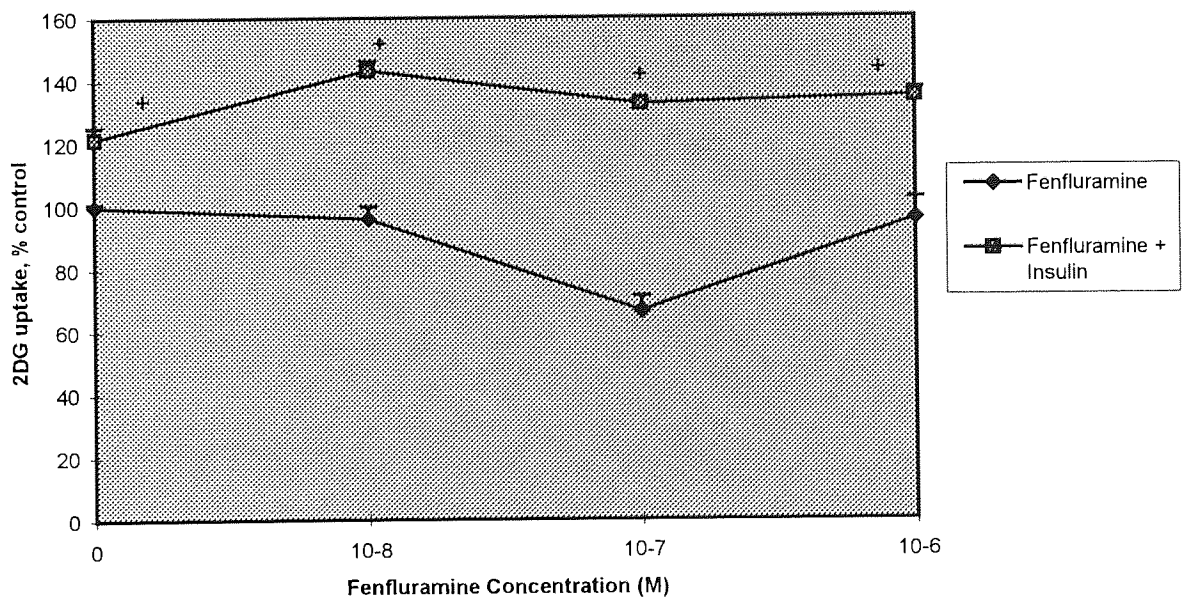


Figure 6.12e Effect of nordexfenfluramine \pm insulin 10^{-8} M on 2DG uptake in differentiated 3T3-L1 cells. **Figure 6.12f** Effect of phentermine \pm insulin 10^{-8} M on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to nordexfenfluramine and phentermine for 24h at concentrations 10^{-8} - 10^{-6} M. Results expressed as mean values \pm SEM (n= 6).
 * p<0.05 versus control, + p<0.05 versus nordexfenfluramine and phentermine alone.

Figure 6.12e

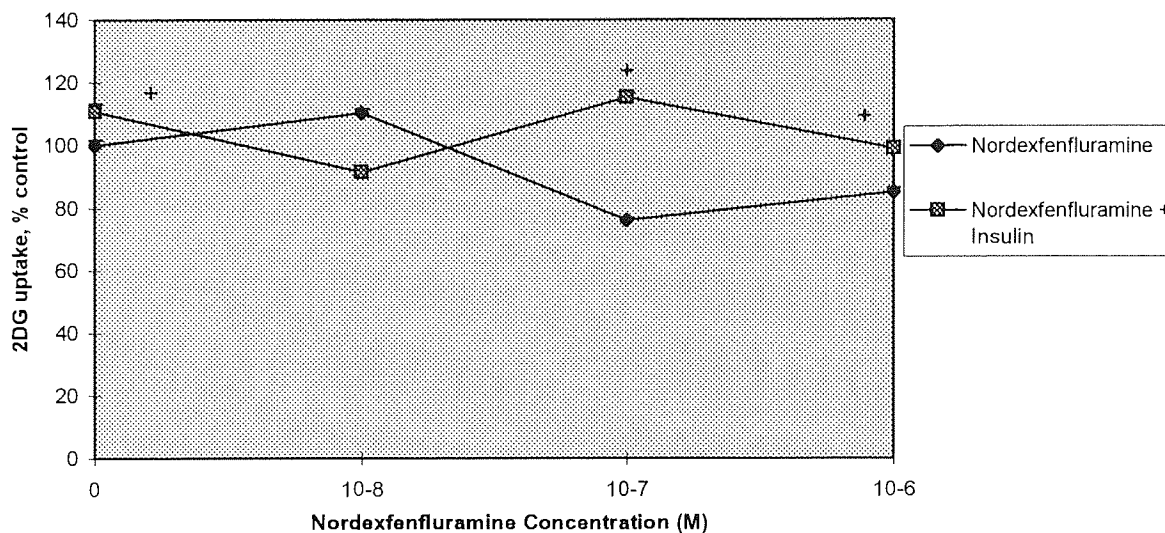


Figure 6.12f

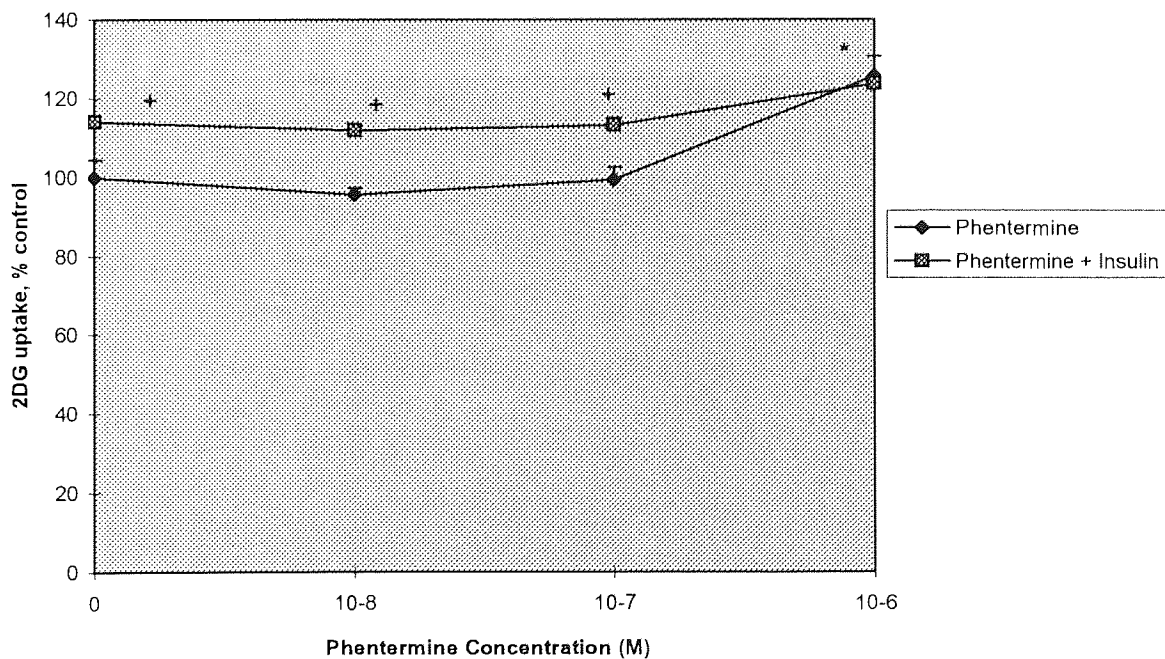


Figure 6.12g Effect of serotonin \pm insulin 10^{-8} M on 2DG uptake in differentiated 3T3-L1 cells. **Figure 6.12h** Effect of dopamine \pm insulin 10^{-8} M on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to serotonin and dopamine for 24h at concentrations 10^{-8} - 10^{-6} M. Results expressed as mean values \pm SEM (n= 6). * p<0.05 versus control, + p<0.05 versus serotonin and dopamine alone.

Figure 6.12g

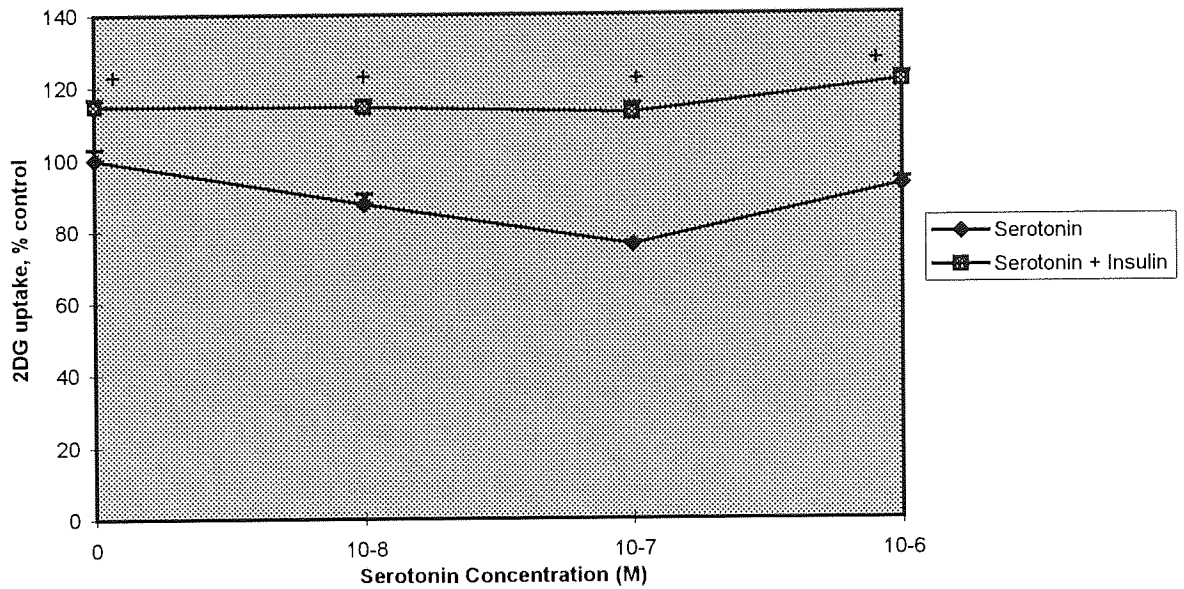


Figure 6.12h

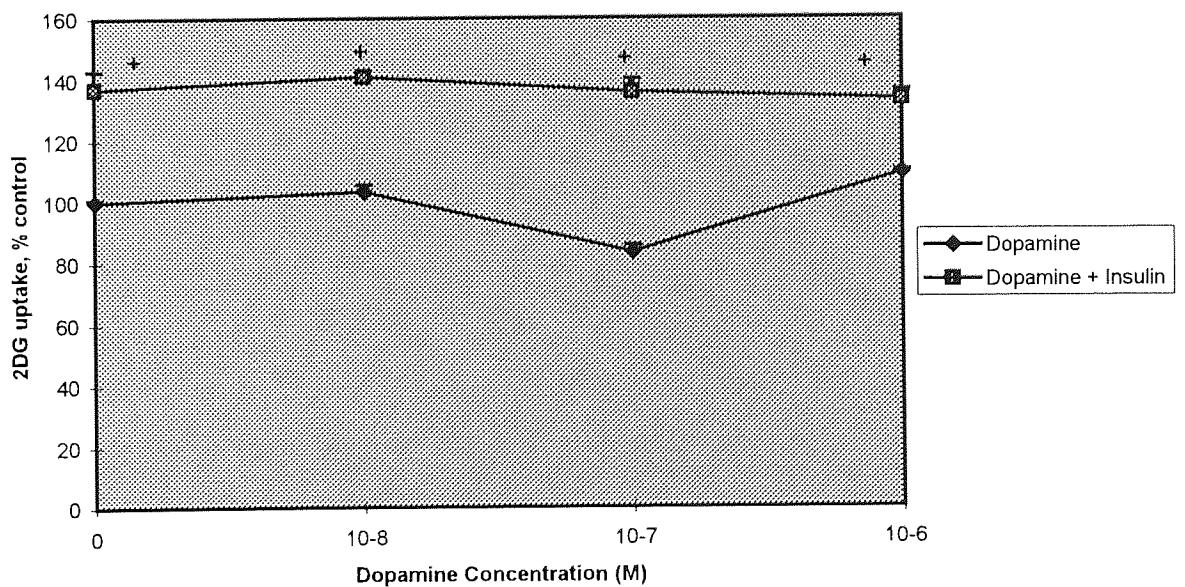
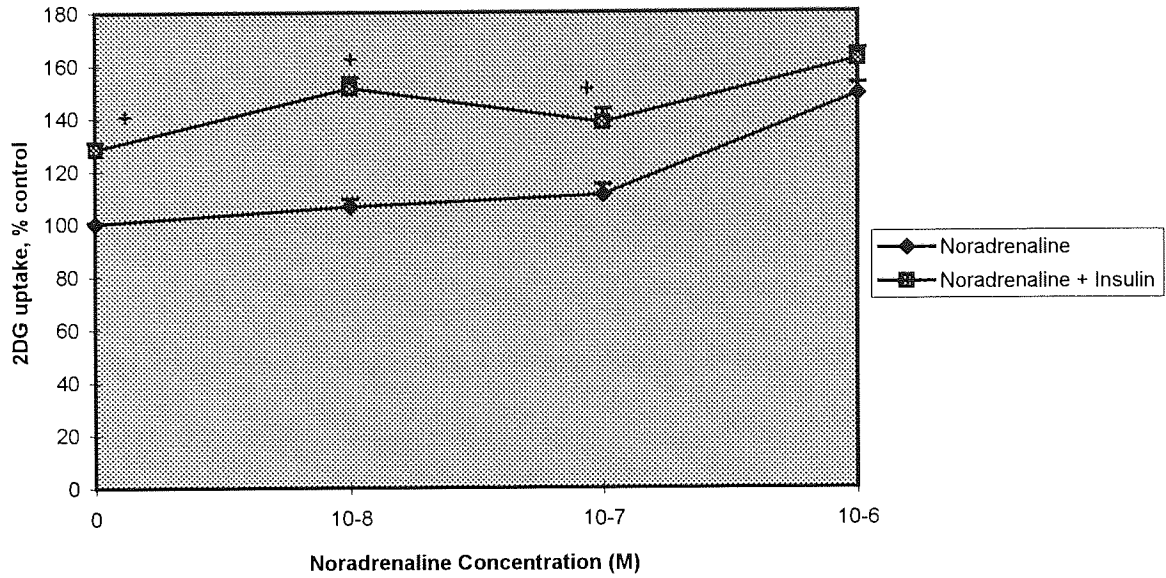


Figure 6.12i Effect of noradrenaline \pm insulin 10^{-8} M on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to noradrenaline for 24h at concentrations 10^{-8} - 10^{-6} M. Results expressed as mean values \pm SEM (n= 6). * $p < 0.05$ versus control, + $p < 0.05$ versus noradrenaline alone.

Figure 6.8i



6.3.5 Discussion

The results show that the sibutramine M2 can increase 2DG uptake by differentiated 3T3-L1 cells. Sibutramine and M1 were ineffective at increasing basal and insulin-stimulated glucose uptake. Thus suggesting that M2 is the active component of the centrally acting anti-obesity agent.

M2 increased basal 2DG uptake by the 3T3-L1 differentiated cells. Although no insulin was added to the basal M2 stimulated glucose uptakes, a small amount of insulin may be present, since the cells were exposed to a low concentration of insulin in the differentiating medium. With the addition of a sub-maximally stimulating insulin concentration the uptake of 2DG was not increased further than the effect observed with M2 alone. This suggests that M2 directly increased 2DG uptake into the differentiated 3T3-L1 cells independently of a sub-maximally stimulating insulin concentration. However, M2 did not alter the maximally stimulating effect of insulin on 2DG uptake, indicating that M2 is increasing insulin sensitivity. These results are consistent with the improved insulin sensitivity by L6 muscle cells after treatment with the sibutramine metabolites (Bailey *et al.*, 2001), a decrease in hyperinsulinaemia and amelioration of insulin resistance during treatment with sibutramine (Day and Bailey, 1998) and improvements in glycaemia and hyperinsulinaemia during treatment with sibutramine in clinical studies (Day and Bailey, 2002; Fujioka *et al.*, 2000; McNeely and Goa, 1998).

Basal M2 stimulated 2DG uptake was partially reduced by cycloheximide. Cycloheximide is an inhibitor of protein synthesis and here it decreases the translation

of glucose transporters. To support these data, cytochalasin B (blocks sodium-independent glucose transporters), completely inhibited M2 stimulated 2DG uptake. The reduction in 2DG uptake indicates that M2 promotes glucose uptake in the 3T3-L1 differentiated cells via activation of either GLUT1 or GLUT4. In addition, LY 294 002 inhibited M2; therefore it is also likely that the action of M2 on increasing glucose uptake in 3T3-L1 differentiating cells is mediated via PI3-Kinase. The results suggest that M2 activates the PI3-Kinase, which is the major pathway in the mediation of insulin-stimulated glucose transport and metabolism. Following the activation of the PI3-Kinase, there is an activation of the 3-phosphoinositide-dependent protein kinase (PDK-1) that activates the protein kinase B (PKB) and thereby stimulating glucose transport (Shepherd and Kahn, 1999).

Sibutramine induces satiety in rodents by enhancing the activation of 5HT_{2A/2C} receptors and α 1 and β 1 adrenoceptors. It acts as a serotonin noradrenaline reuptake inhibitor (SNRI) and to investigate whether these mechanisms could account for increased glucose uptake in 3T3-L1 differentiated cells, experiments were undertaken with serotonin, noradrenaline and agents known to stimulate release or inhibit reuptake of these substances in nervous tissues (fluoxetine, nisoxetine, phentermine, fenfluramine and nordexfenfluramine) (see chapter 6, table 4).

Nisoxetine and fluoxetine in combination, phentermine and noradrenaline all at maximal concentrations (10^{-6} M) increased basal-stimulated 2DG uptake in the 3T3-L1 differentiated cells. Nisoxetine in combination (selectively inhibit the reuptake of noradrenaline), phentermine (noradrenaline releasing anorectic agent) and noradrenaline all increased 2DG uptake suggesting that M2 may be partially acting on

the 3T3-L1 cells via catecholamine pathways. Although a significantly three- to fourfold greater weight loss was observed with phentermine than with placebo in obese diabetic patients, there was no significant improvement in blood glucose control (Campbell *et al.*, 1977; Gershberg *et al.*, 1977), suggesting that phentermine does not improve glucose control and insulin sensitivity. These data conflict with the above observation where phentermine increases 2DG uptake in the 3T3-L1 cells.

None of the other agents increased glucose uptake, thus suggesting that M2 is not acting on the 3T3-L1 differentiated cells via serotonin. Centrally acting agents, such as dexamphetamine and methamphetamine stimulate dopamine release (McNeely and Goa, 1998). Although it is unlikely that sibutramine and its metabolites could affect dopamine receptors and its release, there was no evidence that dopamine could stimulate 2DG in differentiated 3T3-L1 cells.

In conclusion M2 can act directly on adipose tissue to increase glucose uptake via a pathway involving new protein synthesis and activation of the glucose transporters. This pathway is activated by M2 via the conversion of phosphatidylinositol bisphosphate (PIP₂) to phosphatidylinositol trisphosphate (PIP₃) by PI3-Kinase. The 2DG uptake by M2 is independent of the serotonin reuptake inhibiting properties of sibutramine. However, it may act through the noradrenaline reuptake inhibiting properties of the SNRI agent.

6.4 Section Three: *In vivo* studies using lean and obese-diabetic *ob/ob* mice

M2 effects on the isolated lean and obese mouse adipocytes and the 3T3-L1 cell line have been investigated in sections one and two of this chapter. The latter part of this chapter is focused upon the effects of M2 *in vivo*. The acute and chronic effects of the active metabolite were investigated in lean and *ob/ob* mice.

6.4.1 Method

6.4.1a Acute Effect of Oral and Intra-peritoneal Administration of M2 on Basal Non-Esterified Fatty Acids and Glycaemia in Homozygous Lean Mice

Groups of homozygous lean (+/+) mice of either sex, aged 30 weeks, with a weight range of 35g-40g, were obtained from the Aston University breeding colony (as described 2.2.2). In each group were 6 mice matched for body weight and basal glucose concentration. The control groups received PBS (1ml/kg) administration by oral gavage (po). In addition these control mice were also given an intra-peritoneal (ip) placebo injection. The M2-treated groups (1mg/kg; 10mg/kg) received the drug by either po or ip injection. M2 was dissolved in PBS prior to use. Blood samples for plasma glucose concentration were taken at 0h, 2h, 4h, 8h and 24h after drug and PBS administration. Moreover, blood was collected from the tail tip of the conscious mice at 0h, 4h and 24h for non-esterified fatty acid (NEFA) determination (see section 2.2.4). Food was withheld during the acute study and water was available *ad libitum*. 24h after drug administration food was *ad libitum* and intake was monitored hourly.

6.4.1b Acute Effect of Oral and Intra-peritoneal Administration of M2 on Basal Non-Esterified Fatty Acids and Glycaemia in *ob/ob* Mice

Obese *ob/ob* mice of either sex, aged 10-20 weeks, a weight range of 65g -100g, were obtained from the Aston University breeding colony. The mice were separated into 6 groups; control, M2-treated (1mg/kg po), M2-treated (1mg/kg ip), control, M2-treated (10mg/kg po), M2-treated (10mg/kg ip). In each group were 6 mice matched for body weight and basal glucose concentration. The control groups received PBS (2.5ml/kg) administration by oral gavage (po) and were given a placebo intra-peritoneal (ip) injection. M2 was dissolved in PBS prior to drug administration. Blood was collected from the tail tip of the conscious mice at 0h, 2h, 4h, 8h and 24h for NEFA determination and plasma glucose concentration (as described in section 2.2.4). Food was withheld during the acute study and water was available *ad libitum*. 24h after drug administration the food was returned to the cages and intake was monitored hourly.

6.4.1c Chronic Effect of Oral and Intra-peritoneal Administration of M2 on Basal Non-Esterified Fatty Acids and Glycaemia in Homozygous Lean Mice

Lean mice described in section 6.4.1a were used for the chronic M2 study. Prior to administration with either PBS or M2 (1mg/kg; 10mg/kg) the mice were given a 24h rest period between the acute study and beginning the chronic drug administration study. Similarly to the acute study described above, the M2 was administered by either po or ip injection. The control treated groups received PBS (1ml/kg) by po and

were given a placebo ip injection of vehicle only. Treatment was administered at 11am daily for approximately 7 days. Food and water were *ad libitum* and monitored daily. Body weights of the mice were measured daily for 7 days before, during and for 14 days after drug administration. Blood samples were taken weekly and assayed for glucose and NEFA concentration (2.2.4).

6.4.1d Chronic Effect of Oral and Intra-peritoneal Administration of M2 on Basal Non-Esterified Fatty Acids and Glycaemia in *ob/ob* Mice

Obese *ob/ob* mice described in section 6.4.1b were used for the chronic M2 study. Prior to administration with either PBS or M2 (1mg/kg; 10mg/kg) the mice were given a 24h rest period between the acute study and beginning the chronic drug administration study. Similarly to the acute study described above, the M2 was administered either po or ip. The control treated groups received PBS (2.5ml/kg) by po and were given a placebo ip injection. Treatment was administered at 11am daily for approximately 7 days. Food and water were *ad libitum* and monitored daily. Body weights of the mice were measured daily for 7 days before, during and for 14 days after drug administration. Blood samples were taken weekly and assayed for glucose and NEFA concentration (2.2.4).

6.4.2 Results

6.4.2a Acute Effect of Oral and Intra-peritoneal Administration of M2 in Homozygous Lean Mice

Groups of homozygous lean mice were studied at intervals over 24h, after an po or ip dosage of either 1mg/kg or 10mg/kg M2. There were no significant effects on plasma glucose concentration in the groups of mice receiving a dose of M2 (1mg/kg and 10mg/kg) via po.

However, administration via ip injection significantly reduced glucose concentrations at 8h after dosing with 1mg/kg M2 (7.23 ± 0.27 basal glucose vs 5.61 ± 0.28 4h after dose, $p < 0.05$) (see figure 6.13a). Similarly, a significant reduction in basal glucose concentration at 4h and 8h after ip M2 (10mg/kg) injection was observed (7.43 ± 0.25 basal glucose vs 5.16 ± 0.67 4h after dose vs 3.25 ± 0.30 8h after dose, $p < 0.05$) (see figure 6.13b).

There were no significant effects on plasma NEFA concentration in the groups of mice receiving a dose of M2 (1mg/kg) via po and ip administration (see figure 6.13c). However, administration of M2 (10mg/kg) via ip injection significantly increased the concentration of NEFA at 24h after dosing (see figure 6.13d).

Food was withheld throughout the 24h period and intake was monitored hourly when given back to the mice. There were no significant changes in food intake between the

placebo, M2-po and M2-ip treated mice (6.35 ± 0.10 vs 7.0 ± 0.20 vs 7.5 ± 0.15 g/mouse/day, respectively).

