CERAMIDE AND INSULIN RESISTANCE IN MUSCLE AND ENDOTHELIAL CELLS

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Summary

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Ceramide and insulin resistance in muscle cell and endothelial cells

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Insulin resistance in skeletal muscle plays a major role in the development of type 2 diabetes, and insulin resistance in the endothelium is associated with decreased vascular reactivity. There is preliminary evidence that insulin resistance is associated with increased intramuscular accumulation of the sphingolipid ceramide, and it is possible that increased cellular ceramide could be a generalised factor mediating insulin resistance. The main aim of this study is to examine insulin resistance in cultured cell models of rat muscle L6 cells and human endothelium EAHY cells to determine the effect of ceramide on insulin signalling.

Insulin action was assessed in L6 muscle cells by insulin-stimulated 2-deoxy-D-glucose (2 DG) uptake and in EAHY cells by insulin-stimulated nitric oxide (NO) production.

The key finding of this thesis is that C_2 Ceramide impaired insulin action in L6 muscle cells and EAHY endothelial cells. Palmitate, which is the principal precursor of ceramide synthesis, also impaired insulin action in these cells. Moreover, an inhibitor of ceramide synthesis, Fumonisin B1 slightly but significantly protected cells from these defects.

These findings indicate that ceramide is a factor which could possibly account for or contribute to insulin resistance in a range of tissues and disrupt diverse effects of insulin. Thus ceramide might be a potential drug target to address insulin resistance.

Key words: insulin resistance, C_2 ceramide, 2 DG uptake, NO production, L6 muscle cell, EAHY endothelial cells.

Dedication

For Mum and Dad, with thanks

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Abbreviations

2 DG: -	2-deoxy-[^{3}H]-glucose
C_2 ceramide: -	Carbon two ceramide
DAG: -	Diacylglycerol
DMEM: -	Dulbecco's modification of Eagle's medium
DMSO: -	Dimethyl sulphoxide
EC: -	Endothelial Cell
EDTA: -	Ethylene diamine tetraacetic acid
FFA: -	Free fatty acids
Fb1:-	Fumonisin B1
eNOS: -	Endothelial nitric Oxide synthase
GLUT: -	Glucose transporter
GSK: -	Glycogen synthase kinase
H.A.T.: -	Hypoxanthine-Aminopterin-Thymidine
HBSS: -	Hank`s Balanced Salts Solution
IRS: -	Insulin receptor substrate
KRB: -	Krebs-Ringer buffer
MAP: -	Mitogen activated protein
NO: -	Nitric oxide
NOS: -	Nitric Oxide synthase
PBS: -	Phosphate buffered saline
PDK-1: -	3-phosphoinositide-dependent protein kinase
PH: -	Pleckstrin homology
PI-3 kinase: -	Phosphatidylinositol-3 kinase
PKB/AKT: -	Protein kinase B
PKC: -	Protein Kinase C
PP1: -	Protein phosphatases 1
PP2A: -	Protein phosphatases 2A

ROS: -	Reactive oxygen species
TNFα:-	Tumour necrosis factor alpha
VSMC: -	Vascular smooth muscle cell

Chapter 1:

Introduction

1.1 Overview of this thesis

This thesis sets out a programme of literature review and experimentation to investigate the cellular basis of insulin resistance, particularly to examine the effect of ceramide on insulin signalling.

The first part of this report provides a brief review of current knowledge of the importance of insulin in nutrient homeostasis and other physiological functions. The next section considers the cellular mechanism of insulin action and defines the condition of insulin resistance. The clinical importance of insulin resistance is then discussed to illustrate how defective insulin action is a pathogenic factor that underlies a multitude if different conditions, particularly type 2 diabetes and associated cardiovascular diseases which comprise the 'Metabolic Syndrome'.

The main experimental purpose of this thesis is to investigate potential cellular defects of insulin resistance, particularly the involvement of ceramide. The area of investigation that I have chosen relates to insulin resistance in type 2 diabetes because this is now recognized as a very serious threat to health worldwide. Since skeletal muscle is a major site of insulin resistance in type 2 diabetes, most of the experimental studies reported in this thesis have been undertaken using cultured cell models of muscle cells. Endothelium is also an important site of insulin resistance that has been implicated in diabetic vascular disease. The literature pertaining to this issue

is reviewed and studies have been undertaken using cultured endothelial cells.

Cellular accumulation of ceramide has recently been identified as a defect generally associated with insulin resistance. Excess ceramide could possibly cause a rate limiting interruption to a variety of metabolic actions of insulin: hence ceramide has been selected for more detailed study in this thesis as a potential target for pharmacological therapeutic intervention.

The main experimental methods used in this study are cultured L6 muscle cells and EAHY endothelial cells because they are representative of skeletal and endothelium respectively. Typical experimental design has been to investigate the cells with and without insulin and other test substances and to measure parameters of insulin action such as glucose uptake in muscle cells and NO production by endothelial cells. Measures of cell viability and cell growth have also been considered.

The main results are that ceramide can reduce insulin-stimulated effects both on muscle cells and endothelial cells independently of any interference with cell viability.

The discussion section evaluates these results and the main conclusion is that ceramide is likely to be a generalised cellular factor because it disrupts a variety of

insulin actions, suggesting that ceramide interferes with one or more common cellular pathways of insulin signalling.

1.2 Importance of insulin in nutrient homeostasis

The term 'homeostasis' was coined by Walter B. Cannon to describe the ability of the body to maintain a relatively stable internal environment (Cannon, 1929). Although Cannon was particularly interested in the control of the extracellular fluid, the concept of homeostasis is important in all aspects of physiology including the ability to maintain a supply of nutrient fuels within the internal environment. Nutrient homeostasis is pivotal because all cells constantly need energy, and this in turn requires the body to make continual adjustments to the processes of storage and liberation of fuels to reflect the changes of the body energy supply and demand (Frayn, 1996).

Glucose is probably the most important nutrient-derived fuel. If the extracellular glucose concentration is within the normal range, then most tissues use glucose for their energy source in preference to other sources of energy such as fatty acids and amino acids (Unger, 1977). The brain is almost entirely dependent on an adequate supply of glucose. Therefore it is essential that the extracellular glucose concentration is maintained within a relatively narrow 'normal' range. If the glucose concentration falls too low then a state of clinical hypoglycaemia could occur, with potentially fatal

neuroglycopenia (Marks, 1976; Cryer, 1993; Frier, 2001). However, if the glucose increases too high for too long, this constitutes a condition of diabetes mellitus in which the excess glucose can have a toxic effect (glucotoxicity), with glycation of proteins and osmotic effects that lead to microvascular diseases and contribute to some macrovascular diseases (Pickup and Williams, 2003). Severe untreated insulinopenic (type 1) diabetes will result in acutely life threatening osmotic disturbances and acidotic coma (Eisenbarth et al, 1998).

In general, when the supply of glucose in the diet exceeds the demand for glucose there is a net storage as glycogen. This process is known as glycogenesis. Nevertheless, the size of glycogen stores is limited, and a substantial excess of glucose is transferred into triglyceride stores through lipogenesis. Glycogen is stored mainly in liver and skeletal muscle, and fat is stored mainly in adipose tissue, although some extra fat may be deposited in liver, muscle and many other tissues (Frayn, 1996; Machann et al, 2004).

On the other hand, when dietary intake of glucose is less than the demand for glucose as a metabolic fuel, then stored glycogen is converted back to glucose by glycogenolysis. Since the glycogen stores are limited other metabolic adaptations occur. Triglyceride is broken down to liberate fatty acids as an alternative source of energy for some tissues. The energy from fatty acids is used by liver to produce new

glucose from such substrates as lactate, amino acids and glycerol through gluconeogenesis. These adaptations help to keep the blood glucose concentration stable (Frayn, 1996; Silverthorn et al, 2001).

In conditions of diabetes the plasma glucose concentration is raised because there is inadequate utilization of glucose and usually also excess new glucose production. There are also alterations in lipid metabolism due to increased mobilization of fatty acids as a source of energy instead of glucose. Protein metabolism is also adversely affected with increased protein catabolism. Hence the overall control of nutrient homeostasis is disturbed. Thus diabetes mellitus is a wide range metabolic disorder, and it is usually the result of a malfunction in either the secretion or action of insulin, or both of these problems together (Del Prato, 2002). Hence, insulin is the key hormone involved in the control in nutrient homeostasis, and the action of this hormone is therefore a central theme for this thesis.

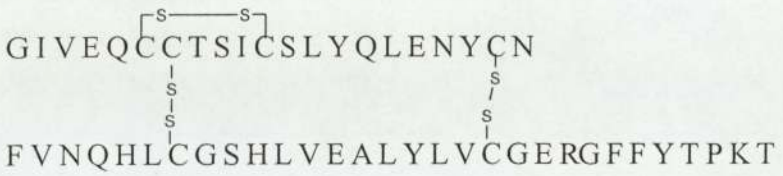
It is well recognized that the control of nutrient homeostasis involves many other hormonal, neural and metabolic factors as well as insulin, and disturbances in many of these factors occur in diabetic states (Stumvoll et al, 2003). Important effects of counterregulatory hormones such as glucagon, adrenaline and glucocorticoids, plus vagally-mediated control pathways, nutrient partitioning in various tissues, the actions of cytokines and many other factors contribute to normal nutrient homeostasis and

show defects in diabetic states (chapter 11 in Pickup and Williams, 2003; Jéquier, 1995). The scope of this thesis does not allow detailed consideration of the interactions of all of these factors, but these factors will be considered where they could help in the development of the present hypothesis and the interpretation of the data.

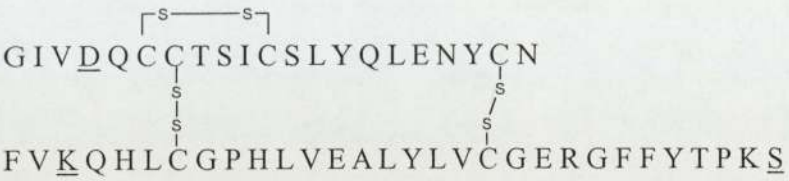
1.3 Control of insulin secretion

Insulin is secreted from pancreatic β cells. The formation of insulin by the β cell is a continuous process that changes in rate in response to the demand for insulin. Transcription of the insulin gene is under the chronic control of the nutrient environment of the β cell, particularly reflected through the rate of glucose metabolism, but also involving the metabolism of other nutrients and the influence of neural and hormonal factors (Docherty, 2000). Translation of the insulin mRNA gives rise to pre-proinsulin which is quickly cleaved to proinsulin in the rough endoplasmic reticulum cisternae of the β cells. In man there is a single proinsulin cleaved by the enzymes PC2 and PC3 mainly in the Golgi apparatus and immature granules to give insulin plus C-peptide (Orci, 1984). In rat and mouse there is differential splicing which results in two proinsulins which are cleaved to give two insulins (insulin I being the predominant form compared with insulin II) (Smith, 1966). The structures of these insulins are shown in Figure 1.1.

Human insulin



Rat/mouse insulin I



Rat/ mouse insulin II

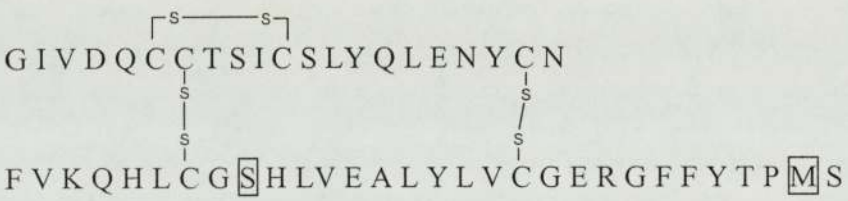


Figure1. 1. Structure of human insulin, and rat/mouse insulin I and II. Letters refer to the amino acid residues. S-S refers to disulphide bonds between cysteine residues. Single underscore shows differences of rat insulin I from human insulin. Frame shows differences of rat insulin from insulin I.

Insulin hexameric crystals aggregate with zinc in storage granules, and C-peptide and some other peptides (mostly islet amyloid polypeptide, also called IAPP or amylin, and β granin) are located around the insulin crystals within the granules. Newly formed granules are mainly stored within the β cell, but some are quickly degraded if there is little demand for insulin secretion. If there is a strong stimulus for insulin

secretion, then the granules appear to be moved rapidly to the cell surface in readiness for release by exocytosis (Clark et al, 2004). It is believed that there is a spatial organization of granules into a 'labile' compartment located near to the membrane for immediate (first phase) insulin release, and another compartment probably located further from the membrane for more long-term storage of granules. The latter granules are then moved into the labile compartment in response to demand to secrete more insulin, so-called second phase insulin release (Rorsman and Renstrom, 2003).

1.3.1 Glucose

The major determinant of insulin secretion in mammals is the blood glucose concentration, though factors such as other nutrients, metabolic, endocrine and neural factors can also influence the β cell. Insulin is constantly secreted at a low basal level, and rises from this level after a meal when the circulating concentration of glucose or other stimuli increase. Glucose is transported in to β cells by GLUT-2 glucose transporters protein. In β cells, this glucose is phosphorylated to glucose-6-phosphate, and then metabolized via glycolysis and the Krebs cycle to produce ATP (Figure 1.2). Thus the generation of ATP within the β cell reflects changes in the glucose concentration (or other nutrient fuels) in the extracellular fluids. ATP acts at the membrane of the β cell to cause ATP-sensitive K^+ channels to close. Accordingly the cell depolarizes voltage-gated Ca^{2+} L-type channels open, and Ca^{2+} entry occurs. The rise in the intracellular free calcium concentration activates several enzymes, such as

pathways, but particularly the calmodulin pathway move granules so that they fuse with the cell membrane and empty their content by exocytosis into the interstitial fluid (Flatt, 1999; Rutter, 2004).

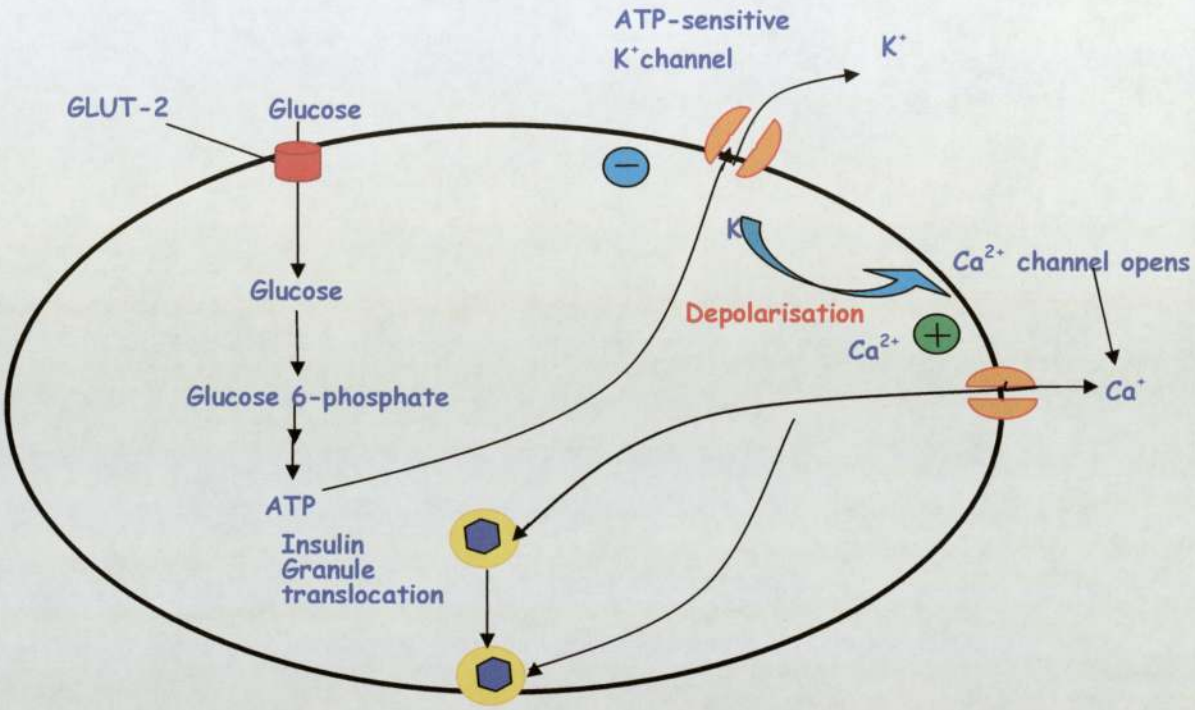


Fig.1. 2. The main mechanism of insulin secretion stimulated by glucose

It should not be forgotten that although glucose is the main controller of insulin secretion, long-term exposure to hyperglycaemia can damage β cells (so-called glucotoxicity) by non-enzymatic glycation of cellular proteins, altering their structure and preventing the protein from functioning correctly (Brownlee, 2003).

secretion, long-term exposure to hyperglycaemia can damage β cells (so-called glucotoxicity) by non-enzymatic glycation of cellular proteins, altering their structure and preventing the protein from functioning correctly (Brownlee, 2003).

1.3.2 Amino acids and fatty acids

In addition to glucose, most amino acids are effective stimulators of insulin secretion in the absence of glucose. Leucine, and analogues such as 2-ketoisocaproic acid are metabolized by the β cell, raise ATP levels which then close K-ATP channels and open voltage-gated Ca^{2+} channels as described above. Arginine, it has been suggested affects calcium channels by directly depolarizing the β cell membrane (Cejvan et al, 2002).

Fatty acids, especially in the short-term, can initiate insulin secretion by providing a nutrient source for ATP production, and by providing a source for signalling intermediates such as inositol trisphosphate (IP_3) and diacylglycerol (DAG) which potentiate insulin secretion by mobilization of calcium within β cells (Girard, 2003). However, it should be noted that long-term exposure to raised concentrations of fatty acids can leave a toxic effect on the β cell by releasing oxygen free radicals which destroy membranes by peroxidation of membrane lipids and interfere with biochemical pathways by oxidizing other molecules within cells (so-called lipotoxicity) (Yaney and Corkey, 2003).

1.3.3 Hormones and nerves

In addition to nutrients, various hormones, nerves, metabolites and other agents in the internal environment of the islets of Langerhans affect insulin secretion.

There are at least three sites for the modulation of insulin secretion by these agents Firstly, they can affect ion channels (Hellman et al, 1992); secondly, they may exert effects on the mobilization of intracellular calcium stores; thirdly, they may modify Ca^{2+} sensitivity within the β cell (Berggren et al, 1994). Other possible sites include the biosynthesis of insulin, which can affect insulin secretion, and the availability of energy (ATP level) within the cell.

The list of hormones and neural transmitters that contribute to the control of secretion of insulin, includes glucagon, GLP-1 (glucagon-like peptide-1), gastric inhibitory peptide (GIP), opioids, somatostatin and islet amyloid polypeptide (IAPP), vasoactive intestinal peptide (VIP), gastric releasing polypeptide (GRP) pancreastatin, galanin and neuropeptide Y, pituitary adenylate cyclase-activating peptide (PACAP), acetylcholine and adrenergic transmitters (Holst, 1992).

Glucagon is a powerful stimulator of insulin secretion, and glucagon mostly has the opposite metabolic effect of insulin (Lefèbvre, 1995). It regulates cellular metabolic pathways by activating adenylate cyclase through a stimulatory GTP-binding protein

(Gs) (McDermott and Sharp, 1994) then generating cyclic AMP production within β cells (Ammala et al, 1993). Cyclic AMP can activate PKA, which alters Ca^{2+} mobilization and the activity of calcium-sensitive enzymes. Glucagon release itself is stimulated by many factors including raised amino acids and nutrients, hormones, and neurotransmitters, as well as fatty acids, GIP, gastrin, and VIP. There may be an evolutionary reason for releasing glucagon with a meal due to eating high protein-low carbohydrate meals, so that the glucose level does not fall while the amino acids are being transferred into cells. The balance between glucagon and insulin secretion is complex (Flatt, 1996), but the most reported feature is that low glucose stimulates glucagon to help raise glucose levels and prevent hypoglycaemia. Also glucagon stimulates insulin secretion: this is a small effect when glucose levels are low and insulin levels are basal.

GLP-1 is a posttranslational product of the proglucagon gene (Mojsov, 1986) and it is released in response to ingestion of glucose or a mixed meal. It is a potential new treatment for type 2 diabetes (Holst, 2002). Clinical trials have shown that in addition to stimulating insulin secretion and normalizing blood glucose, GLP-1 also reduces the rate of gastric emptying and increases satiety (Zander et al, 2002). GLP-1 acts through specific β cell receptors which lead to activation of adenylate cyclase and elevation of cAMP (Flatt et al, 1990). However, other possible actions have been suggested, such as possible effects on membrane ion channels (Gromada et al, 1995).

GIP (gastric inhibitory polypeptide) is a peptide that is synthesized in intestinal K cells and secreted primarily in response to the ingestion of glucose or fat (Kieffer and Habener, 1999). It is a glucose-dependent stimulator of insulin secretion. The principal action of GIP seems to be the stimulation of glucose-dependent insulin secretion after enteral nutrient ingestion (Tseng et al, 1999). It acts by binding target receptors in the β cell plasma membrane, which activates adenylate cyclase to generate cAMP (Blomhoff et al, 1987).

Somatostatin (SRIF) released from islet D-cells reduces the secretion of insulin and glucagon by a local "paracrine" action (Efendic, 1980).

IAPP, also called amylin, a peptide is colocalized with insulin in β cell secretory granules (Lukinius et al, 1989) and is secreted with insulin in response to glucose.

IAPP delays the postprandial rise in plasma glucose levels by slowing the rate of gastric emptying (Young et al, 1995), suggesting that amylin, as another β cell hormone, may coordinate with insulin in regulating blood glucose homeostasis. There are many claims that IAPP can decrease insulin secretion and contribute to β cell failure in diabetes, however this is still a controversial issue (Clark, 2004).

Pancreastatin (PST) is a peptide found in the islets that increased insulin-stimulated

glycogen synthesis. It is released in parallel with insulin in response to glucose and a number of other secretagogues (Ostenson et al, 1989). In fact, high PST plasma levels have been found in type 2 diabetes (Funakoshi et al, 1990) and gestational diabetes (Sánchez-Margalet, 1998), but the significance of this is unclear.

VIP (vasoactive intestinal peptide) may play a role in the control of insulin secretion by increasing the cytosolic calcium ion concentration of β cells, but a physiological role of VIP in the control of insulin secretion is debatable.

GRP (gastric releasing polypeptide) is released by electrical stimulation of the vagus nerve and considered as a powerful stimulator to the secretion of insulin, glucagon and somatostatin (Holst et al 1992). It binds to specific β cell receptors (Wahl et al, 1992) leading to activation of phospholipase C and generation of IP_3. In addition, it may contribute to the depolarization and increase of Ca^{2+} influx (Wahl et al, 1994).

The classical cholinergic and adrenergic neurotransmitters acetylcholine and noradrenaline are involved in the physiology and control of insulin secretion, as well as in the endocrine and paracrine effects. Acetylcholine binds with muscarinic receptors which activate PLC to enhance insulin secretion via increased mobilization and influx of Ca^{2+} (Adeghate et al, 2004). In β cells, alpha-adrenoreceptors predominate, which reduces adenylate cyclase activity and reduce insulin secretion.

This effect predominates over the smaller number of beta-adrenoreceptors predominates which act via adenylate cyclase to promote insulin secretion. Autonomic neurotransmitters may influence insulin secretion indirectly by vascular effects that alter blood flow to the pancreas or alter the distribution of blood within the pancreas. Autonomic activity additionally stimulates glucagon secretion which is an insulin secretagogue as noted above.

Although there are various defects of insulin secretion in type 2 diabetes and there is an absence (or near absence) of insulin in type 1 diabetes, the end problem for glucose metabolism is essentially the same, namely that the cells receive insufficient glucose to function normally. The common diagnostic feature is that blood glucose concentrations are elevated. In type 1 diabetes the lack of insulin is clearly the cause of the hyperglycaemia. However in type 2 diabetes, where there is both insulin resistance and β cell dysfunction eventually leading to insulin deficiency it is not always possible to know how much of the hyperglycaemia to attribute to insulin resistance and how much to β cell dysfunction (Kahn, 2003).

1.3.4 How does insulin 'direct' fuel selection?

In the human body there are two contrasting metabolic states. When a person absorbs a meal and the food is digested, this is the fed state. When there are no nutrients available to be used by tissues, this is the fasting state. Insulin is crucial to direct

excess nutrients into storage in the fed state, and also to a lesser extent reduced insulin levels influence the release of stored nutrients in the fasting state.

In the fed state, glucose absorbed after a meal is taken directly to the liver, where part is metabolized, part is stored as glycogen, and a small part is converted into fatty acids and directed to triglyceride synthesis. The remaining glucose that is not taken up by the liver services other organs. In particular glucose contributes to the supply of the energy for brain, muscle and most other organs and tissues. The remainder is stored as glycogen in muscle and lipid in adipose tissue (Frayn, 2003).

The uptake of glucose by muscle and adipose tissue is dependent largely on insulin, whereas some tissues such as brain, renal medulla and blood cells take up glucose independently of insulin. The uptake of glucose by liver is largely independent of insulin, but the storage as glycogen is strongly stimulated by insulin. The different dependencies of tissues on insulin relates largely to their expression and distribution of different isoforms of the glucose transporters, so that the main insulin-sensitive isoform (GLUT4) is mostly expressed by muscle and adipose tissue, hence their high dependence on insulin (Weicket and Pfeiffer, 2006).

As plasma glucose concentrations begin to fall several hours after a meal, this means that the body shifts away from the fed state to the fasting state. In the fasted state,

nutrients are mobilized from storage and used as a source of energy. In order to maintain the plasma glucose concentration within a normal or acceptable level, glycogen in liver is mobilized through glycogenolysis to produce glucose. Glycogen stored in muscle can be used as a source of glucose within the muscle cells, but it cannot transfer into blood glucose directly because it does not express a form of glucose-6-phosphatase at the cell membrane. Hepatic glycogenolysis represents the initial fast response to falling blood glucose levels, and it is stimulated by glucagon and adrenaline. As an individual passes into a fasting state, adipose tissue breaks down its triglyceride stores into fatty acids. These are released into the blood and taken up by liver and muscle to be used as an energy source. Fatty acids are also a source of energy for many other tissues, but not brain which relies on either glucose or the breakdown of fatty acids to ketones. To make new glucose (gluconeogenesis), which occurs mostly in liver, the liver uses firstly pyruvate and lactate. Subsequently, gluconeogenesis uses glycerol and protein (broken down into amino acids mainly from muscle and transferred to liver) to make glucose (Frayn, 2003).

In these two states, insulin holds an important regulatory position. Insulin promotes the anabolism of glucose by enhancing glycolysis, glycogenesis and lipogenesis and preventing the breakdown of glycogen and fat to ensure that metabolism is normal. If insulin is absent or deficient, nutrient homeostasis becomes chaotic and diabetes mellitus follows.