Figure 6.13a Plasma concentrations of glucose in lean mice during 24h after administration of M2 (1mg/kg). **Figure 6.13b** Plasma concentrations of glucose in lean mice during 24h after administration of M2 (10mg/kg). Results expressed as mean values \pm SEM (n= 6). * p<0.05 versus control (placebo). + p<0.05 versus 0h M2-1P

Figure 6.13a

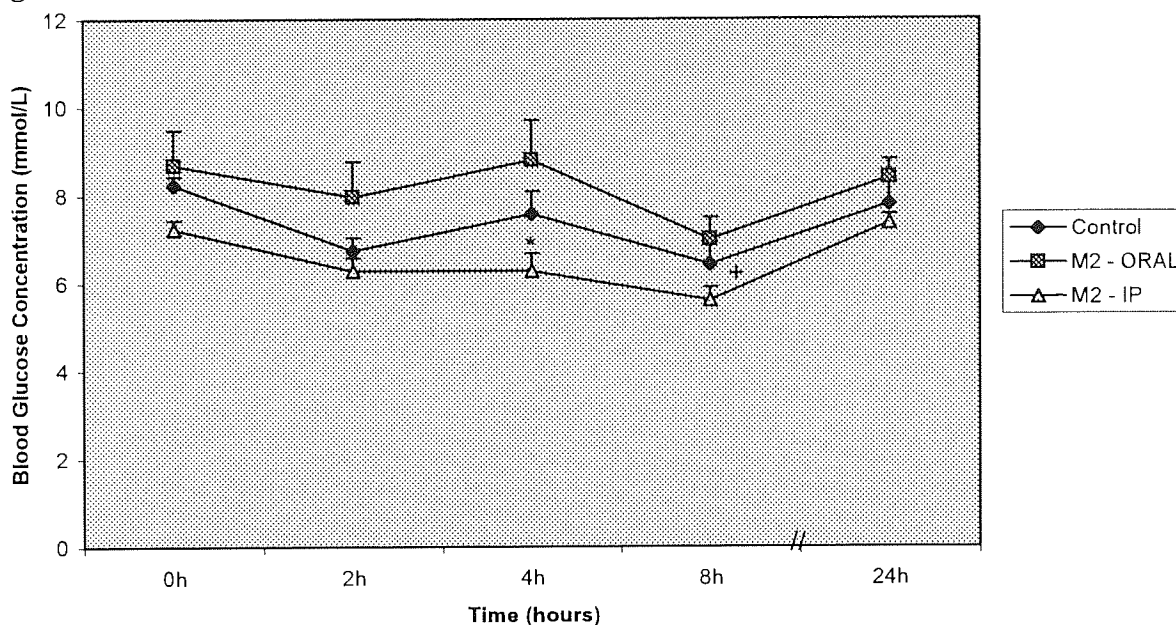


Figure 6.13b

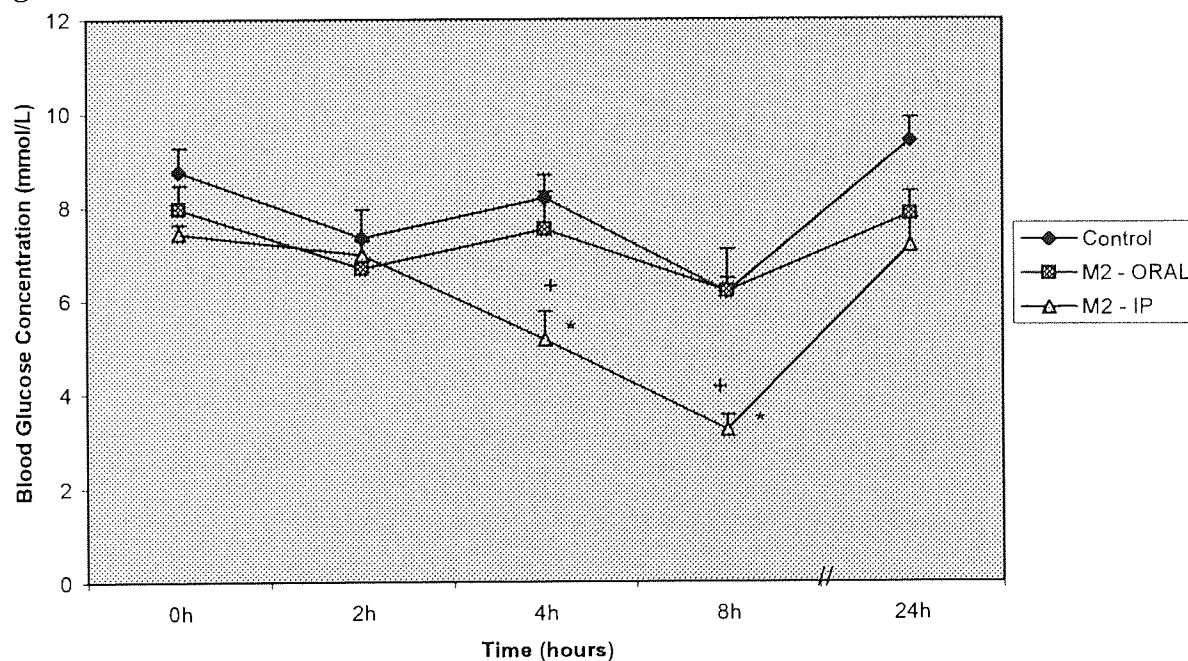


Figure 6.13c Plasma concentrations of NEFA in lean mice during 24h after administration of M2 (1mg/kg). **Figure 6.13d** Plasma concentrations of NEFA in lean mice during 24h after administration of M2 (10mg/kg). Results expressed as mean values \pm SEM (n= 6). * p<0.05 versus control (placebo). + p<0.05 versus 0h M2-1P

Figure 6.13c

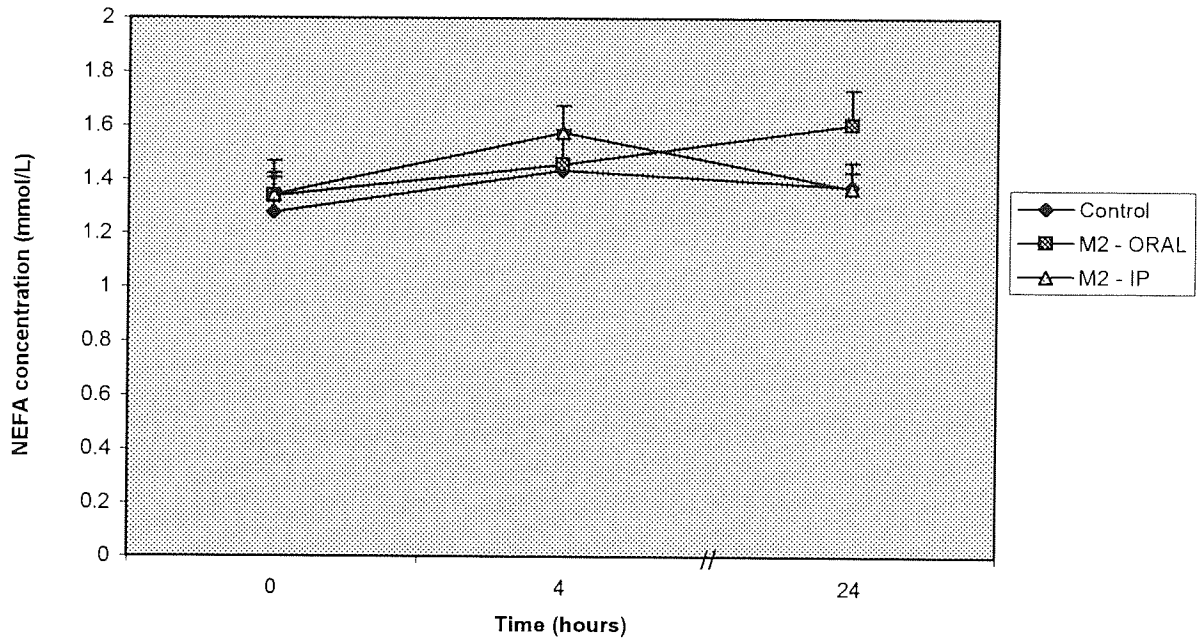
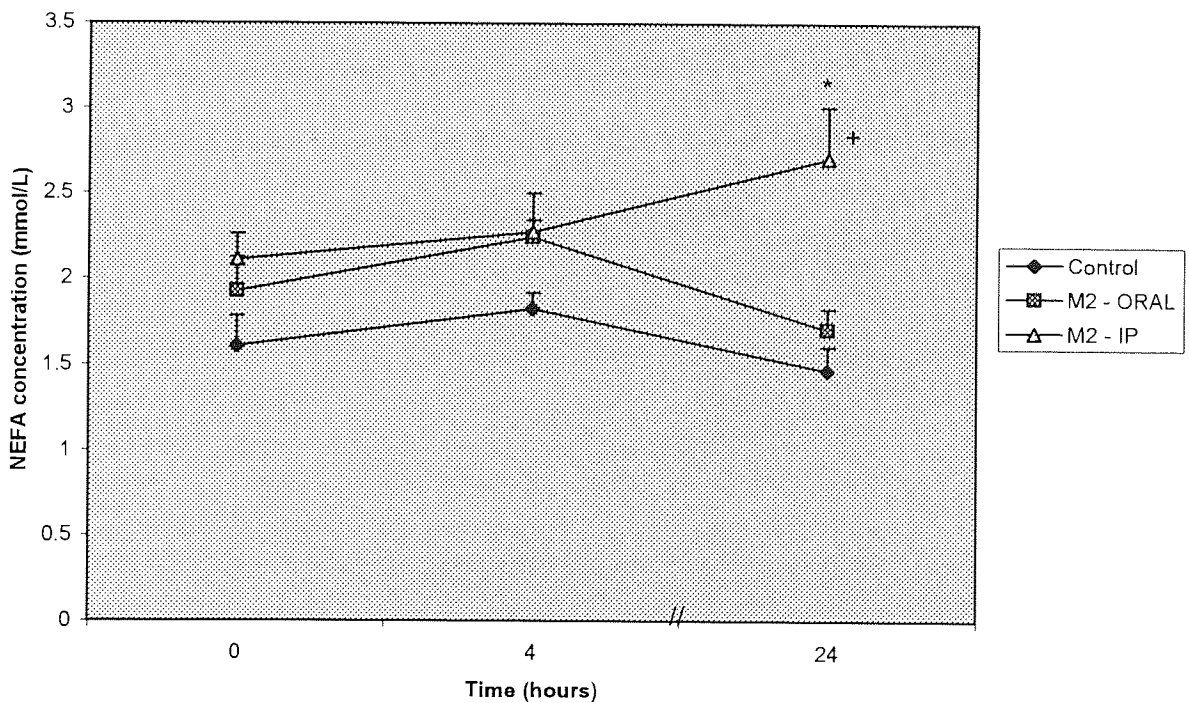


Figure 6.13d



6.4.2b Acute Effect of Oral and Intra-peritoneal Administration of M2 in *ob/ob* Mice

To investigate a possible acute effect of M2, groups of *ob/ob* mice were studied at intervals over 24h, after an po or ip dosage of either 1mg/kg or 10mg/kg. There were significant effects on plasma glucose concentration in the groups of mice receiving a dose of M2 (1mg/kg and 10mg/kg) via the ip route. There were no significant effects on plasma glucose concentration in the po administered mice at both dosages (see figure 6.14a and 6.14b).

Plasma NEFA concentrations were significantly increased in *ob/ob* mice treated with M2 (1mg/kg and 10mg/kg). A more pronounced effect was observed 24h after dosing with M2, particularly at 1mg/kg dose (see figures 6.14c and 6.14d).

As above, food was withheld throughout the study period and intake was monitored hourly for 24h, when the food was given back to the mice. There were no significant changes in food intake between the placebo, M2-po and M2-ip treated mice (8.85 ± 0.25 vs 8.65 ± 0.10 vs 8.7 ± 0.25 g/mouse/day, respectively).

Figure 6.14a Plasma concentrations of glucose in *ob/ob* mice during 24h after administration of M2 (1mg/kg). **Figure 6.14b** Plasma concentrations of glucose in *ob/ob* mice during 24h after administration of M2 (10mg/kg). Results expressed as mean values \pm SEM (n= 6). * p<0.05 versus control (placebo).

Figure 6.14a

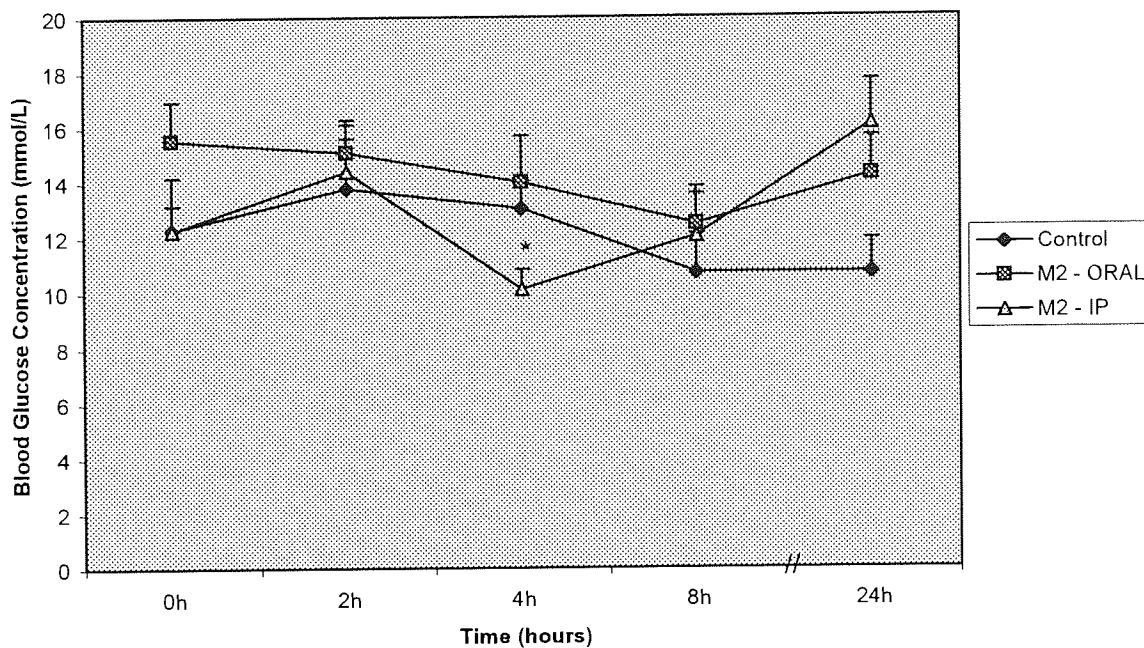


Figure 6.14b

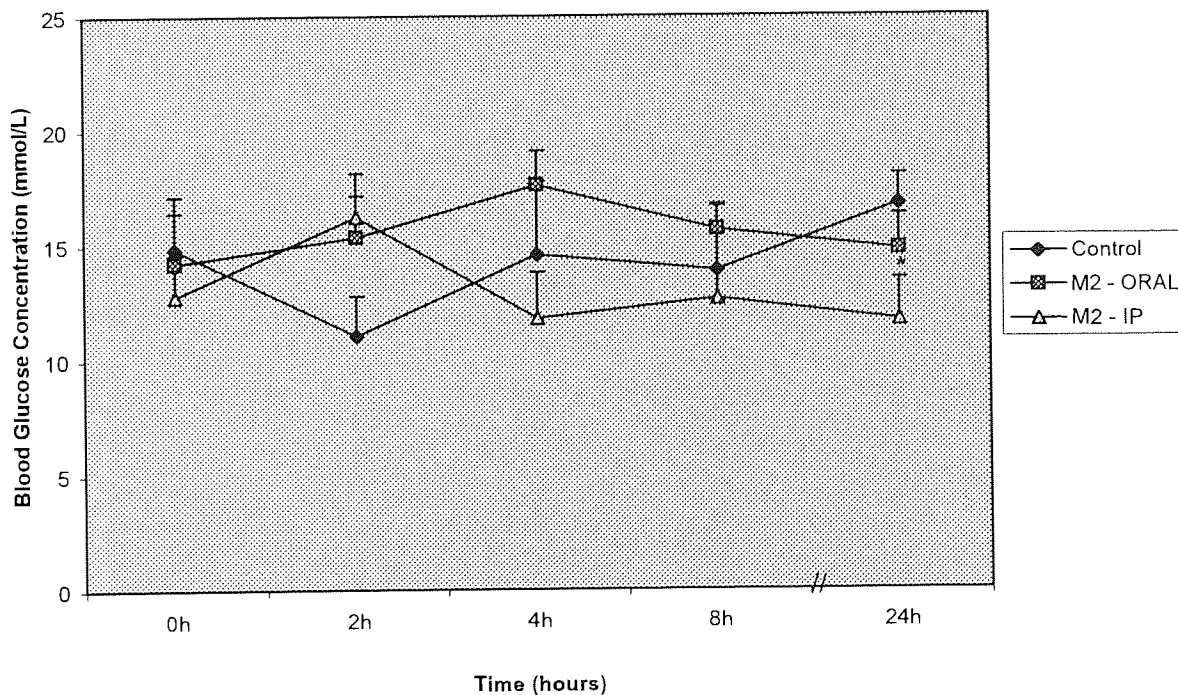


Figure 6.14c Plasma concentrations of NEFA in *ob/ob* mice during 24h after administration of M2 (1mg/kg). **Figure 6.14d** Plasma concentrations of NEFA in *ob/ob* mice during 24h after administration of M2 (10mg/kg). Results expressed as mean values \pm SEM (n= 6).
 * $p < 0.05$ versus control (placebo). + $p < 0.05$ versus 0h M2

Figure 6.14c

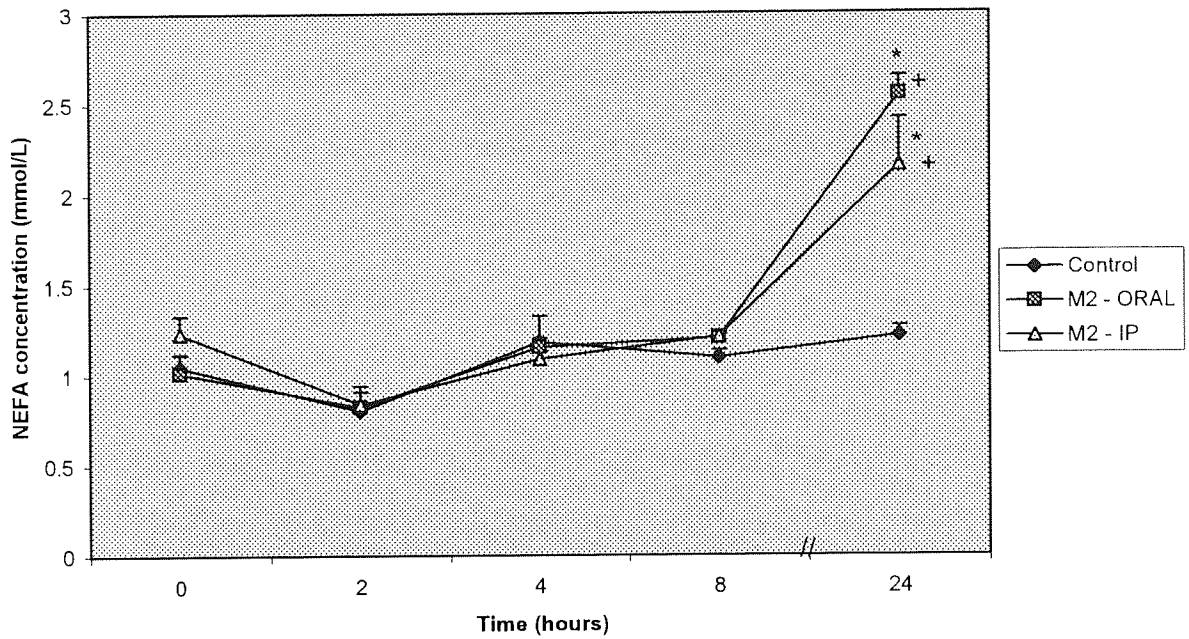
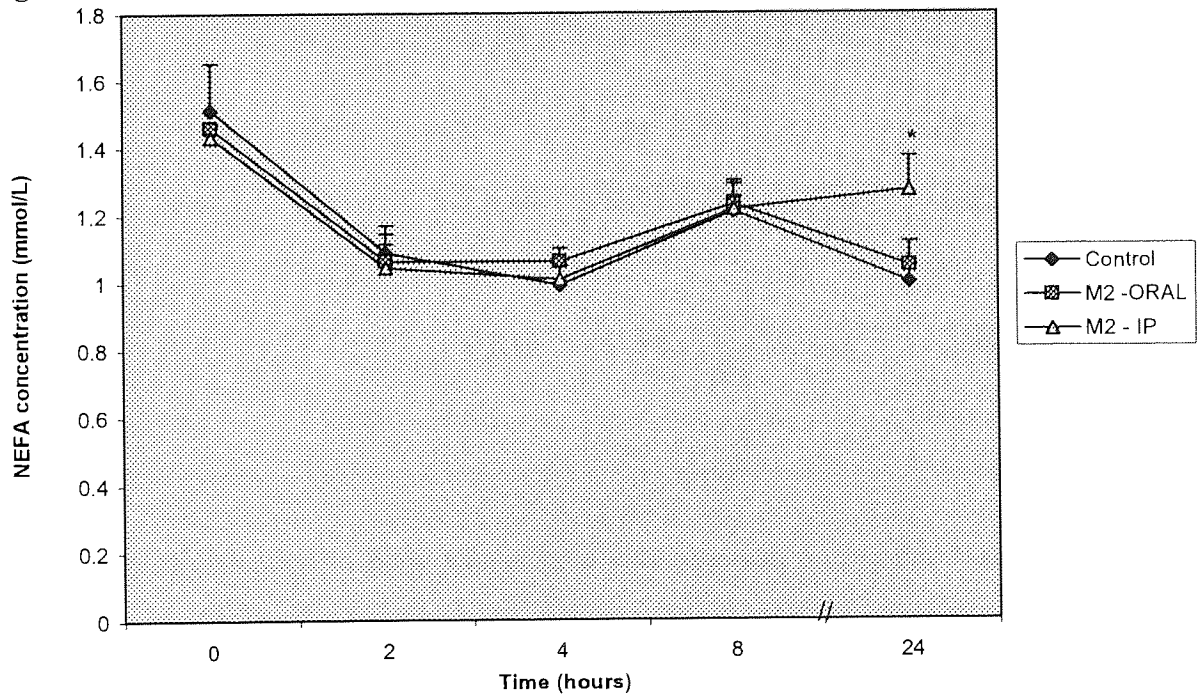


Figure 6.14d



6.4.2c Chronic Effect of Oral and Intra-peritoneal Administration of M2 in Homozygous Lean Mice

Administration of M2 (1mg/kg and 10mg/kg) for 7 days did not significantly alter body weight gain in the lean mice administered M2 by either the oral or intra-peritoneal route (see table 8). Although a reduction in body weight was observed in the M2 intra-peritoneally and orally treated mice (10mg/kg), the result proved to be insignificant.

Table 8: Body Weights (g) of Lean Mice

	M2 (1mg/kg)			M2 (10mg/kg)		
	Start	End	Change in body weight Sig.	Start	End	Change in body weight Sig.
Control	38.03 ± 0.94	38.9 ± 1.79	+ 0.87 NS	39.7 ± 1.85	40.01 ± 1.77	+ 0.31 NS
M2 - Oral	39.61 ± 0.76	40.07 ± 0.89	+ 0.46 NS	41.97 ± 0.87	41.15 ± 1.29	- 0.82 NS
M2 - IP	39.9 ± 0.67	41.15 ±0.84	+ 1.25 NS	41.38 ± 0.74	39.93 ± 1.40	- 1.45 NS

In addition, a reduction in body weight gain was observed during the 7 day M2 (10mg/kg) treatment period, however this also proved to be insignificant (6.15b). M2 at 1mg/kg had no effect on body weight (see figure 6.15a). Mean daily food intake

was not significantly altered in the M2 (1mg/kg and 10mg/kg) treated and the control groups (data not shown).

Glucose concentrations (measured at the end of the study) were unaltered by oral and intra-peritoneal M2 administration (1mg/kg and 10mg/kg) in lean mice (1mg/kg: 8.11 ± 0.54 control vs 7.82 ± 0.37 M2 - Oral vs 7.52 ± 0.20 mmol/L M2 – IP, NS; 10mg/kg: 7.85 ± 0.35 control vs 8.53 ± 0.21 M2 - Oral vs 8.23 ± 0.75 mmol/L M2 – IP, NS).

NEFA concentrations (expressed at mmol/L) were significantly increased by oral and intra-peritoneal M2 administration (1mg/kg) in lean mice (Oral: 1.33 ± 0.14 baseline vs 1.69 ± 0.12 end of study, $p < 0.05$; IP: 1.34 ± 0.09 baseline vs 1.89 ± 0.13 end of study, $p < 0.05$). Similarly, NEFA concentrations were significantly increased by intra-peritoneal M2 administration (10mg/kg) in lean mice (IP: 2.11 ± 0.15 baseline vs 2.70 ± 0.35 end of study, $p < 0.05$). Oral administration of M2 at 10mg/kg did not significantly alter NEFA concentrations.

Figure 6.15a Chronic effect of M2 (1mg/kg) on body weight of lean mice. **Figure 6.15b** Chronic effect of M2 (10mg/kg) on body weight of lean mice. Mice received either M2 (po or ip) or PBS (control). Results expressed as mean values \pm SEM (n= 6).

Figure 6.15a

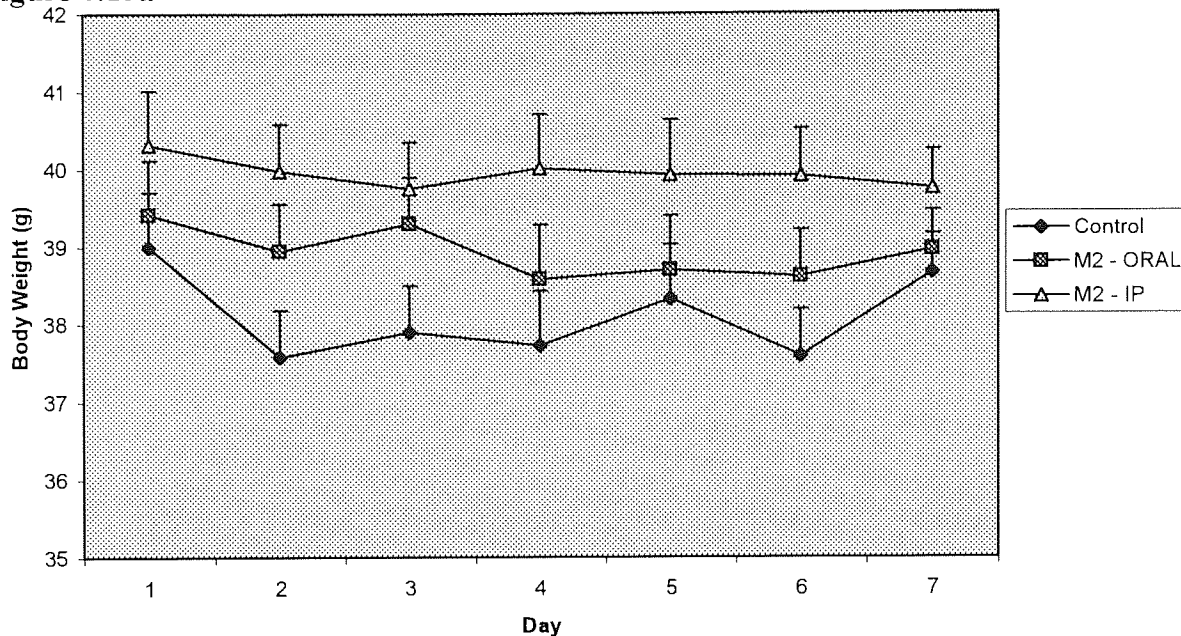
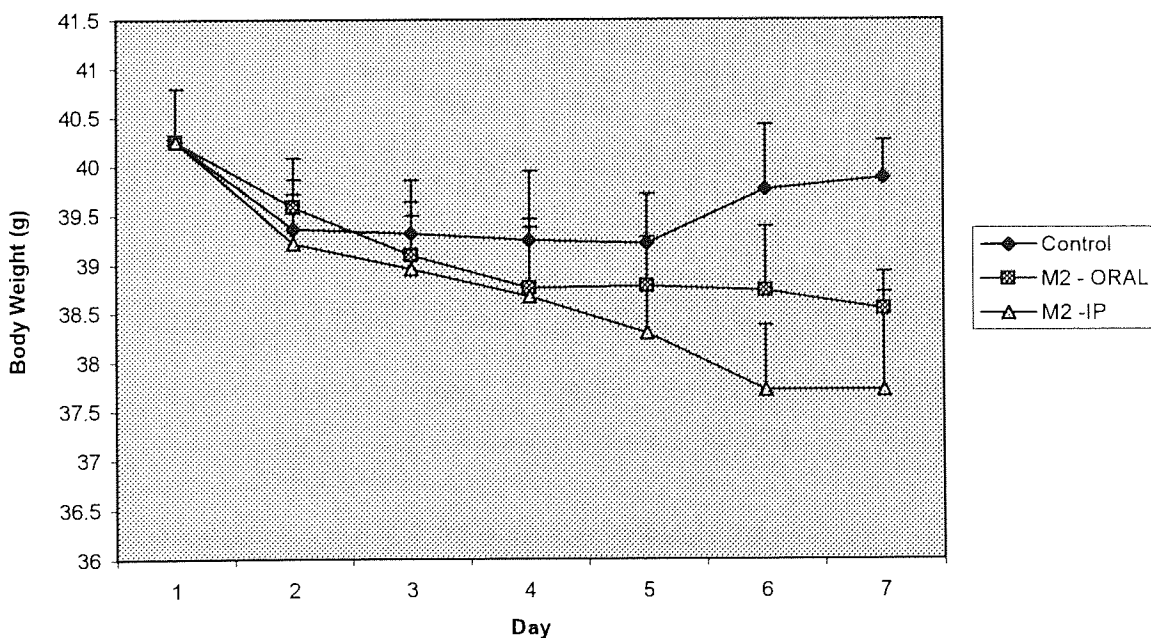


Figure 6.15b



6.4.2d Chronic Effect of Oral and Intra-peritoneal Administration of M2 in *ob/ob* Mice

M2 (1mg/kg and 10mg/kg) for approximately 7 days did not significantly alter body weight gain in the obese mice administered M2 by oral or intra-peritoneal route (see table 9). Although a reduced body weight gain was observed in the intra-peritoneal mice receiving M2 (10mg/kg), the result proved to be statistically non-significant.

Table 9: Body Weights (g) of *ob/ob* Mice

	M2 (1mg/kg)			M2 (10mg/kg)		
	Start	End	Change in body weight Sig.	Start	End	Change in body weight Sig.
Control	77.93 ± 4.05	91.78 ± 2.47	+ 13.85 NS	85.3 ± 4.85	92.68 ± 3.0	+ 7.38 NS
M2 - Oral	81.9 ± 2.04	92.17 ± 86.22	+ 10.2 NS	82.43 ± 3.9	90.63 ± 4.30	+ 8.5 NS
M2 - IP	73.0 ± 3.24	86.22 ± 2.27	+ 13.22 NS	87.20 ± 2.5	91.56 ± 2.34	+ 4.36 NS

M2 at both concentrations proved to have no significant effect on body weight in the *ob/ob* mice during the M2 treatment period (approximately 7 days) (see figures 6.16a and 6.16b). Mean daily food intake was not significantly altered in the M2 (1mg/kg) treated and the control groups (data not shown). However, during oral and intra-

peritoneal treatment with the higher dose of M2 (10mg/kg), food intake was significantly reduced versus the control group. A significant result was observed on day 4-6 of M2 treatment (po and ip) (see figure 6.17).