1.3.5 Effects of insulin on vascular tissue

Insulin exerts many effects on vascular tissue. Some of these effects are direct effects, mainly via the endothelium, but there are also many indirect influences, mainly via the metabolic effects of insulin and possible also via renal and autonomic mechanisms (Cleland et al, 1998).

The metabolic effects of insulin that affect vascular function include the general metabolic action of insulin to increase lipogenesis and decrease lipolysis. These effects are, in principle, likely to lower the circulating concentration of FFAs and TG, and should help to reduce lipid accumulation in the vascular wall and thereby reduce atherogenesis. However insulin can increase TG-rich VLDL production by the liver, so the balance of overall effect of insulin on the vascular tissue via lipid metabolism is uncertain. Excess dietary lipids, which cause increased incomplete lipid metabolism leading to increased reactive oxygen species (ROS) can damage vascular tissue and increase susceptibility to atherosclerosis. While raised cholesterol concentrations (especially small dense LDL particles) are well recognised in the pathogenesis of atherosclerosis, there is no strong evidence linking insulin to the cholesterol pathways (Stehouwer et al, 1997).

Renal sodium retention and increases in autonomic activity after insulin

administration can, in theory leave effects on the vasculature that are likely to increase plasma volume and blood pressure (Baron, 1994). However these effects appear to be relatively small and they are generally out-weighed by the direct actions of insulin on the vasculature to increase vasodilatation and blood flow. For example insulin infusion in normal human volunteers increases blood flow to limbs associated with reduced vascular resistance (increased vasoreactivity) (Steinberg and Baron, 2002). Moreover, the greater the general insulin sensitivity of an individual, as measured by insulin-stimulated glucose disposal, the greater the increase in blood flow. This has been interpreted to indicate that the vasodilatory effect of insulin may be to assist increased glucose uptake by muscle (discussed below), but it can also indicate that insulin can improve both blood flow and glucose metabolism independently.

Conversely, individuals with insulin resistance are highly susceptible to detrimental vascular changes, including an increased prevalence of raised blood pressure. These are epitomised by the "Metabolic Syndrome" (also known as the insulin resistance syndrome) in which insulin resistance is identified as a core component (Reaven, 1993). This is considered in relation to type 2 diabetes in section 1.5, and the area is discussed in more detail in the final discussion of this thesis.

1.3.6 Effects of insulin on endothelium

Endothelium appears to be the main site of action of insulin on the vasculature.

Endothelial cells express insulin receptors on their luminal surface and those are functionally linked to the PI 3-kinase pathway and the MAPK pathway.

In endothelium these pathways are important for nitric oxide production and the control of cytokines and other secreted substances (Hsueh and Law, 1999). The vascular smooth muscle (VSM) also responds directly to insulin, although there is much controversy over its normal physiological action on this tissue. It has been difficult to segregate effects directly on the VSM from effects mediated via the endothelium. The main contentions concern the clinical control of blood pressure and susceptibility to atherosclerosis, and these are reviewed elsewhere and extend beyond the remit of this thesis (Reaven, 1998).

Though the exact mechanism of the transport of insulin remains debated (Steil et al, 1996), in endothelial cells insulin transport across the endothelial barrier appears to be mediated by IR (Insulin receptor) (King and Johnson, 1985). Then, it exerts a vasodilator action via activation of endothelial NO synthase (eNOS): one of three isoforms of NO synthase. Additionally, it has been reported that shear stress may also regulate eNOS by this pathway (Kim and Corson, 2001). This is shown in figure 2. Insulin increases NO production via Akt. The process of insulin-mediated NO production is therefore fundamental to the normal function of the present project and thesis. Moreover, it is reported that the vasodilatory effect of insulin is impaired in

individuals with insulin resistance (Baron, 1993; Natali, et al. 1994; Mather, 2000). Insulin-induced increase in blood flow through skeletal muscle has been suggested to contribute significantly to the increase in glucose uptake by skeletal muscle (Baron, 1994). Indeed, the highest concentration of GLUT 4 glucose transporters is located in those regions of skeletal muscle plasma membranes most closely abutting capillaries.

In addition to the action of insulin described above, it is noteworthy that insulin acts on the vascular wall via anti-inflammatory effects to cause a suppression of intranuclear nuclear factor-κB (NF-κB), monocyte chemoattractant protein-1 (MCP-1) and C-reactive protein (CRP) (Aljada et al, 2001), which will all serve to reduce the risk of or rate of atherogenesis, as well as an increase in cellular inhibitor κB (IκB), which binds NF-κB to prevents its translocation into nucleus (Baldwin, 1996). These effects could be related to the ability of insulin to induce the release of NO (Zeng and Quon, 1996), and are probably anti-atherogenic in the long-term (Brand et al, 1996).

1.4 Cellular mechanism of insulin action

Many laboratories in the past 20 years have explored the principal features of insulin action at the cellular and molecular level (Lizcano and Alessi, 2002).

Insulin-signalling pathways

Insulin initiates its biological effects by activating at least three major signalling

pathways, one involves phosphatidylinositol 3-kinase (PI-3 kinase) and the other involves a RAS/mitogen-activated protein kinase (MAP kinase) cascade. Other signalling pathways may also be activated by insulin receptors, but these are as yet unclear.

Insulin signal pathway:

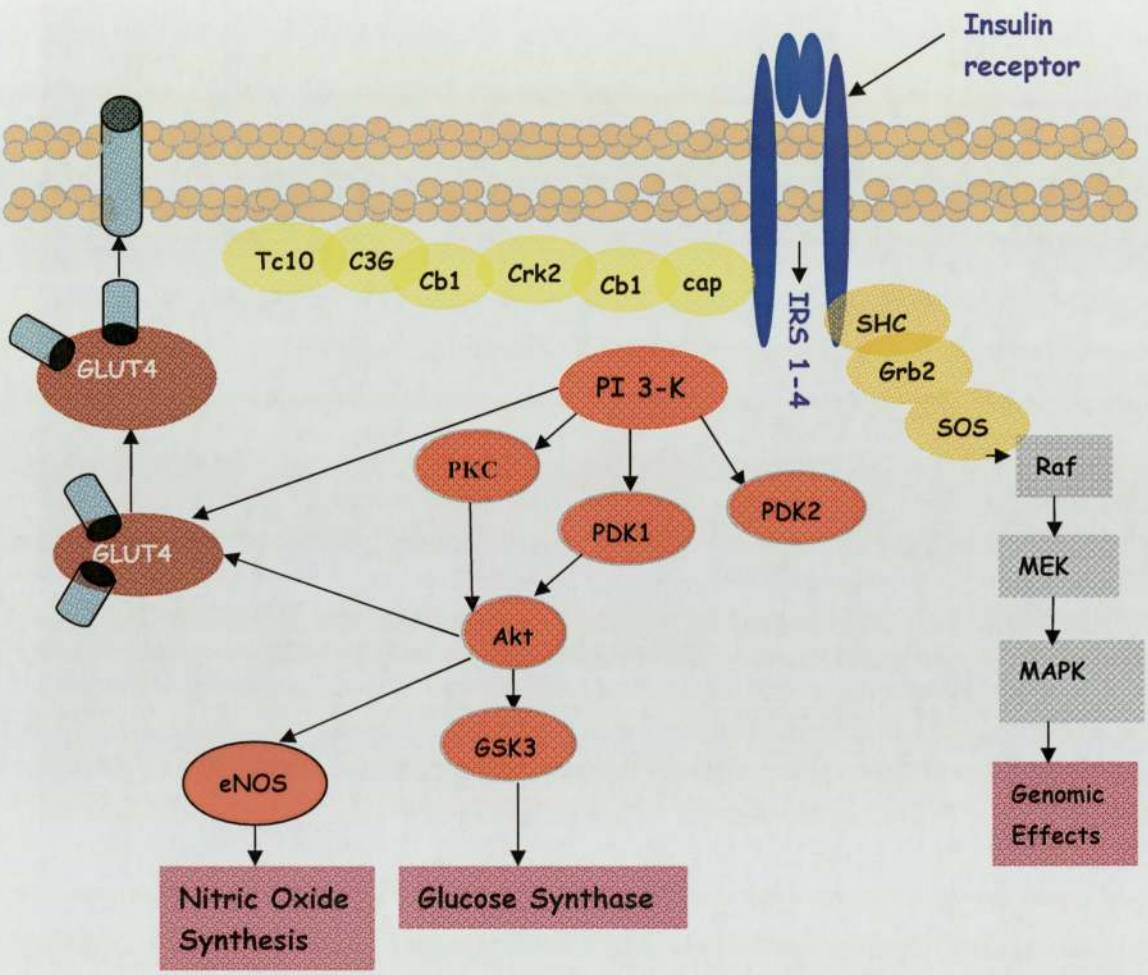


Fig.1.3 .Principal components of insulin signalling pathway. Three major pathways from the activated IRS (insulin receptor substrates) are PI 3-K (phosphoinositide 3-kinase), the CAP/Cb1/Tc10 and the MAPK pathway.

a. PI-3 kinase pathway:

The Insulin receptor (IR) consists of two α- and two β-subunits. Insulin binds to the extracellular α-subunit, changing the conformation of α-subunit, which then changes the conformation of the β-subunits so that the β-subunits are activated. The conformational changes to the intracellular region of the β-subunits expose a tyrosine kinase domain, which is activated by intermolecular transphosphorylation reactions (White, 2003).

The activated receptor acts as a tyrosine kinase and phosphorylates various intracellular proteins, including members of the ***insulin-receptor substrates (IRS)*** protein family, which contains at least four members, namely IRS-1, IRS-2, IRS-3, and IRS-4, (Sun et al, 1991) and probably also SOS and Grb-2. IRS-1 knockout mice display a growth retardation, as well as insulin resistance in peripheral tissues and impaired glucose tolerance (Accili and Joshi, 1996). IRS-2 knockout mice exhibit insulin resistance in both muscle tissues and liver. Compared with them, IRS-3 and IRS-4 knockout mice have normal growth and metabolism so IRS-3 and IRS-4 are probably not vital for normal insulin action (Liu et al, 1999).

Phosphoinositide 3-kinases (PI 3-kinase) produce 3'phosphorylated phosphoinositides, especially PtdIns (3, 4, 5) P3, which can bind to the PH (Pleckstrin homology) domains of a variety of signalling molecules by altering their activity or

subcellular localization (Lietzke et al, 2000). They also act as second messengers to transmit insulin signals in the membrane. Treatment with either of the PI-3 kinase inhibitors wortmannin or LY294002 blocks insulin's effects on glucose metabolism, while expression of constitutively active forms of PI-3 kinase stimulates them. In single-cell assays, microinjection of dominant negative forms of PI-3 kinase or inhibitory PI-3 kinase antibodies block GLUT4 translocation. The PI-3 kinase gene encodes two proteins (p85 α and p110 α) that serve as regulatory and catalytic subunits (Virkamaki, 1999). Disruption of all proteins results in lethality shortly after birth, whereas the targeted disruption of the gene p85 α leads to a viable but hypoglycaemic animal with improved insulin sensitivity shows that this subunit is normally suppressed by the other subunit (Terauchi et al, 1999). Moreover, PI-3 kinase also possesses serine kinase activity, and both the regulatory and catalytic subunits of the enzyme can interact with other signalling proteins, such as Akt/PKB, atypical PKC isoforms (ζ,λ), and phosphoinositide-dependent kinases (PDK-1 and PDK-2) (Alessi et al, 1997).

Protein kinase B (AKT), also known as **Akt**, is a serine/ threonine kinase that plays an important part in insulin downstream signalling (Bellacosa et al, 1991) including Akt-1 and Akt-2. It possesses a PH domain that can interact with PtdIns (3,4,5) P3, promoting membrane targeting of protein and catalytic activation. Mice deficient in isoform Akt-2 exhibit mild insulin resistance in muscle (Cho et al, 2001). Akt-1 and

Akt-2 may mediate the insulin signal to glucose uptake, GLUT4 translocation, glycogen synthesis, lipogenesis, and protein synthesis. Stimulation by Akt of glucose uptake and GLUT4 translocation is insensitive to being inhibited by wortmannin (Kim et al, 1999), suggesting that Akt activates insulin signalling pathways downstream of PI-3 kinase. Akt is responsible for the phosphorylation of the Thr^{308} site on **PDK-1** (Alessi, 1997). Akt has a key function in relaying the PI-3 kinase signal that mediates the effects of insulin on glucose uptake and utilization in skeletal muscle and adipocytes. Akt actives **glycogen synthase kinase-3 (GSK-3)** (Cross, 1995), which has been implicated in the control of many cellular processes, including glycogen and protein synthesis, and the modulation of transcription factor activity. Akt also promotes translocation of GLUT4 glucose transporters into the cell membrane.

The IRS/PI 3- kinase pathway also activates **PKC-ζ/λ**, which has a role to play in insulin signalling and glucose transport activation (Standaert et al, 1997). Insulin causes the phosphorylation of PKC-ζ by PDK-1, and the relocation of PKC-ζ to GLUT4 vesicles. These studies suggest that both Akt and PKC-ζ have functions for GLUT4 translocation (White, 1998).

b. The CAP/Cbl/TC10 Pathway:

When PI-3 kinase inhibitors are used to block the stimulation of glucose transport by insulin, some increase in insulin-stimulated glucose transport still exists. This

indicates that PI 3-kinase activates another parallel complementary pathway for insulin- stimulated glucose transport (Pessin and Saltiel, 2000), sometimes referred to as the 'alternative pathway' for insulin-stimulated glucose transport.

The pathway diverges from PI 3-kinase pathway via tyrosine phosphorylation of Cb1 (Robin et al, 1997) through a process that does not involve IRSs. Cb1 phosphorylation requires an adaptor protein CAP, which has three SH-3 domains. CAP is responsible for relocation of the CAP/Cb1 compound to lipid raft domains. Flotillin is anchored in a lipid raft domain via its interaction with caveolin. When CAP/Cb1 binds to the flotillin, tyrosine phosphorylated Cb1 binds to the Crk-2/C3G complex by the SH-2 domain of Grk-2 (Chiang et al, 2001), and Grk-2 also has a SH-3 domain by which it may interact with C3G. C3G is a guanyl nucleotide exchange factor for TC10 and other small molecular weight GPT-binding proteins. TC10 is a member of a GTPases family (Chiang et al, 2001).

Many experiments suggest that the CAP/Cb1/TC10 pathway makes an important contribution to insulin signal transduction to cause an increase in glucose transport. Disruption of the CAP SH-3 domain results in failing to bind to Cb1, which blocks the recruitment of Cb1 to lipid rafts. This appears to be involved in the translocation of GLUT-4 from its intracellular storage pool to the plasma membrane (Ducluzeau et al, 2002). Importantly, the glucose transport step is rate-limiting for insulin-stimulated

glucose uptake and metabolism in peripheral tissues.

c. The MAPK Pathway:

The MAPK pathway is also activated by insulin, via both SHC and Grb2 association with the insulin receptor. The MAPKs do not play a major role in mediating the insulin signal response, but the increased MAPK activity seems to contribute to the development of insulin resistance. On the other hand, p38MAPK has been considered as a positive regulator of insulin action because of its capability to increase the uptake of glucose by the plasma membrane-localised GLUT4 transporter which is shown in fig 1.3 (Somwar et al, 2002).

1.5 Clinical implications of insulin resistance

If there is insufficient insulin, or the action of insulin is impaired (insulin resistance), then the concentration of glucose will become increased in the blood, creating a condition of diabetes mellitus.

Type 2 diabetes has become a very common disease with at least 150 million patients worldwide. This number of patients is expected to exceed 300 million by the year 2025 (Withers et al, 1998).

Over 90% of all diabetic patients have type 2 diabetes mellitus. Unlike type 1 diabetes, where the disease results from the destruction of insulin secreting pancreatic β cells,

the pathology of type 2 diabetes involves both insulin resistance and abnormalities of insulin secretion (Kadowaki et al, 1984; Kahn, 2003). The development of type 2 diabetes results from both genetic and environmental factors.

According to the majority of studies, peripheral insulin resistance probably represents the earliest event (Reaven, 1995; Kahn, 2003), but this is initially compensated by enhanced insulin secretion. Later β cells are no longer able to keep pace with the increased insulin needs, and a relative lack of insulin occurs. Insulin resistance appears to continue to rise, which accelerates hepatic of glucose and also reduces glucose utilization in muscle (Fig.1.4). Insulin resistance also allows more lipolysis which can cause release of fatty acids that can be used instead of glucose. Eventually the extent of insulin resistance levels out, but the β cells show a persistent decline in function, causing hyperglycaemia to continue to rise.

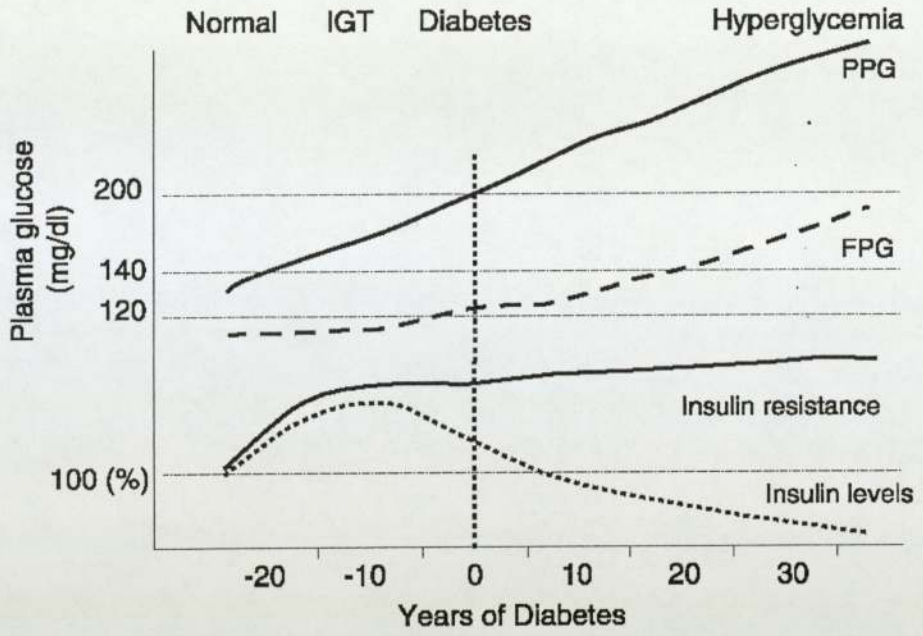


Fig.1.4 A representation of type 2 diabetes: hyperglycaemia develops after insulin resistance appears and when insulin levels are no longer maintained. 100% is an arbitrary value for a normal (non-diabetes) individual. IGT=impaired glucose tolerance; FPG=fasting plasma glucose; PPG=postprandial plasma glucose. This diagram is adapted from International Diabetes Centre (IDC), Minneapolis, MN.

Insulin resistance is very common, almost a universal finding in type 2 diabetes, which plays a cardinal role in its pathogenesis (Kahn, 1994; DeFronzo, 1998). It may be defined in generic terms as 'a failure to respond to an ordinary level of insulin'. Insulin resistance is usually associated with a number of health disorders, such as obesity, raised blood pressure, impaired glucose tolerance, type 2 diabetes, and various cardiovascular diseases. Insulin resistance is both a precursor and continuing underlying feature of the pathology of those diseases. Many individuals (about 25% of the adult population) are thought to have insulin resistance to degrees similar to those seen in patients with diabetes and this gives these individuals a higher risk of getting

those diseases (Taylor and Arioglu, 1998). The insulin resistance is seen in all main major target tissues, i.e. skeletal muscle, liver, fat, endothelium, but it does not affect all actions of insulin in all tissues, indicating that it is heterogeneous, and suggesting that different of cellular defects contribute to insulin resistance in different tissues. Muscle is of particular importance because this is a major site of insulin-stimulated glucose uptake, and the defects of insulin action precede a clinical diagnosis of type 2 diabetes, and continue to participate throughout the natural history of the disease.

From what we have discussed above, we may realize that insulin resistance represents an early and sustained component of type 2 diabetes.

Moreover, as we know, the main atiology for death and for a great percent of morbidity in patients with diabetes is vascular disease (Standl et al, 1996; Stern, 1995). Type 2 diabetes affects both small and large vessels by microangiopathy and macroangiopathy respectively. Even more, macroangiopathy is manifested by enhanced atherosclerosis, which in patients with diabetes is multifactorial and leads to a series of interactions including hyperglycaemia, hyperlipidaemia, oxidative stress and hyperinsulinaemia (Calles-Escandon et al, 1999). Endothelial dysfunction has been found in patients with type 2 diabetes (Hogikyan et al, 1998). Moreover, there is growing evidence to demonstrate the coexistence of insulin resistance and endothelial dysfunction. Insulin-induced vasodilation, which is particularly mediated by NO

release (Steinberg, 1994), is impaired in obese individuals who display insulin resistance (Steinberg, 1996). Hence the particular research in insulin resistance is in this thesis.

There are many genes, enzymes, and other signalling kinases associated with insulin resistance. (table.1)

Table 1 Phenotypes of mice lacking certain gene products

Targeted gene deletion	Growth	Insulin resistance	Diabetes
IR	**death within a week**	**YES (mild peripheral)**	**YES**
IRS-1	**growth retardation**	**YES (peripheral tissues)**	**NO**
IRS-2	**Slightly reduced**	**YES (peripheral & liver)**	**YES**
P85 subunit (PI-3 kinase)	**ND**	**Improved insulin sensitivity**	**NO**
Akt-1	**growth retardation**	**Normal**	**NO**
Akt-2	**Normal**	**YES(liver & peripheral muscle)**	**YES**

Table.1: This table was adapted from information in the reference: Hennige and Ha¨ring. Drug Discovery Today: disease model, 2004; IR: insulin receptor; IRS: insulin receptor substrate; Akt: AKT protein kinase B; PI-3 kinase: phosphatidylinositol 3 kinase; ND: not determined

It is unlikely that complete loss of expression of these genes is responsible for type 2 diabetes in humans. However, an alteration in the expression levels of these and other genes are involved in insulin action (DeFronzo, 1997; Bajaj and DeFronzo, 2003).

Thus many possibilities exist as to the causes of insulin resistance.

1.6 Genetic factors of insulin resistance

Though genetic factors appear to play a leading role in pathogenesis of type 2 diabetes, specific individual genetic defects of insulin itself or insulin receptors are relatively rare. A single defect is unlikely to explain the heterogeneity of insulin resistance, and it is more likely that there is altered expression of several genes. The mix of genes and different levels of expression could then account for heterogeneity. It is recognized that a positive family history of diabetes mellitus considerably increases the risk of insulin resistance, indicating that genetic factors are involved (Howard et al, 1998). So far it appears that the genetic basis for most forms of type 2 diabetes is probably small changes in expression of genes involves in post receptor insulin signalling and in the transport and metabolism of glucose. Probably the altered levels of expression of these genes create a susceptibility to reduced insulin action. Thus when adverse environmental (acquired) influences are added to this, the two problems (genetic susceptibility and adverse environmental factors) then precipitate insulin resistance.

A list of genes has been proposed, as candidates that contribute to insulin resistance, but evidence to prove their involvement is limited (table 1).

1. 7 Acquired factors of insulin resistance

Consistent with its heterogeneous presentation, insulin resistance is characterized by defects at many levels. These include a decreased number of receptors with decreased kinase activity and decreases in the activity of insulin receptor substrate-1, (IRS-1) and IRS-2, phophatidylinositol 3-kinase (PI-3 kinase) and also abnormalities of glucose transporters translocation (DeFronzo, 1997). Several environmental factors are known to cause or aggravate insulin resistance in susceptibility individuals. These include a high calorie intake especially fat and fructose, less physical activity, and over-production of some hormones, such as growth hormones, glucocorticoids, and glucagon.

Additionally, insulin resistance is associated with elevated plasma levels of free fatty acid (FFA) (Kim et al, 2001), which is a characteristic of obesity and type 2 diabetes (Boden et al, 2001). Many studies suggest that the oversupply of lipid to peripheral tissues might result in insulin resistance (McGarry, 2002). First, insulin-resistant subjects frequently display signs of abnormal lipid metabolism including increased circulating FFA concentrations, and elevated intracellular lipid levels (Stumvoll, 2004). Then, peripheral tissues, which are experimentally exposed to lipids, decrease their sensitivity to insulin. These observations have prompted the hypothesis that increased availability of lipids to and within peripheral tissues cause insulin resistance, perhaps by promoting the accumulation of one or more fat-derived metabolites capable of

inhibiting insulin action, so called lipotoxicity (Unger, 1995).

The molecular mechanisms linking FFAs to the inhibition of insulin action remain unclear. The glucose-fatty acid (Randle) cycle indicates that glucose and lipids serve as competitive substrates for oxidation, mainly in muscle. Thus raised circulating FFAs, through their increased availability to muscle, produce an alternative fuel, resulting in its less use of glucose, leading to hyperglycaemia. So disturbed function of the glucose-fatty acid cycle is a mechanism by which excess FFAs impair insulin action and glucose metabolism in skeletal muscle. Studies in cultured cells and rodent models of obesity or insulin resistance indicate that fatty acids also accumulate in muscle tissue and disrupt one or more early steps in insulin signal transduction (Yu et al, 2002; Reynoso et al, 2003).

FFAs have been shown to impair endothelium-dependent vasodilation (Roden et al, 1997). Damage of vascular cells by proatherosclerotic risk factors leads to endothelial dysfunction (Baumgartner-Parzer et al, 2001).

In humans, FFA-mediated endothelial dysfunction could relate to reduced availability of L-arginine or NO (Steinberg et al, 2000) and to oxidative stress (Pleiner et al, 2002). In vitro, selected FFAs have been shown to modulate endothelial expression and secretion of adhesion molecules (Bates et al, 1995) and coagulation, vasoactive

mechanisms, and inflammatory factors. However, FFAs directly affect endothelial apoptosis (Meerarani et al, 2000). This is a key element of endothelial dysfunction as well as macrovascular disease, including diabetic retinopathy and atherosclerosis (Dimmeler et al, 2002). The mechanistic basis of these phenomena is largely unknown. Answering this question could be very important because endothelial cells undergoing apoptosis seem to impair the integrity of the endothelial monolayer and its barrier function, to trigger atherosclerotic plaque erosion and plaque rupture, and to favour coronary thrombosis by their proadhesive and procoagulatory activity (Dimmeler et al, 2002). Note that the cell's apoptotic death results from the loss of intracellular and extracellular survival signals and from the increase of death signals. These include the multifunctional, chaperone-like molecule clusterin (Humphreys et al, 1999) and regulators of vascular tone such as the vasoconstrictor endothelin-1 (Shichiri et al, 1997) and endothelial NO synthase (Sata et al, 2000), the latter catalyzing the release of the vasodilator NO from L-arginine.

In addition, cell cycle regulatory molecules such as cyclins and inhibitors of cyclin-dependent kinases are involved in apoptosis and cell cycle progression (Im et al, 2001). The present study was therefore designed to characterize the background of a potentially detrimental action of insulin resistance in the endothelium, which itself appears to be related to the total plasma FFA concentrations.

Although the exact molecular mechanisms linking the insulin receptor to GLUT-4 translocation are uncertain, several pathophysiological events are well recognized as potential causative factors that can induce insulin resistance. Two events in particular are obesity and hyperglycaemia (Reaven, 1995). With regard to obesity, it has been postulated that adipose tissue secretes factors that impair insulin action. For example, tumor necrosis factor (TNF-α) and interleukin-6, which are produced by adipose tissue, can cause insulin resistance in other tissues such as muscle (Hotamisligil, 1993). TNF-α stimulates sphingomyelinase activity which in lipid raft domains can produce ceramides in cells (Dressler et al, 1992), and there is now emerging evidence that ceramides could mediate the decrease in insulin sensitivity. It has been reported that TNF-α has effects on both protein kinases and phosphatases, and may constitute an important component of the stress response in various tissues. There is also a possible relationship between ceramide signalling and the activation of various signalling pathways, such as those that involve PI-3 kinase and Akt (Peraldi, 1997).

1.7.1 Ceramide

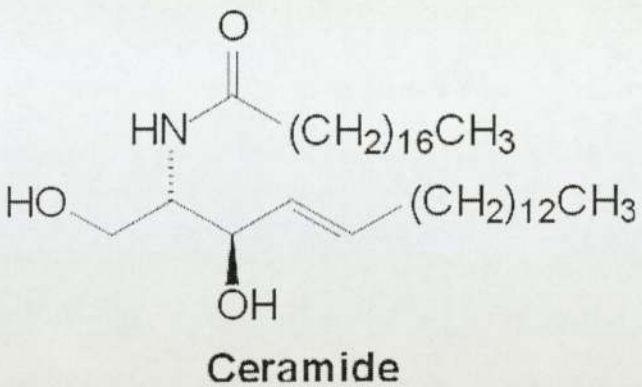


Fig 1.5. Structure of ceramide

Ceramide (N-Acyl-D-erythro-sphingosine, Fig 1.5) is a structural component of mammalian glycolipids, phospholipids, and sphingomyelin (Kolter and Sandhoff, 1998). It is also a second messenger and has effects on protein kinases and phosphatases. It has been implicated in the regulation of diverse cellular responses including cellular differentiation, the pathogenesis of insulin resistance and cell death (Mathias et al, 1998; Adams et al, 2004). The outcome of the effect induced by ceramide seems to be largely dependent on the cell line used as well as on the experimental setup. Moreover, the state of the cell cycle could have an impact on the signalling cascades. A signalling pathway involving Akt kinase leading to the activation of NF-κB has been proposed (Davis, 1993; Hannun and Obeid, 1995) as well as selective induction of the MAP pathway (Verheij et al, 1996). Although several downstream targets for ceramide have been suggested, only cathepsin D,

protein kinase C- ζ, phospholipase A2, and CAPP (ceramide activated proteinphosphatase) have been demonstrated to be activated by this lipid *in vitro* (Kolesnick and Golde, 1994; Kolesnick et al, 2000).

1.7.2 Synthesis of ceramide

Ceramide is at the centre of sphingolipid metabolism, and it serves as the first point of significant accumulation of sphingolipids in the de novo synthesis pathway (Smith and Merrill, 2002). In cells ceramide is formed by the hydrolysis of sphingomyelin by sphingomyelinase (SMase) to yield ceramide and a water-soluble phosphocholine (Hannun, 1994; Jarvis et al, 1996) or alternatively by de novo synthesis of ceramide. *De novo* biosynthesis is essential because dietary sphingolipids are mostly catabolized; control of the *de novo* pathway is also critical because most of its intermediates are highly bioactive.

De novo synthesis of ceramide is initiated on the surface of the cytosolic leaflet of the endoplasmic reticulum by serine palmitoyltransferase (Weiss and Stoffel, 1997), in a reaction forming ketosphinganine from L-serine and fatty acyl-CoA (see Fig.1.6), which combine to produce 3-oxosphinganine. The enzyme involved is serine palmitoyltransferase (SPT). The regulation of SPT is not clearly understood, but the reaction requires adequate availability of both serine and palmitoyl-CoA. SPT is inhibited by a number of agents, and several selective inhibitors have emerged,

including sphingofungins, lipoxamycins and ISP1/myriocin (Miyake et al, 1994; Schmelz et al, 1998).

Three reactions follow 3-oxosphinganine to produce ceramide: additionally ceramide can be synthesized by the reverse reaction of ceramidase (CDase, sphingomyelinase) (Bawab et al, 2001). Within those reactions, dehydroceramide synthesis is the target for many inhibitors (Vesper et al, 1999), such as Fumonisin B 1 (Fb1). The last step of ceramide synthesis: desaturases conversion of dehydroceramide to ceramide is an important reaction because ceramide is active in inducing apoptosis (Hannun and Obeid, 2002), so excess ceramide is usually contrary to healthy cell survival. A cyclopropene analogue of ceramide potently inhibits the desaturase (Triola et al, 2001).

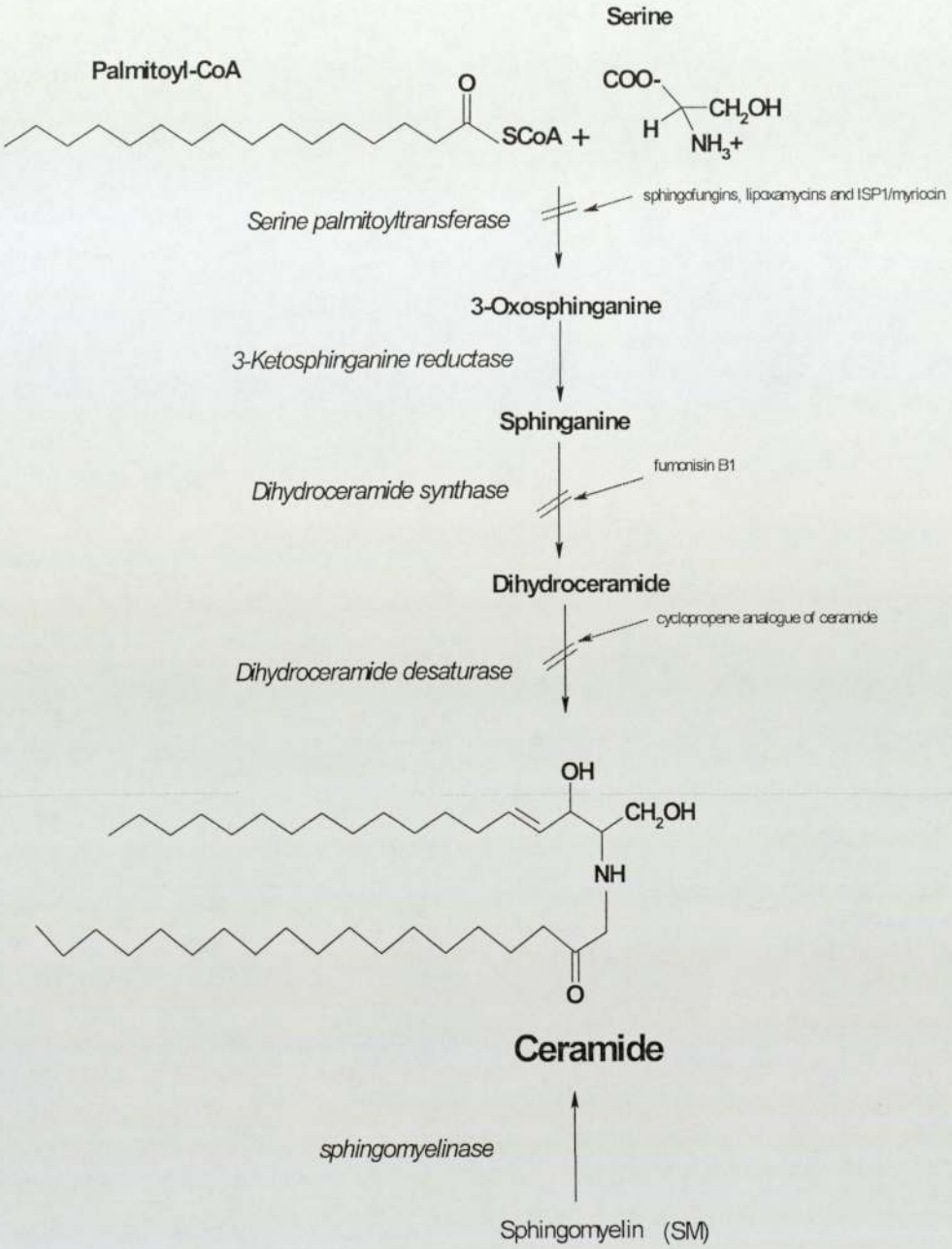


Fig.1.6 Biosynthesis pathway of ceramide showing sites of physiological inhibitors

1.7.3 Degradation of ceramide

Ceramide serves as the precursor for major sphingolipids such as sphingomyelin (SM) or glucosylceramide (see Fig.1.7). The breakdown of complex sphingolipids results in

the formation of ceramide through the action of either sphingomyelinases (SMases) or glycosidases. The breakdown of ceramide proceeds through the action of ceramidases (CDases) and glucosylceramide synthase (GCS). The resulting sphingolipids serve as substrates for sphingosine kinase to form sphingosine 1-phosphate phosphatase (S1P) or they are recycled into ceramide and complex sphingolipids through the action of ceramide synthases.

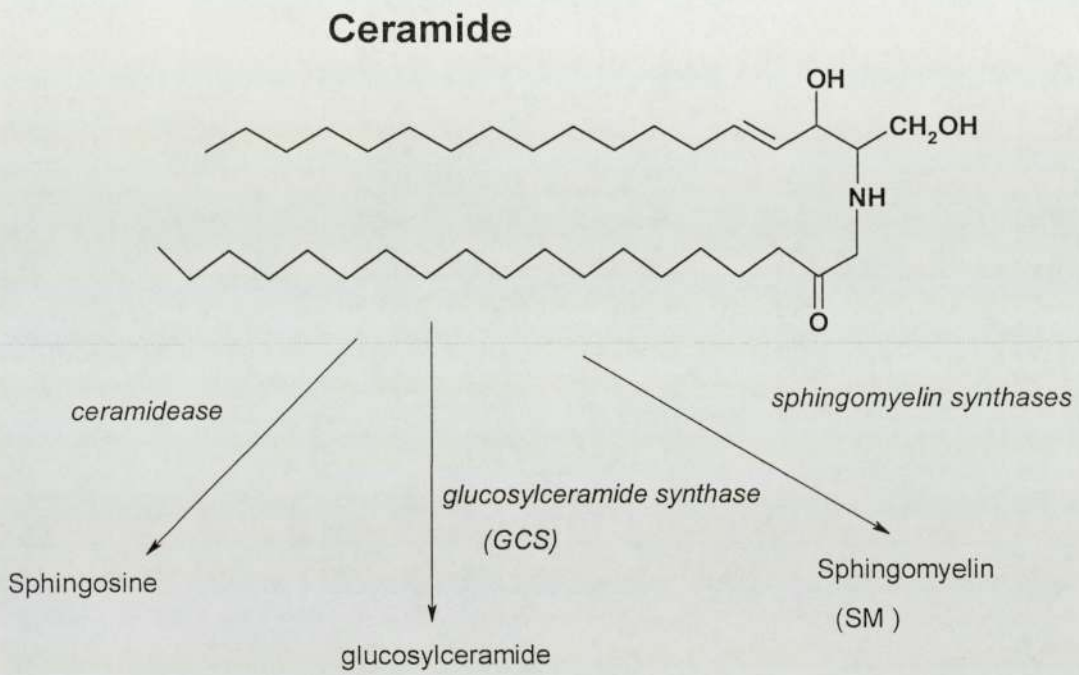


Fig 1.7 Pathways of ceramide metabolism

The inhibitors of GCS and of CDases have been shown to cause an increase in ceramide levels; however, they cause apoptosis and also arrest the cell cycle. There is a similar effect of the overexpression of SMases (Hannun et al, 2001).

1.7.4 Function of Ceramide

Functionally, ceramide has been proposed as a "coordinator" of stress responses (Hannun, 1996), as it has been found that many inducers (e.g.TNF-α, heat, UV radiation) of a stress response result in ceramide accumulation, usually as a result of activation of SMases (Andrieu-Abadie et al, 2001) or the *de novo* synthesis pathway. However, sometimes ceramide accumulates as a result of inhibition of ceramide breakdown (through SM synthase or CDases).

Nevertheless, there is substantial evidence on the role of ceramide in mediating key cellular activities. For instance, fumonisin B1 has been shown to inhibit various aspects of apoptosis, and the overexpression of CDase have been shown to attenuate the induced level of ceramide which occurs in response to TNF-α (Strelow et al, 2000). Thus, a role of ceramide has been indicated in regulating apoptosis.

1.7.5 Pathologies of Ceramide

Numerous studies have identified direct targets for ceramide action. These include the ceramide-activated proteins PP1 and PP2A, which have been proposed to mediate many actions of ceramide. For example, inhibitors of these two proteins have been shown to inhibit the ability of ceramide to cause dephosphorylation of several cellular proteins including PKC, Akt, and Bcl-2 (Chalfant et al, 2002). Cathepsin D was discovered as a ceramide-binding protein.

There are many diseases in which disturbances in ceramide metabolism have been noted, such as neuropathies, and cancer (Hannun and Obeid, 2002). Most recently several studies have begun to implicate that ceramide and defects of sphingolipid metabolism in the pathogenesis of diabetes and its complications (Summers et al, 1998).

1.7.6 Ceramide and insulin resistance

The concentration of cellular ceramide is elevated in muscle of insulin resistant animals (Turinsky et al, 1990; Adams, 2004). Cellular ceramide levels are also raised to a similar extent in response to various cell stresses and injury such as heat shock, UV radiation, and exposure to oxidants as mentioned in the preceding section (Basu and Kolesnick, 1998). Moreover, starvation and prolonged exercise can also induce ceramide production. One reason for this is the metabolic coupling of ceramide with other bioactive lipids such as ceramide-1-phosphate, sphingosine and sphingosine-1-phosphate, which may trigger ceramide metabolism and signalling (see 1.7.4).

Although insulin resistance appears to underlie the pathogenesis of a wide range of metabolic abnormalities related to the "metabolic syndrome" (section 1.4), a general role of ceramide dysfunction in these abnormalities has not previously been considered. Recently, after this research programme had begun, a review article drew

attention to the possibility that ceramide could participate widely in the development of insulin resistance and associated vascular complications in diabetes and states of excess glucocorticoids (Summers and Nelson, 2005). The arguments advanced within that review are similar in many respects to those described in this thesis, although the present thesis has been developed and investigated in different ways to the ideas presented by Summers and Nelson. This research programme has focused mainly on the insulin signalling steps routing through Akt, and has given less attention to glucocorticoid signalling activity. The relative importance of the present approach compared with that offered in the recent review by Summers and Nelson (Summers and Nelson, 2005) will be considered in detail in the general discussion in chapter 7.

It has been demonstrated in cultured adipocytes that ceramide inhibits Akt activation and GLUT-4 translocation (Peraldi et al, 1996). Ceramide may also inactivate certain members of the protein kinase C family (Lee et al, 1996). Recently, several reports have supported this notion by demonstrating negative regulation of ceramide on the PI-3 kinase signalling pathway. First it was reported that ceramide specifically inhibits PI-3 kinase, which in turn results in the loss of Akt activation (Zundel and Giaccia, 1998). Other reports demonstrated negative effects of ceramide on Akt activation (Zhou et al, 1998), independently of PI-3 kinase inhibition.

Activation of Akt depends on its phosphorylation at each of two key amino acid

residues, Thr^{308} and Ser^{473} (Alessi et al, 1996). The N-terminal domain of Akt contains a region with pleckstrin homology (PH), which is thought to be critical in allowing the kinase to interact with 3-phosphoinositides (Hemmings, 1997). The activation of Akt is preceded by its recruitment to the plasma membrane (Andjelkovin et al, 1997) and by binding of PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 to the PH domain of Akt. This lipid-binding also serves to induce conformational changes in Akt that allow phosphorylation of its two regulatory sites.

As noted in section 1.7.4, recent studies have implicated ceramide as a substance that prevents the membrane localization of the PH domains for Akt and the general receptor for phosphoinositides-1 (Grp1), which bind PI(3,4,5)P_3. The accumulation of 3'-phosphoinositides was not altered by ceramide, indicating that this inhibition occurs via a step that is unrelated to 3'-phosphoinositide generation. In addition, the results indicated that cells lacking the PI (3,4,5)P_3 phosphatase PTEN retained sensitivity to ceramide. These studies are, however, difficult to interpret since there is evidence that PTEN may have the additional possibility of independent effects on insulin signalling downstream of PI-3 kinase pathways. Thus, ceramide appears to regulate Akt activity not necessarily through PI-3 kinase modulation, but rather by accelerating the dephosphorylation of Akt selectively at Ser^{473}. Serine ^{473} phosphorylation was reduced by treatment with ceramide, whereas Thr^{308} phosphorylation remained unaffected. In further experiments, ceramide appeared to accelerate Ser^{473} dephosphorylation,

suggesting the activation of phosphatases. This suggests that Akt activity may be regulated by ceramide. In addition to activating phosphatases, ceramide possibly also activates atypical PKC ζ (Mathias et al, 1998), but the potential effects of this on insulin action are unresolved.

The interaction between certain PKC isoforms and Akt requires the PH domain of Akt: PKC ζ phosphorylates the PH domain of Akt (Powell et al, 2003), which reduces the ability of the Akt-PH domain to bind 3-phosphoinositides. This enhances the loss in kinase recruitment and activation at the plasma membrane (Powell et al, 2003). These findings further implicate ceramide as potentially important intermediate linking saturated fats to the development of insulin resistance.

1.8 Aims of present study

The principal aim of this thesis is to investigate the hypothesis that excess ceramide acts to disturb insulin action and is an underlying feature of insulin resistance. It is proposed to investigate this hypothesis in isolated cells, particularly muscle cells and endothelial cells, to determine the effects of ceramide on insulin-mediated glucose transport and insulin-mediated NO production. The main approach will be to manipulate the synthesis of ceramide to determine whether endogenously produced ceramide is both sufficient and responsible for the inhibition of insulin signalling.

While clinical studies, animal models and cell models play a pivotal role for investigating insulin signalling pathways, this study has been restricted to isolated cells for reasons of time availability and ethical constraints. To undertake these studies, experiments will be conducted using two cell models of insulin action: glucose uptake by L6 rat muscle cells and NO production by (human) EAHY endothelial cells.

Muscle has been selected because it is the major metabolic tissue of the body for glucose metabolism, and it is also an important tissue that depends on insulin signalling for its energy supply. Thus the first part of this study will be to characterize insulin action on L6 muscle cells at Aston University, and then to use these cells to investigate whether ceramide has an effect on insulin signalling and glucose transport.

Endothelial cells (ECs) are the interface between circulating blood and vascular smooth muscle cells (VSMC). However the EC is not a simple barrier, as it has a critical role in overall vascular homeostasis. For the EC, NO is a key mediator, thus it is proposed to use ECs to test the effect of ceramide on insulin-stimulated NO production.

Appropriate use of inhibitors to block selected steps in the pathways of insulin signalling and ceramide production and degradation in the muscle and endothelial cultured cells will provide the means to test the hypothesis that excess ceramide

disturbs insulin action leading to insulin resistance.

Chapter 2:

Materials and Methods

2.1 Suppliers of materials

All the chemicals used were purchased from BDH Ltd, Poole, UK, Fisons, Leicester, UK and Sigma Chemical Company, Poole, Dorset, UK unless otherwise stated. Radionuclide chemicals were supplied by Amersham International plc., Buckinghamshire, UK.

The medium and supplements for cell culture were obtained from GibcoBRL/Life Technologies, Paisley, Scotland. The rat L6 skeletal muscle cell line was purchased from the European Culture Collection Porton Down; endothelial EAHY human cell line was purchased from ATCC (American Type Culture Collection). Ceramide and dehydroceramide were supplied by BIOMOL International Research Laboratories, Exeter, UK. Composition of solutions reported in this chapter are given in appendix 1.

2.2 Cell culture

2.2.1 Cell culture medium and supplements

a. L6 muscle cell

DMEM (Dulbecco's Modification of Eagle's Medium) was obtained as 500ml sterile 1x solutions containing 0.11g/l sodium pyruvate. DMEM was supplemented with the following additions; antibiotic/antimycotic solution containing: 100units/ml penicillin G sodium, 10μg/ml streptomycin sulphate, 25μg/ml amphotericin B (as fungizone in

0.85% saline), L-glutamate (1mM), and Foetal Calf Serum (FCS) 25 ml (5%).

The supplements were sterilised by filtration (0.2μm, SASTEDT, Leicester, UK) and added to 500ml basic medium. Supplemented medium was stored at 5°C for a maximum of four weeks. FCS was stored in 25ml aliquots at -20°C. Glutamate was dissolved as a 200mM stock solution and stored in 5ml aliquots at -20°C. Antibiotic solution was stored as 5ml aliquots at –20°C.

b. EAHY endothelial cell

DMEM was obtained as described above (2.2.1a) containing 0.11g/l sodium pyruvate and was supplemented with 100 units/ml penicillin G sodium, 10mg/ml streptomycin sulphate, 25μg/ml amphotericin B (as fungizone in 0.85% saline) and Hypoxanthine-Aminopterin-Thymidine (H.A.T.) (10 ml). This medium was further supplemented with FCS 50 ml (10%).

The supplements were sterilised by filtration as above and stored at 5°C for a maximum of four weeks. FCS needed to be heat inactivated by treatment at 60°C for 30 min before storing in 50ml aliquots at -20°C. H.A.T. was prepared as a 200mM stock solution and stored in 10ml aliquots at -20°C. Antibiotic solution was stored as 5ml aliquots at -20°C as above.

2.2.2 Maintenance and propagation of cell lines

a. L6 muscle cell

The L6 cell line was routinely cultured in $75cm^2$ flasks with 20ml DMEM supplemented with 5% (v/v) FCS and (1mM) glutamate. Cells were maintained as a monolayer in a humidified atmosphere of 5% carbon dioxide, 95% air at 37°C. Cells were passaged when approximately 80% confluent to prevent contact inhibition.

DMEM medium was replaced with 5ml trypsin solution (trypsin-EDTA, containing 0.5g porcine trypsin, and 0.2g EDTA in Hanks' balanced salt solution (HBSS) from Sigma), shaken and incubated at 37°C for up to 10 min, until all cells were detached. Then 5 ml fresh medium was added to the cell suspension to terminate the effect of trypsin. The trypsin β cell suspension then was centrifuged at 800 rpm (MSE Mistral 2000, Fisher Scientific Loughborough, UK) for another 5 min to pellet the cells. Cells were disaggregated and resuspended in 10 ml fresh medium of which 1 ml was transferred to a fresh flask with 19 ml pre-warmed fresh medium. The remaining cell suspension was diluted and used for experiments. It took approximately 3 days for the cells to reach confluence.

Cells were used at passage 15-38. At p38 and higher passages, a significant decline in insulin-stimulated glucose uptake and a lower speed of growth were observed.

b. EAHY endothelial cell

The EAHY cell line was routinely cultured in $75cm^2$ flasks with 30ml DMEM supplemented with 10% (v/v) FCS and 10ml H.A.T. Cells were maintained as a monolayer in a humidified atmosphere of 5% carbon dioxide, 95% air at 37°C. Cells were passaged when approximately 80% confluent to prevent contact inhibition.

To passage cells the DMEM medium was removed and cells were washed with 5ml of PBS-EDTA (EDTA 2.5 g/l in Ca^{2+} and $Mg^{2}+$ free PBS). Then PBS was replaced with 5ml trypsin solution (trypsin-EDTA, containing 0.5g porcine trypsin, and 0.2g EDTA in HBSS), and incubated at 37°C for up to 5 min, until all cells were detached. Then 5 ml fresh medium was added to the cell suspension to terminate the effect of trypsin. The trypsin-cell suspension then was centrifuged at 1200 rpm (MSE Mistral 2000, Fisher Scientific Loughborough, UK) for another 5 min to pellet the cells. Cells were disaggregated and re-suspended in 10 ml fresh medium of which 1 ml was transferred to a fresh flask with 29 ml pre-warmed fresh medium. The remaining cell suspension was diluted and used for experiments. It took approximately 4 days for the cells to reach confluence.

Cells were used at passage 15-40. At p40 and higher passages, a significant reduction in the rate of growth was observed.

2.2.3 Cell seeding for experiment

a. L6 muscle cell

After the process of trypsinisation, the cell concentration was adjusted after a cell count using a haemocytometer. The concentration was 12.5 times greater than required (typically 12.5×10^6 cells per well). Then 4 ml of cell suspension was added to 46 ml of medium. 1 ml cells were seeded in 24 well plates (16mm well containing 10^6 cells in 1 ml medium) for 2 DG-uptake experiments. Cells were grown to confluence and serum starved with 0.5% FCS supplemented DMEM 24 h to decrease basal glucose uptake and it also induces differentiation and fusion of myoblasts into myotubes to prevent further division.

b. EAHY endothelial cell

Cells were seeded in 96 well plates (4mm well containing 10^5 cells in 100µl medium) for nitric oxide determination experiments. Cells were grown to confluence and serum starved with 2% FCS supplemented DMEM for 4 h to decrease basal NO production.

2.2.4 Growth curves

The rate of cell growth was assessed by counting the number of cells at daily intervals using a haemocytometer. An example of a typical growth curve for L6 muscle cells and EAHY endothelial cells is shown in Fig 2.1a &b.

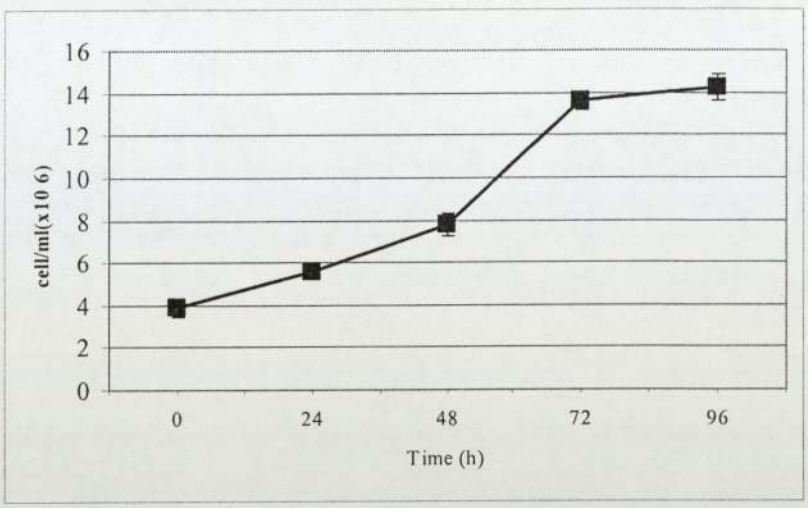


Fig 2.1.a L6 muscle cell growth and fusion. Cultured L6 cells were trypsinised daily and growth was determined by counting the number using haemocytometer. At 72h, approximately 90% of the cells had fused to form myotubes.