Blood glucose concentrations were unaltered by chronic dosing of M2 (10mg/kg) in the *ob/ob* mice treated by either oral or intra-peritoneal injection (16.8 ± 4.08 control vs 19.7 ± 4.6 M2 – Oral vs 11.2 ± 1.48 M2 – IP, NS). In addition, oral administration of M2 at 1mg/kg did not alter basal glucose concentration (12.8 ± 1.79 control vs 14.78 ± 2.15 M2 - Oral, NS). However, glucose concentrations were significantly reduced in the *ob/ob* mice treated with 1mg/kg M2 via intra-peritoneal injection (12.26 ± 0.94 basal glucose vs 7.1 ± 1.35 7 days after last dose vs 9.16 ± 0.90 mmol/L 14 days after last dose, $p < 0.05$).

NEFA concentrations were not significantly altered during M2 (1mg/kg and 10mg/kg) po and ip treatment in the *ob/ob* mice. Similarly, at the end of the studies (approximately 2 weeks after last M2 dose), NEFA concentrations were not significantly changed (data not shown).

Figure 6.16a Chronic effect of M2 (1mg/kg) on body weight of *ob/ob* mice. **Figure 6.16b** Chronic effect of M2 (10mg/kg) on body weight of *ob/ob* mice. Mice received either M2 (po or ip) or PBS (control). Results expressed as mean values \pm SEM (n= 6).

Figure 6.16a

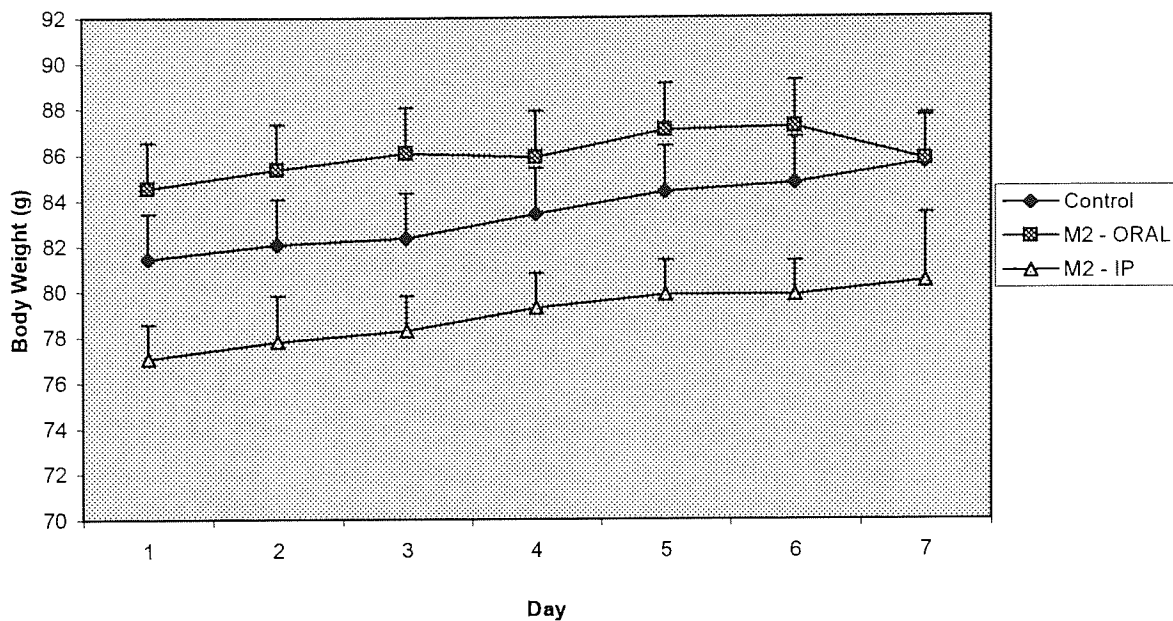


Figure 6.16b

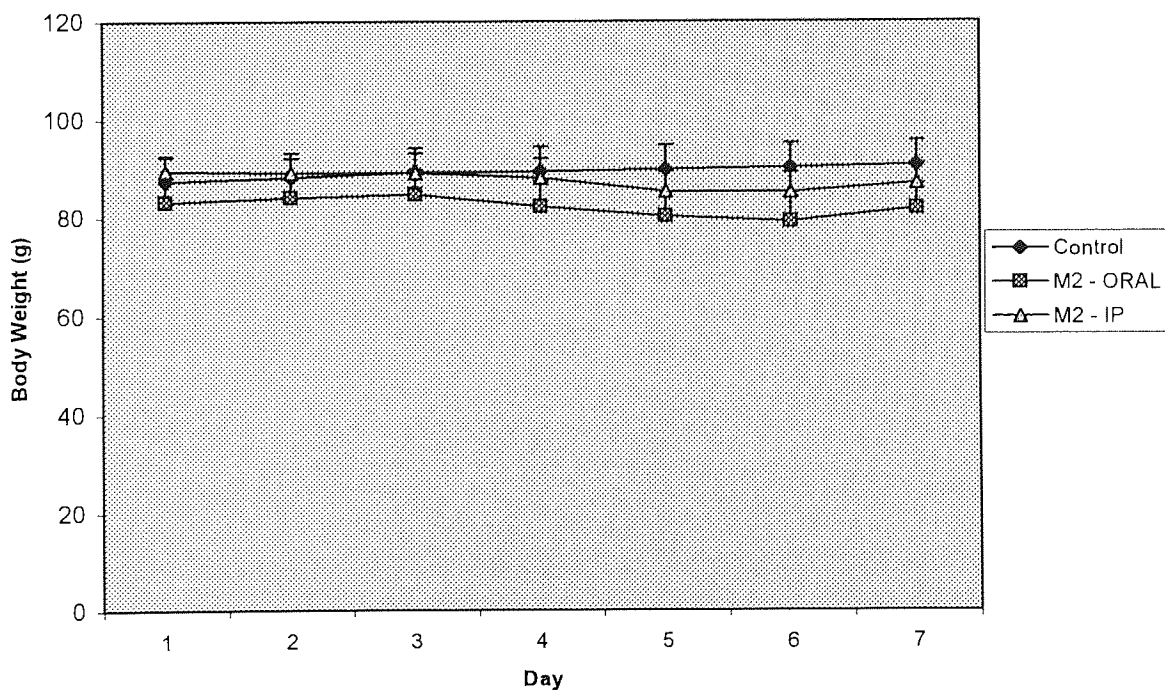
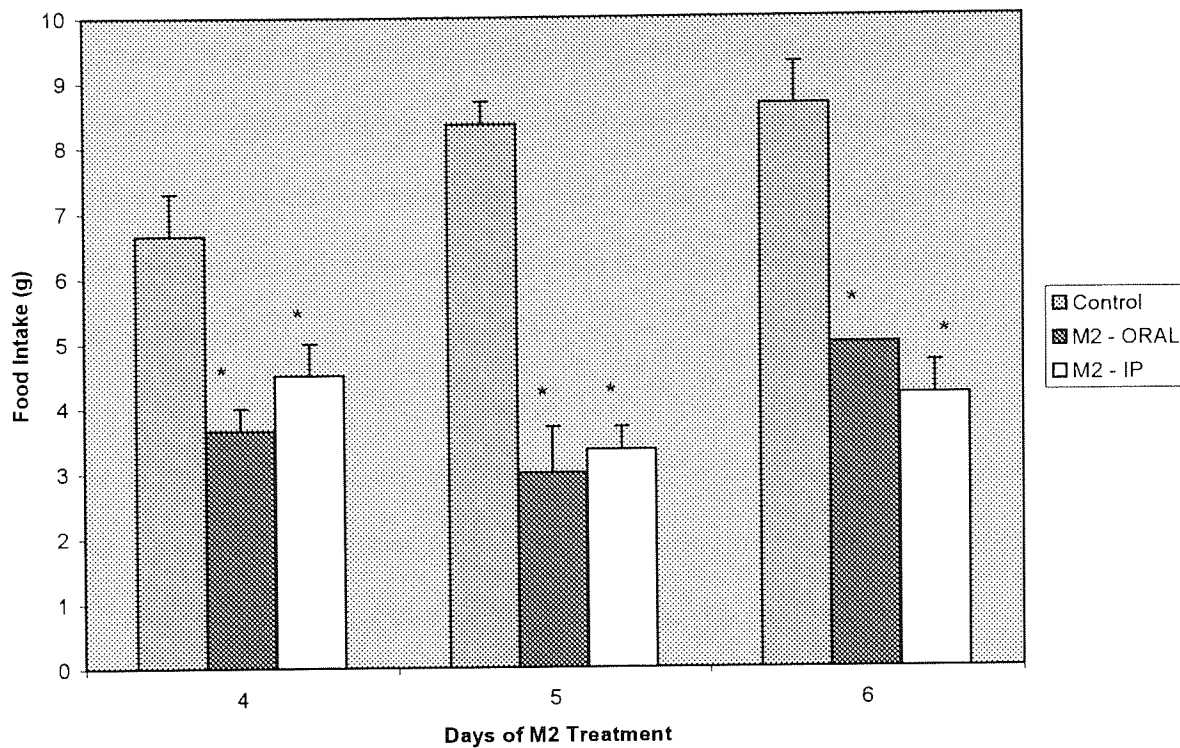


Figure 6.17 Chronic effect of M2 (10mg/kg) on food intake (g) of *ob/ob* mice. Mice received either M2 (po or ip) or PBS (control). Results expressed as mean values \pm SEM (n= 6). * $p < 0.05$ versus control.



6.5 Discussion

The results clearly show that M2 (the primary metabolite of the anti-obesity agent sibutramine) has acute and chronic effects in lean and obese-diabetic *ob/ob* mice. Chronic M2 treatment (10mg/kg) given via intra-peritoneal injection in both lean and *ob/ob* mice reduced body weight gain. The reduced body weight gain was not observed with 1mg/kg M2 treatment, suggesting that a higher dose is required in both lean and obese mice.

There were no significant acute changes in food intake in the lean and *ob/ob* mice. However, a significant effect was observed during oral and intra-peritoneal administration of M2 (10mg/kg) in *ob/ob* mice during 4-6 days after chronic dosing. This effect was not observed in the lean mice. These results suggest that the higher dose of M2 is required to observe the satiety effects associated with sibutramine (Halford *et al.*, 1995). In addition, it appears that the *ob/ob* mice are more sensitive to the effects of M2 than the lean mice. Sibutramine is a hypophagic agent and has clearly been demonstrated to reduce food intake in a dose dependent manner (Jackson *et al.*, 1997; Burlet *et al.*, 1994; Fantino *et al.*, 1995). Moreover, in another study, obese *fafa* rats treated with sibutramine (10mg/kg) had a reduced food intake during the first week of treatment but there were no effects thereafter (Connoley *et al.*, 1995).

Body fat mass in the lean and *ob/ob* mice was not measured. However, NEFA concentrations, an indicator of fat metabolism were measured during the acute and chronic M2 studies. Acute treatment with M2 (10mg/kg) via intra-peritoneal injection significantly increased NEFA concentrations in both lean and obese mice 24h after

first administration. This effect was also observed in the obese mice given M2 (1mg/kg) via oral administration. Day and Bailey (1998) have previously reported that sibutramine (5mg/kg) acutely raised the NEFA concentration in *ob/ob* mice after 24h administration. Similarly, a significant increase in NEFA concentrations was observed in lean mice after treatment with M2 (1mg/kg and 10mg/kg) for 7 days via intra-peritoneal injection. Oral administration of M2 at 1mg/kg dose also increased NEFA concentration. These effects however were not observed in obese mice given chronic M2 treatment via both routes and at the same doses. Taken together, these results demonstrate that acute administration of M2 in both lean and obese mice increase adipose tissue mobilisation. M2 acts on the adipose tissue to increase lipolysis within 24h after its first administration. The chronic effects of M2 are more potent in lean mice versus the obese mice. This suggests that a higher dose may be required to create this lipolytic effect during M2 chronic treatment in obese mice. These results demonstrate that M2 is capable of raising NEFA concentrations via adipose tissue lipolysis and it may be the metabolite that is the active component of sibutramine.

Whereas acute intra-peritoneal treatment with 1mg/kg and 10mg/kg lowered blood glucose concentration in lean and obese mice, basal glycaemia was not reduced in those mice treated by oral administration. Chronic treatment with M2 in the lean mice did not reduce glycaemia. However, chronic treatment with intra-peritoneal M2 (1mg/kg) for approximately 7 days significantly reduced blood glucose concentrations in the *ob/ob* mice. These results clearly show that M2 improves glucose homeostasis by lowering basal glycaemia in both lean and obese mice after the first treatment with M2. However, the long-term effects of M2 are only demonstrated in obese mice. The

effects of M2 in lean and obese mice are more potent when administered by intra-peritoneal injection. By administering the drug via this route, the availability of the drug is increased, i.e. during oral administration M2 passes through the gastrointestinal tract and is believed to be partially digested (Jones, personal communication). From previous studies, sibutramine has been shown to lower blood glucose concentration in *ob/ob* mice following an intra-peritoneal glucose tolerance test and it has also been associated with improvements in glycaemic control (Day and Bailey, 1998; McNeely and Goa, 1998). Taken together, these results indicate that M2 lowers blood glucose concentration. The decrease in blood glucose concentration in this study was associated with a reduction in body weight gain and an increase in adipose tissue mobilisation.

The results observed in the lean and *ob/ob* mice are consistent with the *in vitro* work described in section one and two of this chapter. M2 has been shown to increase lipolysis in isolated and cultured adipocytes. In addition, M2 has been shown to stimulate glucose uptake into 3T3-L1 adipocytes and L6 muscle cells (Bailey *et al.*, 2001). In conclusion, the *in vivo* and *in vitro* work performed here demonstrates that M2 increases adipose tissue lipolysis. Basal glycaemia in lean and obese mice is reduced after M2 treatment, which is possibly due to the increased uptake into skeletal muscle and adipose tissue.

Chapter 7: Guanidines

7.1 Introduction

Guanidine derivatives have anti-diabetic effects, as first shown in 1918 when rabbits injected with guanidine became hypoglycaemic (Watanabe, 1918). Many studies since this initial discovery have noted that mono and biguanidine compounds can decrease blood glucose levels (Meglasson *et al.*, 1993; Bischoff *et al.*, 1929). In addition, the insulin stimulatory effect of guanidine derivatives has been demonstrated *in vivo* and *in vitro* (Alsever *et al.*, 1970; Malaisse *et al.*, 1970; Floyd *et al.*, 1966).

One of the most widely used anti-diabetic drugs is a biguanide metformin (Bailey, 1992). Metformin was first introduced in 1957 as an oral glucose-lowering agent to treat type 2 diabetes (Sterne, 1957). The effects of metformin include increasing glucose utilisation in the body without stimulating insulin secretion, without weight gain and without causing overt hypoglycaemia. Due to the latter property, metformin is regarded as an anti hyperglycaemic agent rather than a hypoglycaemic agent. *In vitro* studies have demonstrated effects of metformin on liver, fat and muscle tissues (Bailey, 1992). Metformin has been shown to directly increase glucose uptake into rat skeletal muscle (Frayn and Adnitt, 1972), L6 skeletal muscle cells (Bates, 1999), insulin resistant human muscle (Galuska *et al.*, 1991) and normal rat adipocytes (Matthaei *et al.*, 1991; Jacobs *et al.*, 1986). Hence metformin has a direct effect on muscle to increase glucose uptake, which is anticipated to contribute to the lowering of blood glucose. In addition, metformin has been shown to decrease basal hepatic

glucose output in type 2 diabetic patients, which contributes to the lowering of blood glucose (Jackson *et al.*, 1987).

It has been demonstrated that 3-guanidinopropionic acid is one of the most potent alkyl guanidine derivatives due to its insulin-stimulatory effect (Aynsley-Green and Alberti, 1974). Here, 3-GPA caused increments in plasma insulin (32 μ U/ml) when given at 0.1 mmol/kg, a dose at which guanidine was without effect. In support of its potency, 3-GPA decreased the plasma glucose levels in hyperinsulinaemic, diabetic KKA^y and *ob/ob* mice (Meglasson *et al.*, 1993). It appears that 3-GPA requires circulating insulin to reduce hyperglycaemia since studies in streptozotocin-diabetic rats and Chinese hamsters, which have low circulating insulin levels failed to decrease plasma glucose concentrations (Gerritsen, 1982; Junod *et al.*, 1969). 3-GPA is an endogenous substance found in the plasma of mammals. Normal plasma concentrations in rat are approximately 0.1 μ M. However, a several hundred-fold increase in 3-GPA concentration is required to decrease blood glucose concentrations (Meglasson *et al.*, 1993). In contrast to metformin, the effects of 3-GPA on liver, muscle and adipose tissue have not previously been investigated.

7.2 Section One: *In vitro* lipolysis experiments

The mechanism of action of 3-GPA on adipose tissue has not been investigated previously. 3-GPA may have an effect on the adipose tissue to increase lipolysis. As previously discussed metformin is from the same guanidine family, and the effects of metformin on adipose tissue have been investigated. *In vitro* human adipose tissue

exposed to metformin did not affect lipolysis (Cigolini *et al.*, 1984). Moreover, metformin had no effect on adipose tissue lipolysis in type 2 diabetic patients treated with metformin (Pederson *et al.*, 1989). The action of 3-GPA on the adipose tissue was therefore investigated to compare with the peripheral effects observed with metformin.

7.2.1 Methods

Adipose tissue from lean and *ob/ob* mice was removed and isolated adipocytes (see section 2.3.3) were incubated with 3-GPA (10^{-5} to 10^{-2} M). The adipocytes were incubated for a set time of 2h at 37°C because incubation times over 2h reduce the accuracy of the method due to the occurrence of some cell death (data not shown). At the end of the incubation period the amount of glycerol released into the medium was measured as an indication of lipolysis (see section 2.3.4).

Concentration-response curves for 3-GPA were expressed in mM glycerol/ 10^5 adipocytes for the isolated adipocytes. Data are given as means \pm SEM, n=6 (duplicates analyses from three separate experiments). Significance was assessed by ANOVA post Dunnett's test. Probability levels of $p < 0.05$ were considered to indicate a significant difference.

7.2.2. Results

7.2.2a Effect of 3-GPA on Lipolysis

3-GPA at 10^{-4} , 10^{-3} and 10^{-2} M significantly increased lipolysis by approximately 50% in adipocytes taken from lean mice (see figure 7.1a). Moreover, 3-GPA significantly increased lipolysis in fat taken from *ob/ob* mice (see figure 7.1b). In adipocytes isolated from *ob/ob* mice, 3-GPA (10^{-4} - 10^{-2} M) increased lipolysis by 100-150% ($p < 0.05$). The mean values for glycerol release were higher after exposure to 3-GPA in the obese adipocytes (see figure 7.1b). The lipolytic rate of 3-GPA (10^{-2} M), measured by glycerol release was significantly higher in the obese adipocytes versus the lean adipocytes (0.060 ± 0.002 versus 0.028 ± 0.003 mM glycerol/ 10^5 adipocytes, $p < 0.05$).

Figure 7.1a Lipolytic activity of 3-GPA on lean mouse fat. **Figure 7.1b** Lipolytic activity of 3-GPA on obese mouse fat. Adipocytes were exposed to 3-GPA (10^{-5} - 10^{-2} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (n=6). *p<0.05 versus control.

Figure 7.1a

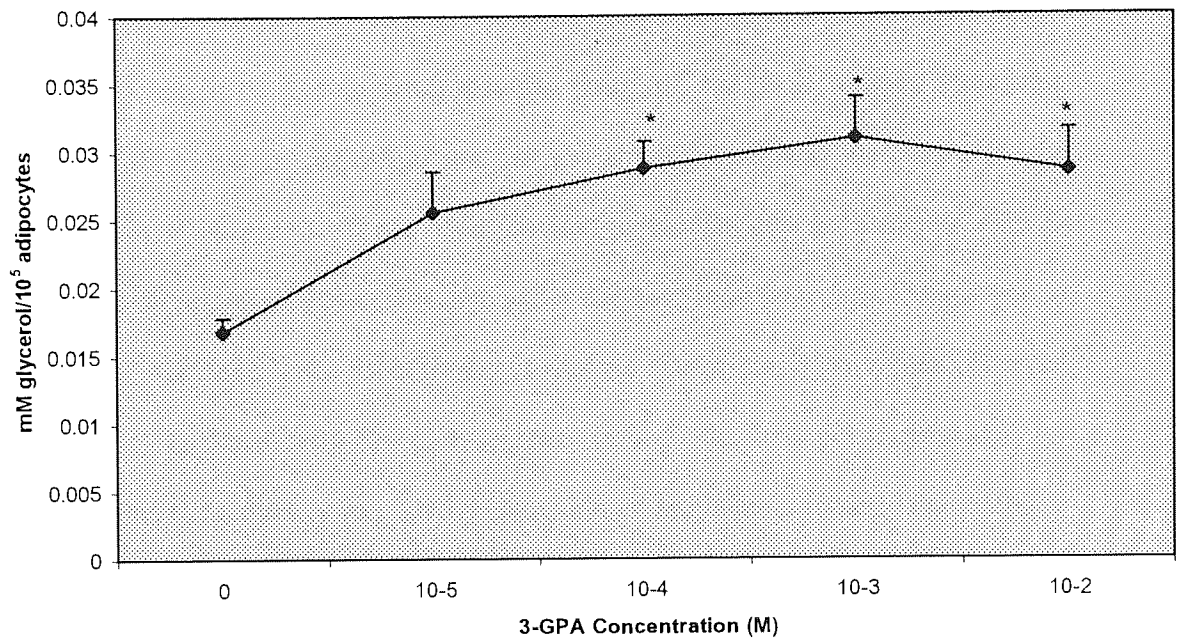
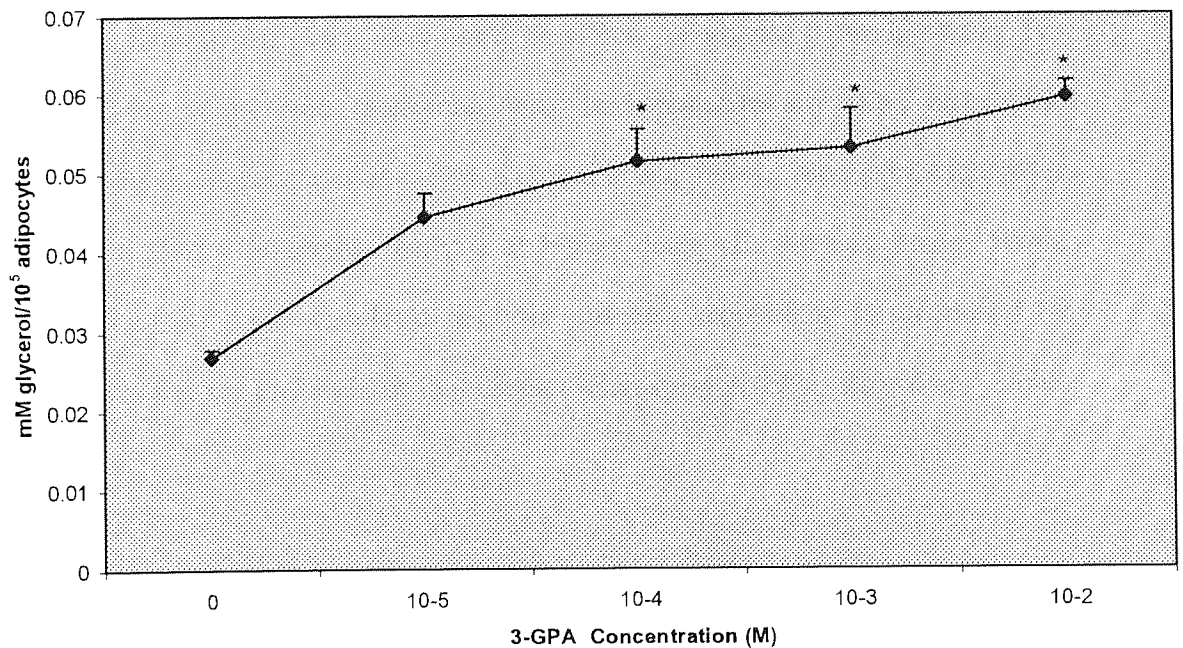


Figure 7.1b



7.2.3 Discussion

3-GPA increased glycerol release from both lean and obese mouse adipocytes isolated from the epididymal and parametrial adipose tissue depots. The mechanism of action of 3-GPA on the adipose tissue was not investigated further due to time constraints. However it might be hypothesised that the action of 3-GPA is through increasing cyclic AMP levels in the adipocyte via the lipolytic pathway described in the introduction of chapter 5. It has been reported previously that mice treated with 3-GPA have an improved glycaemic state and a decrease in body weight (Meglasson *et al.*, 1993). Although it has not been shown that the decrease in body weight observed with 3-GPA is specifically due to a decrease in body fat, it is not unreasonable to consider that the reduction in body weight could be attributable in part to an increase in white adipose tissue lipolysis.

The present result observed with 3-GPA is not consistent with studies of some other guanidine derivatives, which have noted no effects on adipose tissue lipolysis. For example, two studies with metformin failed to demonstrate any effect on adipose tissue lipolysis (Pederson *et al.*, 1989; Cigolini *et al.*, 1984). Moreover, biguanides have been shown to inhibit lipolysis at higher doses (Brown and Stone, 1968). Dibutyryl cyclic AMP-stimulated lipolysis was also reported to be decreased by phenformin but not buformin or metformin (Wiernsperger, 1996; Brown and Stone, 1968).

Taken together, these findings suggest that 3-GPA acts to increase adipocyte lipolysis. This effect may be mediated via a mechanism, which is coupled to the G protein

complex (see chapter 5, section 1). In contrast, biguanides have no lipolytic effect, which suggests that the condensation of two guanidine molecules results in a lack of adipocyte lipolysis.

7.3 Section Two: *In vitro* glucose uptake experiments

L6 cells (see chapter 4) provide an *in vitro* model, which has been used extensively to identify drugs and other agents that increase glucose uptake. The L6 cells therefore enable screening for anti-diabetic drugs which affect glucose uptake into muscle. Here, the L6 cells were used to investigate whether 3-GPA acts directly on muscle cells to improve glucose uptake and insulin action. 3-GPA has been reported to have a similar glucose lowering effect to metformin (Meglasson *et al.*, 1993). Meglasson *et al.* (1993) reported that 3-GPA was an effective glucose-lowering agent in animal models of diabetes. They suggested that it might have advantages over metformin in that it was more likely to facilitate weight loss, and showed little increase in lactate production. The mechanism of action of 3-GPA *in vitro* was evaluated using the L6 muscle cell line, to complement an *in vivo* study in obese diabetic *ob/ob* mice (see section three).

7.3.1 Method

L6 muscle cells were grown (see section 2.5.1), characterised (see chapter 4) and when confluent were plated out for ³H2DG uptake experiments (see section 2.5.3). The cells were plated out at a concentration of 1×10^7 cells/ml per well, and the 3-GPA was added to the cells at concentrations of 10^{-10} - 10^{-3} M, thus a concentration curve for the effect of the guanidine derivative was established. As a comparator in the present study a metformin (10^{-5} - 10^{-2} M) concentration-response curve and an insulin concentration-response curve were performed on the L6 cells (see chapter 4).

The effect of 3-GPA on 2DG uptake by the L6 cells over time (10min, 1h, 4h, 8h and 24h) was also carried out to show the time dependency of the drug. 3-GPA was added to the cells with and without insulin and with and without glucose to measure whether the hyperinsulinaemia and hyperglycaemia would affect the drug's mechanism of action on the L6 cells. At the end of the incubation period, the effect of 3-GPA on 2-deoxyglucose transport by the cells was measured.

Concentration-response curves were expressed as % of control values. Data are given as means \pm SEM, n=6 for each concentration and test compound (duplicates analyses from three separate experiments). Significance was assessed by ANOVA, post Dunnett's test and Student's paired t-test. Probability levels of $p < 0.05$ were considered to be significant.

7.3.2 Results

7.3.2a Effect of 3-GPA on Basal and Insulin-Stimulated Glucose Uptake in L6 muscle cells

In the absence of added insulin, 24h incubation with 3-GPA at 10^{-5} and 10^{-4} M increased mean values for 2-deoxyglucose uptake into the L6 muscle cells by 12% and 11%. However, these values were not statistically significant from the controls (see figure 7.2a). Addition of a sub-maximally stimulating insulin concentration (10^{-8} M) alone increased glucose uptake by approximately 20%. Addition of 3-GPA in the presence of 10^{-8} M insulin for 24h significantly increased 2-deoxyglucose uptake by

the L6 muscle cells compared with insulin alone by a further 20-40% at all concentrations of 3-GPA (see figure 7.2a). The effects observed at 24h were not observed at the 10min, 1h, 4h and 8h incubation times (data not shown).