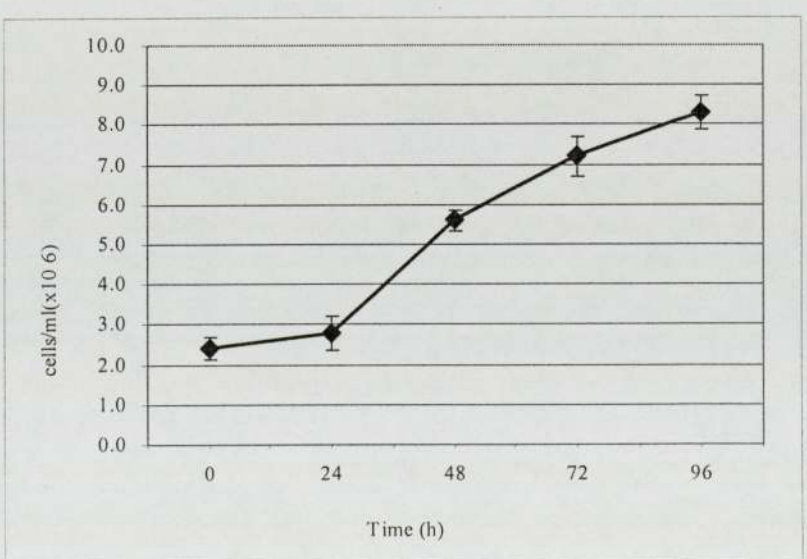


Fig 2.1.b This shows the growth rate of EAHY endothelial cells. After 96h, the cells had become approximately 90% confluent.

2.3 Analyses

2.3.1 2-Deoxy-[^{3}H]-glucose uptake into cells

Glucose uptake was assessed using the tritiated non-metabolised glucose analogue 2-deoxy-D-glucose (2 DG) which is taken up by the cells in an identical manner to glucose, phosphorylated but not metabolised further (Klip et al, 1986).

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

The principle of using the non-metabolised analogue 2 DG to estimate glucose uptake is explained in the section 3.3.1. The uptake experiment was carried out at room temperature (22°C) rather than at body temperature (37°C) to slow down the rate of uptake. This allows more accuracy for the washing steps to terminate the experiment.

Also, the lower temperature results in less 2 DG being taken up by the cells (than if carried out at 37°C), so this ensures that the cells are not saturated with the accumulated 2 DG. Preliminary studies at 22°C have shown that 2 DG uptake is linear over 10 min, even with a maximally stimulating (10^{-6}M) concentration of insulin (Bates et al, 1999).

2.3.2 Nitric oxide determination (Griess reagent)

An indirect way of measuring NO is by the Griess reagent which determines the amount of nitrite and/or nitrate, because the total amount of NO is determined as the change in the sum of nitrite and nitrate.

The Griess reagent was purchased from Sigma including N-(1-naphthyl) ethylenediamine dihydrochloride, sulfanilic acid in 5% H_3PO_4, and Nitrate reductase. The method provides a simple and accurate characterized colorimetric assay for nitrite, and nitrate has been reduced to nitrite by nitrate reductase (Dawson and Dawson, 1995). Nitrite reacts with the Griess reagent to form a purple Azo derivative that can be monitored by absorbance at 590 nm. The related reactions are shown in Fig 2.2 a, b, c. The Griess reaction has been characterized extensively in many previous studies (Promega Technical Bulletin, 2005) and is widely used to measure NO release into culture medium. The present assay has a detection limit of sensitivity for sodium nitrite that is down to about 2.5 μM, and sodium nitrite solution was used as standard

for the present assay. The assay is also linear up to above 100 μM.

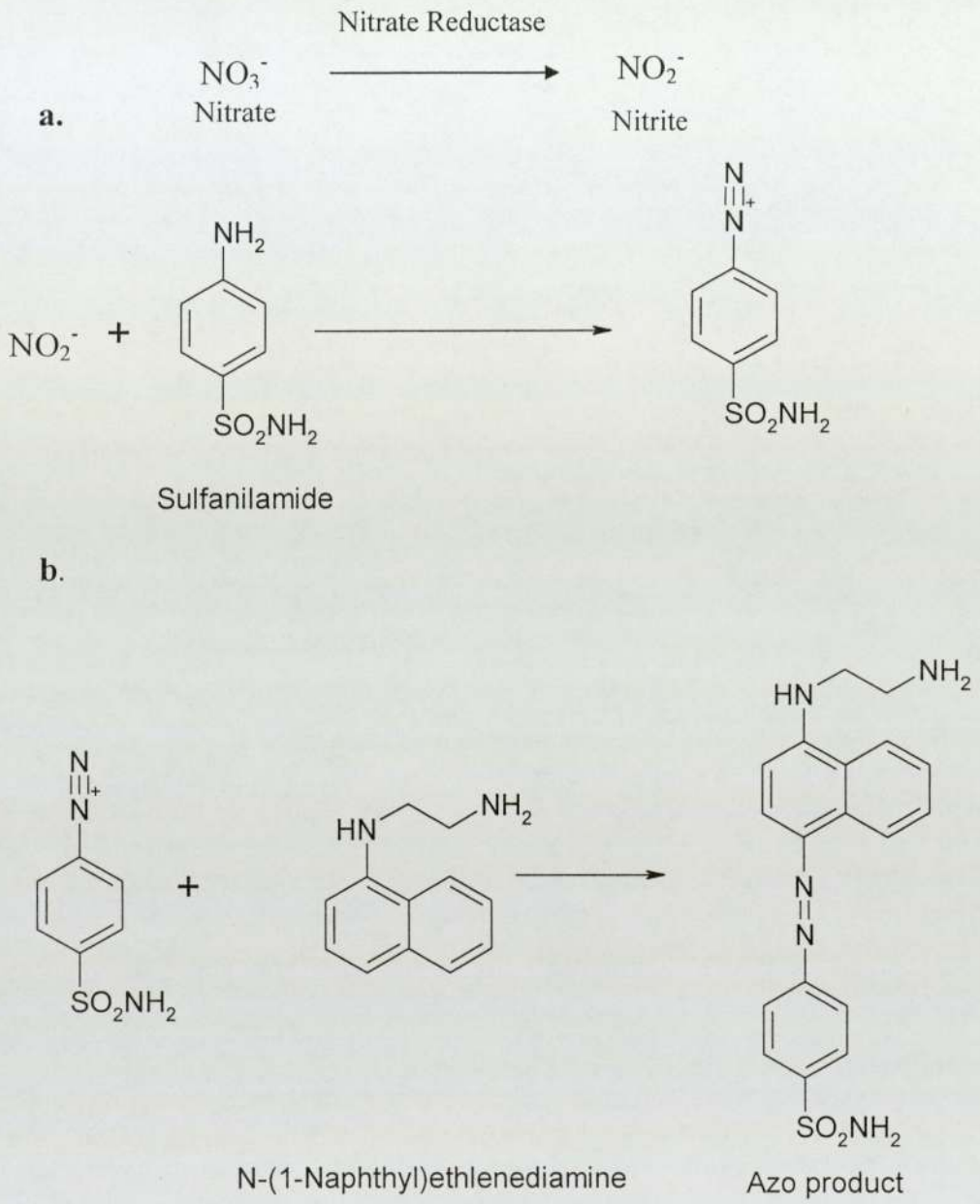


Fig2.2 a, b and c This shows the enzymatic and chemical reactions involved in the formation of a coloured Azo product to measure nitrate and nitrite using the Griess reaction.

Cells were seeded in 96 well plates and grown to confluence, then incubated with additional 50µl/well Griess Reagent. (This procedure must be protected from light, so plates were covered in aluminium foil). For this study the effect time period of insulin action on endothelial cells is short, usually requiring ≤5 min incubation. After 30 min, the colour of the solution had faded and the absorbance was measured by plate reader at 590 nm. Preliminary time course studies and temperature studies suggested that the procedure gave optimal insulin stimulation of NO release from EAHY cells at about 2-3 min at 22°C. This is considered further in chapter 4.

2.3.3 Cell viability assessment

MTT assay

The MTT assay can be used as a measure of cell viability. It is based on the activity of mitochondrial dehydrogenase enzymes to break the tetrazolium ring of a pale yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). This results in formation of dark blue-purple formazan product which quickly crystalizes and mostly unable to leave the cells, causing the cells to accumulate the blue-purple crystals. The crystals can be released and dissolved when the cells are solubilised with DMSO and the colouration can be quantified spectrophotometrically.

The number of healthy, 'viable' cells is directly proportional to the account of blue-purple formazan product (Mosmann, 1983). When metabolic disturbance or

other adverse events disrupt mitochondrial function and/or lead to apoptosis there is little or no blue-purple formazan formation.

For the further present studies, the MTT tetrazolium dye was obtained from Sigma and dissolved into a stock 50mg in 10 ml PBS solution. Then the solution was filtered once to make the final MTT solution and stored in the dark at 4°C. For experiments, the stock was diluted 10 times in PBS.

Cells were seeded in 96 well plates (typically 2000 cells/well), grown to confluence then the medium was removed and cells were incubated with 150µl diluted MTT assay per well for 90 min at 37°C. After that the solution was replaced with 150µl DMSO per well to solubilise the cells and formazan, and incubated for another 90 min. Absorbance was detected by plate reader using a filter at 590 nm.

Trypan Blue assay

Trypan blue is now widely accepted as a reliable way to count cell numbers as well as to examine cells viability. It is based on the principle that live cells do not take up trypan blue, whereas dead cells do. This is due to loss of ability to exclude the dye when membrane competence is lost as the cell becomes moribund and dies. Thus dead cells can be stained blue which can be observed under the microscope (Freshnay, 1987).

Cells were grown to confluence in 24 well plates and incubated with test compounds as determined by experimental design. 150µl 0.4% (w/v) trypan blue solution and 150µl PBS was added to the cell monolayer for 5 min. The staining solution was removed by washing with PBS and the monolayer was photographed using a Nikon F301 microscope at x10 magnification. Although this method only provides a semi-quantitative assessment of cell viability, it usefully demonstrates the effect of compounds used on cell survival.

2.4 Statistical Analyses

Data were expressed as mean ± SEM or ± SD, 'n' indicated the number of the experiments. All statistical analyses were performed using the statistical package available on Aston University internet which is a version of 'Graphpad'. Differences were considered to be significant for $p<0.05$ (Dr P.J. Hanson, undergraduate course manual in statistics for Human Biology).

2.4.1 Analysis of Variance

A one way ANOVA test (analysis of variance) was used to test whether there was a significant difference among multiple groups. This one way test relies on the whole groups defined as a single factor.

2.4.2 Student's 't' test

Student's 't' test was used to compare 'paired' or 'unpaired' data (with the appropriate version of the test) to determine whether there was a significant difference between them.

2.4.3 The Bonferroni adjustment

Bonferroni adjustment was used in multiple comparison procedures to calculate an adjusted probability (0.05) to avoid a type 1 statistical error. This calculation would guarantee an appropriate α value. A given α value maybe appropriate for each individual comparison (not for the set of all comparisons). In order to avoid a host of extra positive results, the α value needs to be lowered to account for the number of comparisons being performed. Groups of data were therefore compared using Student's t-test with a Bonferroni correction.

Chapter 3:

Characterisation of rat L6 skeletal muscle cell line

3.1 Introduction

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

The rat L6 cell line has been used as a model for the study of glucose uptake into skeletal muscle (Klip et al, 1984) and to assess insulin-mimetic properties of pharmacological agents (Bailey et al, 2004). In summary, the L6 cell line is used as a model of skeletal muscle.

3.2 L6 Cell growth and fusion

L6 cells were cultured as described in 2.2.2a, and were allowed to settle down to form a monolayer overnight. When cells were nearly confluent, they were differentiated into myotubes as described in section 2.2.3. Cell number was counted at interval of 24 hours up to 72 hours. Then cells were removed with trypsin-EDTA as used for passaging (section 2.2.2) and counted with haemocytometer with adjustment for the dilution factors. A typical growth curve is shown in Fig 2.1a. After 24 hours, the

monolayer was photographed using a Nikon F301 microscope (figure 3.1).

Fig 3.1 This picture shows differentiated L6 cells grown to near confluence for 72 hours and then differentiated in 0.5% FCS for another 24 hours.

3.3 Insulin-stimulated 2-Deoxy-[^{3}H]-Glucose Uptake in L6 cells

The general procedure for this assay is given in section 2.3.1

3.3.1 2 DG uptake

2 DG uptake has been used extensively in assessing cellular or whole tissue insulin resistance and is used here to determine glucose uptake into cultured muscle cells (Bailey et al, 2001).

2 DG is a glucose analogue which is taken up into cells by glucose transporters. The 2 DG is then phosphorylated by hexokinase to form glucose 6-phosphate. In the glycolytic pathway there is the isomerisation of glucose 6-phosphate to fructose 6-phosphate by phosphoglucoisomerase. This reaction requires a C-2 group and C-5 hydroxyl group. 2 DG however lacks the keto C-2 group thereby preventing the isomerisation reaction. The 2 DG method therefore relies on detection using the ^{3}H radionuclide-labelled 2 DG which enables detection of the internal glucose concentration. Preparations to ensure linearity, accuracy and reproducibility of the technique have been considered in section 2.3.1.

3.3.2 Experimental design

To establish the normal function of L6 muscle cells, experiments were undertaken to measure glucose uptake (2 DG uptake) in response to insulin.

Bovine insulin (Sigma) was dissolved in PBS (20µl HCl may be required in 10 ml buffer) to make 10^{-4} M insulin stock. Low serum medium was used to dilute to a required concentration. L6 cells were grown to confluence in 24 well plates and serum starved for 24 hours. Cells were then incubated with insulin (10^{-8}M-10^{-6}M) for 30 min, 4 hours and 24 hours. Experiments were undertaken in multiples of six wells. Glucose uptake was measured using the 2 DG-uptake method as described in Chapter 2. Uptake of 2 DG was expressed as the percentage change from control (100%). Data

are presented as mean ±SEM and compared using Student's 't' test. Probability values of $p<0.05$ were considered to be significant.

3.3.3 Results

The absolute account of 2 DG uptake under basal conditions was 29-44 pmol/10^5 cell/10 min. When the effects of different agents are studied, the basal value (control) is shown as 100%, and the effects of the agents are shown as % of control.

Insulin increases 2 DG uptake both in a concentration dependent manner and time dependent manner by L6 cells (Fig 3.2).

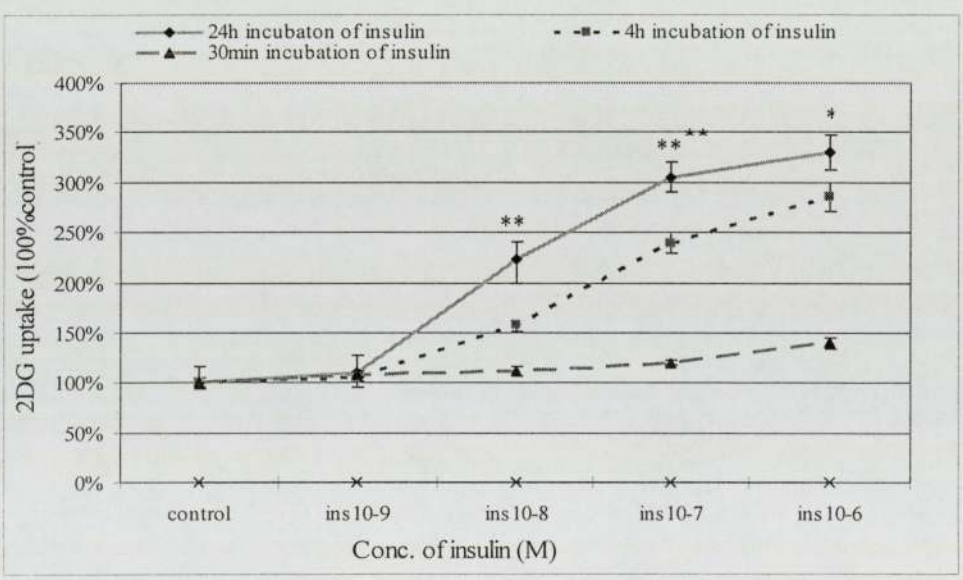


Fig 3.2 This graph demonstrates the concentration and time dependent curve for insulin-stimulated glucose uptake by L6 cells. 2 DG values for 30 min at 10^{-7} and 10^{-8} M insulin are significantly different from same insulin concentration at 4 hours and 24 hours incubation ($p<0.01$). *$p<0.05$, **$p<0.01$ versus 4 hours. Values are mean± SEM, n=6.

Treatment with the same concentration of insulin for different time periods produced qualitatively very similar results. However the shorter time period (30 min) of exposure to insulin was associated with a slightly lower mean maximum glucose uptake rate (per 10 min) than the longer time periods (4h and 24h). This was an increase above basal of 40% at 30 min, 180% at 4 hours and 230% at 24 hours. These incremental values at 10^{-6} M insulin were statistically significantly different between 30 min, 4 hours and 24 hours (*$p<0.05$). However at the shorter time period the concentration-response curve was slightly displayed to the right as indicated by a higher EC50 of 5.24×10^{-8}M at 4hours compared with 2.16×10^{-8}M at 24 hours. This difference was statistically significant (**$p<0.01$). Moreover at insulin concentrations that sub-maximally stimulate glucose uptake, there was a significantly greater uptake of glucose after exposure to insulin at 24 hours than 4h (glucose uptake values at 10^{-8} and 10^{-7} M insulin were greater at 24hours than 4hours, $p<0.01$ at each concentration). Thus the ability of insulin to increase glucose uptake in a concentration-dependent and time-dependent manner has been confirmed.

3.4 Effect of C_2 ceramide on basal and insulin-stimulated 2-Deoxy-[^{3}H]-glucose uptake in L6 cells

The typical amount of ceramide within the cell has been established in primary rat cerebella granule cells at about 0.22nmol/10^6 cells (Prinetti et al, 2000). In the present study we are initially investigating the effect of ceramide at pharmacological

concentrations (1-150μM).

3.4.1 Experimental design

C_2 Ceramide (Biomol) was dissolved in dimethyl sulphoxide (DMSO) at 20mM, stored at -20°C in the dark, and diluted with culture medium to a concentration of 400μM immediately before use. The preparation procedure of cells was similar to that described previously. The concentration of C_2 ceramide was initially chosen to be 100μM, and we choose 2 hours as the normal experimental time period because C_2 ceramide is able to enter cells within this time, but it is not significantly degraded within this time. Additional experiments was undertaken at 4 hours and 1 hour and with a range of ceramide concentrations, however, the results show significant activity of the ceramide at each of these times and at ceramide concentrations of 10μM and alone.

3.4.2 Results

Exposure to ceramide (100μM) for 2 hours reduced insulin-stimulated glucose uptake throughout the range of insulin concentrations tested (10^{-8}-10^{-6} M). The reduction was by about 50% at 10^{-8} M to 80% at 10^{-6} M insulin, and the EC50 was 5.24×10^{-8} M compared with an EC50 of 2.16×10^{-8} M for the effect of insulin only (see fig 3.3).

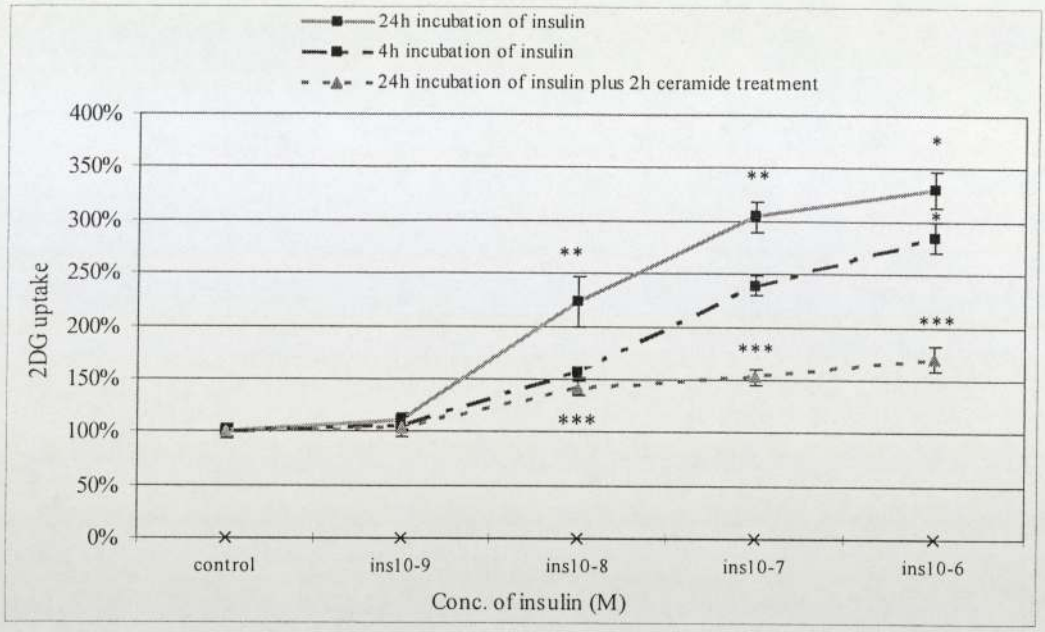


Fig 3.3 Effect of insulin (10^{-8}-10^{-6}M) for 24 hours and 4 hours on glucose uptake in muscle L6 cells. The initial effect of insulin plus C_2 ceramide on uptake of 2 DG is also shown. Values for control (no insulin and no ceramide) are expressed as 100%, and values after exposure to insulin and ceramide (100μM) are expressed relative to the control value. Values are expressed as mean ± SEM, n=6. *p<0.05, **p<0.01 versus 4 hours insulin and 24 hours insulin. Insulin plus ceramide significantly (***p<0.01) reduced 2 DG uptake compared with 24 hours insulin.

Obviously it is evident from Fig 3.3 that exposure to ceramide (100μM) for 2 hours reduced insulin-stimulated glucose uptake at each of the insulin concentrations tested at 10^{-8}M and above. All of these differences were statistically significant (***p<0.01).

In the following experiments of this series, we changed the concentration of C_2 ceramide to investigate a value from 1μM to 150μM as well as the treatment period of both ceramide and insulin. The reasons for this change were to determine whether lower concentrations of ceramide could affect basal and insulin-stimulated glucose

uptake, and whether different periods of exposure to ceramide would influence these effects.

3.5 Effect of ceramide on basal and insulin-stimulated glucose uptake: effect of time and concentrations

3.5.1 Experimental design

L6 muscle cells were grown as described previously and 10^5 cells in 1 ml were seeded in 24-well plates and grown to confluence in the above medium and same atmosphere as previously. Then the medium was changed to DMEM containing 0.5% FCS for another 24 hours to induce differentiation and fusion of myoblasts into myotubes and to prevent further division. Myotubes were then incubated with C_2 ceramide, then addition of insulin (10^{-6} M) in the last 30 min. The concentration range of C_2 ceramide is from 1μM to 150μM, and the time periods are 1, 2, 4 hours. The concentration and treatment time of insulin is 10^{-6}M and 30 min respectively. Basal glucose uptake was similar to previous studies (about 30-45 pmoles glucose/10^5cells/10 min). Results are shown below (Fig 3.4).

3.5.2 Results

Under the same conditions, when we changed the treatment period between 1, 2, and 4 hours, with or without 30 min exposure to 10^{-6}M insulin, we found that ceramide alone (10μM) increased 2 DG uptake by 15-18% between 2 to 4 hours exposure (see

Fig 3.4), while 1 hour seems too short for ceramide to induce this effect. Insulin alone (10^{-6}M) produced a typical increase in 2 DG uptake.

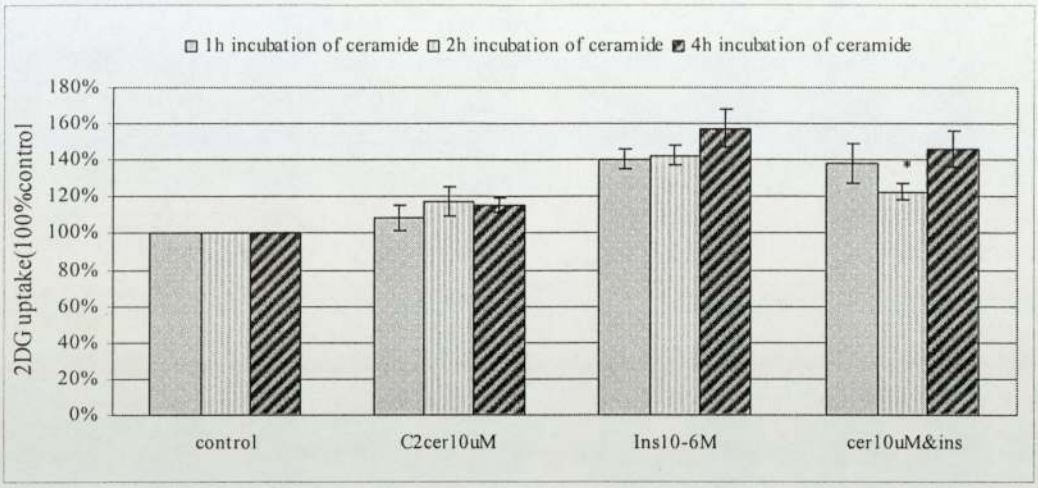


Fig 3.4 Effects of insulin (Ins 10^{-6} M), ceramide (cer 10μM) on glucose uptake in L6 muscle cells. Cells were pre-treated with ceramide for three periods; for 1 hour (...), 2 hours (11), 4 hours (//). Values for control (no insulin and no ceramide) are expressed as 100%, and values after exposure to insulin and ceramide are expressed relative to the control value. Values are expressed as mean ± SEM, n=6. All values for ceramide, insulin and ceramide plus insulin were significantly raised above control ($p<0.05$). Ceramide significantly reduced insulin-stimulated 2 DG uptake at 2 hours incubation versus insulin alone (*$p<0.05$).

Fig 3.4 Indicates that ceramide reduced mean values for the insulin-stimulated 2 DG uptake at 2 hours and 4 hours but this was not statistically significant at 4 hours, but it was just significant (*$p<0.05$) at 2 hours and 10μM concentration of C_2ceramide. It is noted that the effective period of ceramide exposure is not very long, and it is suggested that ceramide will exert its effect within 2 hours, and this effect may have declined slightly by 4 hours.