As mentioned above 3-GPA is a guanidine derivative as is metformin. *In vitro* studies to demonstrate that metformin has anti-diabetic effects have been performed as an active comparator for the 3-GPA experiments. Metformin (10^{-3} and 10^{-2} M) significantly increased glucose uptake into the L6 cells by 68% and 76% (see figure 7.2b). The action of metformin on the cells was apparently independent of the concentration of insulin, in so far a similar effect was evident without added insulin and in the presence of 10^{-8} M insulin.

Meglasson *et al.* (1993) suggested that 3-GPA was only effective in animal models of diabetes, where hyperinsulinaemia and hyperglycaemia exist. Thus to test this hypothesis *in vitro*, the L6 cells were incubated in the presence of high glucose (25mM) and high insulin (10^{-6} M) concentrations with addition of 3-GPA for 24h (see figure 7.2c). The results suggest that a high glucose concentration does not significantly alter 2-deoxyglucose uptake by the L6 cells over 24h, whereas a high insulin concentration increased 2-deoxyglucose uptake as similarly noted in figure 7.2a. Once again, 3-GPA alone had no *in vitro* effect on 2-deoxyglucose uptake by L6 cells at 24h. A decrease in glucose uptake was observed in incubations with 3-GPA at 10^{-3} M (see figure 7.2a and 7.2c), which suggests that the compound is toxic at this concentration and causes cell death.

Figure 7.2a Effect of 3-GPA ± on 2DG uptake in L6 skeletal muscle cells. Cells were exposed to 3-GPA for 24h at concentrations 10^{-10} - 10^{-3} M. Results expressed as mean values ± SEM (n= 6). +p<0.05 versus 3-GPA only.

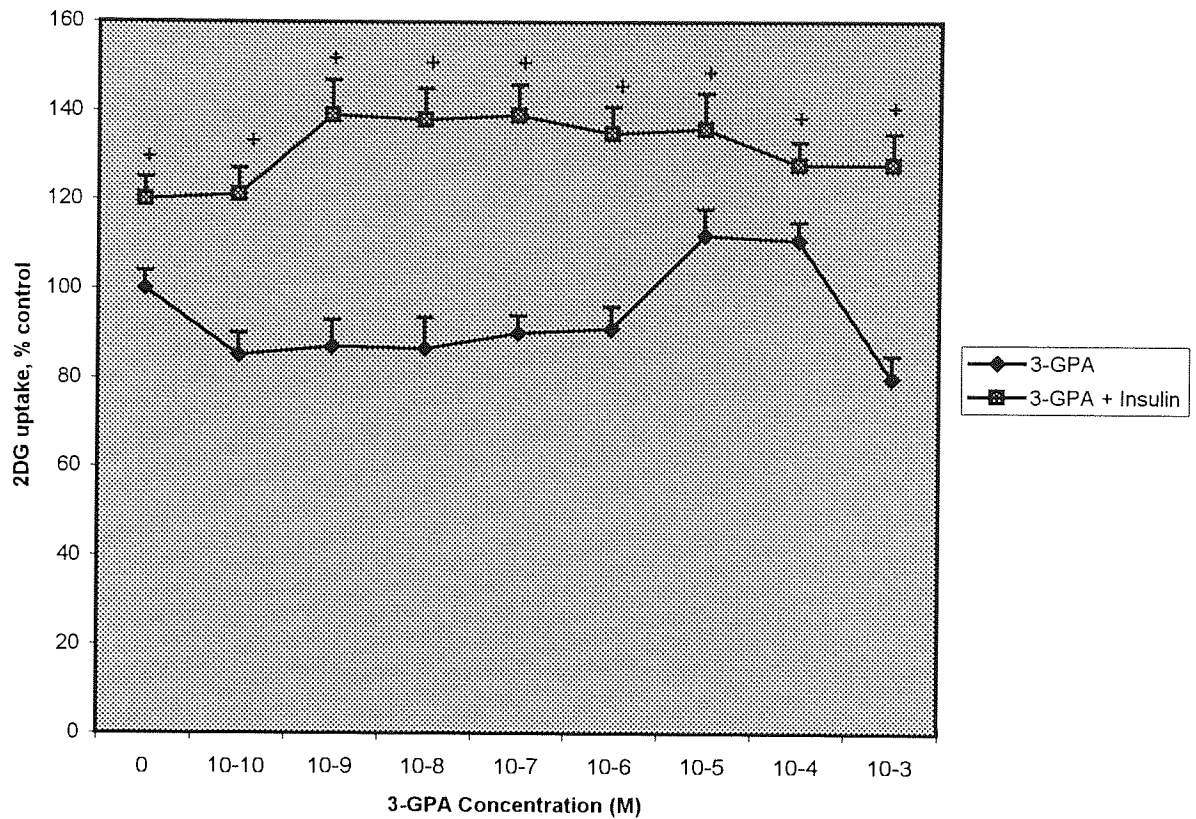


Figure 7.2b Effect of metformin ± insulin 10^{-8} M on 2DG uptake in L6 skeletal muscle cells. Cells were exposed to metformin for 24h at concentrations 10^{-5} - 10^{-2} M. Results expressed as mean values ± SEM (n= 6). * p<0.05 versus control.

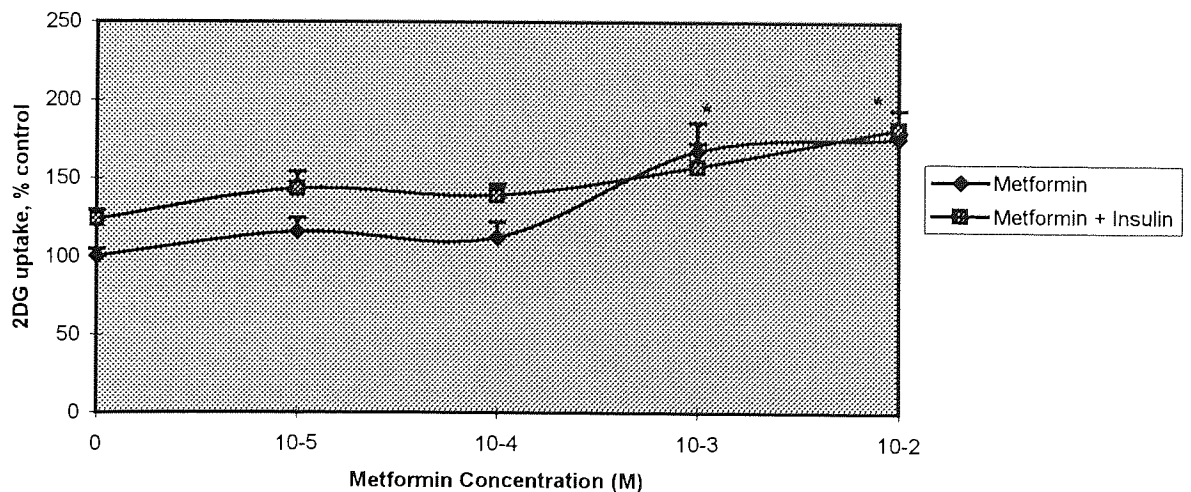
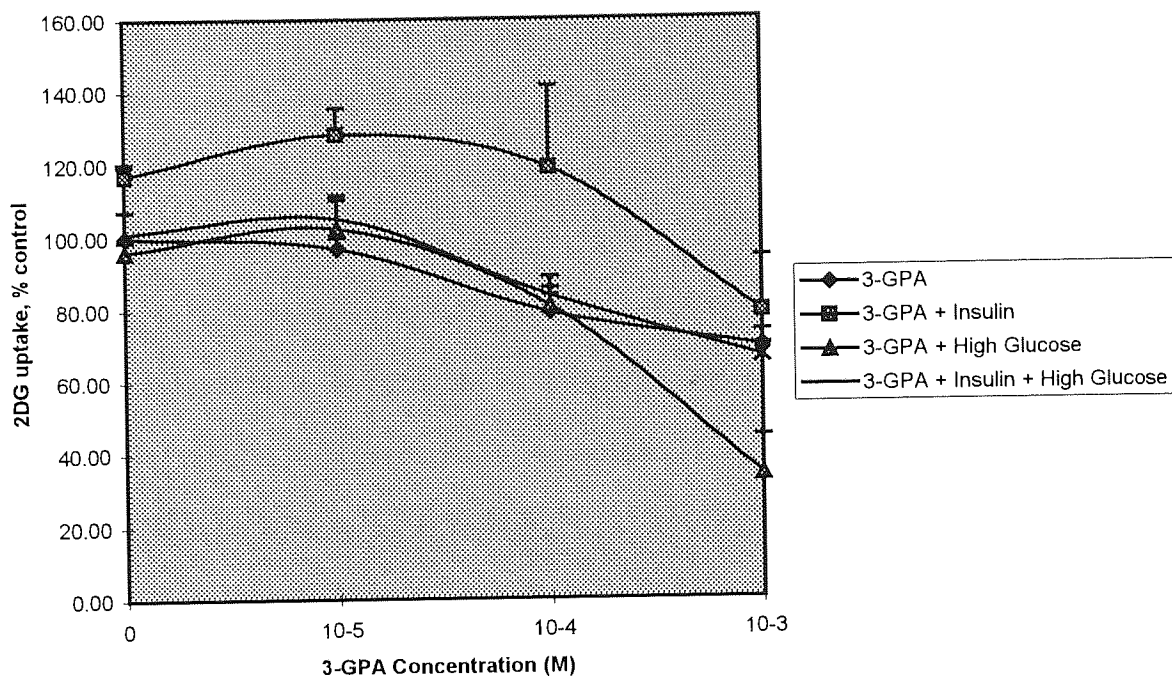


Figure 7.2c Effect of 3-GPA \pm insulin (10^{-6} M), 3-GPA \pm glucose (25mM) and 3-GPA \pm glucose (25mM) and insulin (10^{-6} M) on 2DG uptake in L6 skeletal muscle cells. Cells were exposed to metformin for 24h at concentrations 10^{-5} - 10^{-2} M. Results expressed as mean values \pm SEM (n= 6).



7.3.3 Discussion

The results suggest that 3-GPA in the presence of insulin acts weakly on the L6 cells to increase glucose uptake. 3-GPA enhanced insulin-stimulated (10^{-8}M) glucose uptake during 24h incubation, but did not act independently of insulin at this time point. 3-GPA alone significantly increased glucose uptake in the L6 cells by 12%. In addition, 3-GPA failed to stimulate basal and insulin-stimulated glucose uptake into 3T3-L1 differentiated cells (data not shown). In an *ex-vivo* study, Ren *et al.* (1993) demonstrated that maximally-stimulated 3-*O*-methylglucose transport was 49% higher in muscles of 3-GPA-fed rats as compared with the control group. This suggests that 3-GPA can increase glucose uptake into isolated muscle tissues (exposed to 3-GPA prior to removal from the rat) but not L6 skeletal muscle cells, suggesting that 3-GPA must be able to influence muscle glucose uptake indirectly via other effects *in vivo*.

Metformin (10^{-2}M) increased 2-deoxyglucose uptake into the L6 muscle cells by 76%. This result is consistent with numerous studies that have demonstrated a stimulatory effect of metformin in L6 cells (Bates, 1999; Klip *et al.*, 1992). The effect of metformin on the L6 muscle cells is mediated via a mechanism alternative to PI3-Kinase mediated signalling (Bates, 1999). Here, metformin has been demonstrated to increase basal and insulin-stimulated glucose transport via a pathway involving new protein synthesis (Bates, 1999; Klip *et al.*, 1992).

Taken together, these results suggest that 3-GPA was not as effective as metformin as a stimulant of 2-deoxyglucose uptake into L6 skeletal muscle cells. 3-GPA did not

increase glucose uptake at 24h exposure to the cells. Longer incubation times may be required to observe the 3-GPA insulin-stimulated glucose uptake effects demonstrated by Ren *et al.* (1993). Due to time constraints this could not be undertaken, but it would not be expected that an agent with a rapid (within 24h) effect *in vivo* would take longer than 24h to exert a measurable effect *in vitro* if a direct effect was occurring.

7.4 Section Three: *In vivo* studies using obese-diabetic *ob/ob* mice

3-GPA effects on the isolated lean and obese mouse adipocytes and the L6 muscle cell line have been investigated in section one and two of this chapter. The latter part of this chapter is focused upon the effects of 3-GPA *in vivo*. The chronic effects of the guanidine derivative will be investigated in obese-diabetic *ob/ob* mice.

7.4.1 Method

Obese *ob/ob* mice of either sex, aged 10-20 weeks, a weight range of 65g -100g, were obtained from the Aston University breeding colony. The characteristics of these mice have been described fully in section 2.2.2. The mice were separated into 2 groups; control, 3-GPA treated (500mg/kg). In each group were 6 mice matched for body weight and basal glucose concentration and had a similar age and equal sex destination. The control and 3-GPA groups were caged separately and maintained at 21°C with a 12h light-dark cycle. Fresh water (supplemented with freshly prepared 3-GPA as appropriate) and rodent pellet diet were *ad libitum*.

The 3-GPA was administered in the drinking water at a dose of 500mg/kg/day. Food and water consumption were monitored before, during and after the study. Body weights of the mice were measured daily starting 7 days prior to, during the 28 days when 3-GPA was administered and for 14 days after drug administration. Blood glucose levels in all mice (control and 3-GPA treated) were measured each week using a glucose-oxidase based portable glucose monitor (Glucotrend®, Boehringer

Mannheim). Blood samples taken from the tail tip of the mice were collected each week as described in chapter 2.2.4. The plasma was then stored at -40°C until analysed for insulin and free fatty acid content (described in 2.2.4). At the end of the study, an intra-peritoneal glucose tolerance test (IPGTT) was performed (fully described in section 2.2.5).

7.4.2 Results

7.4.2a Chronic Effect of 3-GPA on Basal Non-Esterified Fatty Acids, Insulin and Glycaemia in *ob/ob* Mice

The 2 groups of obese *ob/ob* mice at the run in period of the study displayed a similar increase in body weight and similar levels of hyperglycaemia. Treatment of the *ob/ob* mice with 3-GPA for 4 weeks prevented further weight gain (see figure 7.3). Body weight gain in *ob/ob* mice treated with 3-GPA for 28 days was $+1.26 \pm 1.98\text{g}$ vs $12.8 \pm 1.92\text{g}$ in the untreated group, $p < 0.0001$. Weight gain returned after treatment was stopped. At the end of the treatment period there was a significantly lower body weight (by 10g, $p < 0.0001$) and a significant decrease in food intake (by 3g, $p < 0.001$) when comparing the 3-GPA treated mice with the control mice (see figure 7.4).

3-GPA however did not produce any significant changes in basal plasma glucose. Initially, for the first 2 weeks, the plasma glucose in the *ob/ob* mice treated with 3-GPA decreased, but then gradually started to increase back to normal (see figure 7.5).

An IPGTT was performed on the *ob/ob* mice to determine whether the 3-GPA had altered glucose homeostasis in the mice that received the drug. After administering glucose to the mice, the blood glucose disappearance rate was similar in the two groups (see figure 7.6). In addition, 3-GPA did not significantly alter the basal hyperinsulinaemia, and elevated free fatty acids of the *ob/ob* mice (data not shown).

In conjunction with the present study, a pair fed study on *ob/ob* mice was conducted for a 28 day period. The mice were given the same amount of food as consumed by the 3-GPA treated mice, which was approximately 7g food per day per mouse (see figure 7.4). Food and water consumption during and after the study were monitored. Body weights of the mice were measured daily for the 28 days and for 14 days after the study. The mice consumed all of the food given and water consumption was at a normal intake rate (about 5ml/day/mouse). Weights of the mice increased gradually during the study, and once normal feeding was resumed, their weights increased markedly (see table 1).

Table 1: Body weights (g) of *ob/ob* mice

	BASELINE	28 DAY STUDY	14 DAYS AFTER STUDY
CONTROL MICE	84.2 +/- 1.83	97.1 +/- 2.02	99.8 +/- 2.50
PAIR FED MICE	79.76 +/- 1.86	90.6 +/- 2.69 *	99.5 +/- 3.93
3-GPA TREATED MICE	84.6 +/- 1.69	85.86 +/- 2.28 **	91.8 +/- 4.45

*p<0.05 versus control (ANOVA, post Dunnett's test)

**p<0.05 versus control and pair fed mice (ANOVA, post Dunnett's test)

The results above show that 3-GPA had a significant affect on body weight and by controlling the food intake in the pair fed mice, the average weight of the mice was also significantly less than the control mice. The glucose levels in the pair fed *ob/ob* mice were also analysed each week and an IPGTT was performed at the end of the study. There was no change in the basal hyperinsulinaemia, hyperglycaemia, elevated free fatty acids and glucose intolerance of the pair fed *ob/ob* mice (data not shown).

Figure 7.3 Chronic effect of 3-GPA (500mg/kg) on body weight of *ob/ob* mice. Mice received either 3-GPA (drinking water) or water (control). Results expressed as mean values \pm SEM (n= 6). *p<0.05 versus control.

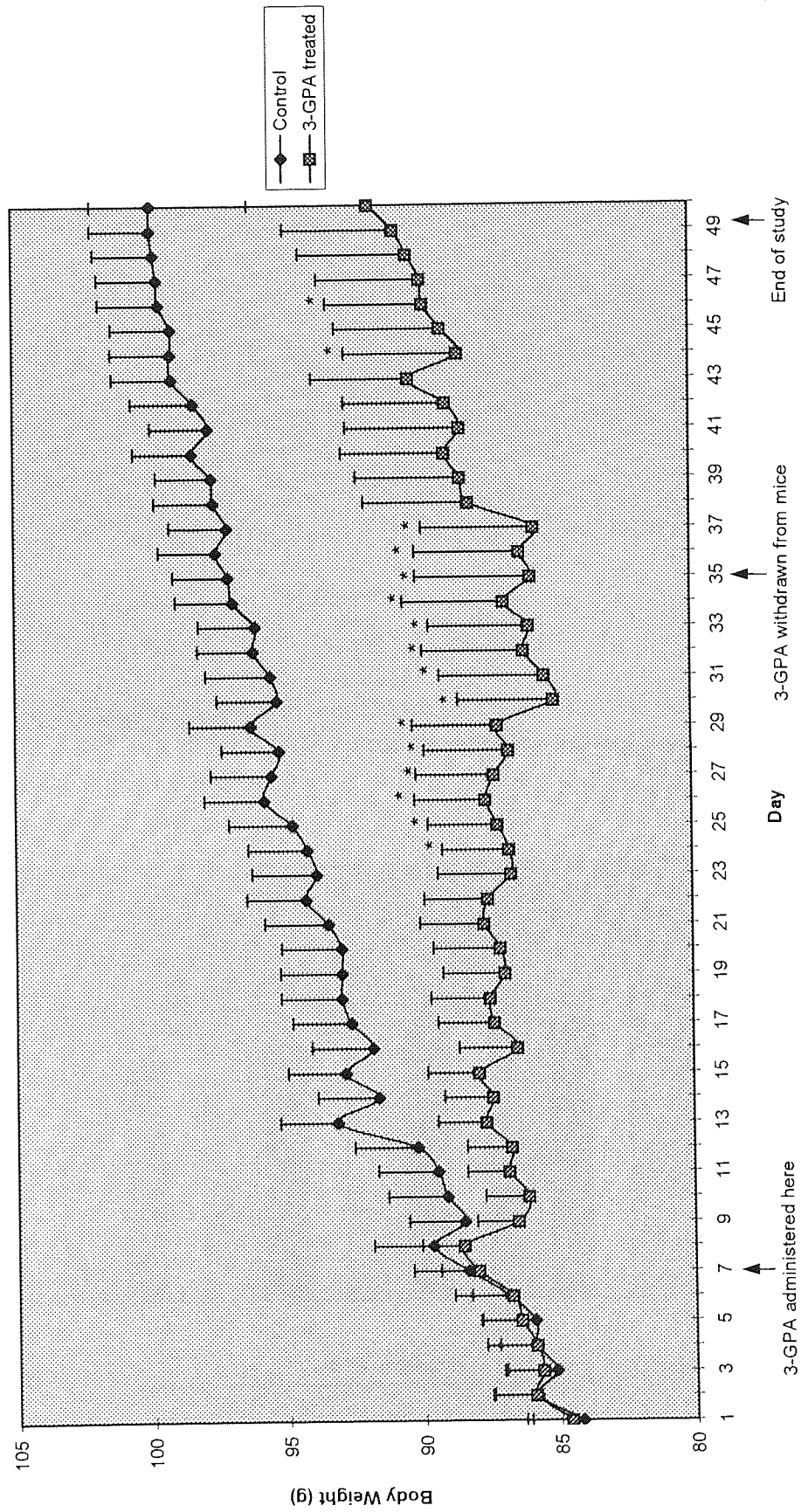


Figure 7.4 Chronic effect of 3-GPA (500mg/kg) on food intake (g) of *ob/ob* mice. Mice received either 3-GPA (drinking water) or water (control). Results expressed as mean values \pm SEM (n= 6). * p<0.05 versus control.

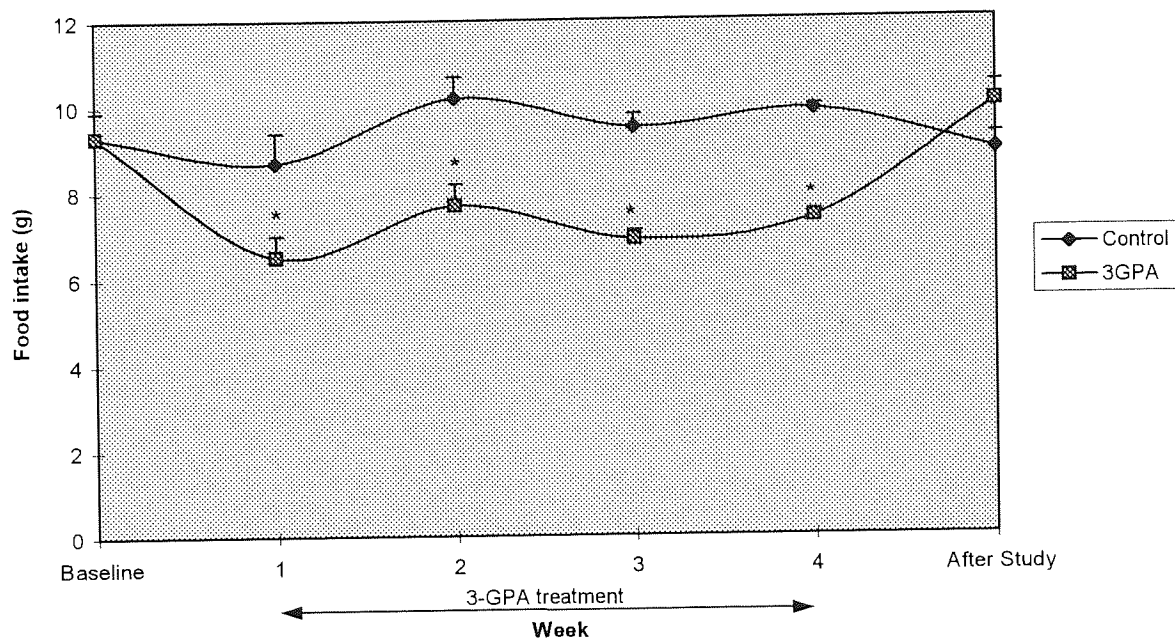


Figure 7.5 Plasma concentrations of glucose in *ob/ob* mice during chronic 3-GPA treatment (500mg/kg). Mice received either 3-GPA (drinking water) or water (control). Results expressed as mean values \pm SEM (n= 6).

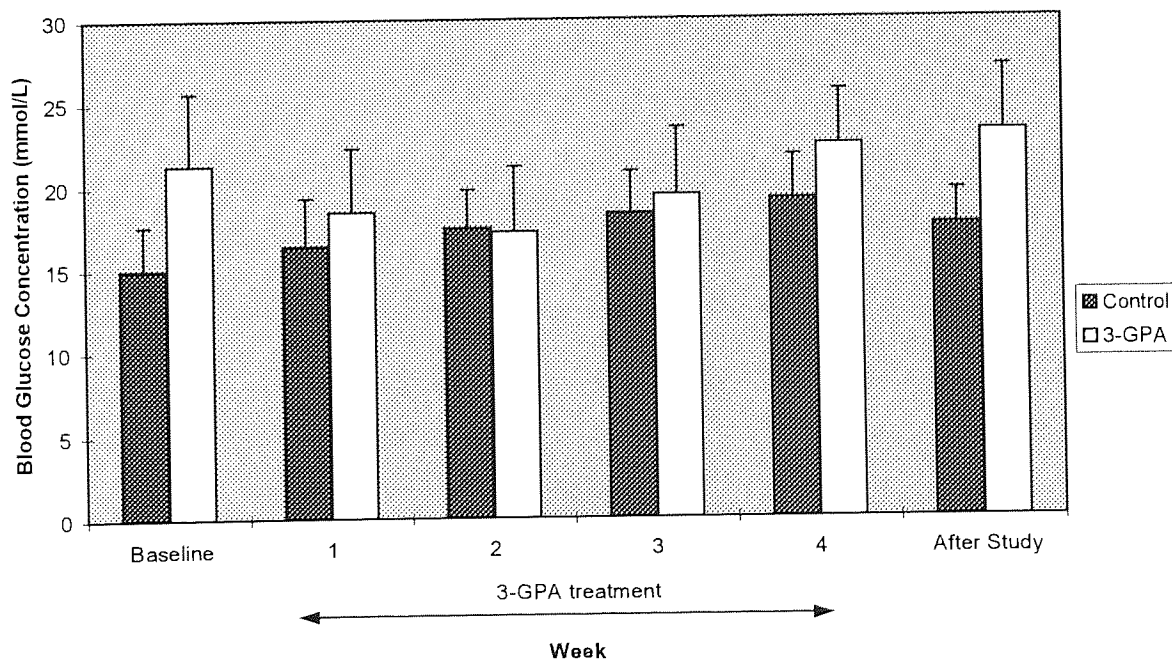
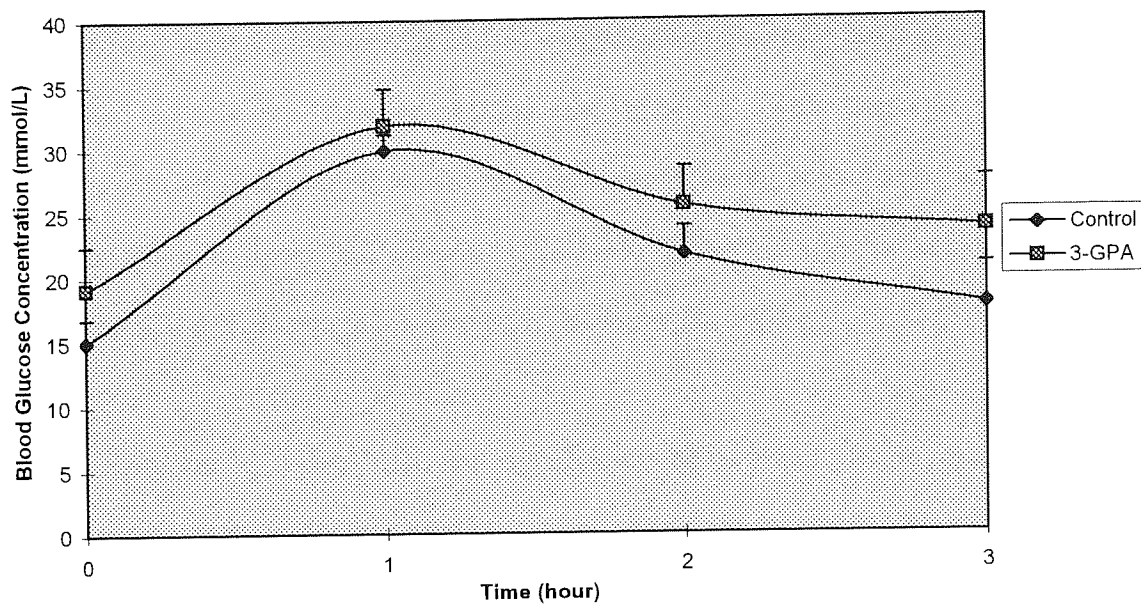


Figure 7.6 Plasma concentrations of glucose in *ob/ob* mice following an IPGTT. Mice received either 3-GPA (500mg/kg in the drinking water) or water (control). Results expressed as mean values \pm SEM (n= 6).



7.4.3 Discussion

The results indicate that 3-GPA reduces obesity and hyperphagia in obese-diabetic *ob/ob* mice associated with increased adipocyte lipolysis (see chapter 7, section 1). A study in pair-fed mice showed that the lack of weight gain in the *ob/ob* mice could not be attributed entirely to decreased feeding (see table 1). This contrasts with metformin, which has been studied with the same experimental procedures and did not significantly alter lipolysis (Pederson *et al.*, 1989; Cigolini *et al.*, 1984) or long-term body weight or food intake in *ob/ob* mice (Bailey *et al.*, 1986).

In the present study, 3-GPA did not significantly alter basal plasma glucose concentrations in obese mice, probably due to the extremely severe insulin resistance. This finding is consistent with the data presented by Aynsley-Green and Alberti (1974), who observed that blood glucose was unaffected by 3-GPA. By the stage of the *ob/ob* syndrome at which obesity, hyperglycaemia and insulin resistance are well established, modest changes in body weight do not appear to produce significant alterations in the hyperphagia or insulin resistance. However these data are not consistent with another finding, which demonstrated blood glucose lowering properties of 3-GPA (Meglasson *et al.*, 1993). Meglasson *et al.* (1993) observed a decrease in the plasma glucose concentrations in *ob/ob* and KKA^y mice. In addition, the effects were more potent than with metformin treatment in the same mice.

Thus the present study certainly endorses the view that 3-GPA is not as effective an anti-diabetic agent in *ob/ob* mice as reported for metformin (Bailey, 1992). However,

3-GPA does decrease body weight in the *ob/ob* mice through a decrease in food intake and as such might be a useful anti-obesity drug.

Chapter 8: Mazindol

8.1 Introduction

Pharmacological treatments used for obesity include noradrenergic and serotonergic agents and can be divided into three groups: (1) drugs that reduce food intake, (2) drugs that increase energy expenditure, and (3) drugs that exert other effects on metabolism (Bray, 2000).

Mazindol is one of the older weight loss medications approved clinically as a short-term adjunct to non-pharmacologic treatments for obesity. It is a centrally acting appetite suppressant and has been shown to suppress feeding in humans and rodents by stimulating catecholaminergic pathways and directly suppressing neurons in the lateral hypothalamus (Mattei and Carlini, 1995; Inoue 1995; Inoue *et al.*, 1992). Mazindol plus diet therapy in severely obese women was shown to be a useful means of treating severe obesity (Yoshida *et al.*, 1994). In addition, studies in obese-diabetic patients and diabetic rats treated with mazindol have shown a significantly greater weight loss and an improvement in glucose tolerance when compared with placebo (Scheen and Lefebvre, 2000; Chun *et al.*, 1998).

Mazindol appears to reduce body weight in obesity by decreasing the white adipose tissue depots. Yoshida *et al.* (1996) demonstrated in obese-diabetic yellow KK mice a significant reduction in the amount of WAT in subcutaneous, mesenteric and retroperitoneal regions. In addition, mazindol decreased the levels of blood glucose and serum insulin in the same mice, indicating a potential role for this drug in the

treatment of type 2 diabetes mellitus. To evaluate the anti-diabetic effects of mazindol, Nagai *et al.* (1994) investigated its pharmacological action. Mazindol was shown to stimulate 2-deoxy-D-glucose transport into sarcolemmal vesicles of the gastrocnemius muscle *in vitro* and that this stimulation was blocked by cytochalasin B.

8.2 Section One: *In vitro* lipolysis experiments

From the Yoshida *et al.* (1996) study the results demonstrate a reduction in WAT depots in the obese-diabetic yellow KK mice treated with mazindol for 2 weeks. The decrease in WAT depots could be attributable in part to an increase in white adipose tissue lipolysis. Thus this present study investigates possible direct metabolic effects of mazindol using freshly isolated white adipocytes from lean and obese-diabetic *ob/ob* mice and cultured 3T3-L1 preadipocytes.

8.2.1 Methods

Adipose tissue from lean and *ob/ob* mice was removed and isolated adipocytes (see section 2.3.3) were incubated with mazindol (10^{-12} - 10^{-5} M) for 2h at 37°C. The mechanism for the lipolytic effect of mazindol was studied in cultured 3T3-L1 preadipocytes. The 3T3-L1 preadipocytes were grown to confluence in 24 well plates (see section 2.4.1) and incubated with fluoxetine, nisoxetine, serotonin, fenfluramine, nordexfenfluramine, phentermine, and dopamine (10^{-8} - 10^{-6} M) for 24h at 37°C (see

chapter 6, table 4). The concentration of glycerol released into the medium was measured enzymatically using the method of Wieland (1974) (see section 2.3.4).

Concentration-response curves for the lipolytic effects of mazindol, fenfluramine, nordexfenfluramine, phentermine, serotonin, fluoxetine, dopamine and nisoxetine were expressed in mM glycerol/ 10^5 adipocytes for the isolated adipocytes and mM glycerol/ 10^6 preadipocytes per ml for the 3T3-L1 preadipocytes. Experiments were undertaken in duplicate on at least 3 occasions. All data were expressed as mM glycerol released \pm standard error. Significance was assessed by ANOVA post Dunnett's test. Probability levels of $p < 0.05$ were considered to indicate a significant difference.

8.2.2. Results

8.2.2a Effect of Mazindol on Lipolysis

In adipocytes isolated from lean mouse epididymal and parametrial fat pads, incubation with mazindol (10^{-10} - 10^{-5} M) increased lipolysis (measured by glycerol release) by 150-250% ($p < 0.05$) in a concentration-related manner up to (10^{-6} M) (see figure 8.1a). In adipocytes isolated from *ob/ob* mice, mazindol (10^{-8} - 10^{-5} M) increased lipolysis by 50-75% ($p < 0.05$) (see figure 8.1b).

The mechanism for the lipolytic effect of mazindol was studied in cultured 3T3-L1 preadipocytes. Whereas mazindol (10^{-7} - 10^{-5} M) increased lipolysis by about 75-150% by the cultured 3T3-L1 preadipocytes (see figure 8.1c), fenfluramine, nordexfenfluramine and phentermine did not significantly alter lipolysis. Also, serotonin, fluoxetine, dopamine and nisoxetine were without a significant effect on lipolysis in these cells (see figure 8.1d).

Figure 8.1a Lipolytic activity of mazindol on lean mouse fat. **Figure 8.1b** Lipolytic activity of mazindol on obese mouse fat. Adipocytes were exposed to mazindol (10^{-12} - 10^{-5} M) for 2h at 37°C . Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (n=6). * $p < 0.05$ versus control.

Figure 8.1a

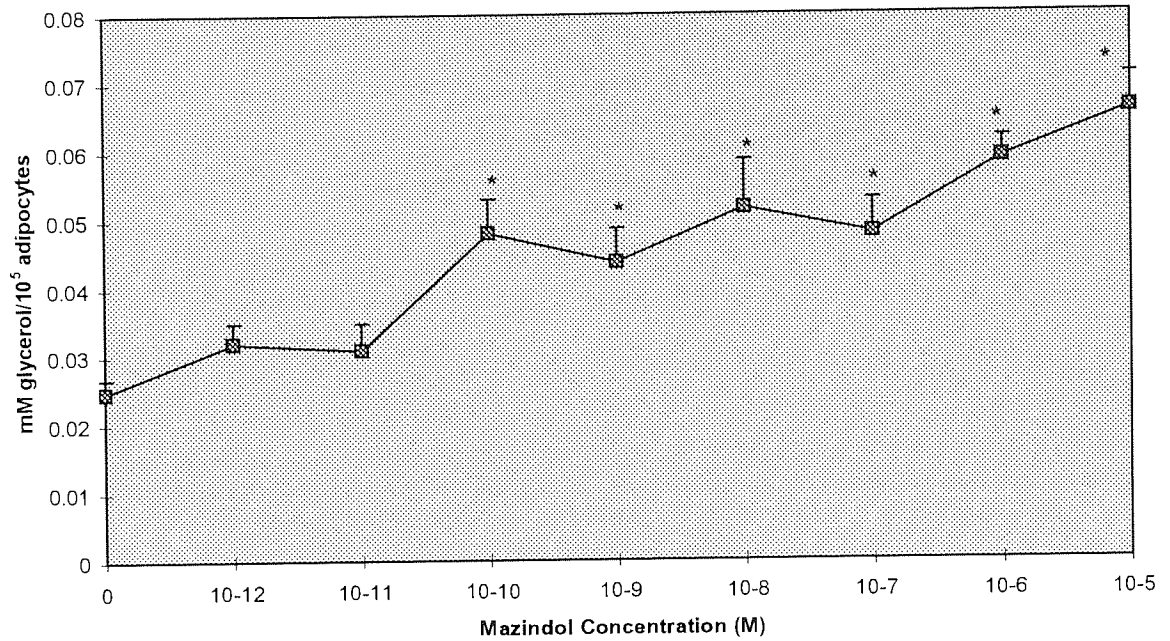


Figure 8.1b

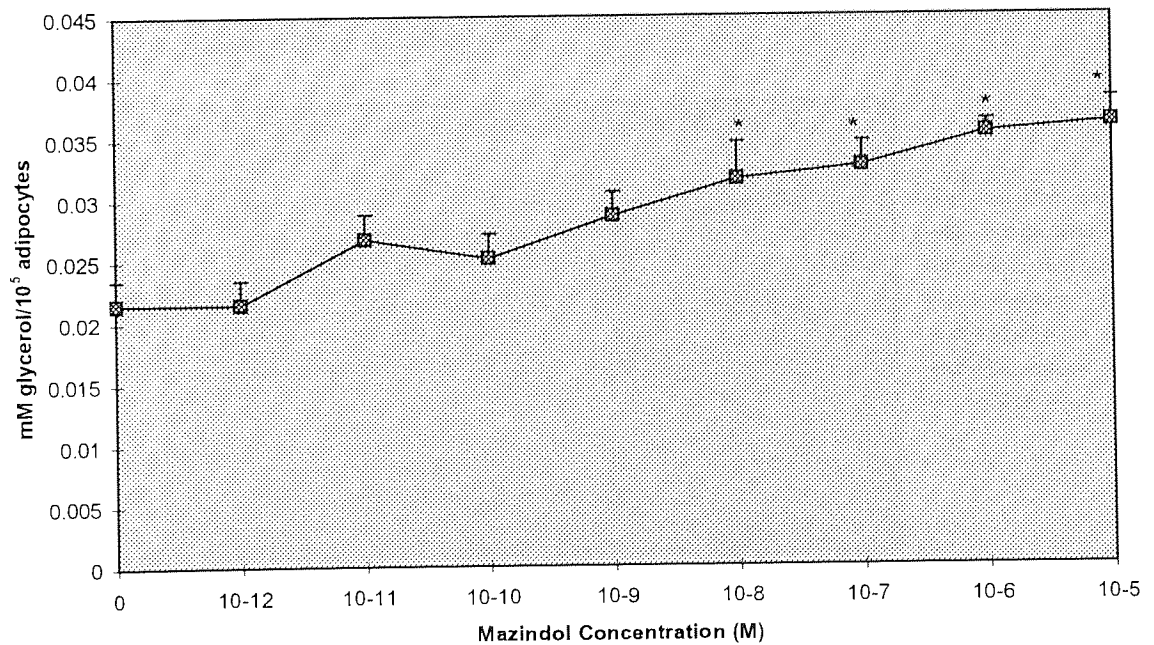


Figure 8.1c Lipolytic activity of mazindol on 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were exposed to mazindol at 10^{-10} - 10^{-5} M for 24h at 37°C. Results expressed as mM glycerol/ 10^6 preadipocytes. Mean values \pm SEM (n=6). *p<0.05 versus control.

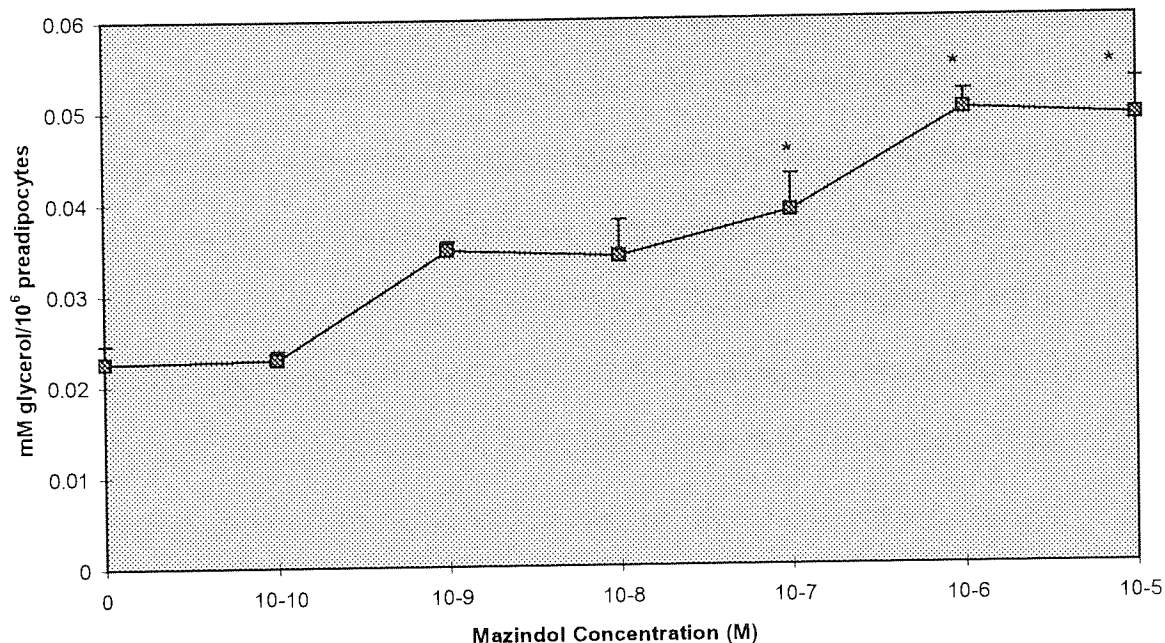
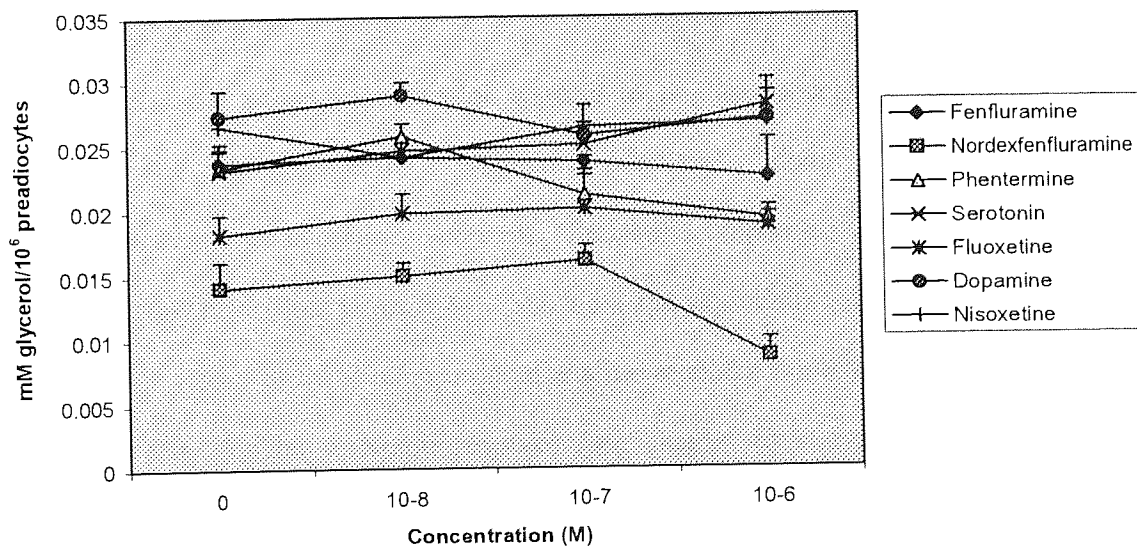


Figure 8.1d Lipolytic activity of fenfluramine, nordexfenfluramine, phentermine, serotonin, fluoxetine, dopamine and nisoxetine on 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were exposed to all agents at 10^{-8} - 10^{-6} M for 24h at 37°C. Results expressed as mM glycerol/ 10^6 preadipocytes. Mean values \pm SEM (n=6).



8.2.3 Discussion

The results indicate that mazindol can act directly on the adipocyte to increase lipolysis. Mazindol was more effective at increasing lipolysis in adipocytes isolated from lean mice compared with the obese-diabetic *ob/ob* mice. Possible explanations for the reduced lipolytic response of mazindol on the obese adipocytes include fat cell size and a decrease in the number of β adrenoceptors on the cell surface membrane. Larger fat cells have a reduced responsiveness to lipolytic hormones, which is possibly due to a reduction in β adrenoceptor numbers or a reduced Gs expression or activity (Arner, 1988; DiGirolamo and Fried, 1987). During characterisation of the lean and obese mouse adipose tissue (see chapter 5), a much-reduced lipolytic stimulus of isoproterenol was observed in obese mouse adipocytes versus the lean mouse adipocytes, which reaffirmed the impaired lipolytic responses of these adipocytes (Lemonnier, 1972). Mazindol action on the obese adipocytes was reduced by approximately 50% versus the lean adipocytes, which supports a reduction in either receptors on the cell membrane or a defect in the lipolytic pathway.

The mechanism of action of mazindol appears to be independent of serotonin, dopamine and norepinephrine reuptake inhibition since fenfluramine, nordexfenfluramine, phentermine, serotonin, fluoxetine, dopamine and nisoxetine were without any effect (see figure 8.2d). Mazindol and sibutramine share a common mechanism of action in that both are noradrenaline reuptake inhibitors. However, sibutramine also inhibits serotonin reuptake (see chapter 6) and shares another common feature with mazindol in that its metabolite (M2) stimulates adipocyte lipolysis (see section 6.2.4). Studies in chapter 6 have confirmed that M2 can act

directly on adipose tissue to increase lipolysis via a pathway involving the β adrenoceptors and components of the lipolytic signalling pathway. Taken together, these results suggest that the action of mazindol on adipose tissue is similar to that described for M2.

In conclusion, mazindol is a potentially useful treatment for obesity due to its suppression of appetite via inhibition of neurons in the lateral hypothalamus (Inoue, 1995) and because of its direct metabolic effect to increase adipocyte lipolysis.

8.3 Section Two: *In vitro* glucose uptake experiments

Part One: L6 skeletal muscle cells

Mazindol has been shown to decrease blood glucose levels in obese-diabetic yellow KK mice treated for approximately 2 weeks (Yoshida *et al.*, 1996). The decrease in blood glucose levels could be attributable in part to an increase in glucose uptake into muscle. In one study, it has been demonstrated that mazindol significantly increased glucose uptake into the gastrocnemius muscle *in vitro* (Nagai *et al.*, 1994). In this present study the metabolic effects of mazindol were evaluated. The main objective was to investigate whether mazindol acts directly on L6 muscle cells to improve glucose uptake and insulin action.

8.3.1 Method

L6 muscle cells were grown to confluence in 24 well plates seeded at 1×10^7 cells/ml (see section 2.5.1). Once confluent the myoblasts were serum starved (0.5% foetal calf serum) to induce differentiation and fusion into myotubes. Myotubes were incubated with mazindol (10^{-10} - 10^{-5} M) \pm insulin (10^{-8} M) at 1h, 4h, 8h and 24h. Known inhibitors of the intracellular signalling pathway of insulin-stimulated glucose transport in L6 skeletal muscle cells (see chapter 4) were used to determine the possible mechanisms by which mazindol increases glucose transport. The effect of LY 294 002 (10^{-8} - 10^{-5} M) and cycloheximide (10^{-8} - 10^{-5} M) (table 1) on glucose uptake was determined in the presence of mazindol (10^{-7} M) and insulin (10^{-7} M).

Table 1: Inhibitors of the Insulin-Stimulated Glucose Signalling Pathway

Compound	Site of Action
Cycloheximide (10^{-8} - 10^{-5} M)	Inhibits protein synthesis and therefore blocks the synthesis of glucose transporters
LY-294 002 (10^{-8} - 10^{-5} M)	Inhibitor of PI3-Kinase

Glucose uptake by the cells was assessed using the non-metabolised glucose analogue 2-deoxyglucose (2DG) (see section 2.5.3). Cell monolayers were washed with glucose-free Krebs buffer at 22°C, then incubated in 1ml of Krebs buffer supplemented with 0.1mM 2DG and 2D[³H]G (0.2μCi/ml) for 10min at 22°C. The buffer was then aspirated and cells were washed twice with ice-cold Krebs buffer. 0.5ml of 1M NaOH was pipetted into the culture dishes and left for 1h. The cell digest was transferred to vials containing 4ml of scintillant (HiSafe 3) and ³H counted.

Experiments were undertaken in multiples of 3 wells on at least 3 occasions. All data were expressed as mean values ± standard error. Uptake of 2DG was expressed as % change compared with the control (100%). Significance was assessed by ANOVA (post Dunnett's test) and Student's unpaired t-test. Probability levels of p<0.05 were considered to be significant.

8.3.2 Results

8.3.2a Effect of Mazindol on Basal and Insulin-Stimulated Glucose Uptake in L6 muscle cells

As shown in figure 8.2a, mazindol (10^{-10} - 10^{-5} M) did not significantly alter the basal uptake of 2DG by L6 muscle cells. However in the presence of a sub-maximally stimulating concentration of insulin (10^{-8} M), mazindol (10^{-7} - 10^{-5} M) increased 2DG uptake by 50-150% ($p < 0.05$). These effects were observed up to 24h, being optimal at 8h. During 8h incubations, mazindol (10^{-7} M) significantly shifted a typical insulin concentration-response curve (10^{-9} - 10^{-5} M) to the left. EC_{50} values changed from 2×10^{-8} M in absence of mazindol to 2×10^{-9} M in its presence, $p < 0.05$, (see figure 8.2b). Mazindol (10^{-7} M) also increased the maximum insulin (10^{-5} M)-stimulated uptake of 2DG.

As shown in figure 8.2c, the inhibitor of protein synthesis cycloheximide (10^{-8} - 10^{-5} M), caused a significant concentration-dependent inhibition of insulin (10^{-7} M) + mazindol (10^{-7} M)-induced 2DG uptake by 25-150% ($p < 0.05$). Inhibition of phosphatidylinositol 3-kinase (PI3-Kinase) activity using LY 294 002 (10^{-8} - 10^{-5} M) did not significantly inhibit insulin (10^{-7} M) + mazindol (10^{-7} M) action (see figure 8.2d).

Figure 8.2a Effect of mazindol ± insulin (10^{-8} M) on 2DG uptake in L6 skeletal muscle cells. Cells were exposed to mazindol for 24h at concentrations 10^{-10} - 10^{-5} M. Results expressed as mean values ± SEM (n=6). +p<0.05 versus mazindol only.

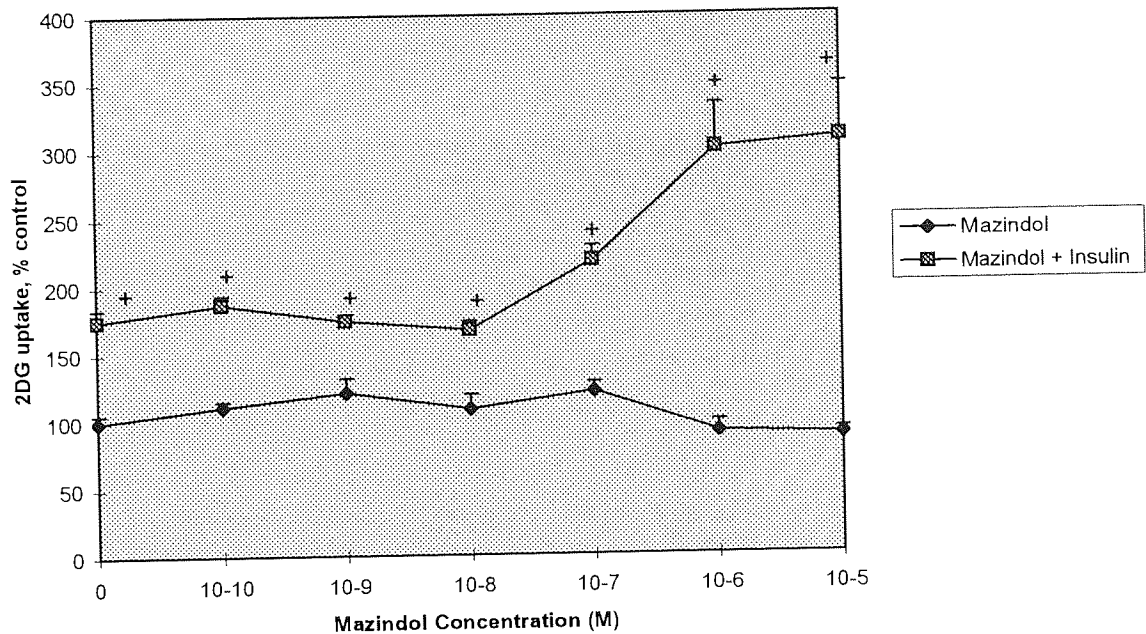


Figure 8.2b Effect of insulin ± mazindol (10^{-7} M) on 2DG uptake in L6 skeletal muscle cells. Cells were exposed to insulin for 24h at concentrations 10^{-9} - 10^{-5} M. Results expressed as mean values ± SEM (n=6). +p<0.05 versus insulin only.

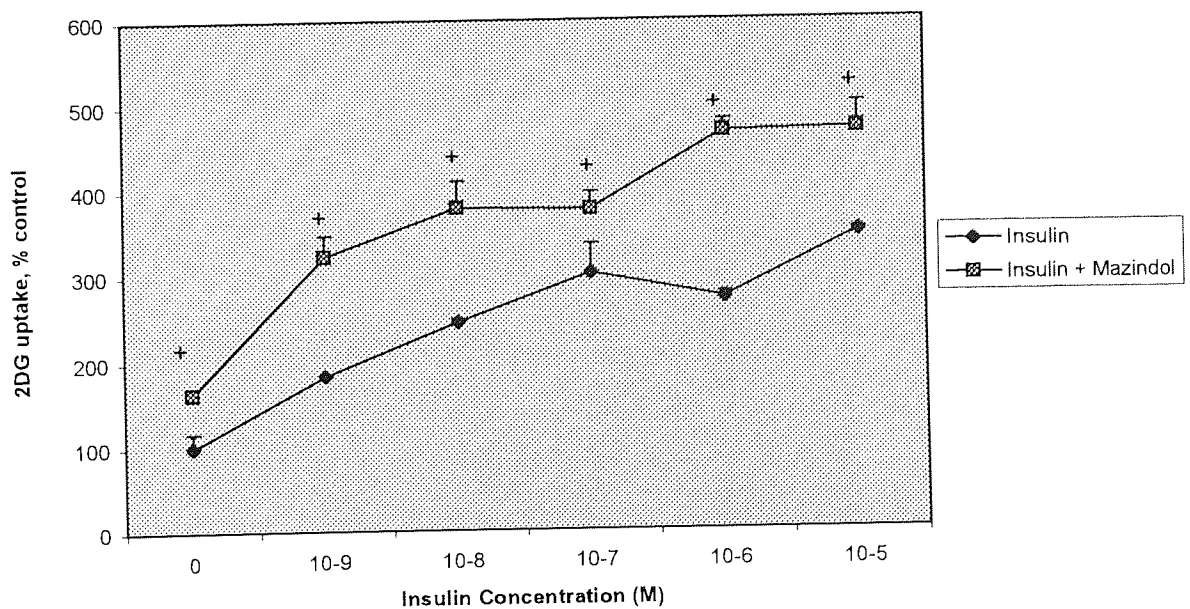


Figure 8.2c Effect of cycloheximide \pm insulin (10^{-7} M) + mazindol (10^{-7} M) on 2DG uptake in L6 skeletal muscle cells. **Figure 8.2d** Effect of LY 294 002 \pm insulin (10^{-7} M) + mazindol (10^{-7} M) on 2DG uptake in L6 skeletal muscle cells. Cells were exposed to cycloheximide and LY 294 002 for 24h at concentrations 10^{-8} - 10^{-5} M. Results expressed as mean values \pm SEM (n= 6). *p<0.05 versus control only.