Since the effect of ceramide on glucose uptake changed between 10 and 100μM, we

treated L6 cells as described previously with intermediate concentrations of ceramide. But this time we lowered the minimum concentration to 1μM and raised the maximum to 150μM. We pre-treated with C_2 ceramide from 1μM to 150μM respectively for 2h, with addition of insulin (10^{-6} M) in the last 30 min. Basal glucose uptake was similar to previous studies (about 25-35 pmoles glucose/10^5cells/10 min) (see fig 3.5& 3.6).

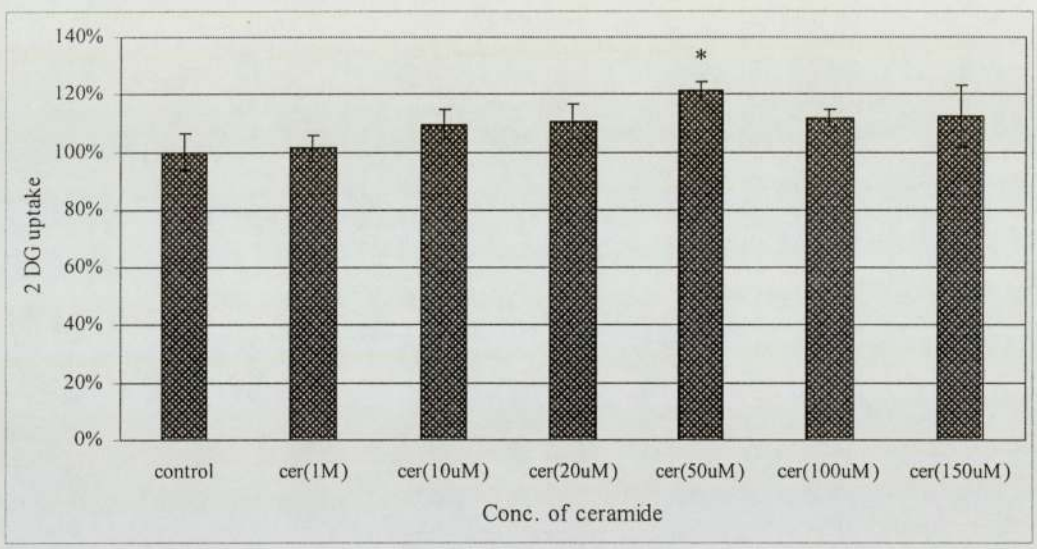


Fig 3.5 Effect of different concentrations of ceramide for 2 hours on basal glucose uptake by L6 muscle cell. Ceramide as indicated as 'cer' in the figure. *$p<0.05$ versus control. Values for control (no insulin and no ceramide) are expressed as 100%, and values after exposure to different concentrations of ceramide are expressed relative to the control value. Values are expressed as mean ± SEM, n=6.

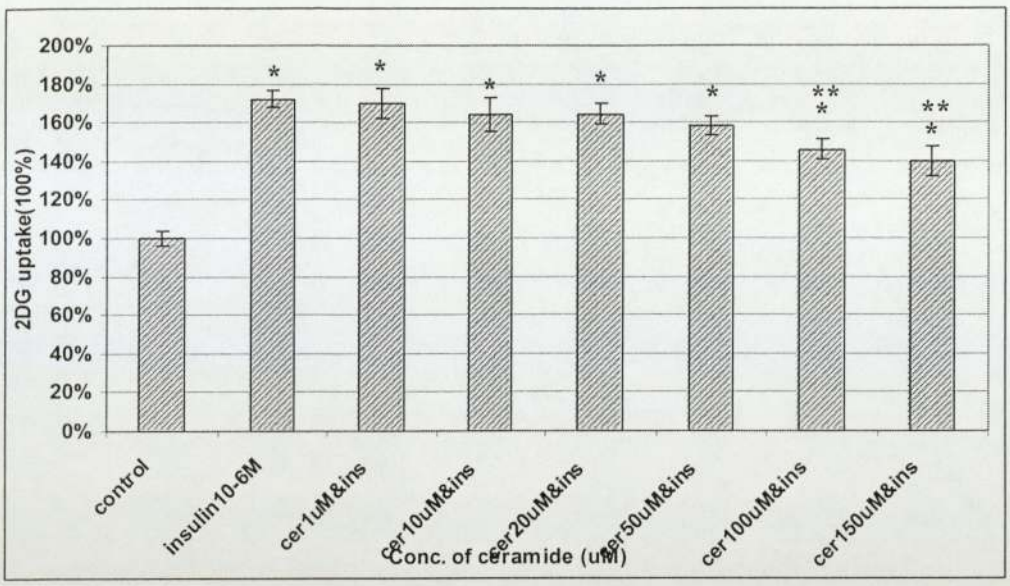


Fig 3.6 Effect of different concentrations of ceramide for 2 hours on insulin-stimulated 2 DG uptake by L6 muscle cells. Insulin was added at 10^{-6} M for 30 min as indicated by 'ins' in the figure. Values for control (no insulin and no ceramide) are expressed as 100%, and values after exposure to insulin and ceramide are expressed relative to the control value. Values are expressed as mean ± SEM, n=6. *$p<0.05$ versus control; ** $p<0.05$ versus insulin alone (second column).

Fig 3.6 & 3.7 have shown the effect of different concentrations of ceramide on glucose uptake. It is evident that C_2 ceramide has an effect on both basal glucose uptake and insulin-stimulated glucose uptake by L6 muscle cells. In the absence of added insulin (Fig 3.5) C_2 ceramide alone at 50μM concentration significantly increased 2 DG uptake ($p<0.05$). Although when values for 2 DG uptake were increased by ceramide alone at concentrations above and below 50μM, they did not achieve statistical significance. In Fig 3.6 it can be seen that insulin produced the expected increase in 2 DG uptake. However, insulin-stimulated 2 DG uptake was decreased in a concentration-dependent manner with the higher ceramide

concentrations (100 and 150μM) producing a significant ($p<0.05$) suppression of insulin-stimulated 2 DG uptake compared with insulin alone. Lower ceramide concentrations (10-50μM) reduced the mean value for insulin-stimulated 2 DG uptake, but this did not achieve statistical significance. However, this may be a non-specific effect as described in the following section; therefore the next experiment was undertaken with dehydroceramide.

3.6 Effect of dehydroceramide on glucose uptake

It is important to establish that the effects of ceramide are specific effects on L6 cell function. Thus the non-reactive ceramide analogue dehydroceramide (100μM) was tested. Myotubes were incubated with C_2 dehydroceramide (100μM) for 2 hours, with addition of insulin (10^{-6}M) for another 30 min. Glucose uptake was assessed by uptake of 2 DG as previously. Results were shown in fig 3.7.

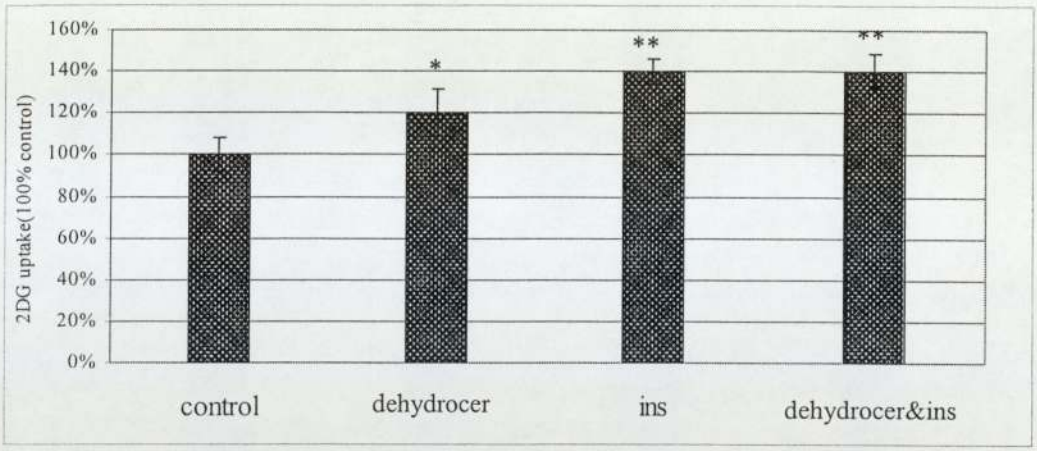


Fig 3.7 Effect of dehydroceramide (100μM) on glucose uptake by L6 muscle cells for 2 hours. Values for control (no insulin and no dehydroceramide) are expressed as 100%, and values after exposure to insulin and dehydroceramide are expressed relative to the control value. Values are expressed as mean ±SEM., n=6. *p<0.05, **p<0.01 versus control.

Dehydroceramide increased basal glucose similarly to ceramide (by 10-20%, *p<0.05 versus control), thus the effect of ceramide on basal glucose uptake may be a non-specific effect of the experimental procedure or an effect that is not associated with the normal biochemical effects of ceramide in cells. Conversely, dehydroceramide did not influence the effect of insulin on glucose uptake dehydroceramide plus insulin versus insulin treatment only). This indicated that the previously observed reduction of insulin-stimulated glucose uptake by ceramide is likely to be attributable to a direct and specific effect of ceramide itself.

3.7 Toxicity Study

3.7.1 MTT assay

L6 muscle cells were grown to nearly confluent in the medium described previously

and 10^5 cells in 100µl were seeded in 96-well plates and grown to confluence. Cells were pre-treated with C_2 ceramide and C_2 dehydroceramide (100µM) for 2 hours, and then toxicity was measured by MTT assay. 50µl MTT solution (5mg:10ml) per well was added to each well and incubated for 90 min. After this the medium was removed and replaced with DMSO 50µl per well, and incubated for another 90 min. Absorbance was then measured using a spectrophotometric plate reader at 590 nm. The principle of the MTT assay and details of the practical procedure are given in the methods section (2.3.3). Results are shown in Fig 3.8.

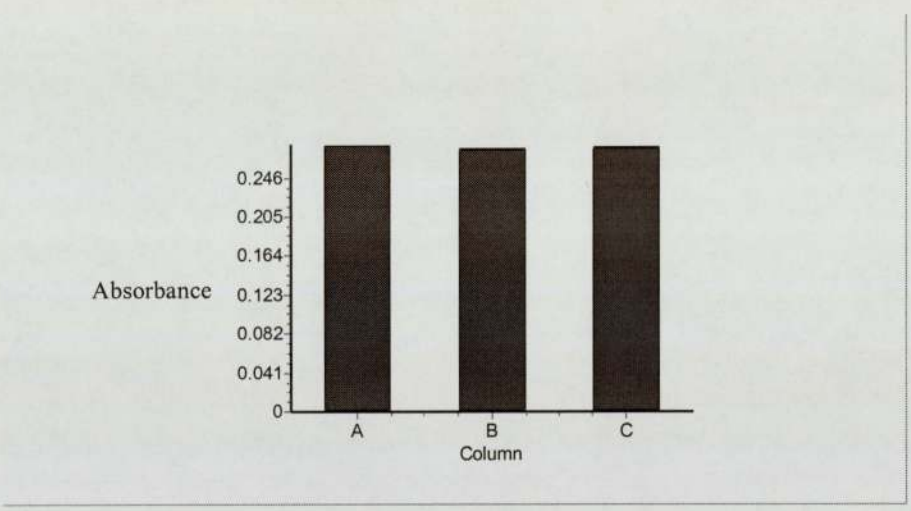


Fig 3.8 Toxicity of ceramide in L6 muscle cells. Values for control A (no chemical) are compared with cells exposed to 100µM ceramide B, and 100µM dehydroceramide C. Values are expressed as mean ± s.e.m., n=32.

The standard error was very small, showing a very close similarity of cell viability in each group. The ceramide and dehydroceramide did not have any substantial detrimental effect on the viability of the L6 cells as assessed by the MTT assay. Because the standard errors were so small, a statistically significant reduction in

viability was recorded ($p<0.05$) with both the ceramide and dehydroceramide. This was trivial and similar for the ceramide and dehydroceramide, and would appear to be insufficient to account for the effect of ceramide and dehydroceramide observed in previous experiments.

3.7.2 Trypan Blue assay

L6 cells were grown to confluence in 24 well plates and incubated with C_2 ceramide and C_2 dehydroceramide (100μM) for 2 hours. 1ml 0.4% (w/v) trypan blue solution and 1ml PBS was mixed and 300μl was added to the cell monolayer for 5 min. The staining solution was removed and the monolayer was photographed using a Nikon F301 microscope at×10 magnification. This method provides an appropriate quantitative assessment of cell viability, and demonstrates the effect of compounds used on cell. The percentage of cells showing no evidence of any blue staining was expressed; this represents the viable cells. As typically noted with L6 cultures, 80% of the cells are living by the trypan blue method (Bailey and Turner, 2004). The presence of ceramide and dehydroceramide (100μM) did not significantly alter cell viability by the trypan blue test (Fig 3.9).

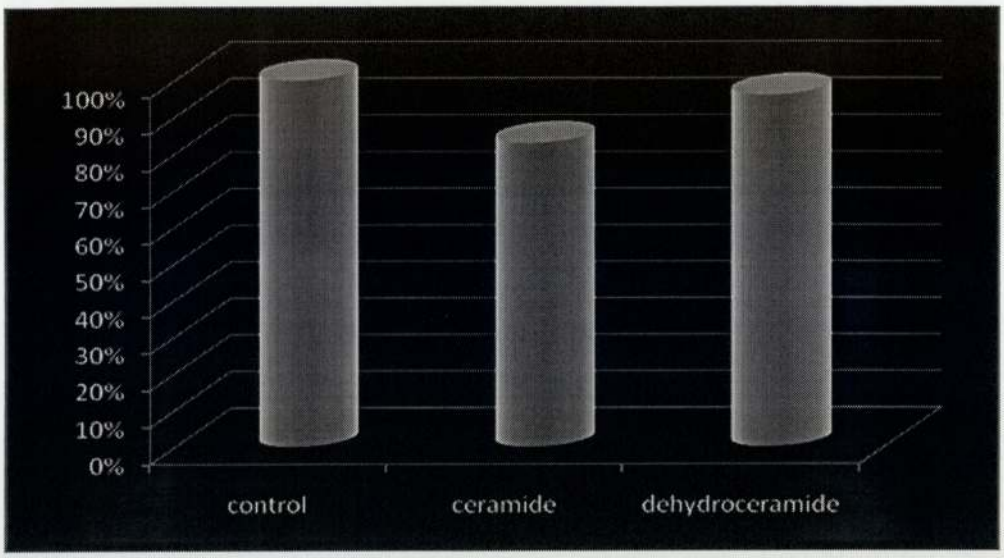


Fig 3.9 Toxicity of ceramide (100μM) and dehydroceramide (100μM) for 2 hours incubation on L6 muscle cells assessed by the typan blue assay. The culture showed a high viability of the cells, and cell viability was not significantly altered by the ceramide and dehydroceramide.

3.8 Discussion

The first set of experiments described in this chapter show that differentiated L6 myotubes took up 2 DG at a basal rate of about 29-44 pmol/10^5 cell/10min under the conditions of study (at room temperature). This is consistent with previous studies reported in the literature (Bates et al, 2002; Bailey et al, 2001) and therefore indicates that the cells were functioning normally. Moreover, the L6 myotubes showed increased glucose (2 DG) uptake in response to added insulin in a concentration-dependent and time dependent manner as noted by others (Bates et al, 1999). This confirms the functional integrity of the L6 cells as insulin-responsive myotubes. The myotubes take longer to respond to insulin than skeletal muscle in vivo, and the extent of insulin-stimulated glucose uptake may not be as great (Baron

et al, 1988). However the qualitative changes are the same as in skeletal muscle in vivo. The L6 cells should achieve maximal or near maximal stimulation of 2 DG uptake by 10^{-7}-10^{-6}M insulin (Bailey et al, 2001), and the absolute amount of 2 DG uptake increased with duration of exposure to insulin between 30 min and 24 hours. By 24 hours, 10^{-6}M insulin usually increased 2 DG uptake by about 300% compared with basal (100%). These observations are consistent evidence that the L6 muscle cells express insulin receptors (Moon et al, 2003) and that these receptors are functionally coupled via insulin receptor substrates with the PI-3 kinase-PDK-Akt post-receptor signalling pathway to increase transporters into plasma membrane (Klip et al, 1984).

Although a proportion of these glucose transporters will be isoform GLUT4, it appears that other isoforms are present. For example, GLUT1 is the principal transport protein for most cells (Montessuit and Thorburn, 1999). These are especially important for brain cells, which enable glucose to be transported at very low blood concentration. On the other hand, adipose cells and muscle cells have GLUT4 as a major glucose transporter protein, which requires insulin for its translocation into the plasma membrane and for its action. These GLUT4 transporters appear to transport 2 DG similarly to glucose itself, and the effect of insulin stimulation is to increase the number of mainly GLUT4 glucose transporters that are translocated into the plasma membrane to enable the increased 2 DG (glucose) uptake (Sarabia et al, 1992).

This effect of ceramide on L6 muscle cells was comparable to other reported studies (Hajduch et al, 2001; Chavez et al, 2003). Pre-treated with C_2 ceramide for 2 h, we observed a concentration-dependent reduction in insulin-stimulated glucose uptake, and the maximal observed inhibition was by 35% at 100μM ceramide. However an effect of ceramide was also be seen at the concentration of 10μM which produced a reduction of about 10%. Ceramide did not produce a measurable decrease in insulin-stimulated glucose uptake at a concentration at 1μM, and the maximum functional concentration was 100μM. The concentration of 150μM did not produce a greater decrease in insulin-stimulated glucose uptake. However, as discussed later, the range of C_2 ceramide concentrations (10-100μM) has been used by other investigators to cover what is regarded as the physiological and pharmacological concentration range for a effect of this agent (Hajduch et al, 2001). To try to explain how ceramide could decrease insulin action in the muscle cells could be a physical event and/or a chemical event. From a physical viewpoint it is relevant to note that ceramide mostly accumulates against the cytosolic surface of the plasma membrane. This could prevent normal access of the substances from the cytosol to the inside surface of the insulin receptor-postreceptor signalling (through IRS proteins, PI-3 kinase, PDK and Akt) take place at the interface of the inner surface of the plasma membrane and the cytosol. Therefore too much ceramide at the inner surface of the plasma membrane could prevent the normal movement of insulin-signalling intermediates to and from the membrane, and consequently decrease insulin action. Excess ceramide might also

physically interfere with the translocation of GLUT4 preventing attachment of microvesicles (which carry GLUT4 in their membranes) into the plasma membrane. Although we do not have any evidence for these possible physical effects of ceramide, they could be contributing to the inhibitory effect of ceramide on insulin-stimulated glucose uptake.

There is evidence in the literature that supports the possibility that ceramide affects one or more biochemical events of postreceptor insulin signalling. It has been suggested that ceramide might disturb the signalling steps between PI-3 kinase and Akt (Summers et al, 1998). The PI-3 kinase, PDK, Akt pathway is the main pathway through which insulin stimulates translocation of GLUT4-containing microvesicles into the plasma membrane. Therefore, such as effect of ceramide could explain, either in part or in total, the decrease in insulin-stimulated glucose uptake produced by C_2 ceramide. Since cells were pre-treated with dehydroceramide had no effect on insulin-stimulated glucose uptake, this indicates that the effect of ceramide to decrease insulin-stimulated glucose uptake was a specific effect.

Contrary to insulin-stimulated glucose uptake, ceramide might slightly increase basal glucose uptake. However when the muscle cells were pre-incubated with an inactive ceramide analogue, dehydroceramide, this also slightly increased basal glucose uptake. Therefore the effect of ceramide on basal glucose uptake may be an artefact of the

experiment as indicated by the same effect of the inactive dehydroceramide.

It is possible that the ceramide could have a physical effect (i.e. not an active biochemical effect) to create an increase in glucose uptake. For example ceramide might physically slow down the internalisation of GLUT4 vesicle formation and movement out of the membrane. So if more GLUT4 is prevented from leaving the membrane this might contribute to the very small increase in apparently basal glucose uptake. While there might be a trivial effect on cell viability, this does not appear to be a generalized toxic effect of the concentration of ceramide studied, as indicated by viability during trypan blue exclusion tests. However the very slight decrease in mitochondrial viability mediated by a slight decrease in the MTT test could theoretically slightly increase anaerobic glucose metabolism.

To investigate whether the observed changes with ceramide could be a cause of insulin resistance, we set up another model of insulin action by EAHY endothelial cells which will be considered in next chapter.

Chapter 4:

Characterisation of the human EAHY endothelial cell line

4.1 Introduction

The previous chapter has described an initial set of experiments to investigate the effect of ceramide on insulin action in a cell model of skeletal muscle. The parameter measured was insulin-stimulated 2 DG uptake. The general hypothesis being tested in the present programme is that ceramide has a wide-spread effect on insulin action. Therefore it is appropriate to test the effect of ceramide on a different parameter of insulin action in a different tissue. The tissue selected, as described in chapter 1 (section 1.9) is the endothelial cell line EAHY926, and the parameter measured is insulin-stimulated nitric oxide (NO) production (section 2.3.2). The receptor-post-receptor signalling pathway that controls insulin-stimulated NO production appears to follow the same kinase cascade that also controls insulin-stimulated glucose uptake (chapter 1, section 1.4). This appears to be a common early signalling pathway leading from insulin receptor to insulin receptor kinase action to IRS phosphorylation to the PI-3 kinase-PDK-Akt cascade, as described in chapter 1(section 1.9) and reviewed in the literature (Hartell et al, 2005).

4.1.1 EAHY cells

The EAHY cell line was chosen because it shows many of the typical features of endothelial cells, including insulin responsive NO production. The EAHY cells line was originally derived by fusing human umbilical vein endothelial cell with the permanent cell line A549 (Edgell et al, 1983).

The cells are very flat, about 1-2μm thick, and they have a central nucleus. There are few relatively permanent cell lines that express most of the morphological and functional features of human endothelial cells. We set up the experiments here by using EAHY as a model to see whether the NO production is associated with ceramide-induced insulin resistance.

Endothelial cells (ECs) form the inner lining of a blood vessel and provide an anticoagulant barrier between the vessel wall and blood. In addition to its role as a selective permeability barrier, the endothelial cell is a unique multifunctional cell with critical basal and inducible metabolic and synthetic functions (Sumpio et al, 2002). Since the actions of ECs are multiple and involved in many systems, it is important that they maintain normal function. Increasing evidence has demonstrated the coexistence of insulin resistance and endothelial dysfunction (Petrie et al, 2003). Furthermore it was reported that early abnormalities in vascular reactivity and reduced EC activation occur in individuals at risk of developing type 2 diabetes (Caballero et al, 1999).

NO is believed to be a key mediator of many of the vascular functions of endothelial cells and its cellular concentration has been regarded as a standard for the measurement of EC dysfunction (Moncada and Higgs, 1993). Thus it is valuable to research NO production as a model to assess insulin resistance in endothelial cells.

EAHY cells were cultured as described in 2.2.2b, and were allowed to settle down to form a monolayer overnight. Cells were counted using a haemocytometer, adjusted for dilution factors, and transferred into 96 well plates as described below.

4.2 Nitric Oxide Determination

4.2.1 Introduction

Nitric oxide was measured indirectly by the Griess reagent which can measure the sum of nitrite and /or nitrate as described in section 2.3.2. Because the total amount of NO is determined as the change in the sum of nitrite and nitrate, a very short effect period of insulin on endothelial cells has been selected; therefore the results of this experiment were measured initially at intervals over a time course of 10 min. The absorbance of product of the Griess reaction was measured using a plate reader with a filter at 590 nm as in section 2.3.2.

4.2.2 Experimental design

EAHY endothelial cells were incubated to nearly confluent at 37°C in 95% air and 5% CO_2 in medium containing 25mM glucose. The medium was supplemented with 10% FCS, 100U/ml of penicillin G, 100µg/ml of streptomycin and 10ml of H.A.T. For each experiment, 10^5 cells in 100µl were seeded in 96-well plates and grown to confluence in the above medium and atmosphere. Then the medium was changed to DMEM containing 2% FCS for 4h to complete differentiation. The methods have been

described in chapter 2.

4.2.3 Results

The first full experiment determined the time dependent ability of insulin (10^{-6}M) to act on EAHY endothelial cells to alter NO production as shown in fig 4.1.

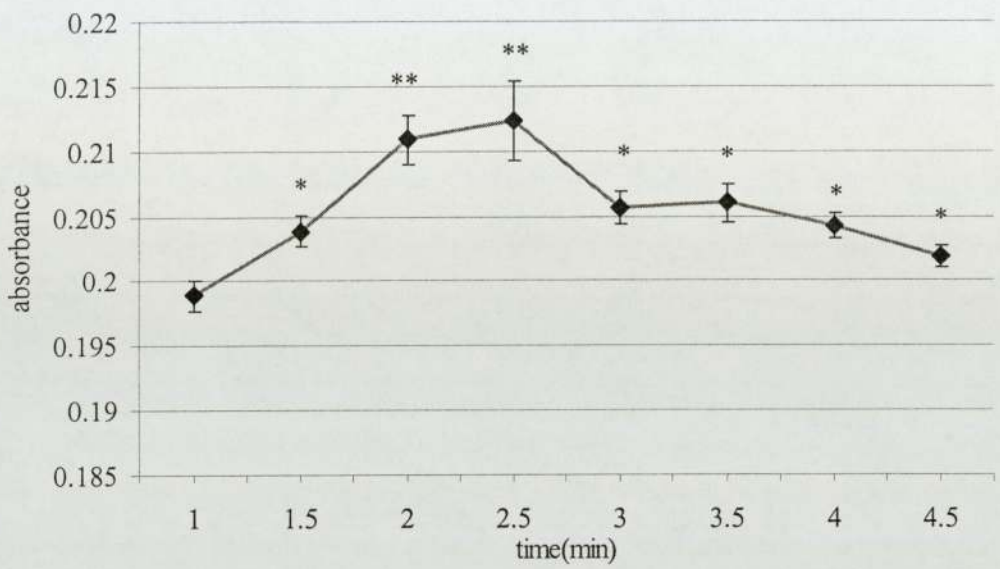


Fig 4.1 Time-dependent effect of insulin (10^{-6}M) on NO production by EAHY endothelial cells. Values are expressed as mean ± s.e.m., n=8. All values were significantly increased at times 1.5-4.5 min compared with 1.0 min (*p<0.05; **p<0.01).

This indicates that insulin (10^{-6}M) increased NO production by endothelial EAHY cells. The effect was maximal at 2-3 min and declined thereafter. These data were all obtained under the present experimental conditions; although the experiments were run several times to increase the 'n'. However the technique requires rapid manual input that prevented the complete experiment from being undertaken on a single occasion, so the data were pooled from several identical experiments conducted in series.