Figure 8.2c

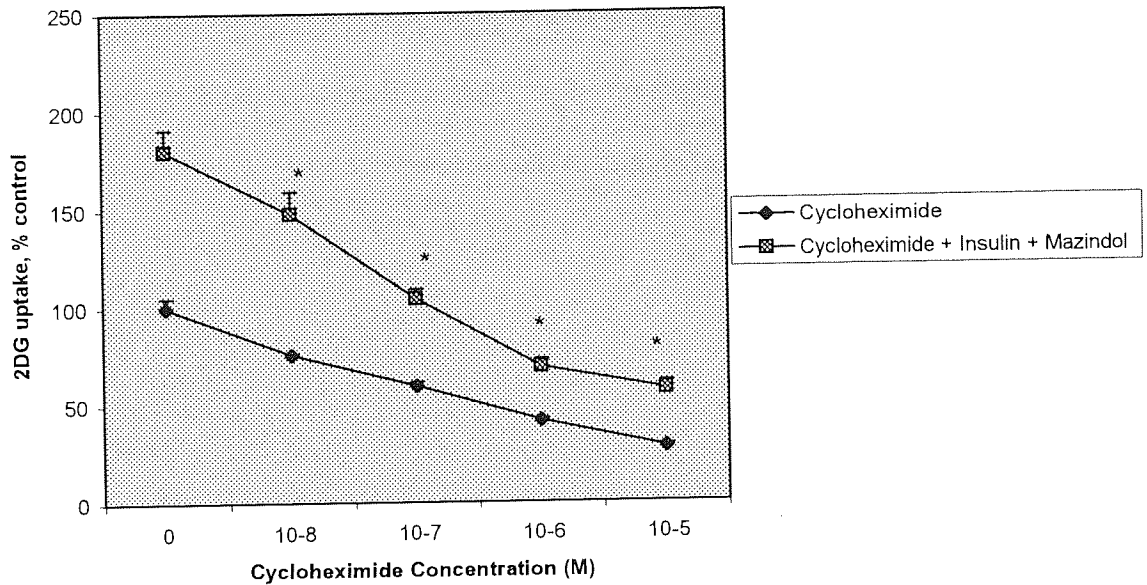
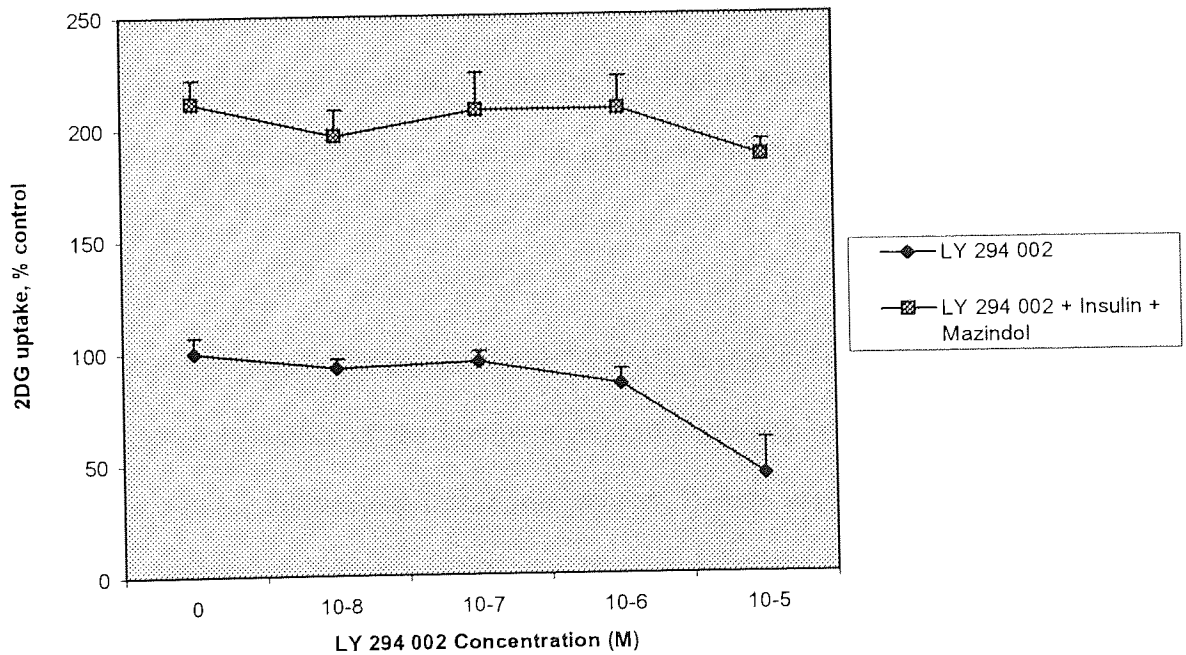


Figure 8.2d



Part Two: 3T3-L1 differentiated cells

The bulk of insulin-stimulated glucose uptake is metabolised in skeletal muscle (Bonadonna and Bonora, 1997). Glucose is also taken up by fat under insulin-stimulated conditions, however to a much lesser degree than the muscle. Mazindol directly increases insulin-stimulated glucose uptake into cultured L6 skeletal muscle cells. The present study evaluates mazindol action on the adipose tissue to determine if it acts directly on 3T3-L1 differentiated cells to improve glucose uptake and insulin action.

8.4.1 Method

3T3-L1 cells were grown in 100mm culture dishes (see section 2.4.1) and characterised according to the methods described in chapter 3. Once confluence was reached the preadipocytes were differentiated into adipocytes (see section 2.4.3). Adipocyte phenotype was confirmed using the Oil Red O staining technique (see 2.4.3). Cells were seeded at 10^7 cells/ml with a final concentration of 8×10^7 cells/ml (see chapter 3).

The cells were incubated with mazindol (10^{-10} - 10^{-5} M) \pm insulin (10^{-8} M) for 24h. In addition, known inhibitors of the insulin-stimulated signalling pathway of glucose transport in 3T3-L1 differentiated cells (see chapter 3) were used to determine the possible mechanisms by which mazindol increases glucose transport. The effect of LY 294 002 (10^{-8} - 10^{-5} M), cycloheximide (10^{-8} - 10^{-5} M) and cytochalasin B (10^{-9} - 10^{-

⁶M) on glucose uptake was determined in the presence of mazindol (10^{-6} M) and insulin (10^{-8} M). At the end of the incubation period, the effect of the test compounds on glucose transport into the cells was measured by ³H 2DG uptake (as described 2.4.4).

Concentration-response curves for mazindol were expressed as % of control values. Data are given as means \pm SEM, n=6 for each concentration and test compound (duplicates analyses from three separate experiments). Significance was assessed by ANOVA, post Dunnett's test and Student's paired t-test. Probability levels of $p < 0.05$ were considered to be significant.

8.4.2 Results

8.4.2a Effect of Mazindol on Basal and Insulin-Stimulated Glucose Uptake in 3T3-L1 differentiated cells

As shown in figure 8.3a, mazindol (10^{-10} - 10^{-5} M) significantly increased the basal uptake of 2DG by 50-100% in the differentiated 3T3-L1 cells. In the presence of a sub-maximally stimulating concentration of insulin (10^{-8} M), mazindol (10^{-8} - 10^{-5} M) further increased 2DG uptake by 100-150% ($p < 0.05$) (see figure 8.3a).

Inhibition of phosphatidylinositol 3-kinase (PI3-Kinase) activity using LY 294 002 (10^{-8} - 10^{-5} M) did not significantly inhibit insulin (10^{-8} M) + mazindol (10^{-6} M) action (see figure 8.3b). The inhibitor of protein synthesis cycloheximide (10^{-8} - 10^{-5} M),

caused a significant concentration-dependent inhibition of insulin (10^{-8}M) + mazindol (10^{-6}M)-induced 2DG uptake by approximately 85% ($p < 0.05$). Cytochalasin B (10^{-9} - 10^{-5}M), the inhibitor of glucose transporters, significantly decreased insulin (10^{-8}M) + mazindol (10^{-6}M) stimulated glucose uptake by 85-100%.

Figure 8.3a Effect of mazindol ± insulin (10^{-8} M) on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to mazindol for 24h at concentrations 10^{-10} - 10^{-5} M. Results expressed as mean values ± SEM (n= 6). *p<0.05 versus control only; +p<0.05 versus mazindol only.

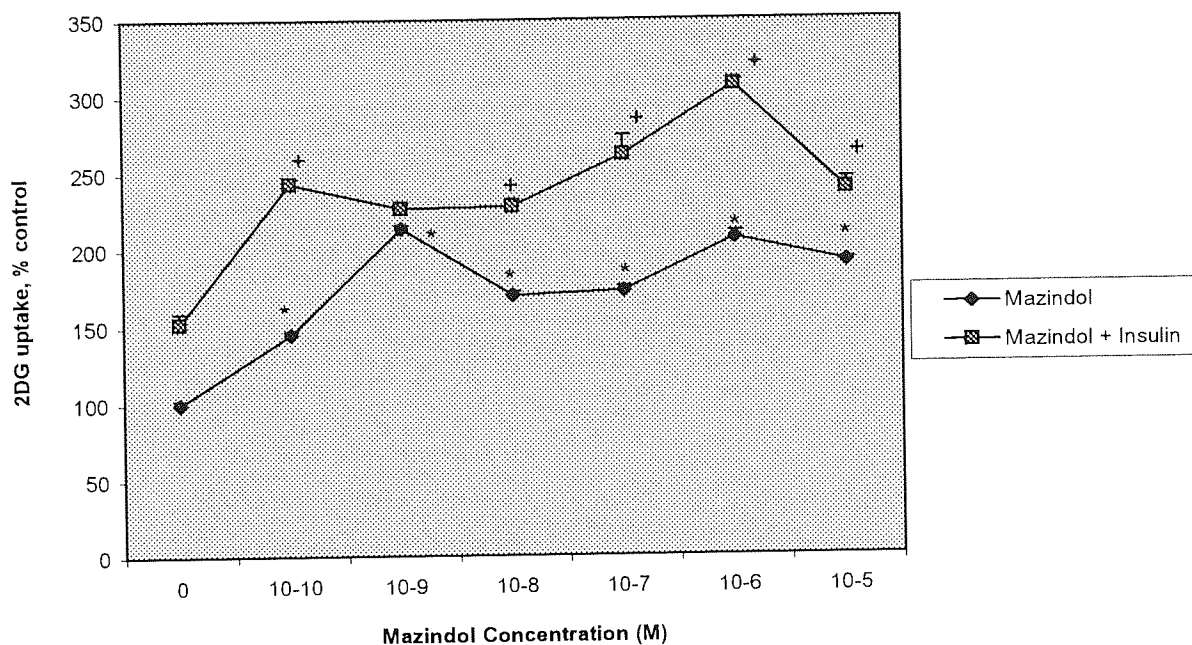


Figure 8.3b Effect of LY 294 002 ± insulin (10^{-8} M) + mazindol (10^{-6} M) on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to LY 294 002 cycloheximide for 24h at concentrations 10^{-8} - 10^{-5} M. Results expressed as mean values ± SEM (n= 6).

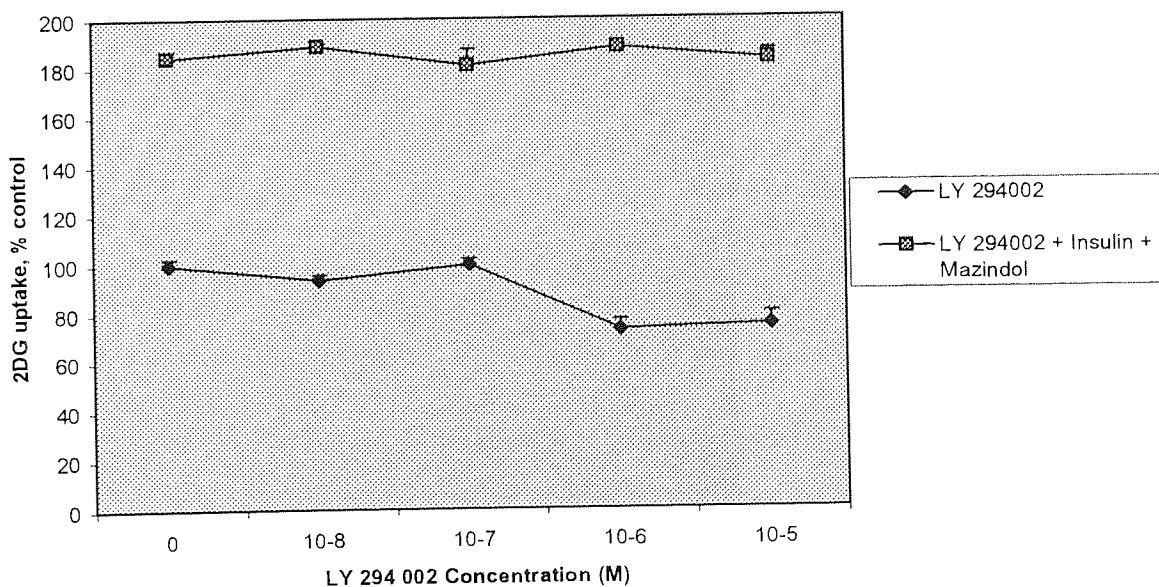


Figure 8.3c Effect of cycloheximide \pm insulin (10^{-8} M) + mazindol (10^{-6} M) on 2DG uptake in differentiated 3T3-L1 cells. **Figure 8.3d** Effect of cytochalasin B \pm insulin (10^{-8} M) + mazindol (10^{-6} M) on 2DG uptake in differentiated 3T3-L1 cells. Cells were exposed to cycloheximide and cytochalasin B for 24h at concentrations 10^{-8} – 10^{-5} M. Results expressed as mean values \pm SEM (n= 6). *p<0.05 versus control only.

Figure 8.3c

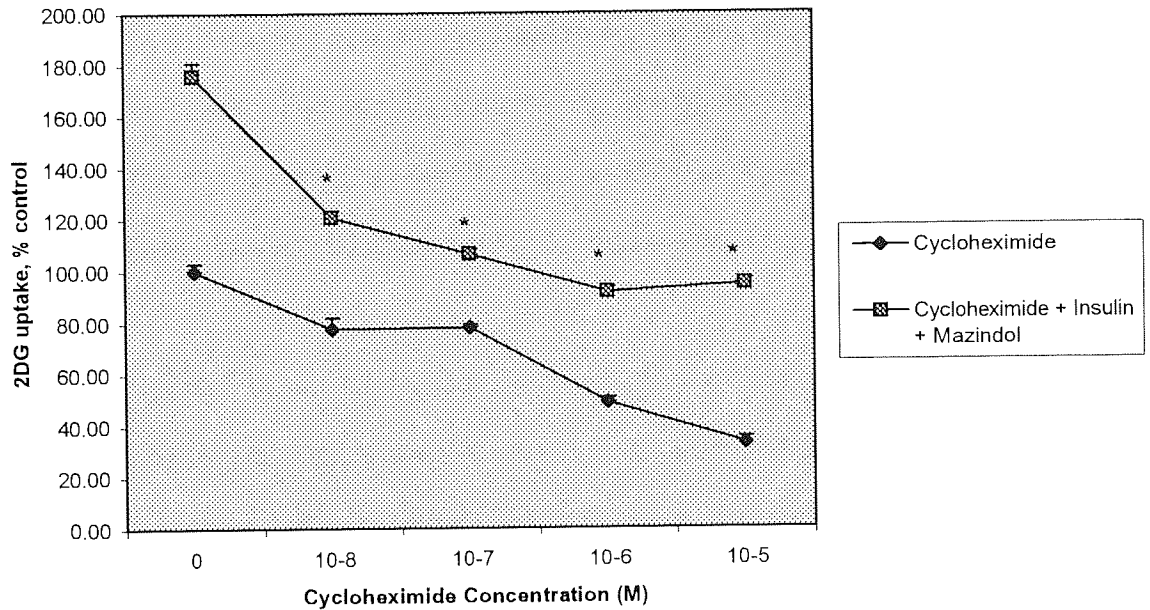
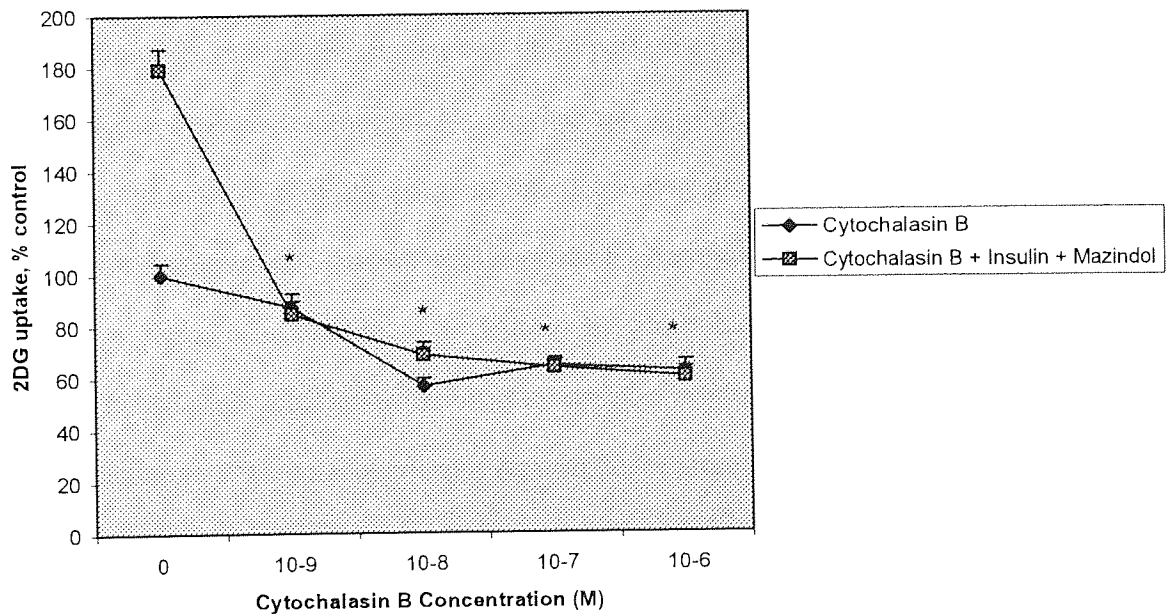


Figure 8.3d



8.5 Discussion

The present study demonstrates that mazindol can act directly on muscle and adipose tissue to increase glucose uptake. This is in agreement with the study by Nagai *et al.* (1994), where mazindol stimulated 2DG transport into the gastrocnemius muscle. In the same study, 2DG uptake was not increased in epididymal adipose tissue *in vivo* 90min after intragastric administration of mazindol. This result conflicts with the data observed in the present study where mazindol directly increased both basal and insulin-stimulated glucose uptake into the 3T3-L1 differentiated cells.

Mazindol stimulated basal and insulin-stimulated glucose uptake in the 3T3-L1 differentiated cells. In the L6 skeletal muscle cells, mazindol increased insulin-stimulated 2DG uptake, but had no effect on basal glucose uptake in these cells. This result suggests that mazindol has a more potent *in vitro* effect on the differentiated 3T3-L1 cells.

As confirmed by the present studies and those performed by Nagai *et al.* (1994) the mechanism of action of mazindol is to increase glucose uptake via a pathway involving the activation of glucose transporters and new protein synthesis. Mazindol + insulin-stimulated 2DG uptake was abolished by cycloheximide, an inhibitor of protein synthesis. In addition, cytochalasin B, which blocks sodium-independent glucose transporters completely inhibited mazindol + insulin-stimulated 2DG uptake. The reduction in 2DG uptake indicates that mazindol promotes glucose uptake in the 3T3-L1 differentiated cells and L6 skeletal muscle cells via activation of the glucose transporters (GLUT1 or GLUT4). This pathway is activated by mazindol distally to

the conversion of phosphatidylinositol bisphosphate (PIP₂) to phosphatidylinositol trisphosphate (PIP₃) by phosphatidylinositol 3-kinase (PI3-Kinase), since the inhibitor of PI3-Kinase (LY 294 002) was without effect on mazindol + insulin action in both these cells.

The 2DG uptake by mazindol in the 3T3-L1 differentiated cells, appears to be independent of serotonin and dopamine (see section 6.3.4c). In the studies described in section 6.3.4c, the differentiated 3T3-L1 cells were exposed to serotonin, noradrenaline, dopamine and agents known to stimulate release or inhibit reuptake of these substances in nervous tissues (fluoxetine, nisoxetine, phentermine, fenfluramine and nordexfenfluramine) (see chapter 6, table 4). Serotonin, fluoxetine, fenfluramine, nordexfenfluramine and dopamine had no effect on 2DG uptake in these cells. In contrast, nisoxetine (in combination), phentermine and noradrenaline all increased 2DG uptake into these cells, which suggests that mazindol may be acting on the 3T3-L1 differentiated cells via a catecholamine pathway.

Studies performed by Bailey *et al.* (2001) have demonstrated that these serotonergic, noradrenergic and dopaminergic agents (see chapter 6, table 4) were without effect on 2DG uptake in L6 skeletal muscle cells. This suggests that mazindol action in these cells is independent of serotonin, noradrenaline and dopamine.

In conclusion, mazindol can improve insulin sensitive 2-deoxyglucose uptake by cultured 3T3-L1 differentiated cells and L6 skeletal muscle cells, suggesting potential anti-diabetic properties of this agent.

Chapter 9: Human adipose tissue studies

9.1 Introduction

This chapter focuses on agents that have been shown in the preceding chapters to affect adipocyte lipolysis. These agents are M2 (chapter 6), 3-GPA (chapter 7) and mazindol (chapter 8) and have been demonstrated to increase lipolysis in lean and obese-diabetic *ob/ob* isolated adipocytes and 3T3-L1 preadipocytes. In support of these lipolysis studies, omental and subcutaneous adipose tissue biopsies were obtained from adult patients undergoing elective surgery (see section 2.3.2) and the adipocytes were isolated and used to study the effects of these compounds on adipocyte lipolysis. Firstly, the lipolytic properties of the omental and abdominal subcutaneous adipocytes was characterised and evaluated using isoproterenol and/or noradrenaline (data not shown). The respective degree of α and β adrenoceptors expression on human adipocytes is discussed in detail in chapter 5.

From the previous studies performed in chapter 6, it was demonstrated that M2 (sibutramine metabolite) could act directly on adipose tissue to increase lipolysis. The mechanism of action of M2 was identified as a pathway involving the β adrenoceptors and components of the lipolytic signalling pathway, notably PKA. It was demonstrated that M2 also activates the p38 MAP kinase pathway, which is an important downstream target of the β adrenergic/cAMP/PKA signalling pathway in adipocytes.

The findings in chapter 7 demonstrate that 3-GPA acts to increase adipocyte lipolysis. The effect of 3-GPA may be mediated via a mechanism, which is coupled to the G protein complex (see chapter 5.1).

Chapter 8 studies indicate that mazindol acts on the adipose tissue to stimulate lipolysis. Although the mechanism of action of mazindol was not studied in detail in chapter 8 due to time limitations, it was suggested that the action of mazindol on the adipose tissue could be similar to that described for M2.

9.2 Effect of M2, 3-GPA and Mazindol on Glycerol Release by Lean and Obese Diabetic and Non-Diabetic Human Adipocytes

9.2.1 Methods

Adipose tissue (~2g) from lean and obese diabetic and non-diabetic adults was removed (as described 2.3.2) and the isolated adipocytes (see section 2.3.3) were incubated with M2 (10^{-10} to 10^{-5} M), 3-GPA (10^{-6} to 10^{-2} M) and mazindol (10^{-10} to 10^{-5} M). M2 (supplied by Knoll Pharmaceuticals), 3-GPA and mazindol were dissolved in PBS by sonication for approximately 1h prior to use. Experiments on the isolated adipocytes were at 37°C for a 2h incubation period. Concentration of glycerol released into the medium, which is indicative of triacylglyceride breakdown, was measured enzymatically (see 2.3.4).

The body mass index (BMI) was used to classify and assess the fat mass of the adult subjects in the following studies. Advantages and disadvantages of defining obesity using the BMI and other methods are discussed in chapter1, section 1. Table 1 illustrates the cut-off points proposed by a WHO expert committee for the classification of overweight in women and men.

Table 1: Classification of overweight

BMI *	WHO classification	Description
<18.5	Underweight	Thin
18.5-24.9	-	Normal
25.0-29.9	Grade 1 overweight	Overweight
30.0-39.9	Grade 2 overweight	Obesity
40.0	Grade 3 overweight	Morbid obesity

*BMI is the weight in kilograms divided by the square of the height in metres.

(Adapted from Kopelman, Obesity as a medical problem. *Nature*. 2000, Vol. 404, page 636).

To establish that the protocol functions efficiently to provide metabolically viable adipocytes the cells were exposed to isoproterenol (10^{-6} M) and/or noradrenaline (10^{-6} M) with each concentration response curve performed for M2, 3-GPA and mazindol (data not shown).

Concentration-response curves for M2, 3-GPA and mazindol were expressed in mM glycerol/ 10^5 adipocytes. Data were given as means \pm SEM, n=6 (duplicates analyses from three separate experiments, (unless stated otherwise)). Significance was assessed by ANOVA and a post Dunnett's test. Probability levels of $p < 0.05$ were considered to indicate a significant difference.

9.2.2 Results

9.2.2a Effect of M2 on Glycerol Release by Lean and Obese Diabetic and Non-Diabetic Human Adipocytes

In normal adults, M2 (10^{-7} - 10^{-5} M) significantly increased lipolysis by 300% in adipocytes isolated from the omental adipose tissue depot (see figure 9.1a). A total of 9 patients were studied and all were within the normal BMI range of 18.5-24.9. Similarly, adipocytes isolated from the subcutaneous regions of these normal individuals were also responsive to M2 (see figure 9.1b). There was a concentration-dependent increase in lipolysis by 200% in the subcutaneous adipocytes, however a significant effect was only observed at 10^{-5} M M2 concentration.

M2 (10^{-5} M) caused a substantial increase in mean values for lipolysis in omental adipocytes isolated from underweight (by 100%), overweight (by 75%) and obese (by 100%) subjects, however these apparent effects proved to be statistically non-significant (see figure 9.1a). In contrast, M2 (10^{-6} - 10^{-5} M) caused a significant increase in lipolysis by adipocytes isolated from the subcutaneous regions of underweight and obese subjects by approximately 150% and 200%. Adipocytes

isolated from the subcutaneous regions of the overweight subjects also appeared to be responsive to M2, however this proved not to be a statistically significant effect (see figure 9.1b). The omental adipose tissue taken from obese-diabetic adults was unresponsive to the effects of M2. There was no change observed in the basal lipolysis of these isolated omental adipocytes (see figure 9.1a).

Figure 9.1a Lipolytic activity of M2 on human fat. Omental adipocytes were exposed to M2 (10^{-10} - 10^{-5} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (number in parenthesis in text box). * $p < 0.05$ versus control.