4.3 Effect of C_2 ceramide on insulin stimulated nitric oxide release

4.3.1 Experimental design

When EAHY endothelial cells were grown to nearly confluent in the medium described previously, 10^5 cells in 100µl were seeded in 96-well plates and grown to confluence, then the medium was changed to low serum (2% FCS) for 4 hours. During this serum starvation period cells were pre-treated with C_2 ceramide for 2 hours. Then we added the Griess Reagent and measured the absorbance immediately after addition of insulin (10^{-6} M) by the plate reader using a filter at 590 nm.

4.3.2 Results

Incubation of endothelial cells with insulin (10^{-6}M) increased NO production. C_2 ceramide decreased insulin stimulated NO production in a concentration dependent manner. 150µM was the highest concentration of ceramide used and this produced a significant decrease in both basal and insulin stimulated NO production at the 2 hours incubation time period investigated (**$p<0.01$ versus control). The ceramide concentrations lower than 100µM (from 10-100µM still had a significant effect on the reduction of insulin stimulated NO production (See fig.4.2).

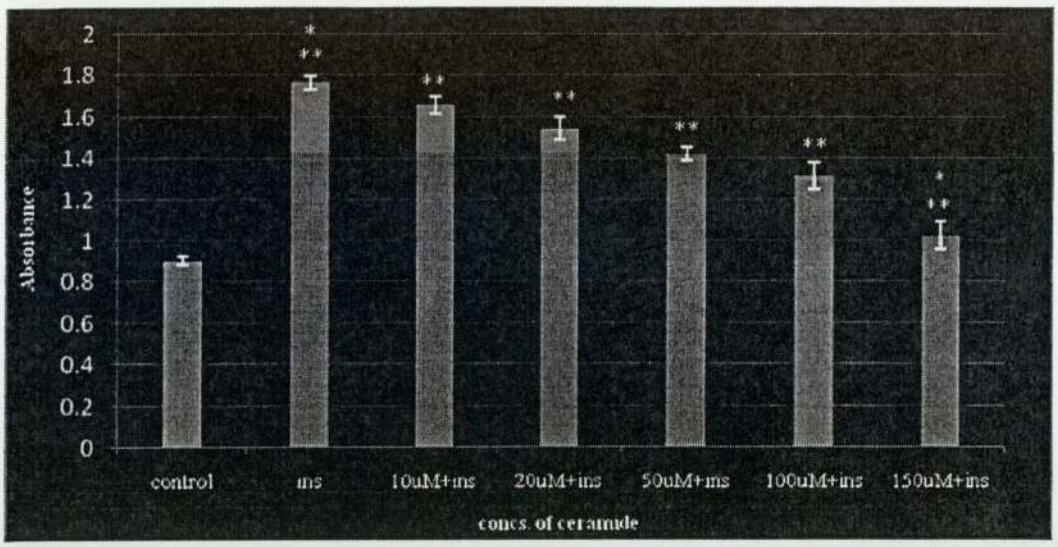


Fig 4.2 Effect of different concentrations of ceramide (2 hours) on insulin (10^{-6}M) stimulated NO production by human endothelial EAHY cells. Values are expressed as mean ± s.e.m., n=8. *p<0.01 vs control; **p<0.01 insulin v.s 10μM /20μM /50μM /100μM /150μM ceramide plus insulin.

However, C_2 ceramide alone seems not to affect basal NO production (see fig 4.3).

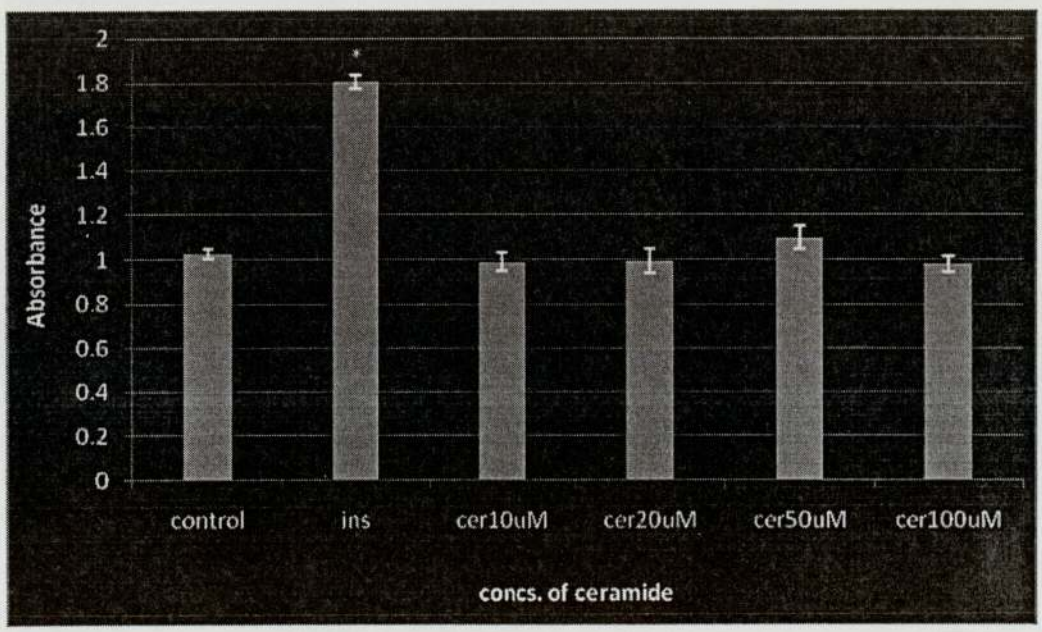


Fig 4.3 Effect of different concentrations of C_2 ceramide (2 hours) on basal NO production by human endothelial EAHY cells. Values are expressed as mean ± s.e.m., n=8. *p<0.01 vs control.

4.4 Effect of dehydroceramide on nitric oxide production

4.4.1 Introduction

As we described before, it is necessary establish that the effects of ceramide are specific on EAHY endothelial cell function. Thus the non-reactive ceramide analogue dehydroceramide (100μM) was tested here again.

4.4.2 Experimental Design

Cells were incubated with C_2 dehydroceramide (100μM) for 2 hours, then with addition of insulin (10^{-6}M) for another 5 min. NO production was assessed with Griess Reagent as above. We compared the results from cells treated with ceramide (66.7μM), dehydroceramide (100μM), insulin (10^{-6}M), ceramide plus insulin, and dehydroceramide plus insulin. Results were shown in Fig 4.4.

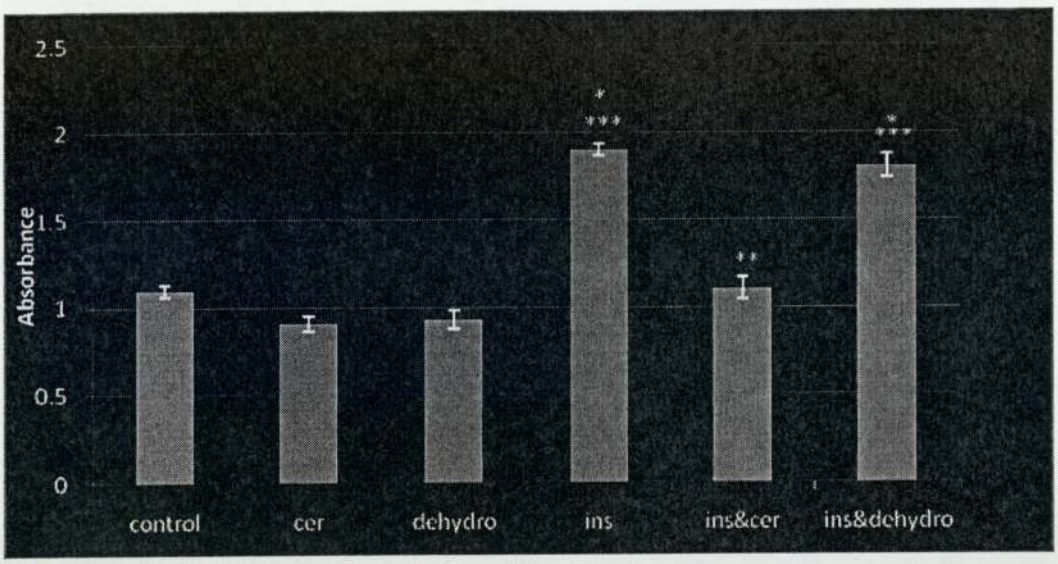


Fig 4.4 Effect of dehydroceramide (100μM), ceramide (66.7μM) and insulin (10^{-6}M) on nitric oxide production by EAHY endothelial cells. Cells were exposed to ceramide and dehydroceramide for 2 h and insulin for 5 min. Values are expressed as mean ± s.e.m., n=8. *P<0.05 vs control. **P<0.05, insulin v.s. all other groups except dehydroceramide with insulin. ***P<0.01 vs ceramide, dehydroceramide and insulin with ceramide.

Although ceramide may slightly reduce basal NO production by EAHY endothelial cells, dehydroceramide produced a similar small effect. This suggested that the effect of ceramide on basal NO production may be a non-specific effect of the experimental procedure or some other influence that is not due to the metabolic influence of ceramide. However, the lack of effect of dehydroceramide on insulin-stimulated NO production indicated that ceramide exerts a specific suppressive effect on insulin-stimulated NO production. This is analogous to the situation seen with ceramide on basal and insulin-stimulated glucose uptake by L6 cells.

4.5 Toxicity Study

4.5.1 MTT assay

EAHY endothelial cells were grown to nearly confluent in the medium described previously and 10^5 cells in 100ul were seeded in 96-well plates and grown to confluence then changed to low serum (2% FCS) for 4h. During this serum starvation period we pre-treated with C_2 ceramide and C_2 dehydroceramide (100 μM) for 2 hours, and then measured toxicity by MTT. 50μl MTT solution (1:10) per well was added to each well and incubated for 90 min. After this the medium was removed and replaced with DMSO 50μl per well and incubated for another 90 min. Absorbance was then measured using a plate reader at 590 nm. The principle of the MTT assay and details of the practical procedure are given in the methods section. Results are shown in Fig 4.5.

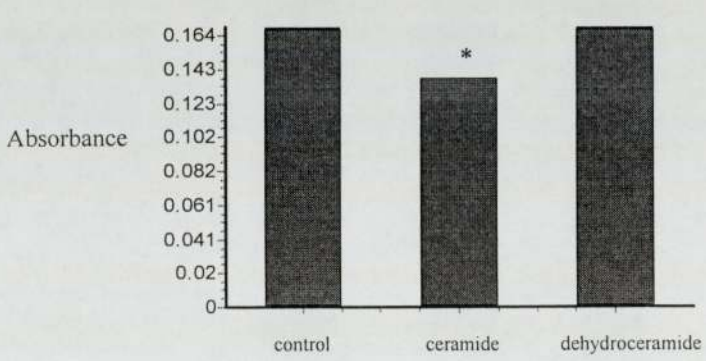


Fig 4.5 Toxicity of EAHY endothelial cells using the MTT assay. Values for control, are compared with cells pre-treated with C_2 ceramide * $p<0.01$; Values are expressed as mean ± s.e.m., n=32. Error bar is too small to see, just attach it the top.

These results show that cells exposed to dehydroceramide retained full viability. There was a small 19% reduction in the mean number of viable cells after exposure to ceramide. This was slightly significant, but the effect of ceramide on the cells does not appear to be explained by loss of normal cell viability because ceramide alone had little effect on basal NO production and was not different to dehydroceramide.

4.5.2 Trypan blue assay

Cells were grown to confluence in 24 well plates and incubated with C_2 ceramide and C_2 dehydroceramide (100μM) for 2 hours. 1 ml 0.4% (w/v) trypan blue solution and 1 ml PBS was mixed and 300μl was added to the cell monolayer for 5 min. The staining solution was removed and the monolayer was photographed using a Nikon F301 microscope at ×10 magnification. This method provides an approximate quantitative assessment of cell viability, and demonstrates the effect of compounds used on cell survival.

From the images observed from the microscope we can estimated that about 20% of cells incubated with ceramide were dead, and less than 5% of cells were affected by dehydroceramide. Since it was difficult to clearly visualise faint blue colouration of cells in this experiment, numerical values are not given here.

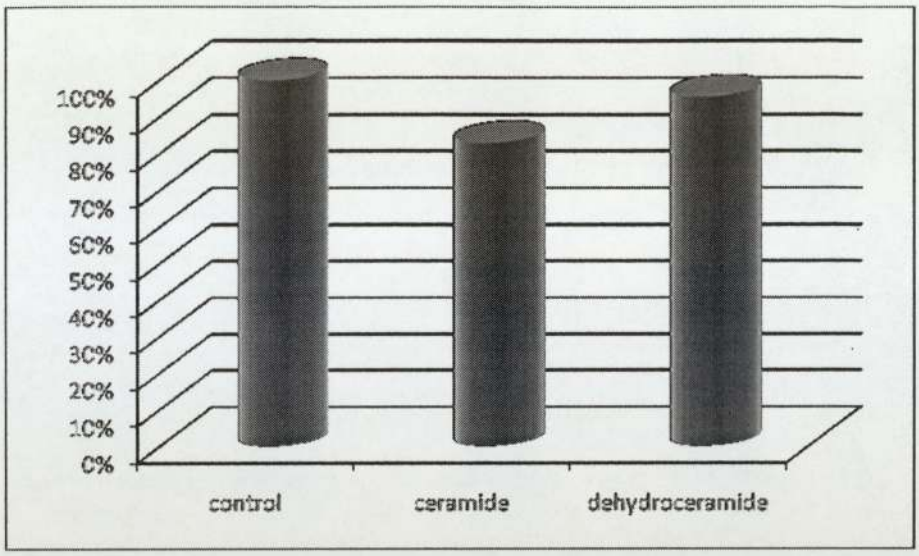


Fig 4.6 Toxicity of ceramide (100μM) and dehydroceramide (100μM) for 2 hours incubation on EAHY cells assessed by the trypan blue assay. The culture showed a high viability of the cells, and cell viability was not significantly altered by the ceramide and dehydroceramide

4.6 Discussion

The experiments described in this chapter focus on the effect of ceramide on NO production by EAHY endothelial cells. Firstly, it is evident that the cells were metabolically competent, as indicated by the toxicity tests with MTT and trypan blue, and by their rapid responsiveness to insulin with increased NO production. The measurement of NO production by EAHY endothelial cells was explored as a model for insulin action. Similar to glucose uptake by L6 muscle cells, the EAHY cells showed a time-dependent stimulation of NO production as well. The activity time of insulin on EAHY cells is quite rapid and short, thus all measurements were made within 5 min. After 5 min the effect of insulin was reduced. We chose an insulin concentration at 10^{-6}M, because this is a maximally effective concentration in the

previous studies. We measured the effect from 1 min to 4.5 min, and we identified a maximal effect at 2 or 2.5 min normally, sometimes a little later at 3 min. The maximal effect was the value selected. This was considered to be a more representative measure of insulin-stimulated NO than either the area under the curve (AUC) or the mean value of measurements at 2-3 min. This also took account of differences in the time of peak effect within the period 2-3 min.

The main finding of this present study with EAHY cells was that C_2 ceramide reduces insulin-stimulated NO production. The effect of ceramide on EAHY endothelial cells was similar to L6 cells where ceramide inhibited insulin-stimulated glucose uptake. Indeed, EAHY cells pre-treated with C_2 ceramide for 2 hours showed a total inhibition of insulin-stimulated NO production at concentration ranges from 10-150μM. The effect of ceramide could be attributed to a fall in insulin-stimulated NO production because NO is rapidly released from the cell. A small reduction was seen in basal NO production as well. However the reduction of ceramide on insulin-stimulated NO production was even lower than basal (Fig 4.2). The maximal observed inhibition was a 60% reduction below basal at 150μM ceramide. The reason for this cannot be explained by the two present experimental designs but suggests that the inhibitory effect of ceramide on NO production is much greater in the presence of insulin. We chose dehydroceramide in presence of insulin to pre-treat EAHY cells for 2 hours, and no effect on insulin-stimulated NO production was observed. Thus the effect of

ceramide on NO production was a specific effect of the metabolically active ceramide.

A possible mechanism of ceramide to reduce the production of NO might involve an influence of ceramide on endothelial nitric oxide synthase (eNOS). However there are no reports in the literature to indicate that ceramide can directly affect eNOS. It has been well established that insulin exerts a direct vasodilator effect via the endothelium by stimulation of eNOS (Hsueh and Law, 1999; Scherrer et al, 1994). eNOS is a multicomponent protein comprising oxygenase and reductase domains which catalyse a reaction by which NO is produced from the amino acid L-arginine, molecular oxygen and NADPH (Andrew and Mayer, 1999) (see fig4.6).

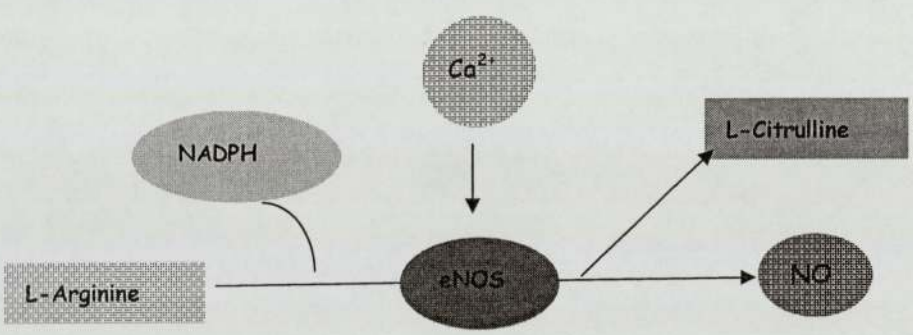


Fig4.7 NO pathway: using this pathway the vascular endothelium synthesizes NO from the terminal guanidino nitrogen atoms of L-Arginine using the soluble enzyme NO synthase.

In the recent years, it has been clear that cellular localization and trafficking of eNOS are important in the regulation of NO production. The signalling pathway of insulin to activate eNOS comprises PI-3 kinase-PDK-Akt, which then leads to the phosphorylation of eNOS at serine and threonine residues (Zeng et al, 2000;

Dimmeler et al, 1999). This pathway may be down-regulated in insulin-resistant states (Jiang et al, 1999). Insulin-resistant individuals exhibit resistance to both the metabolic and some of the vascular actions of insulin (DeFronzo 1987; Hsuch and Mayer, 1999). At the cellular level, the signalling pathway by which insulin mediates glucose uptake and NO production are similar. After binding to cell membrane receptors, insulin promotes GLUT4 translocation in skeletal muscle. In endothelial cells insulin increases eNOS activity through the same signalling cascades, which involves both PI-3 kinase and Akt (Montagnini and Quon, 2000). This lends to support to the hypothesis that disruption of the PI-3 kinase-Akt pathway may be lead to insulin resistance which causes impaired glucose uptake by muscle and endothelial dysfunction.

From the foregoing experiments I suggest that ceramide may slightly reduce the viability of EAHY cells, but the effect (about 20% reduction in viable cells) was much less than the complete inhibition of insulin-stimulated NO production. Therefore the effect of ceramide to prevent insulin-stimulated NO production cannot be attributed (other than a small contribution) to a reduction in the general competence of the cells. Since the inactived analogue dehydroceramide had no measurable effect on insulin-stimulated NO production, this further suggests the view that ceramide is acting through a specific metabolic or biochemical effect to interrupt the insulin signalling pathway. Additional support for this view comes from the experiment

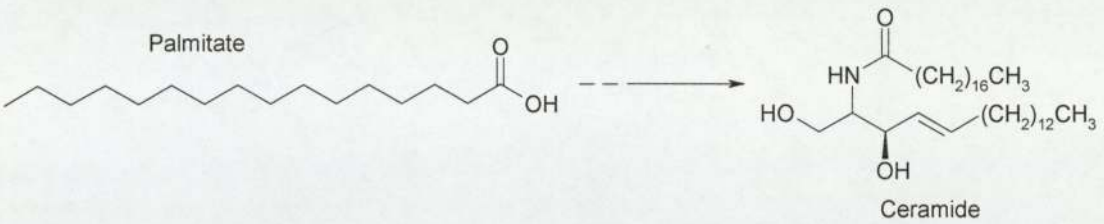
shown in Fig 4.4 in which ceramide exert little or no effect on basal NO production.

To investigate further the possible metabolic or biochemical effect of ceramide on the insulin signalling pathway, it is important to consider related evidence in the literature that palmitate, which is a precursor of ceramide, can induce insulin resistance. This is investigated in the next chapter.

Chapter 5:

Effect of palmitate on L6 muscle cells and EAHY endothelial cell

5.1 Introduction



Palmitate is a term for the salts or esters of palmitic acid. The palmitate anion is the observed form of palmitic acid at physiological pH. Palmitic acid is the first fatty acid produced during lipogenesis and from which longer fatty acids can be produced.

Recent evidence suggests that intracellular accumulation of saturated free fatty acids (FFA) contributes substantially to lipid-mediated cellular damage (Listenberger et al, 2003). This includes muscle cells and vascular endothelial cells. The cellular dysfunction associated with FFA overload, known as lipotoxicity, contributes to cell injury as well as excess triglyceride accumulation, such as in obesity and type 2 diabetes (Eckel et al, 2002). Since palmitate is the main precursor of ceramide synthesis, there is substantial evidence to suggest that much of the lipotoxicity associated with excess fatty acids could be mediated via ceramide (Dyntar et al, 2001).

However there have also been observations that palmitate can promote apoptotic cell death and lipotoxic affects that are independent of ceramide (Listenberger et al, 2001).

The studies described herein have investigated whether the saturated fatty acid palmitate is either an antagonist of insulin signalling and or an inducer of ceramide accumulation in these two cell types: L6 muscle cell and EAHY endothelial cell. The role of palmitate is an important precursor for the synthesis of ceramide is described in section 1.6.4 palmitate provides a convenient way to increase cellular ceramide content. Findings will show whether palmitate can effectively impair insulin-stimulated glucose uptake and NO production. For time limitation, I did not measure the cellular ceramide concentration directly; however the selected concentration of palmitate plus ceramide (lower than maximal functional concentration) produced a maximum effect on insulin signalling. Also at the concentration used in this study, palmitate may be regarded as an effective stimulator of ceramide as shown by others (Liu and Anderson, 1995; Listenberger et al, 2001).

5.2 Experimental design

5.2.1 L6 muscle cells

5.2.1.1 Cell culture

L6 muscle cells were grown as described previously and 10^5 cells in 1 ml were seeded in 24-well plates and grown to confluence in DMEM medium with 5% FCS and the same atmosphere as previously (section 2.1.1). Then the medium was changed to DMEM containing 0.5% FCS for another 24 hours to induce differentiation and fusion of myoblasts into myotubes and to prevent further division.

5.2.1.2 Fatty acid treatment

Palmitate was dissolved in 70% hot ethanol to make up a stock at 150mM. 10% fatty acid free BSA was then used to conjugate with palmitate stock stirring at 37 °C for 1 hour to achieve a concentration at 8mM. Final ratio for palmitate and BSA was 6:1. For the experiment, normal medium was used to dilute to 4mM working solution.

After the differentiation, L6 cells were incubated with palmitate at 0.75mM for 16 hours. This time period was selected to allow long enough for palmitate to fully increase ceramide synthesis, as considered in the discussion section. For other treatments, ceramide and insulin were added for the last 2 hours and last 30 min respectively.

5.2.2 EAHY endothelial cells

5.2.2.1 Cell culture

EAHY endothelial cells were cultured as previously described (section 2.2.2), when they had grown to nearly 80% confluent in the medium. 10^5 cells in 100µl were seeded in 96-well plates and grown to confluence, then the medium was changed to low serum (2% FCS).

5.2.2.2 Fatty acid treatment

The treatment for EAHY endothelial cells was similar to L6 cells. The same palmitate stock and same incubation time period (16 hours) were used for studies with

endothelial cells.

5.3 Results

5.3.1 L6 muscle cells

5.3.1.1 Effect of palmitate on 2 DG uptake

Exposure to palmitate (0.75mM) for 16 hours reduced insulin-stimulated glucose uptake with a high insulin concentration (10^{-6} M) and ceramide (50μM) (see Fig 5.1).

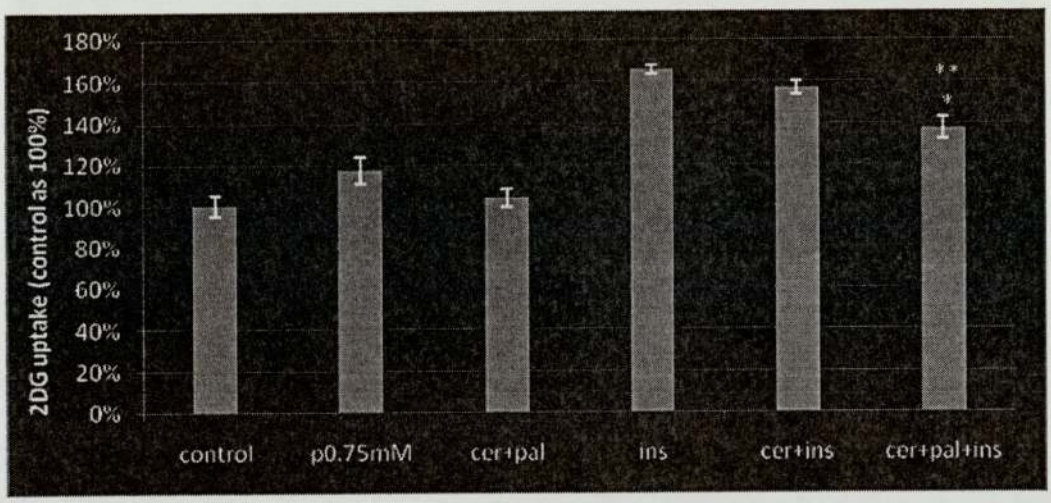


Fig 5.1 L6 myotubes were pre-incubated with palmitate (0.75mM) for 16 hours and, during the last 2 hours of this period, were treated with C_2 ceramide (50μM) and/or the last 30 min of this period, were treated with insulin (10^{-6}M). Cells treated with palmitate (0.75mM), C_2 ceramide (50μM) and insulin (10^{-6}M) show significantly reduced insulin-stimulated 2 DG uptake (*$p<0.01$) compared with insulin alone; And this value was compared with cells pre-treated with C_2 ceramide (50μM) **$p<0.05$. Values are expressed as mean ± SEM, n=16.

Fig 5.1 indicates that 2 DG uptake was slightly increased in the presence of 0.75mM palmitate but absence of insulin and this effect is similar with 50μM C_2 ceramide. Insulin alone (10^{-6}M) produced a typical large increase in 2 DG uptake. Using cells

pre-treated with 0.75mM palmitate, ceramide treatment caused a further reduction on insulin-stimulated 2 DG uptake. It is evident that palmitate may be a stimulator of ceramide which could indicate its effect; although at this stage this must be regarded as a preliminary interpretation. To expand this hypothesis, another cell model has been studied.