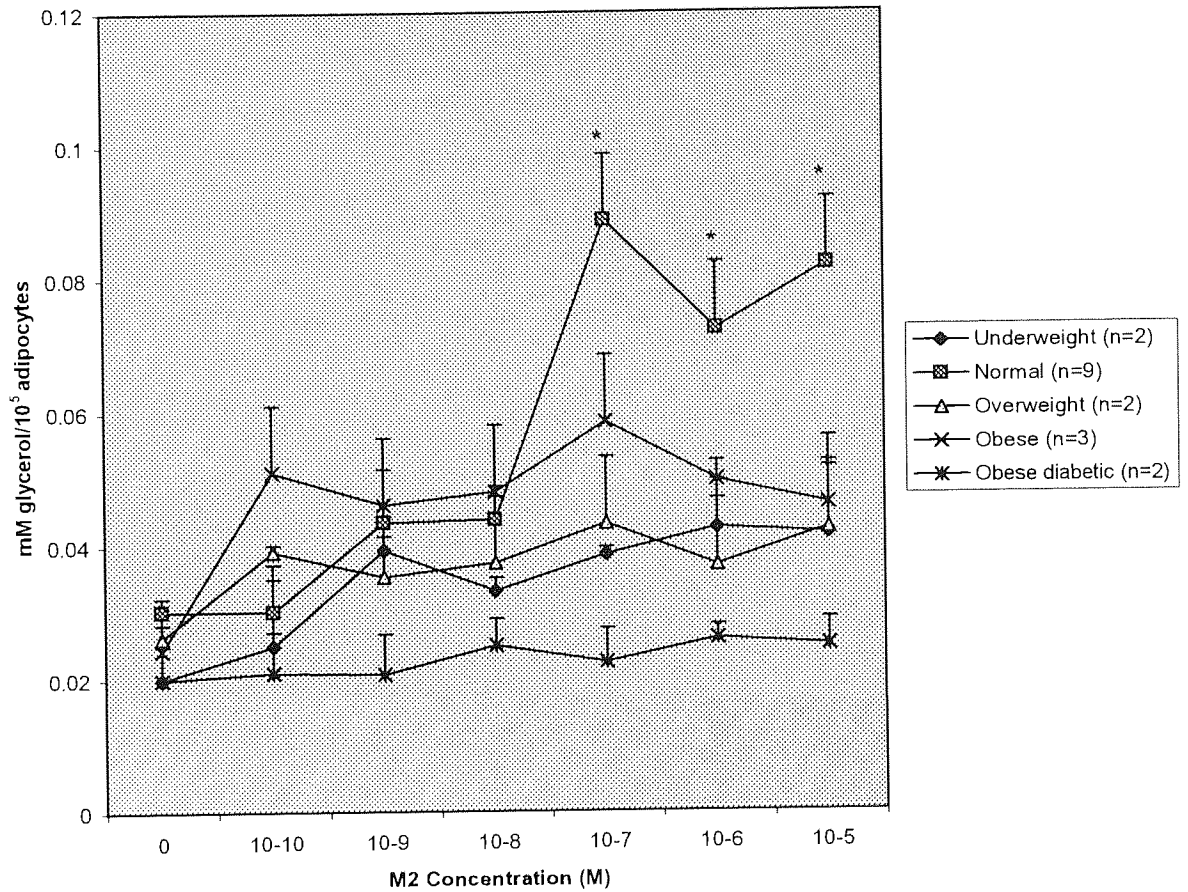
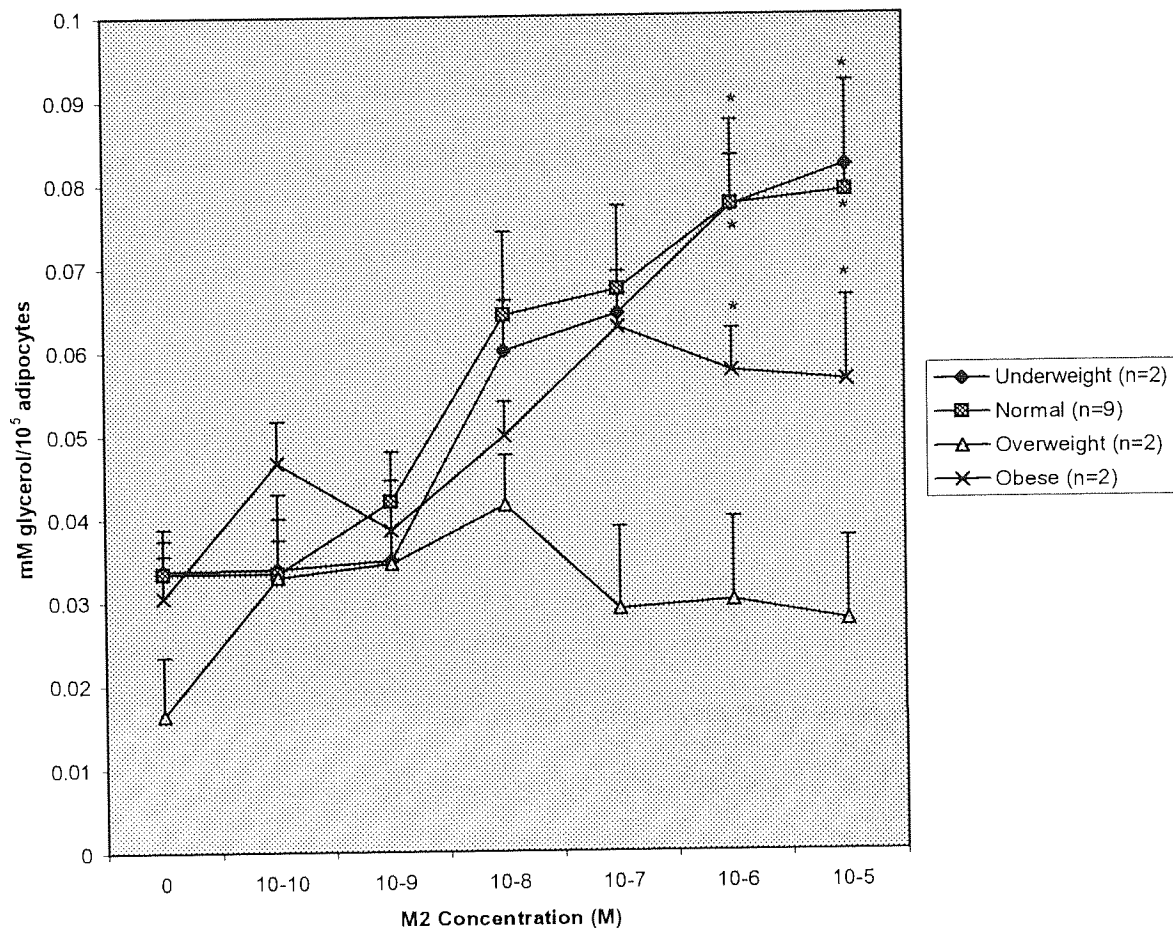


Figure 9.1b Lipolytic activity of M2 on human fat. Subcutaneous adipocytes were exposed to M2 (10^{-10} - 10^{-5} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (number in parenthesis in text box). * $p < 0.05$ versus control.



9.2.2b Effect of 3-GPA on Glycerol Release by Lean Non-Diabetic Human Adipocytes

3-GPA (10^{-4} - 10^{-3} M) caused a concentration-dependent increase in lipolysis by 50-75% by omental adipocytes isolated from adipose tissue biopsies (see figure 9.2c). The adipocytes were isolated from individuals with a normal BMI of 18.5-24.9, who were non-diabetic.

9.2.2c Effect of Mazindol on Glycerol Release by Lean Non-Diabetic Human Adipocytes

An increase in glycerol release by omental adipocytes was observed with mazindol at 10^{-6} - 10^{-5} M (see figure 9.2d). These omental adipocytes were isolated from tissue obtained from non-diabetic individuals with a BMI between 18.5-24.9. Mazindol significantly increased lipolysis by approximately 150-200%.

Figure 9.1c Lipolytic activity of 3-GPA on human fat. Omental adipocytes (from subjects with a normal BMI) were exposed to 3-GPA (10^{-6} - 10^{-2} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (2). * $p < 0.05$ versus control.

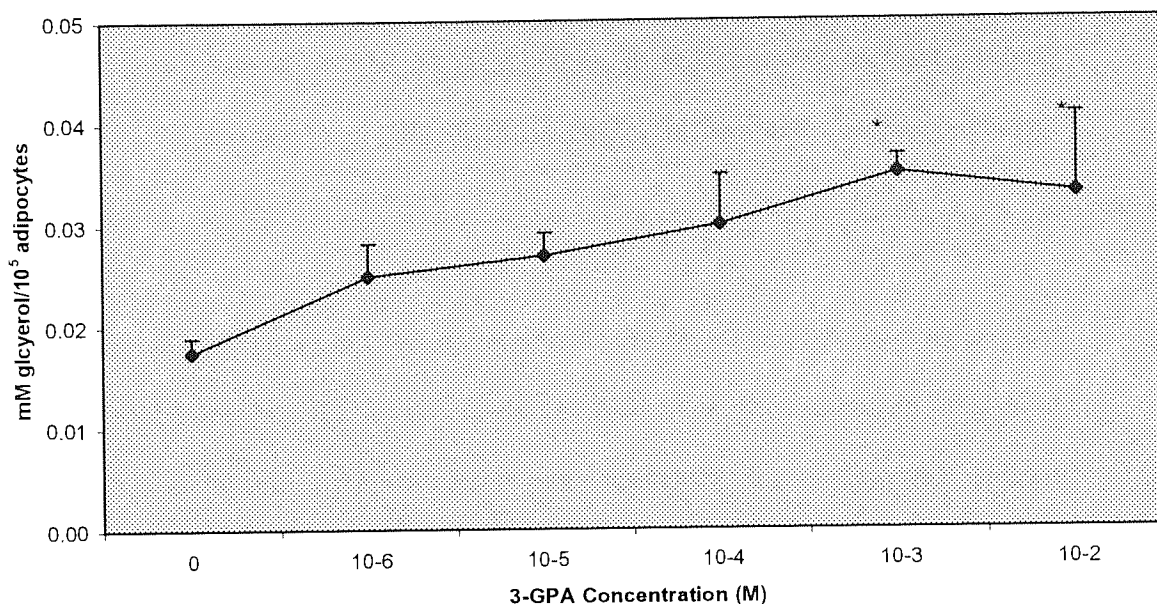
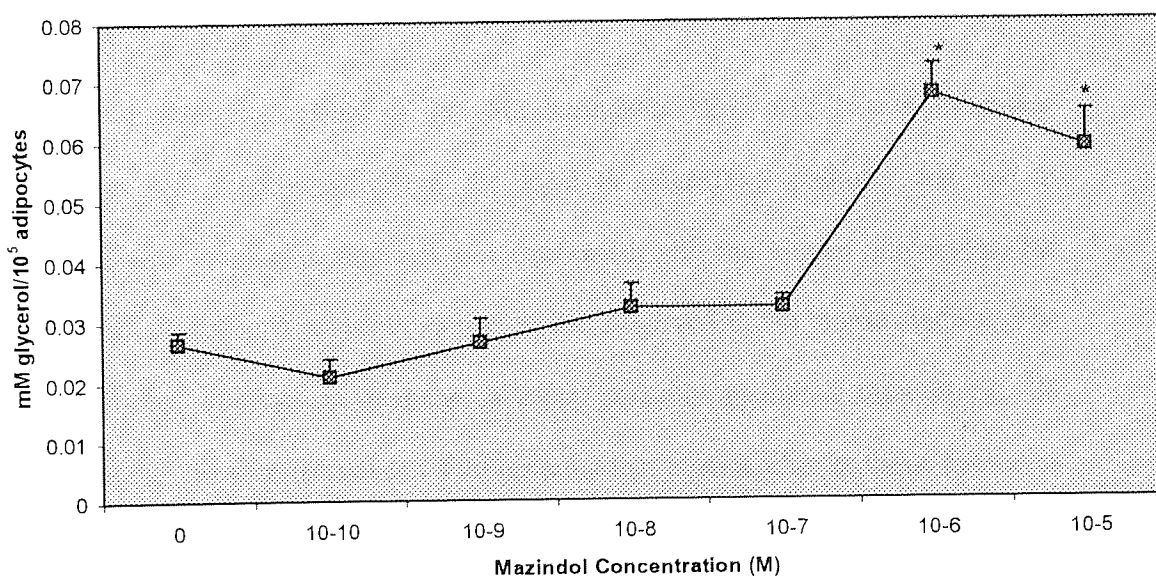


Figure 9.1d Lipolytic activity of mazindol on human fat. Omental adipocytes (from subjects with a normal BMI) were exposed to mazindol (10^{-10} - 10^{-5} M) for 2h at 37°C. Results expressed as mM glycerol/ 10^5 adipocytes. Mean values \pm SEM (2). * $p < 0.05$ versus control.



9.2.3 Discussion

The human adipose tissue studies reaffirm the lipolytic effect of M2, 3-GPA and mazindol. These compounds were effective at increasing lipolysis in lean and obese-diabetic *ob/ob* mice and have been shown in these present studies to increase lipolysis in omental and subcutaneous adipocytes isolated from lean and obese non-diabetic subjects.

The studies performed on M2 (sibutramine metabolite) provide further evidence that this compound acts directly on adipose tissue to stimulate the metabolism of TAG to yield FFA and glycerol. M2 was clearly more lipolytic in those adipocytes isolated from lean non-diabetic subjects. A much less pronounced lipolytic effect was observed in adipocytes isolated from obese non-diabetic subjects. The reduced lipolytic response of M2 on the obese non-diabetic adipocytes versus the lean subjects suggests a decrease in β adrenoceptor number on the adipocyte cell surface membrane (Bolinder *et al.*, 2000; DiGirolamo and Fried, 1987).

It was suggested in chapter 6 that M2 stimulates FFA mobilisation in mouse adipocytes by directly activating the lipolytic signalling pathway via the β adrenoceptors. Although this was not confirmed with the human adipocytes, the mechanism of action is probably similar to that observed in the mouse studies. The M2-stimulated lipolysis in humans is most probably mediated through the β_2 adrenoceptors because it has been demonstrated that human adipose tissue expresses mainly the β_2 adrenoceptor and that these receptors are the dominant mediators of lipolysis (Enocksson *et al.*, 1995; Galitzky *et al.*, 1993; Lai *et al.*, 1982). In contrast,

mouse adipocytes express mainly the β_3 adrenoceptor subtype and are responsible for mediating M2-stimulated lipolysis (see chapter 6). In addition, the lipolytic rate of M2 on the human adipocytes (75-300%) was generally higher versus the effect observed with the mouse adipocytes (100-200%). This suggests that M2 has a higher affinity for the β_2 adrenoceptor versus the β_3 adrenoceptor or that expression of these receptors on the cell surface membrane is higher on the human adipocytes. The differences in the β adrenoceptor subtypes in mouse and human adipose tissue are discussed in detail in section 5.2.1 and 5.2.2 (found in chapter 5).

The M2-stimulated lipolysis of the omental adipocytes isolated from lean non-diabetic subjects was much higher than that observed with the subcutaneous adipocytes isolated from the same individuals. This substantiates a difference in lipid mobilisation in different adipose tissue depots. In general, with central (omental) adipose tissue FFA turnover is elevated in comparison with peripheral (subcutaneous) adipose tissue. These regional differences in adipocyte metabolism can be explained by the increase in density of specific receptors on the cell surface membrane, i.e. the β adrenoceptors (Bjorntorp, 2000).

The lipolytic effect of M2 was not reproduced in adipocytes isolated from obese diabetic subjects. Both subjects were receiving insulin injections for treatment of their diabetes. The adipocytes were therefore exposed to insulin *in vivo*, probably at higher than normal circulating concentrations (i.e. hyperinsulinaemia), which will accentuate the extent of insulin resistance. Since insulin would itself be expected to prevent lipolysis it is possible that the pre-operative injection of insulin would have been sufficient to override any effect of the agents tested, preventing their lipolytic

effect. Although adipocytes have a low density of insulin receptors (Bolinder *et al.*, 1983) the antilipolytic effect of insulin is a sufficiently powerful inhibitor of FFA release from adipose tissue and that this would provide a feasible explanation for the lack of effect of M2 in the obese-diabetic patients in the present study.

3-GPA and mazindol both stimulated adipose tissue lipolysis by adipocytes isolated from lean non-diabetic subjects. Due to time constraints the effects of these compounds on adipocytes isolated from obese and obese-diabetic subjects could not be undertaken. It could be hypothesised that these agents would have a reduced response in adipocytes isolated from obese subjects, since it is known that M2 has a reduced lipolytic response due to a reduction in β adrenoceptor number. Similarly, these compounds might be anticipated to have a reduced lipolytic effect on adipocytes isolated from the subcutaneous regions of lean and obese subjects, since these adipocytes have also been shown to have a reduced number of β adrenoceptors (Bjorntorp, 2000; Guo *et al.*, 1997).

Adipose tissue is usually obtained during local and general anaesthesia. Although the effects of anaesthesia were not investigated on the human adipose cells, there is evidence by Large *et al.* (1997) that adrenergic regulation of lipolysis is not significantly influenced by local and general anaesthesia in isolated subcutaneous adipocytes of obese subjects. However, it has been demonstrated elsewhere that thiopentone anaesthesia provokes small disturbances in glucose homeostasis and serum NEFA metabolism in conscious and anaesthetized fasted dogs (Toso *et al.*, 1993), although the effects of this anaesthetic are typically transient. The present studies in human tissue which involved a short-acting barbiturate pre-medication and

a gaseous (fluothane) general anaesthetic gave similar results for the control of lipolysis as seen with adipocytes isolated from non-anaesthetised mice. Thus, the present results suggest that *in vitro* metabolism of human adipocytes is unaffected by the anaesthesia used and does not interfere with the lipolytic action of M2, 3-GPA and mazindol on the adipose cells.

Taken together, the present studies have confirmed that M2, 3-GPA and mazindol are effective at stimulating lipolysis in human adipocytes. Although it is important to screen these compounds for lipolytic properties in mouse adipocytes, there are obvious advantages for using the human adipocytes. Although structurally and morphologically similar, the human adipocytes express a different distribution of subtypes of α and β adrenoceptors versus the mouse adipocytes (see chapter 5). Hence, lipolytic compounds may act on lipolysis with different potencies in human adipocytes versus the mouse adipocytes. The human adipocytes provide an important model for these lipolysis studies since the compounds being tested are potential new therapeutic treatments for human obesity.

Chapter 10: General Discussion

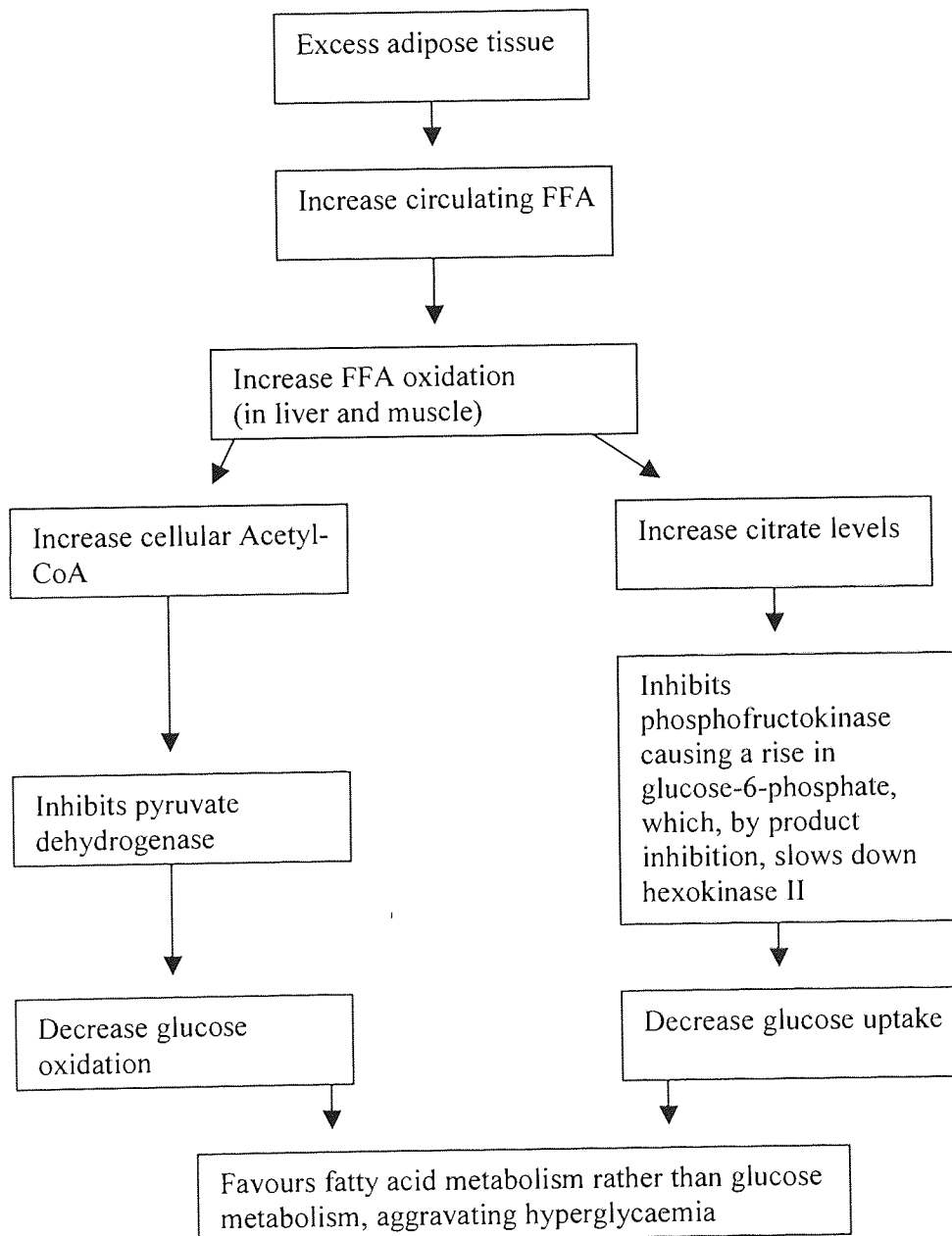
10.1 Introduction

Improving the metabolic environment of an obese diabetic individual is a particularly difficult hurdle to overcome. Obesity alone is associated with increasing insulin resistance and compensatory hyperinsulinaemia, plus various detrimental changes to the lipid profile and other cardiovascular risk factors. One of the latter factors is impaired glucose tolerance (IGT). If the pancreatic β cells are unable to sustain the compensatory hyperinsulinaemia, and deterioration of β cell further occurs, then a state of type 2 diabetes develops together with obesity. The two conditions combined encompass a major threat to health and a major problem for treatment as described in chapter 1.

The complex relationship between carbohydrate and lipid metabolism becomes adversely distorted in obesity, and this is further aggravated with the development of diabetes. Due mainly to the prevailing insulin resistance, the obese-diabetic individual is unable to use the glucose as a fuel; hence free fatty acids (FFA) shed by the lipolysis of white adipose tissue are used as a fuel for the liver (to facilitate gluconeogenesis). Excess FFAs in the bloodstream, especially in the splanchnic bed act to decrease insulin action in the liver. In addition increased fatty oxidation in the liver stimulates pyruvate carboxylase, thereby accelerating gluconeogenesis and hepatic glucose production. Moreover, these FFAs are associated with skeletal muscle insulin resistance as first described by Randle and his colleagues (1963). A schematic representation of a defective Randle (glucose-fatty acid) cycle, which

highlights the key intermediate steps of fatty substrates and the impairment of insulin-stimulated glucose oxidation and glucose uptake, is shown in figure 1.

Figure 1. Schematic representation of the consequences of defective function of the Randle cycle



(Adapted from Bonadonna and Bonora, Glucose and free fatty acid metabolism in human obesity. *Diabetes Reviews*. 1997, Vol. 5 (1), page 22).

Increasing lipolysis in the obese individual (with or without type 2 diabetes) would chronically raise FFAs. In the short term FFAs would be detrimental to promoting the insulin resistance. However, in the longer term weight loss in obese diabetic individuals has been shown to improve insulin sensitivity. Hence therapeutic approaches to achieve this weight loss are an important primary approach to achieve an improvement in the diabetic state of the obese-diabetic individual.

The main aim of this thesis was to investigate new intervention targets against obesity and diabetes, alone or in combination, by researching novel and established anti-obesity and anti-diabetic agents as therapeutic approaches to increase white adipose tissue lipolysis and/or increased energy expenditure. The underlying hypothesis for this thesis was to treat obesity through increased weight loss, which would improve insulin sensitivity and ultimately have benefits for type 2 diabetes.

The focus of this thesis was primarily the white adipose tissue and hence it was important to develop and characterise methods for isolating and evaluating human and animal adipose tissue and cultured 3T3-L1 preadipocytes. Adipose tissue is an 'endocrine' organ, which is capable of secreting hormones and cytokines. Adipocytes are the main regulator of FFA storage and mobilisation, hence adipose tissue depots were used as models for the study of lipolytic agents. In addition, the adipose tissue plays a role, albeit small in whole-body glucose disposal: thus the adipose tissue models were also characterised for insulin signalling and glucose uptake studies. It was also vital to characterise cultured L6 muscle cells because muscle is responsible for most of the glucose utilisation in the body.

The adipose tissue models were primarily used to study potential lipolytic agents and the muscle cells were used to investigate novel agents that may increase energy expenditure, predominantly through increasing glucose uptake. Ideally, other ways to increase energy expenditure would have been preferable. In the aims set out in chapter 1, it was suggested that stimulation of the uncoupling proteins (UCP) in the muscle would be an ideal way to increase energy expenditure. UCPs act to increase uncoupled thermogenesis and hence generate heat rather than store the energy as (adenosine triphosphate) ATP. Although this thesis did not investigate thermogenic agents, the potential therapeutic benefit of using an agent to stimulate UCP in conjunction with a lipolytic agent will be discussed later on in this chapter.

10.2 Summary of Chapters

The 3T3-L1 cell line was used throughout the studies within this thesis and have been characterised in chapter 3. As preadipocytes, these cells when cultured and grown to confluence were used as a model for studying lipolytic agents. Alternatively the cells were differentiated according to the protocol described by Frost and Lane (1985) for insulin signalling and glucose uptake studies. From the work undertaken on the characterisation of these cells, it was confirmed that adrenoceptors were present on the preadipocytes before the differentiation process, thus these cells were used directly. Studies by Lai *et al.* (1982 and 1981) demonstrated a predominantly higher number of β_1 adrenoceptors. Preliminary studies on these preadipocytes confirmed that these cells were unresponsive to basal and insulin-stimulated glucose uptake studies. Thus it was essential to differentiate the preadipocytes into the adipocytes for

the glucose uptake studies. Cells that have undergone adipocyte differentiation exhibit a 20-fold increase in the number of insulin receptors and an increase in glucose transporters (Reed *et al.*, 1981).

Insulin-stimulated 2DG uptake in differentiated 3T3-L1 cells required the PI3-Kinase component of the intracellular signalling cascades as indicated by the inhibitory effect of wortmannin (or LY 294 002 compound). Insulin stimulation via PI3-kinase has also been shown to activate GLUT1 and GLUT4 transcription and translation in these cells (Cheatham *et al.*, 1994; Sanchez-Margalet *et al.*, 1994). The insulin signalling pathways in these cells was identified and hence provided a suitable tool for screening potential anti-diabetic and anti-obesity drugs.

In chapter 4, the L6 cell culture cell system was evaluated and characterised. Much of the L6 cell characterisation has been described elsewhere (Bates, 1999). Hence this chapter provided selected experiments only, which were important to confirm the key characterisation for each batch of new cells. The L6 muscle cells were responsive to insulin and the effects observed confirmed the features reported by Bates (1999), indicating that PI3-kinase is pivotal to both acute and chronic insulin-stimulated glucose transport. In addition, basal and insulin-stimulated glucose transport via the activation of GLUT1 and GLUT4 transporters appear to be regulated by both protein synthesis and translocation of the transporters to the membrane (Bates, 1999). Thus the preliminary studies described herein confirmed that the L6 skeletal muscle cells were a suitable model for studying insulin signalling and glucose uptake.

There are obvious advantages to using cell culture systems as discussed in some detail in chapters 2, 3 and 4. The 3T3-L1 cells and L6 skeletal muscle cells were responsive to insulin and agents known to inhibit its actions. The cells maintain viability for extended periods of times and the technique allowed an even and rapid accessibility of cells to substrates. The activity of the cells was reproducible, accurate and consistent with that shown by the respective isolated adipocytes and skeletal muscles. Substantiation of this latter feature was considered in chapter 5.