5.3.1.2 Effect of different concentrations of palmitate on 2 DG uptake in L6 cells

Exposure to palmitate (0.25mM, 0.5mM, 0.75mM, 1mM) for 16 hours, with or without insulin (10^{-6}M) shown in fig5.2. Insulin alone again strongly stimulated 2 DG uptake. Palmitate (0.25-1.0mM) did not significantly alter 2 DG uptake although mean values showed a tendency to increase uptake slightly. However palmitate reduced insulin-stimulated 2 DG uptake (fig 5.2)

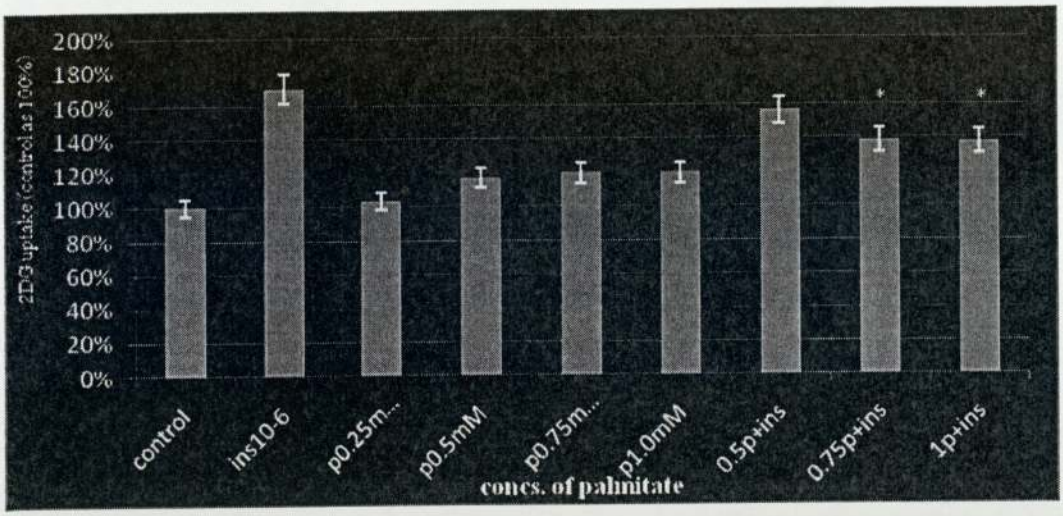


Fig 5.2 L6 muscle cells were pre-incubated with palmitate (0.25mM, 0.75mM, 1mM) for 16 hours and, with/without insulin (10^{-6}M). Both 0.75mM and 1mM palmitate with insulin had a significant effect (*$p<0.05$ versus insulin alone) to reduce insulin-stimulated glucose uptake. Values are expressed as mean ± SEM, n=16.

5.3.2 EAHY endothelial cells

5.3.2.1 Effect of palmitate on endothelial cells

Insulin (10^{-6}M) increased NO production by EAHY cells as previously. Exposure to palmitate (0.75mM) for 16 hours reduced insulin-stimulated glucose uptake treated with a high insulin concentration (10^{-6} M) and ceramide (50μM) (see Fig 5.3).

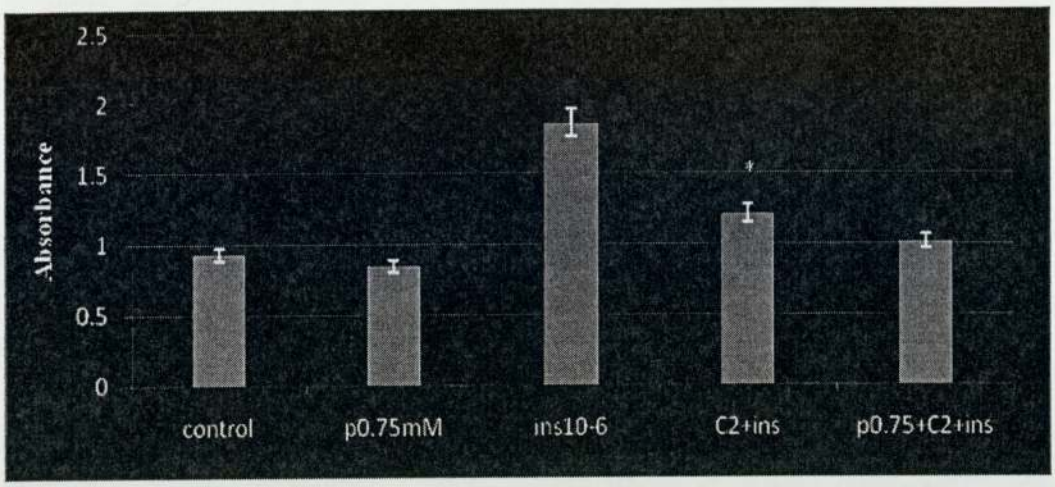


Fig 5.3 EAHY endothelial cells were pre-incubated with palmitate (0.75mM) for 16 hours and, during the last 2 hours of this period, were treated with C_2 ceramide (50μM). Cells treated with palmitate (0.75mM), C_2 ceramide (50μM) with insulin (10^{-6}M) showed significantly reduced insulin-stimulated NO production compared with insulin only, *$p<0.01$; However, the effect of 0.75mM palmitate alone did not significantly change NO production Values are expressed as mean ± SEM, n=16.

Fig 5.3 indicated that the effect of palmitate on basal NO production by endothelial cells was negligible. This was similar to the effect of ceramide. However, ceramide reduced insulin-stimulated NO production as noted in chapter 4 (fig 4.4). If the production of ceramide has been enhanced by pre-treatment of palmitate, this would possibly explain why palmitate plus ceramide produced a similar or slightly greater reduction in insulin-stimulated NO production compared with ceramide.

5.3.2.2 Effect of different concentrations of palmitate on NO production

Endothelial cells were treated by a concentration range from 0.5mM to 1mM palmitate for 16 hours with or without insulin (see fig.5.4).

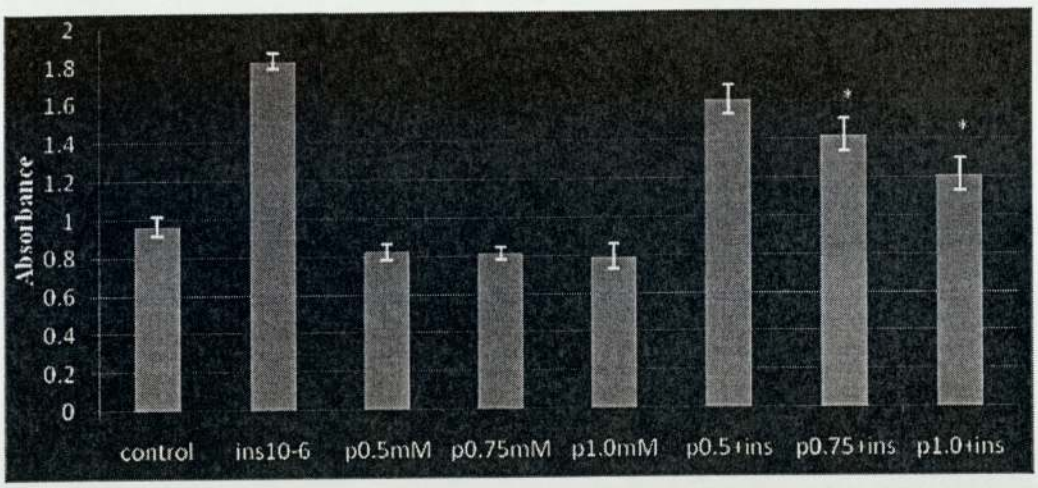


Fig 5.4 EAHY endothelial cells were pre-incubated with palmitate (0.5mM, 0.75mM, 1mM) for 16 hours and, with/without insulin; *p<0.05 versus insulin only; Values are expressed as mean ± SEM, n=16.

Fig 5.4 shows that a range of concentrations of palmitate (0.5mM-1mM) does not significantly affect basal NO production. However, both 0.75mM and 1mM palmitate with insulin had a significant effect (*p<0.05 versus insulin-stimulated NO production) to reduce insulin-stimulated NO production. 0.5mM palmitate did not significantly alter insulin-stimulated NO production.

5.4 Toxicity Study

5.4.1 MTT assay

The procedure to assess cell viability by the MTT method has been described in chapter 2.3.3. The effect of palmitate and ceramide on viability of L6 cells by this method is shown in fig 5.5, EAHY cells is shown in fig 5.6.

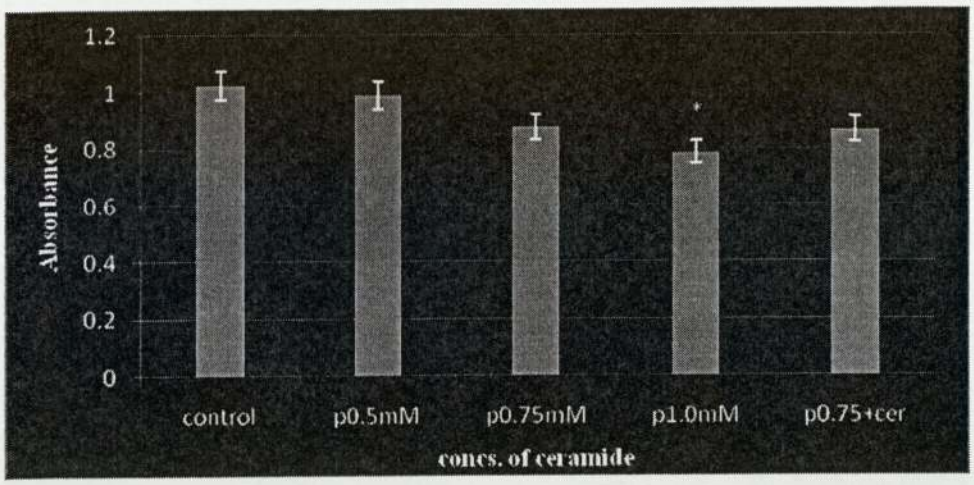


Fig 5.5 Toxicity of palmitate (p) and ceramide (cer) on L6 muscle cells by using the MTT assay. Values are expressed as mean ± s.e.m., n=16. The 0.5mM palmitate, 0.75mM palmitate and 0.75mM palmitate with 50μM ceramide did not have any substantial detrimental effect on the viability of the L6 cells as assessed by the MTT assay. 1mM palmitate had a significant effect on cell viability (*p<0.05 versus control).

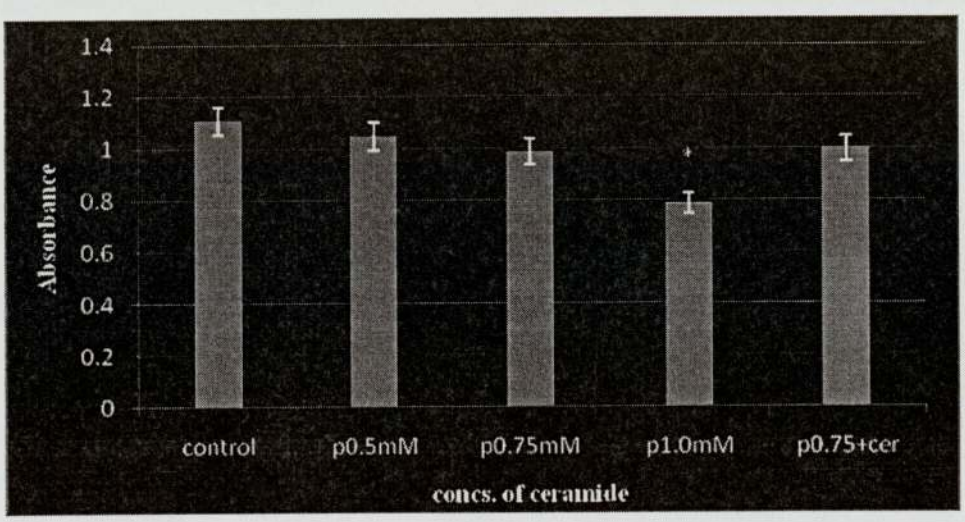


Fig 5.6 Toxicity of palmitate and ceramide on EAHY endothelial cells by using the MTT assay. Values are expressed as mean ± s.e.m., n=16. The 0.5mM palmitate, 0.75mM palmitate and 0.75mM palmitate with 50μM ceramide did not have any substantial detrimental effect on the viability of the EAHY cells as assessed by the MTT assay. 1mM palmitate had a significant effect on cell viability (*p<0.05 versus control).

These results show that cells exposed to palmitate no higher than 0.75mM

concentration retained nearly full viability for both L6 muscle cells and EAHY endothelial cells. However, at 1mM treatment, there was a significant (25%) reduction in the mean number of viable cells (data not shown), hence no other studies were conducted with palmitate concentrations >1mM.

5.4.2 Trypan blue assay

The method to assess cell viability by trypan blue has been described in a previous chapter (2.3.4). From the images observed from the microscope we can estimated 20% of cells incubated with 1mM palmitate were dead, and less than 10% of cells were affected by lower palmitate concentrations. This supports evidence from the MTT assay, and justifies limiting the high palmitate concentration to 1mM.

5.5 Discussion

This chapter reports studies to test the hypothesis that the saturated fatty acid palmitate can reduced insulin signalling in two separated cell types where insulin controls very different effects, namely glucose uptake by L6 muscle cells and NO production by EAHY endothelial cells. Palmitate is a primary substrate for the synthesis of ceramide. However, time did not allow me to measure the effect of palmitate on intracellular ceramide levels, so I suggested that palmitate was likely to raise ceramide levels as noted in other cell types. The effect of palmitate in the presence and absence of C_2 ceramide was studied to see if palmitate can produce a

similar effect to ceramide, and to see whether the effect showed any 'additive' value.

Since it was not clear from the literature what concentration of palmitate to select, several studies were undertaken to determine the effect of a range of palmitate concentrations. Considerable initial difficulty was encountered to get the palmitate into solution, as noted in the methods, but this was eventually accomplished successfully. The methods for 2 DG uptake and NO production were the same as described and characterized in previous chapters, but it was necessary to check firstly to see if palmitate had any toxic effect on L6 and EAHY cells. Concentrations of palmitate at 1mM started to show evidence of toxicity by MTT and trypan blue assays, so high palmitate concentrations were not studied for 2 DG uptake or NO production. 0.75mM palmitate was the highest palmitate concentration that did not appear to affect cell viability during the period of study in these experiments, so this palmitate concentration was chosen for most studies, or was right middle of the range of concentrations used. Previous studies with the literature do not seem to have made detailed toxicity studies, and some of these studied have used palmitate concentrations of 1.5mM or even higher (Werner, 1983; Glatz and Veerkamp, 1982). It is possible therefore that effects observed in these studies could be due at least partly to a toxic effect that reduced cell viability.

The pre-treatment period for palmitate of 16 hours was selected because previous

studies suggested that palmitate achieves its full effect to increase ceramide synthesis and to disturb insulin signalling by about this time (Listenberger et al, 2001). C_2 ceramide was studied at 2 hours as it appears to be about fully effective at this time as discussed earlier (Hajduch et al, 2001).

As noted previously, insulin increased 2 DG uptake by L6 cells and NO production by EAHY cells. This confirms that the cells we responding in a healthy manner as before. Also ceramide reduced or inhibited the effect of insulin as previously.

Palmitate has significantly reduced insulin-stimulated 2 DG uptake by L6 cells and NO production by EAHY cells. However, no significant effect was found on basal 2 DG uptake or basal NO production. Both effects of palmitate could be regarded as similar to or an amplified effect of ceramide. My reason for thinking this is that palmitate plus ceramide together gave a slightly greater effect than ceramide alone to reduce insulin-stimulated 2 DG uptake or NO production. However ceramide did not lower the effect of a high (0.75mM) concentration of palmitate alone. This suggests that 0.75mM palmitate probably has a similar or greater effect to reduce the effect of insulin than 100μM C_2 ceramide. It is possible that palmitate can reduce the effect of insulin by increasing ceramide synthesis and by additional other mechanism. We did not measure the cellular ceramide concentration, but other studies in the literature indicate that the concentration range of palmitate used here (0.5-1.0mM) will increase

intracellular ceramide levels (Chavez et al, 2003). Indeed by adding palmitate to a low concentration of ceramide, it is found that the effect was equal to a higher concentration of ceramide alone. If the saturated fatty acid (palmitate) significantly induced the synthesis of ceramide, we would be limited by the rate-limiting reaction in ceramide biosynthesis, which is the condensation of serine with palmitoyl-CoA, a reaction catalyzed by serine palmitoyltransferase (SPT) (see section 1.5.1). The product, 3-ketosphinganine, is then reduced, acylated, and oxidized in three subsequent reactions to produce ceramide. Palmitate increases ceramide levels by providing precursor palmitoyl-CoA for ceramide synthesis (Merrill et al, 2002) and, in at least one cell type, inducing SPT expression (Shimabukuro et al, 1998). Therefore the limited rate of ceramide synthesis would be a limiting factor for any effect of palmitate that is mediated through ceramide.

Thus, as a stimulator of ceramide, pre-treating with palmitate was equal to treating with a relatively high concentration of ceramide (but within what is believed to be the functional concentration range).

It is tentatively concluded from the studies reported in this chapter that the saturated fatty acid palmitate reduces insulin signalling in two different tissues, and controls two different effects of insulin. This suggests that this effect of palmitate is a widespread inhibitory effect on insulin action. Since palmitate is a key precursor of

ceramide, and the effects of palmitate were at least similar to or slightly greater when added together with ceramide, it is possible that at least some of the activity of palmitate is mediated through ceramide.

Further information on this preliminary interpretation will be acquired in studies reported in the next chapter, using an inhibitor of ceramide synthesis.

Chapter 6:

Effect of Fumonisin B1 on muscle cells and EAHY endothelial cell

6.1 Introduction

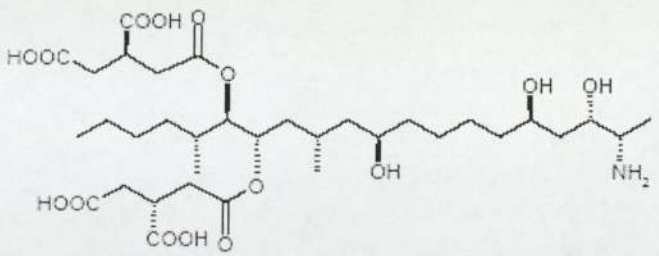


Fig 6.1 Structure of Fumonisin B1 (Fb1)

As described earlier, palmitate impaired both insulin-stimulated glucose uptake and insulin-stimulated NO production, and this effect to some extent, amplified the effect of ceramide alone. Also others have reported that when cells are pre-treated with palmitate (up to 0.75mM), a 3 fold increase of ceramide is observed (Powell et al, 2004; Paumen et al 1997; Chavez et al, 2003). We hypothesized that ceramide was a principal factor which inhibited Akt causing insulin resistance. To test this hypothesis here, we determined whether an inhibitor of *de novo* ceramide synthesis could prevent palmitate's inhibition of insulin signalling.

Briefly, ceramide biosynthesis requires the coordinate action of two enzymes: serine palmitoyltransferase (SPT) and ceramide synthase. SPT catalyzes the initial step, which involves the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine, a sphingolipid that is subsequently reduced to form the sphingoid base sphinganine. Ceramide synthase catalyzes sphinganine acylation, producing

dehydroceramide, which is then converted to ceramide (see Fig 1.6).

Fumonisin B1 (Fb1), is a fungal toxin that inhibits dehydroceramide synthase and it is a key enzyme in the *de novo* ceramide synthesis pathway (Schroeder et al, 1994). Here we use Fb1 to investigate whether it could prevent the inhibiting effect of palmitate on insulin-stimulated glucose uptake by L6 muscle cells and NO production by EAHY endothelial cells.

6.2 Experimental design

6.2.1. Cell culture

L6 muscle cells and EAHY endothelial cells were grown as described previously and were seeded in 24-well plates/96-well plates respectively as noted in section 2.1.1 & 2.2.2.

6.2.2 Fatty acid treatment

Palmitate stock was prepared as described in section 5.2.1.1 and used at the working concentrations of 0.5 and 0.75mM as indicated in sections 6.3 and 6.4.

6.2.3 Treatment with Fumonisin B1

Fumonisin b1 was dissolved in a solvent mixture of acetonitrile/water (1:1) to make up a stock at 3.68mM before experiments. Normal medium was used to dilute this to

460μM as working solution. 50μM Fb1 and 0.75mM palmitate were used to treat both L6 and EAHY cells for 16 hours.

6.2.4 2 DG uptake and NO production

2 DG uptake by L6 cells was measured after exposure to test substances for 30 min. NO production was measured within 5 min as described in section 2.3.2 and 4.2.1.

6.3 Results

6.3.1 L6 muscle cells

6.3.1.1 Effect of Fb1 on 2 DG uptake

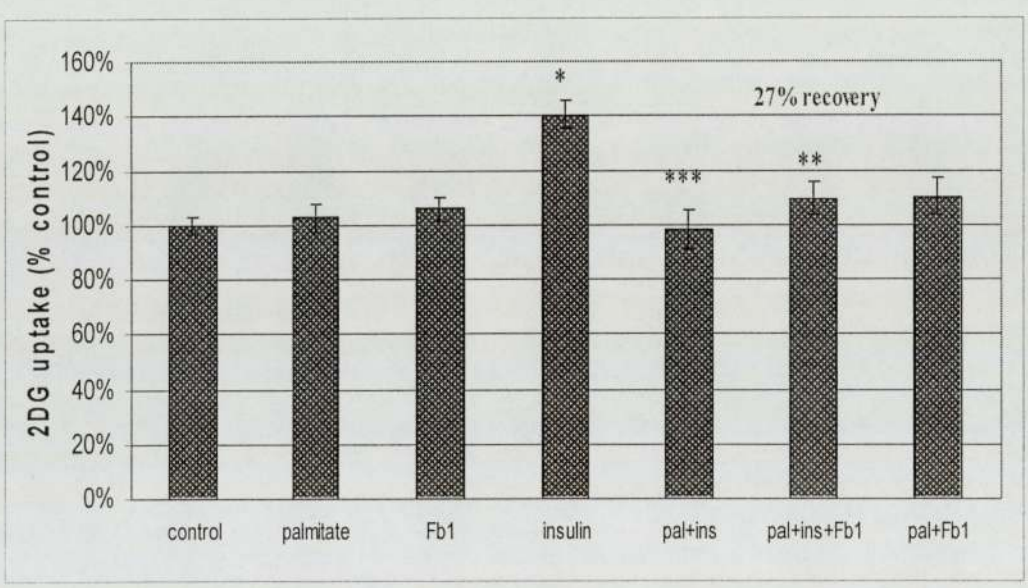


Fig 6.2 Effect of Fb1(50μM), palmitate (0.75mM), with or without insulin (10^{-6}M) on 2 DG uptake by L6 muscle cells. Cells were pre-incubated with Fb1 and palmitate for 16 hours, and insulin 30 min. Pre-treating with 10^{-6}M insulin alone significantly increased 2 DG uptake by 40% *$p<0.001$ versus control. Palmitate 0.75mM reduced insulin-stimulated NO production by 30% ***$p<0.001$ versus insulin. The treatment of Fb1 significantly prevented approximately 27% of the defect in insulin-stimulated 2 DG uptake **$p<0.01$ versus 0.75mM palmitate with insulin. Values are expressed as mean ± SEM., n=16.

Fig 6.2 indicated that Fb1 and palmitate alone did not significantly alter the basal glucose uptake. However, palmitate significantly reduced insulin-stimulated glucose uptake by approximately 30%. This effect then was slightly but significantly reduced in magnitude by pre-treating with Fb1. Since a 40% increased 2 DG uptake was observed in the presence of insulin as similarly noted in previously results, we may assume the cells were healthy and responsive.

6.3.2 EAHY endothelial cells

6.3.2.1 Effect of Fb1 on endothelial cells

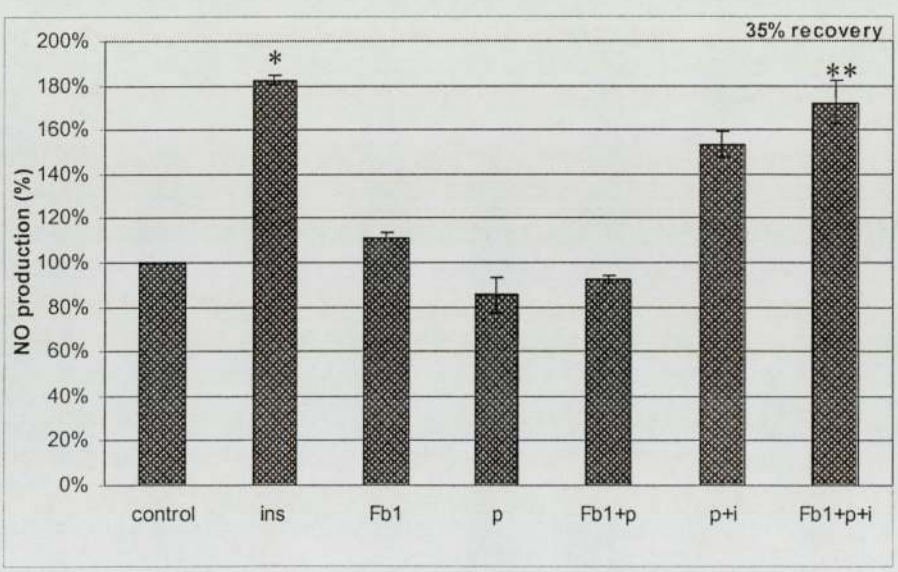


Fig 6.3 EAHY endothelial cells were pre-incubated with Fb1 50μM, palmitate 0.75mM for 16 hours, with/without insulin; Pre-treating with 10^{-6}M insulin significantly increased NO production by 80% *p<0.001 versus control. The treatment of Fb1 was significantly preventing approximately 35% of the defect in insulin-stimulated NO production**p<0.001 versus 0.75mM palmitate with insulin. Values are expressed as mean ± SEM, n=16.

Fig 6.3 shows that 50μM Fb1 alone did not significantly alter NO production.

Palmitate alone (0.75mM) also did not significantly alter NO production, nor the two agents together. However, as shown in previous chapters, insulin (10^{-6}M) increased NO production, and this was reduced by 37% by palmitate. The reduction by palmitate was significantly smaller in the presence of Fb1, such that Fb1 prevented most of the effect of palmitate, so that the palmitate inhibition of insulin-stimulated NO production was only 35% of the effect of palmitate without Fb1. Since insulin treatment still obtained an 80% increased NO production compared with control, we may regarded the cells as healthy and responsive.

6.4 Toxicity Study

6.4.1 MTT assay

The procedure to assess cell viability by the MTT method has been described in chapter 2.3.3. The effect of palmitate and Fb1 on viability of L6 cells by this method is shown in fig 6.4, EAHY cells are shown in fig 6.5.