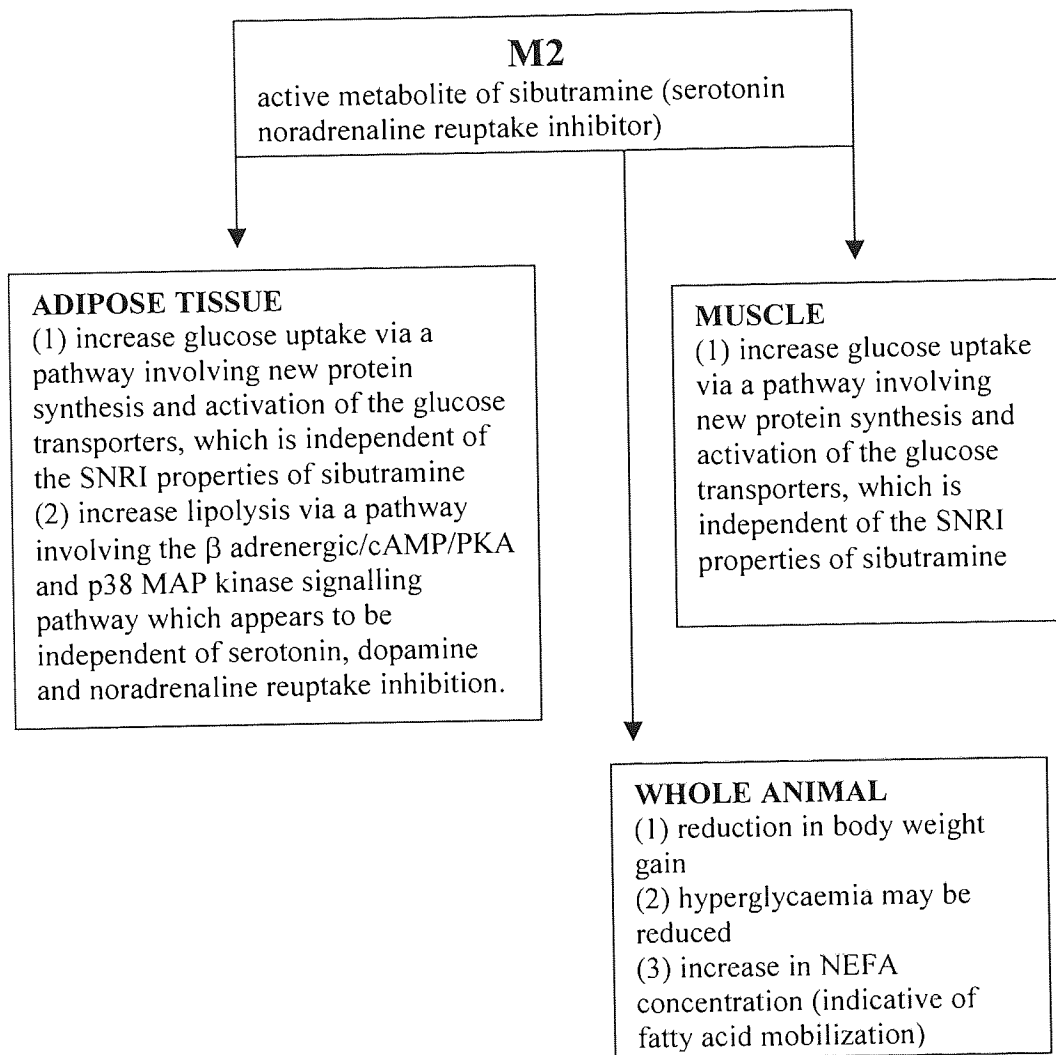
In chapter 5, the lean and obese mouse adipocytes were characterised and evaluated as a model for studying the effects of agents on white adipose tissue lipolysis. These adipocytes were exposed to a range of α/β adrenoceptor agonists and antagonists to establish the degree of β_1 , β_2 and β_3 adrenoceptor expression on the lean and obese mouse cell membrane. The characterisation studies confirmed that β_1 and β_2 adrenoceptors play a minor role in the lipolysis of white adipose tissue (Germack *et al.*, 1997; Hollenga and Zaagsma, 1989). The β_3 adrenoceptor was demonstrated to be the most important receptor involved in mouse adipocyte lipolysis through the studies reported in chapter 5. A much-reduced lipolytic stimulus of agents specific for the various subtypes of the β adrenoceptor was demonstrated in obese mouse adipocytes compared with the lean adipocytes, which reaffirmed the impaired lipolytic responses of these adipocytes (Lemonnier, 1972). Characterisation of the adipose tissue was important to demonstrate viability and functional integrity of the white adipose tissue preparation prior to evaluating and screening the novel and established agents.

The studies performed in chapter 6 have identified a potent lipolytic agent. The compound identified is M2, the metabolite of sibutramine (an anti-obesity agent currently used in USA and Europe). From the work undertaken in the preceding chapters and by Bates (1999), it can be concluded that M2 is the active component of the serotonin noradrenaline reuptake inhibitor, sibutramine. Bates (1999) and Bailey *et al.* (2001) have demonstrated that M2 has anti-diabetic properties because it was able to stimulate the uptake of 2DG in skeletal muscle and reduce insulin resistance and hyperglycaemia in some diabetic states. Stimulating glucose uptake into muscle is a vital step to controlling the hyperglycaemia associated with type 2 diabetes. To reaffirm the studies performed by Bailey *et al.* (2001) and Bates (1999), M2 was investigated on the adipocytes for its ability to stimulate glucose uptake. M2 was able to increase glucose uptake into the adipocytes in a similar pathway described by Bates (1999) for the L6 skeletal muscle cells.

From the studies in this thesis it became clear that M2 is not only active at the muscle. In chapter 6 it was confirmed that M2 not only stimulates glucose uptake into the adipocytes but it also directly acts on the adipocyte to stimulate lipolysis. The mechanism of action of M2 on the adipocytes is via a pathway involving the β adrenoceptors and components of the lipolytic signalling pathway. Inhibitors of these signalling components (protein kinase A and p38 MAP kinase) were able to reduce the M2 stimulated lipolysis on the adipocytes. It has been suggested by Hutchinson *et al.* (2002) that the β_3 adrenoceptor is able to influence insulin signalling via activation of PI3-kinase in Chinese hamster ovary cells expressing the $\beta_3\alpha$ and $\beta_3\beta$ subtypes of the receptor.

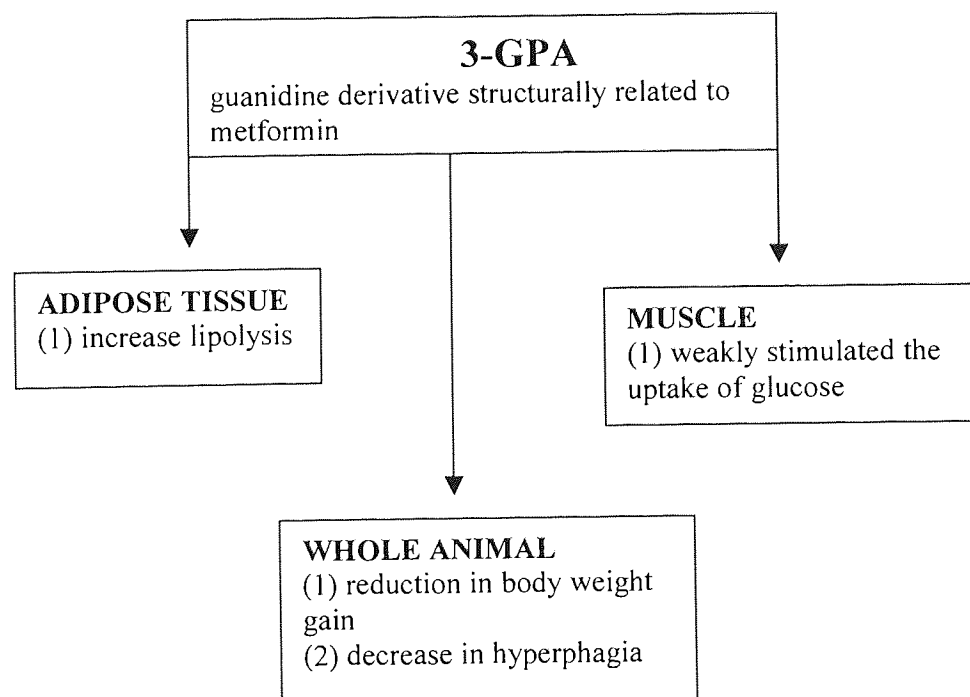
An *in vivo* study in chapter 6 confirmed that in the whole animal M2 increases adipose tissue mobilisation and reduces basal glycaemia in lean and obese-diabetic mice. The reduction in basal glycaemia indicates the removal of glucose into insulin responsive tissues, i.e. skeletal muscle and white adipose tissue. The increase in blood NEFA concentrations following M2 treatment is indicative of white adipose tissue lipolysis. Taken together, these results indicate that M2 has both anti-diabetic and anti-obesity properties (see figure 2). From the work undertaken as part of this thesis, it has clearly been demonstrated that M2 would be beneficial in the treatment of an obese diabetic individual.

Figure 2: Summary of M2 actions affecting glucose and lipid metabolism in adipose tissue and muscle



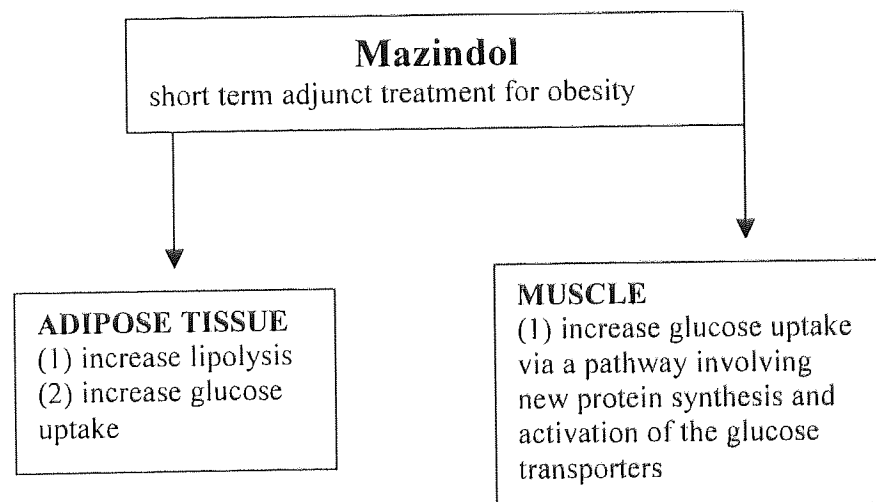
3-GPA, a guanidine derivative was shown in chapter 7 to reduce body weight gain and decrease hyperphagia in obese-diabetic *ob/ob* mice. In addition 3-GPA stimulated lipolysis by isolated adipocytes. The guanidine derivative appeared to act weakly on the L6 muscle cells to stimulate glucose uptake, suggesting that this agent would not be sufficiently potent to be a useful therapeutic agent in the treatment of type 2 diabetes. Indeed unlike the comparator metformin (one of the world's leading drugs in the treatment of type 2 diabetes), 3-GPA did not display anti-diabetic properties. Because 3-GPA is found naturally in the body, it is unlikely to have toxic and CNS side effects versus other obesity treatments and might therefore be useful to treat obesity.

Figure 3: Summary of 3-GPA actions affecting glucose and lipid metabolism in adipose tissue and muscle



The work described in chapter 8 demonstrated that mazindol, an obesity treatment currently used in Japan, increased WAT lipolysis by stimulating FFA mobilisation. Mazindol actions are clearly similar to M2, since both agents are capable of stimulating glucose uptake into differentiated 3T3-L1 cells and L6 muscle cells (see figure 4). The two compounds are related in that both have noradrenaline reuptake inhibitor properties. Studies in this thesis have shown that M2 and mazindol actions appear to be independent of fenfluramine, nordexfenfluramine, phentermine, serotonin, fluoxetine, dopamine and nisoxetine.

Figure 4: Summary of mazindol actions affecting glucose and lipid metabolism in adipose tissue and muscle



Chapter 9 reaffirmed that the three lipolytic agents identified using the lean and obese adipocytes and 3T3-L1 preadipocytes (M2, 3-GPA and mazindol) were capable of stimulating lipolysis in human adipocytes isolated from lean, obese and obese-

diabetic individuals. Human adipocytes express mainly the β_2 adrenoceptor (Lai *et al.*, 1982) with a possibility for the β_3 adrenoceptor mediating some lipolysis (Van Harmelen, 1997; Galitzky *et al.*, 1997; Sennitt *et al.*, 1998). Rodent adipose tissue expresses a higher proportion of β_3 adrenoceptors versus the β_1 and β_2 . The three lipolytic agents are capable of increasing lipolysis via either of the β adrenoceptors in rodent and human adipose tissue and therefore could be therapeutically meaningful.

10.3 Ideal Therapeutic Treatment of the Obese-Diabetic Individual

The original aims set out in chapter 1 describe the concept of the ideal treatment for obesity and type 2 diabetes alone or in combination. From the work undertaken in this thesis, three compounds have been identified and evaluated and proved to be potential anti-obesity and anti-diabetic therapeutic treatments. The three compounds were capable of enhancing lipolysis in white adipose tissue, which would be essential to decrease fat mass of an obese individual. To treat the type 2 diabetes of a non-obese or obese individual it is essential to control the hyperglycaemia. From the studies performed in this thesis, two compounds (M2 and mazindol) were effective at increasing basal and insulin-stimulated glucose uptake into the muscle and adipose tissue. By stimulating the uptake of glucose into the insulin sensitive tissues, there is a reduction in hyperglycaemia, which should result in a decrease in hyperinsulinaemia and an improvement in insulin sensitivity in the obese individual with the early stages of type 2 diabetes.

The therapeutic benefit of using a thermogenic agent to stimulate uncoupling proteins (UCPs) would be potentially ideal in the treatment of an obese diabetic individual. UCPs are good candidates through which to increase energy expenditure and increase overall metabolic rate (Fleury *et al.*, 1997). When activated, the UCPs promote proton transport and consequently decrease the proton electrochemical potential gradient across the inner mitochondrial membrane. This uncouples oxidative thermogenesis of adenosine diphosphate (ADP) to adenosine triphosphate (ATP), leading to the generation of heat (Cassell *et al.*, 1999). Fasting, glucocorticoids, thyroid hormones and leptin increase the expression of UCP1 and 3 (Weigle *et al.*, 1998; Ohno *et al.*, 1990; Gong *et al.*, 1997). UCP3 is predominantly expressed in skeletal muscle with UCP1 being expressed in brown adipose tissue and UCP2 expressed in numerous tissues (Schrauwen, 1999).

Combining a lipolytic agent with a thermogenic agent would be an ideal treatment for obesity and type 2 diabetes. When the lipolytic agent stimulates lipolysis, FFAs are mobilised from the TAG and released into the bloodstream. FFAs are putative ligands for peroxisome proliferator-activated receptors, which have been shown to stimulate UCP expression (Schrauwen *et al.*, 1999). Although a thermogenic agent was not identified as part of this research, M2 may play a role in stimulating the expression of UCPs (McNeely and Goa, 1998). Sibutramine therapy is known to prevent the reduction in thermogenesis that accompanies increased metabolic efficiency during weight loss (Day and Bailey, in press). As its principal metabolite, M2 is presumed to be the main substance responsible for this effect. M2-stimulated lipolysis was reduced by an inhibitor of p38 MAP kinase, an enzyme that is required for the β adrenoceptor dependent increase in UCP1 expression in brown adipocytes

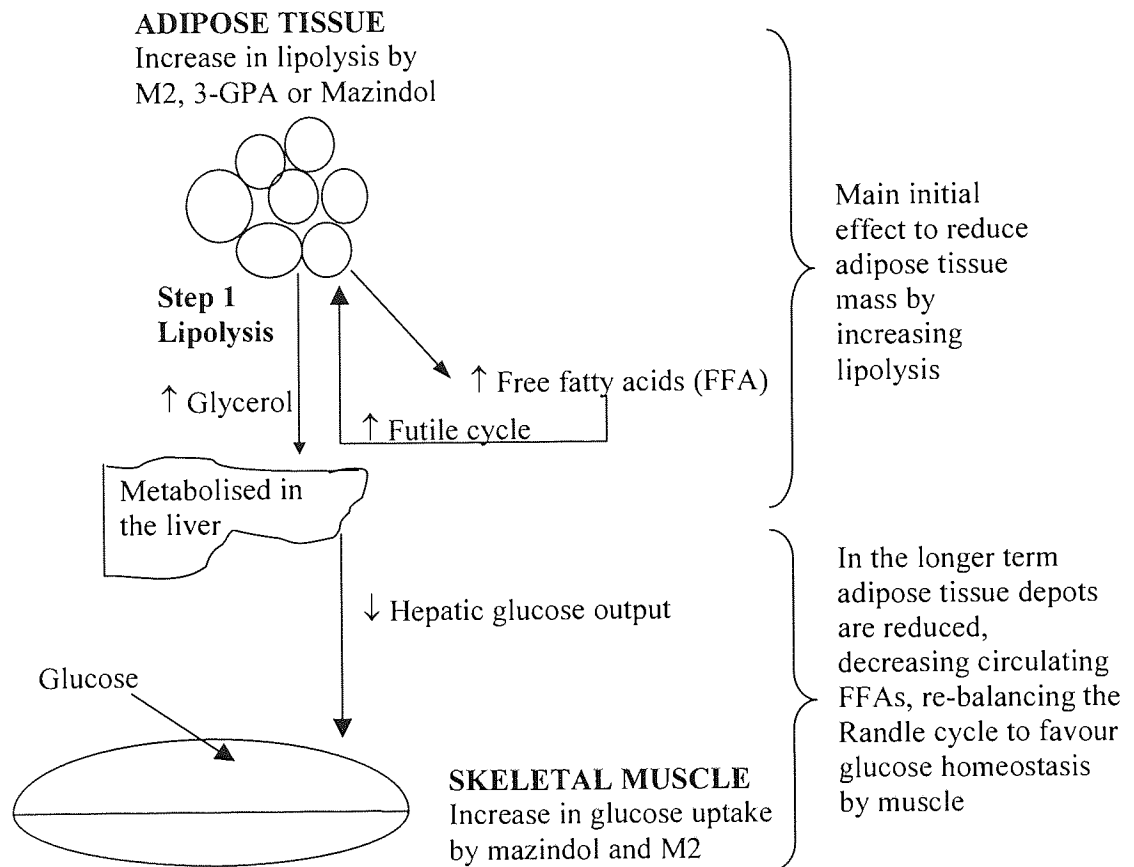
(Cao *et al*, 2001). Although not investigated here M2 may have thermogenic properties and could stimulate the UCPs.

Future work to follow on the research presented in this thesis would include the investigation of novel thermogenic agents that rely on increased utilisation of FFAs to stimulate UCPs. In addition, future studies could include the investigation into combination treatment therapy (i.e. anti-obesity agent with an anti-diabetic agent) and the benefits of this therapeutic approach for the obese-diabetic individual.

10.4 Conclusions

This thesis has provided novel information demonstrating that M2, mazindol and 3-GPA are potential therapeutic treatments for obesity with or without type 2 diabetes. The three compounds are clearly lipolytic and both M2 and mazindol have known satiety effects e.g. neural tracts affecting the hypothalamic satiety 'centre'. In addition, the agents are capable of increasing glucose uptake into insulin sensitive tissues. The experimental programme detailed herein has also delineated the cellular mechanisms involved in the lipolytic and glucose uptake effects of M2. The conclusions of this thesis are summarised schematically in figure 5 showing that the lipolytic effects of M2, mazindol and 3-GPA provide a basis for implementing the starting hypothesis of this thesis for the treatment of obesity, namely treating the obesity through increased weight loss, which would improve insulin sensitivity and ultimately have benefits for type 2 diabetes.

Figure 5: Conclusions of The Present Work



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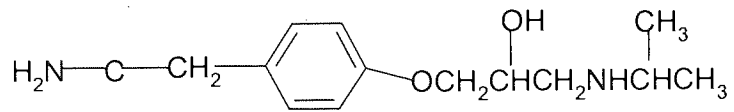
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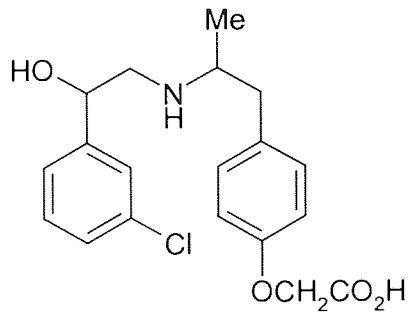
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Appendix I: Chemical Structures

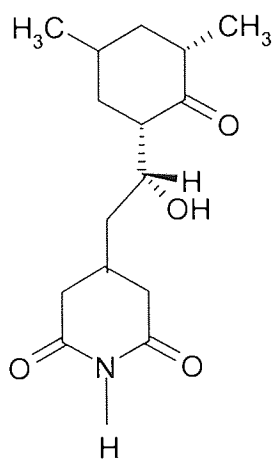
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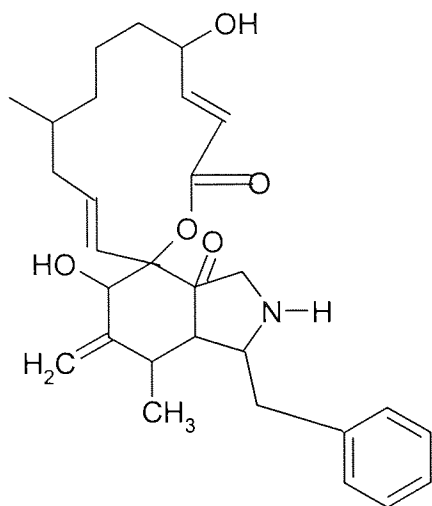
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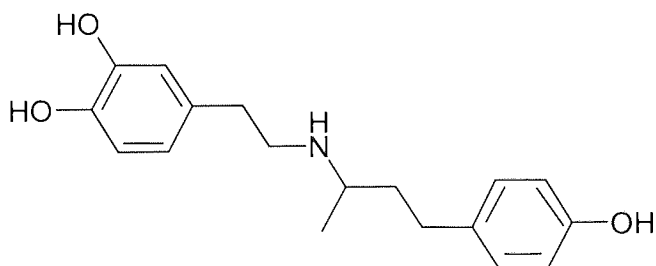
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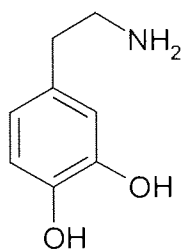
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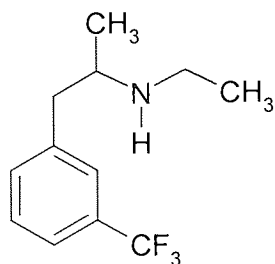
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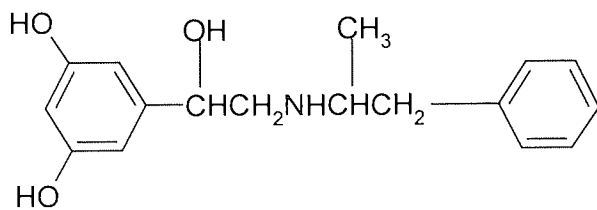
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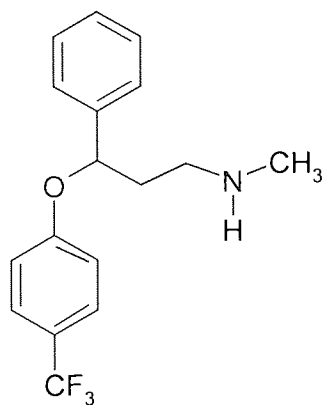
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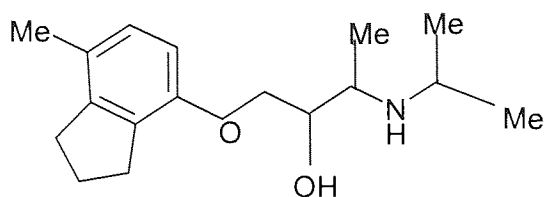
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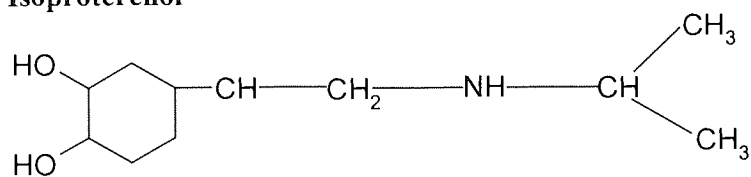
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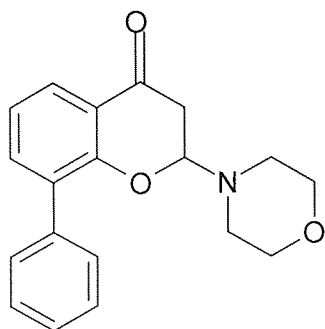
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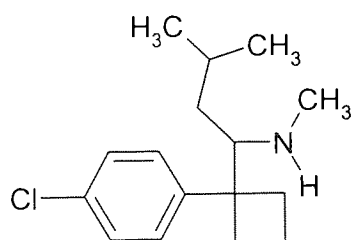
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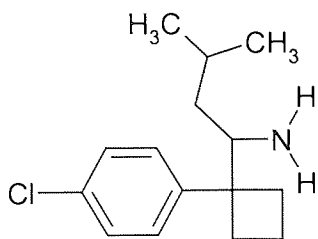
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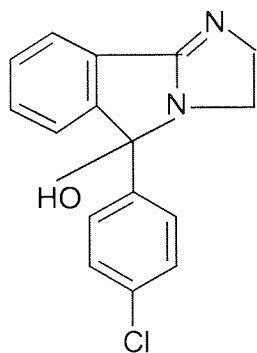
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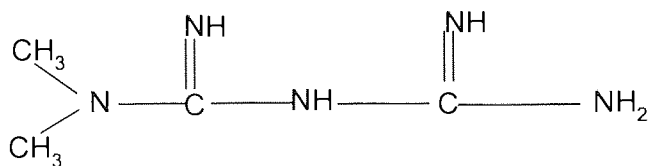
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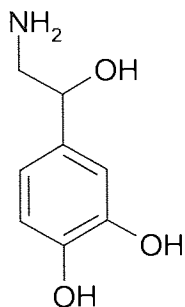
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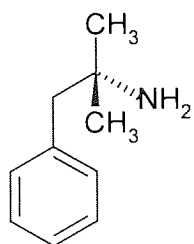
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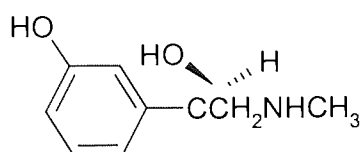
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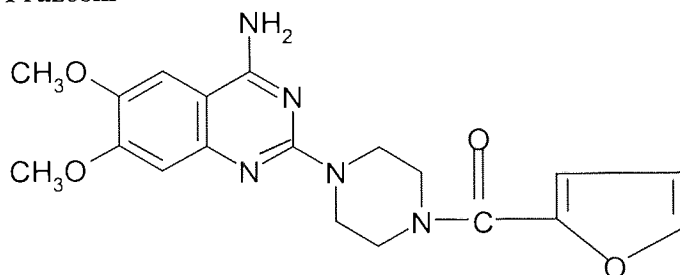
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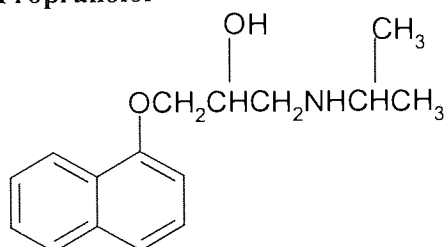
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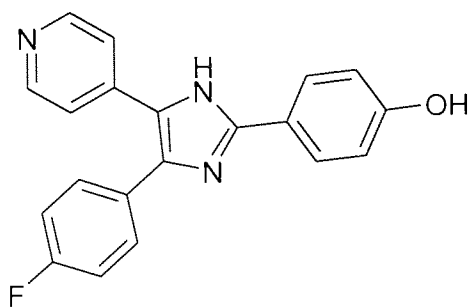
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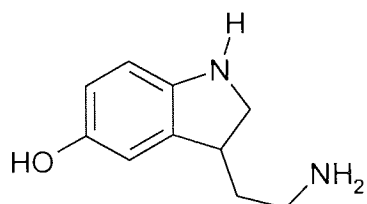
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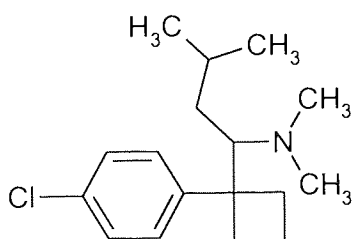
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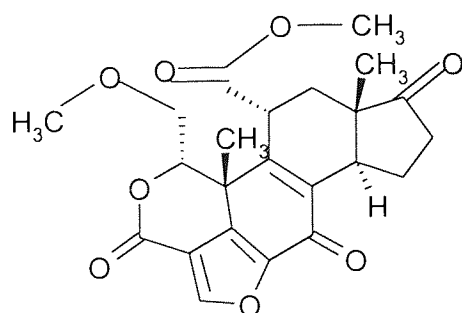
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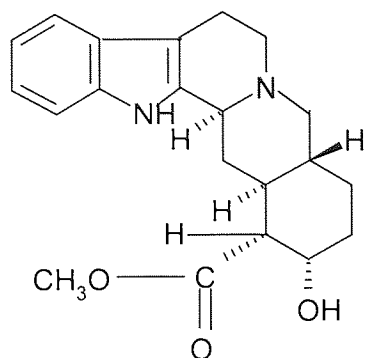
Sibutramine



Wortmannin



Yohimbine



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