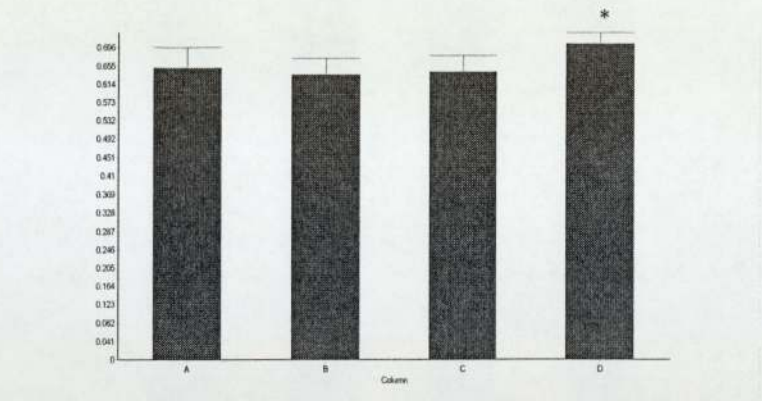


Fig 6.4 Toxicity of Fb1and palmitate on L6 muscle cells using the MTT assay. Column A, control; Column B, 0.5mM palmitate; Column C, 0.75mM palmitate; Column D, 50μM Fb1. Values are expressed as mean ± SEM., n=16. The 0.5mM palmitate and 0.75mM palmitate did not have any substantial detrimental effect on the viability of the L6 cells as assessed by the MTT assay. However, 50μM Fb1 had a very small but significant effect to increase MTT reduction (*p<0.05 versus control).

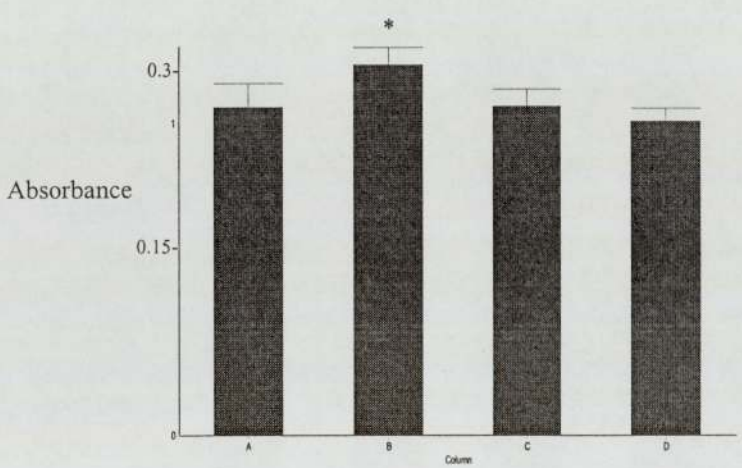


Fig 6.5 Toxicity of palmitate and ceramide on EAHY endothelial cells by using the MTT assay. Column A, control; Column B, 50μM Fb1; Column C, 0.5mM palmitate; Column D, 0.75mM palmitate. Values are expressed as mean ± SEM, n=16. The 0.5mM palmitate, 0.75mM palmitate did not have any substantial detrimental effect on the viability of the EAHY cells as assessed by the MTT. 50μM Fb1 had a small but significant effect to increase MTT reduction(*p<0.05 versus control).

The results above have shown that there was no significant reduction with the treatment of Fb1 and lower concentration of palmitate. Moreover, the absorbance of the treatment with Fb1 was even higher than control. This is consistent with the view that endogenous ceramide production may be causing a small amount of apoptosis. When Fb1 is present this reduces endogenous ceramide synthesis, resulting in a very small but significant increase in cell viability as measured by MTT.

6.4.2 Trypan blue assay

The method to assess cell viability by trypan blue has been described in a previous chapter (2.3.3). From the images observed from the microscope we can estimate more than 90% cells are healthy when treating with Fb1 alone, about 10% reduction by pre-treating with 0.75mM palmitate, and no significant reduction in 0.5mM palmitate. The palmitate results are consistent with data reported in chapter 5.

6.5 Discussion

This chapter reports studies to test the hypothesis that the inhibitor of ceramide synthesis (Fb1) can improve insulin actions in two separate cell types which are L6 muscle cells and EAHY endothelial cells. Herein, the inhibitor we chose, Fb1 inhibits ceramide synthesis in all cells a previously studied (Shimabukuro et al, 1998; Petrache et al, 2005). From the results noted above, it significantly protected both L6 cells And EAHY cells from the inhibition of palmitate. Fb1 also appeared to significantly

improve cell viability.

The present experiments show that the cells were responding normally, because they produced extra 2 DG uptake and NO production when challenged with insulin as shown in previous chapters. The trypan blue and MTT assays confirmed the viability of the cells.

Fb1 has been studied previously in a range of cell types. It inhibits synthesis of dehydroceramide and therefore prevents ceramide production. It is more than 50% effective in reducing ceramide synthesis in other cells at concentration of 10-50μM. Therefore a concentration of 50μM Fb1 was used in the present study which is likely to inhibit ceramide synthesis by at least half (Puglielli et al, 2003). This concentration of Fb1 does not appear to be toxic in other cells. In this study I checked with the MTT and trypan blue exclusion methods and confirmed that 50μM Fb1 was not toxic. In fact 50μM Fb1slightly increased viability of the cells. This could be explained in the context of normal cell apoptosis. Ceramide is believed to increase apoptosis (Pettus et al, 2002). Since Fb1 reduces ceramide synthesis, this would explain how Fb1 could produce small increases in cell viability.

Palmitate reduced insulin-stimulated 2 DG uptake and NO production as shown previously. Palmitate is the major substance for ceramide synthesis, so this could

account for at least in part for the effects of palmitate. Fb1 would theoretically be expected to prevent or reduce the actions of palmitate via ceramide. This was the case in the present studies where Fb1 reduced the inhibition effect of palmitate on insulin-stimulated NO production by 37% and insulin-stimulated 2 DG uptake by 27%.

From the effects of ceramide we can conclude because Fb1 slightly increased viability and slightly increased basal activity in L6 and EAHY cells, it is likely that Fb1 is reducing ceramide synthesis so that there is a reduced effect of endogenous ceramide. Fb1 did not produce significant effects on basal measurements in the cell models, but it partly prevented the inhibitory effects of palmitate on the actions of insulin. This is all consistent with the concept that endogenous ceramide reduces the effects of insulin on glucose uptake and NO production similarly to the effect of exogenous C_2 ceramide. Thus the present chapter suggests the data obtained with C_2 ceramide in the previous chapters.

In conclusion to this chapter, Fumonisin B1 has been seen to protect the two cell models from the defects of palmitate.

Chapter 7:

General Discussion

7.1 Discussion

The background to this research programme was to look at potential causes of insulin resistance. The general aim was to investigate whether there is a common feature that could account in part or more generally for the development of insulin resistance within different tissues and apply to different actions of insulin. The introduction develops the hypothesis that ceramide could be a widespread factor contributing to insulin resistance. The rationale for this is set out in chapter1 sections 1.7.4 to section 1.7.6. The main evidence is cellular accumulation of ceramide has been identified as a defect generally associated with insulin resistance. Excess ceramide could possibly cause disturbances that impair a variety of metabolic actions of insulin.

From the evidence above I took the working hypothesis that excess ceramide accumulation could be an important factor of insulin resistance in skeletal muscle and endothelium; and the inhibition of ceramide could provide a means to improve insulin actions. I then proposed the following questions: **a**. Could ceramide affect basal and/or insulin-stimulated glucose uptake as well as NO production? **b**. Could these effects be in a concentration and/or time dependent manner? **c**. Could these effects of ceramide be amplified by adding its main synthesis precursor (palmitate)? **d**. Could an inhibitor of ceramide synthesis prevent palmitate inhibition of insulin signalling?

To answer these questions I selected two tissues with two different key actions of insulin. These were: L6 cells and insulin-stimulated 2 DG uptake, and EAHY cells

and insulin-stimulated NO production. I selected these cells and actions of insulin because glucose uptake and NO production are fundamental and very different physiological actions of insulin. Also, muscle has been selected because it is the major metabolic tissue of the body for glucose metabolism, and it is important because the body depends on insulin signalling for its cellular energy supply. Endothelium has been chosen because endothelium has an established and crucial role in maintaining vascular homeostasis which depends on insulin signalling to release NO.

The key findings of this thesis are:

a. C_2 ceramide reduces insulin-stimulated glucose uptake by the L6 muscle cells and NO production by EAHY endothelial cells.

b. The effect of C_2 ceramide occurs in a concentration and time dependent manner.

c. The free fatty acid palmitate impedes two different actions of insulin and these effects are even greater when added together with ceramide.

d. Pre-treatment with an inhibitor of ceramide synthesis was able to block the inhibitory effects of palmitate on insulin action in two model system.

Control studies with dehydroceramide

The effect of ceramide on L6 muscle cells was comparable to other reported studies (Hajduch et al, 2001; Chavez et al, 2003). The inhibition of ceramide could not be seen on basal glucose uptake. Conversely to insulin-stimulated glucose uptake, ceramide might slightly increase basal glucose uptake. To investigate whether these effects of ceramide are metabolic effects or physical effects, the same experiments were performed with an inactive ceramide analogue, dehydroceramide. There was no effect of dehydroceramide on insulin-stimulated glucose uptake. However the effects of ceramide on basal glucose uptake may be an artifact of the experiments as indicated if by the same effect occurring with the inactive dehydroceramide. Thus the effect of ceramide was a specific effect to reduce insulin-stimulated glucose uptake.

Ceramide concentration study

Selecting a concentration of ceramide that is representative of the normal "intracellular" concentration is difficult to determine. The basal amount of ceramide within cells has been established in primary rat cerebella granule cells at 0.22 nmol/10^6 cells (Prinetti et al, 2000). Accurate data for the intracellular concentrations of ceramide in other cell models has been difficult to acquire, but it is suggested that other cells such as muscle and endothelium will contain very approximately similar

amounts of ceramide in the normal range (Hyde et al, 2004; Mathias et al, 1998). To produce a relatively "physiological" alteration to the intercellular ceramide pool by incubating cells with C_2 ceramide is difficult to assess. I selected the concentration range 10μM-100μM, considering that under the basal condition, total ceramide concentration was increased nearly two fold in insulin resistance subjects (Adams et al, 2004), but probably most of the extracellular exogenous ceramide will not gain access to the intracellular compartment. Other studies in cultured cells, including studies with L6 cells have exposed these cells to concentrations of 100μM C_2 ceramide to produce what they claim to be physiologically relevant effects (Hyde et al, 2004).

Interaction of ceramide with insulin signalling

Studies have identified the key targets for ceramide action including protein phosphatases 1 (PP1) and protein phosphatases 2A (PP2A) which are activated by ceramide in *vitro*. Increasing evidence shows that inhibitors of these phosphatases will inhibit the ability of ceramide to cause the dephosphorylation of several cellular proteins (Chalfant and Hannun, 2002). This could be a possible mechanism for ceramide to disrupt the insulin signalling pathway.

Thus ceramide might be reducing insulin-stimulated glucose uptake in muscle by

blocking the activation of Akt "downstream" of PI-3 kinase, either by inhibiting its translocation to the cell membrane (Powell et al, 2003) or/and by promoting the dephosphorylation of Akt via protein phosphatases PP1 and PP2A (Teruel et al, 2001; Chalfant and Hannun, 2002). However, the inhibitory effect of C_2 ceramide on Akt translocation is possibly a wide-spread phenomenon, because both ceramide and Akt are present in most cell types but not all; moreover, an inhibitor of C_2 ceramide has been shown to prevent the inhibition on Akt phosphorylation completely (Stratford et al, 2004) (Fig 7.1).

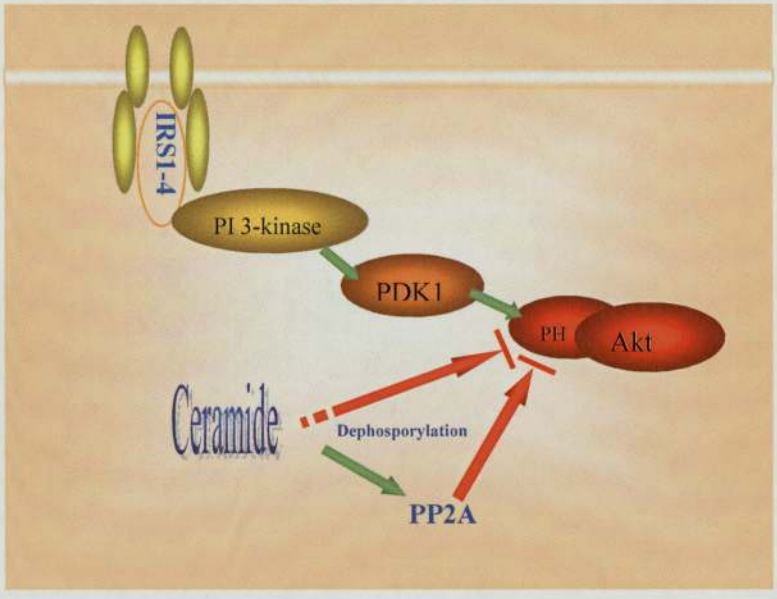


Fig7.1.The possible mechanism of ceramide for the regulation of Akt. Ceramide may be involved in cell signalling events downstream of phosphatidylinositol 3-kinase (PI-3 kinase) by inhibiting the Akt phosphorylation by activation of protein phosphatases such as 2A (PP2A). IRS1, insulin receptor substrate; PDK, phosphatidylinositide-dependent kinase; PH, phosphorylated activated Akt; Akt, protein kinase B.

There is a resent study which has suggested that ceramide could affect insulin signalling by inhibiting Akt via PP1 or PP2A. (Hyde et al, 2004). This was a study in

L6 cells showing that C_2 ceramide (100μM) reduced the sodium dependent system A amino acid transporter (SNAT2). This transporter is under the regulatory influence of insulin which promotes its movement from intracellular microsomes into the plasma membrane. The process is similar to insulin promoting movement of glucose transporters, especially GLUT4, from microsomes into plasma membrane. When the L6 cells were indicated with okadaic acid, which inhibits PP2A, this prevented C_2 ceramide from reducing insulin-stimulated amino acid uptake via SNAT-2. Hyde et al (2004) suggest that ceramide could activate PP2A to inhibit Akt which interrupts insulin signalling. The same mechanism has been proposed for ceramide inhibition of insulin-stimulated glucose uptake (Hajduch et al, 2001), and this fits the proposed mechanism put forward in this thesis.

Insulin also activates mitogen activated protein kinase (MAP kinase) pathways (Saltiel and Kahn, 2001), however when cells were treated with specific inhibitors of MAP kinase, there was no improvement in ceramide reduced insulin sensitivity as well as the reduced phosphorylation of Akt (Schmitz-Peiffer et al, 1999). Several studies have confirmed the regulation of ceramide on Akt signalling pathway, however, the effect is not mediated through the MAP kinase pathway (Hajduch et al, 2001).

Nitric Oxide and ceramide

At the same time, the main finding of EAHY cells was that C_2 ceramide reduces insulin-stimulated NO production. The effect of ceramide on EAHY endothelial cells was similar to L6 cells to inhibit insulin-stimulated glucose uptake. Although the effect of ceramide could be attributed to a fall in insulin-stimulated NO production, a similar but smaller reduction was seen in basal NO production as well. Thus the reduction of ceramide on insulin-stimulated NO production was significantly greater and even longer acting than basal. As noted above, we chose dehydroceramide to pre-treat EAHY cells for 2 h, and no effect was observed. Thus the effect of ceramide on NO production was specific and cannot be dismissed as an experimental artifact.

The possible mechanism of ceramide to reduce the production of NO might be an influence on endothelial nitric oxide synthase (eNOS). It has been well established that insulin exerts a direct vasodilator effect via the endothelium by stimulation of endothelial nitric oxide synthase (eNOS) (Hsueh and Law, 1999; Scherrer et al, 1994). At the cellular level, the effect of C_2 ceramide on insulin mediated NO production appeared to be similar to its effects on glucose uptake. The production of NO from endothelial cells is promoted via the PI-3 kinase pathway (Zeng and Quon, 1996) (Fig 7.2). Another parallel insulin pathway is highlighted by studies using a dominant-negative inhibitor of MAP kinase (Cusi et al, 2000). However, no further

effect has been observed on reduced NO production. It is likely that this intracellular insulin signalling pathway is not an important route for insulin-activated eNOS. The possible mechanism of insulin to mediate increased activity of eNOS has been reported by other studies (Dimmeler et al, 1999; Fulton et al, 1999). They argued that insulin promoted eNOS activity via the activation of PI-3 kinase and Akt. Activated Akt, in turn phosphorylated eNOS, leading to increased in NO production.

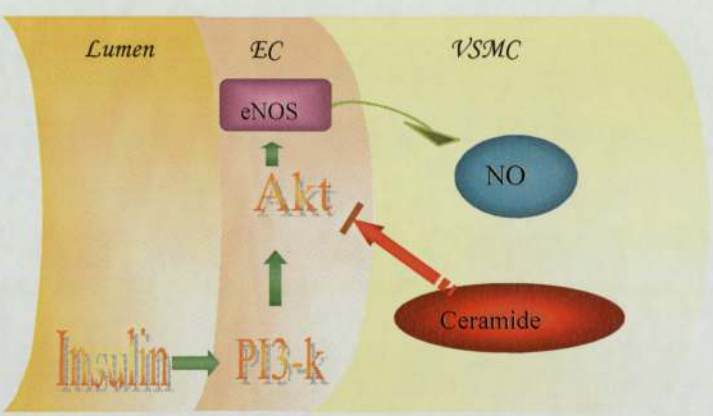


Fig7.2 Ceramide inhibits PI-3 kinase-Akt insulin signalling pathway required for NO production by activation of eNOS. Studied in the literature suggests that excess ceramide resulted in inhibition of endothelial PI-3 kinase pathway insulin signalling in downstream vessels by dephosphorylation of Akt. EC=endothelial cell. PI3-K, Phosphoatidylinositol-3-kinase. eNOS=endothelial nitric oxide synthase. VSMC=vascular smooth muscle cell.

This could possibly involve activations of protein phosphatases (e.g. PP2A) as suggested for Akt inhibition in muscle cells (Fig 7.1).

Endothelial cells (ECs) line the internal lumen and serve as an interface between circulating blood and vascular smooth muscle (VSMC) (Fig7.2). In addition they

serve as a physical barrier between the blood and tissues, the ECs have an established and crucial role in maintaining vascular homeostasis including vasoreactivity response such as increased postprandial blood flow to muscles to facilitate glucose disposal into muscle (Laakso et al, 1992). Since the actions of ECs are multiple and involved in many system, EC dysfunction may affect one or more of these systems. Increasing evidence has demonstrated the coexistence of insulin resistance and endothelial dysfunction (Pinkney et al, 1997). Furthermore it was reported that early abnormalities in vascular reactivity and reduced EC activation occur in individuals at risk of developing type 2 diabetes (Caballero et al, 1999).

Influence of palmitate

In the present experiments we pre-treated cells with palmitate at 0.75mM for 16 hours. As we discussed previously (section 5.5), the effect of palmitate was similar to ceramide. And to some extent, the effect of ceramide could be amplified by adding with palmitate. It is known that palmitate is a precursor for synthesis of ceramide. Unfortunately, for time limitation, it is not clear whether the cellular concentration of ceramide could be increased by pre-treating with palmitate in these two cell models. However others have reported that about 3 fold increase cellular ceramide was observed after treating with palmitate (Chavez et al, 2003; Powell et al, 2004; Paumen et al 1997). This evidence suggests that ceramide could be an important mediator of insulin resistance. It is becoming increasingly apparent that fatty acids are an

important chemical group that contribute to insulin resistance. They appear to do this through multiple cellular effects including alteration to the glucose-fatty acid (Randle) cycle, metabolism to products such as diacylglycerol (DAG) and protein kinase C (PKC) isoforms that impede the action of insulin signalling intermediates (Storz et al, 1999) such as IRS and PI-3 kinase, by increasing insulin receptors deactivation by reducing the breakdown of certain phosphotyrosine phosphatases (Virkamaki et al, 1998).

These data implicate ceramide in insulin resistance possibly resulting from the oversupply of saturated fatty acids. To minimize the possibility we selected an inhibitor Fb1 which is capable of blocking the enzyme (ceramide synthase) required in the ceramide synthesis pathway. Pre-treating L6 myotubes and EAHY cells with Fb1 (50μM), 16 hours, as discussed in chapter 6 partly prevented the effects of ceramide on the action of insulin, supporting the view that palmitate increases ceramide, which then mediates inhibitory effects on insulin action.

Other mediators of ceramide

PKC isoforms and cathepsin have been implicated as possible mediators of ceramide effects. These could affect insulin action: for example, PKC ζ has been reported to underlie the suppressive action of ceramide on Akt (Bourbon et al, 2002; Powell et al,

2003), and Bourbon and Powell also suggested that PKC ζ was 9- fold activated by treating with 100μM C_2 ceramide (Bourbon et al, 2000; Powell et al, 2003). The possible mechanism may involve that PKC ζ could promote the inhibition of Akt by phosphorylation of Akt-PH domain at Thr^{34} (Powell et al, 2003), and then the mediated phosphorylation reduces the ability of the Akt-PH domain to bind 3-phosphoinositides. This process responds to the increased ceramide synthesis.

Ceramide causes increased apoptotic cell death. This is believed to be mediated by a group of enzymes known as cysteine-directed asparagine proteases (caspases) (Krown et al, 1996; Oral et al, 1997). In the present studies the use of Fb1 to reduce ceramide synthesis was accompanied by a slightly increased number of viable cells. However, the present research did not investigate whether the effect involved caspases.

In section 1.4, it was noted that there is a known stimulation of glucose transporter activity via a pathway other than that involving Akt. This is sometimes called the 'alternative' pathway. Its physiological role is unclear. However, since ceramide alone produced a small increase in basal glucose uptake, it is possible that ceramide lipid rafts attached to the cytosolic surface of the plasma membrane could influence this pathway. Whether this could account for any of the basal effects of ceramide was not investigated in this thesis, but it is another area worthy of attention.

Stress, diabetes and metabolic syndrome

Ceramide is clearly an important regulator of metabolic signalling in cells. It is believed that ceramide responds to cytokines which increase SMase activity to raise ceramide production. Therefore ceramide is a response to stress situations (Mathias et al, 1998). Homeostatic responses to stress are going to require many adaptive changes by cells, and this thesis has provided evidence that ceramide affects the insulin signalling pathway to produce an acute suppression of diverse effects of insulin on glucose uptake in muscle and NO production in endothelium. Stress also causes cell death, and ceramide appears to facilitate to this process. The present studies showed that inhibiting ceramide production with Fb1 tended to increase viability whereas increasing ceramide with palmitate tended to reduce viability. Each of these observations supports the general literature suggesting that ceramide is a mediator of insulin resistance and reduced cell viability. Therefore ceramide synthesis pathway could be a possible drug target in order to reduce some of the detrimental effects of diabetes.

Insulin resistance is a pathogenic influence that contributes to several cardiovascular risk factors that are collectively referred to as the metabolic syndrome (section1.6). These factors include abdominal adiposity, raised blood pressure, dyslipidaemia a prothrombotic state as well as hyperglycaemia.

All of these factors contribute to atherosclerosis and cardiovascular disease. Excess glucose and lipid levels, along with raised inflammatory cytokines and reactive oxygen species are all likely to cause stress to cells and are therefore likely to involve ceramide (Summers and Nelson, 2005). Nelson and Summers (2005) proposed that various stress factors increased ceramide, and increased ceramide acted on a wide range of tissues to cause insulin resistance. This thesis has produced evidence that supports this proposal.

Conclusion

Taking all aspects in consideration, the present study shows parallel issues in insulin signalling between diverse tissues and diverse effects of insulin. Ceramide reduced insulin-stimulated glucose uptake in muscle cells and insulin-stimulated NO production by endothelial cells. Thus ceramide could be a potential generalized mediator of insulin resistance.

Thus ceramide might be a potential therapeutic target and finding selected inhibitors of ceramide could be a new direction of medicine research to address insulin résistance.

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Appendix 1

Krebs-Ringer buffer solution

Adapted from:

Krebs, H.A.(1950). Body size tissue Respiration. Biochemica et Biophysica Acta. Vol. 4, pp 249-269.

Compound	Molecular Weight (g)	g/ liter	Required Molarity (mM)
NaCl	58.44	6.902	118.00
KCl	75.56	0.354	5.00
$NaHCO_3$	84.01	2.100	25.00
$MgSO_47H_2O$	246.48	0.290	1.18
KH_2PO_4	136.09	0.324	1.17

This solution was pre-gassed using 5%CO_2; 95%O_2 for approximately 20 minutes.

$CaCl_2$ was added from pre-prepared stock solution for 1.27M. This was added as 1µl/ml of buffer solution.

Nitric Oxide determination (Griess reagent)

The Griess reagent was obtained from Sigma. It comprises N-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilic acid in 5% H3PO4, and nitrate reductase.

MTT assay

The MTT assay (using Thiazdyl Blue Tetrazolium) was obtained from Sigma. The reaction was undertaken using MTT stock at 50mg in 10 ml PBS solution, filtered once to make a working MTT solution and stored in dark at 4°C. For experiments, the working solution was diluted 1 ml MTT into 9 ml PBS to be used.

DMEM medium for cultured L6 cells

DMEM (Dulbecco's Modification of Eagle's Medium) was obtained as 500ml sterile 1× solutions (1x dilution in this context means undiluted) containing 0.11g/l sodium pyruvate. DMEM was supplemented with antibiotic/antimycotic solution at a final concentration of 100 units/ml penicillin G sodium, 10mg/ml streptomycin sulphate, 25µg/ml amphotericin B as fungizone and 1 mM glutamate, and 5% foetal calf serum (FCS).

DMEM medium for EAHY cells

DMEM was obtained as 500ml sterile solutions containing 0.11g/l sodium pyruvate. DMEM was supplemented with antibiotic/antimycotic solution containing 100 units/ml penicillin G sodium, 10mg/ml streptomycin sulphate, 25µg/ml amphotericin B as fungizone, H.A.T. (x50) (50x dilution in this text means 10 ml per 500 ml of

DMEM) and 10% foetal calf serum (FCS).

Hank's Buffered Salt Solution (HBSS)

HBSS is an Intelligent Transport Solutions Company.Established in 1996 with its headquarters at North Andover, MA, HBSS has chartered itself to provide solutions built around state-of-the-art technology for Regional Transport Authorities in specific transportation industry in general. All solutions should be stored at 4°C.

To make HBSS solution, there are 7 steps to be done:

Step1: *Dissolve 8.0g NaCl, 0.4g KCl into 100 ml distilled water to make* ***stock1***;

Step2: *Dissolve 0.358 g Na_2HPO_4 (anhydrous), 0.60 g KH_2PO_4 into 90 ml of distilled Water to make a* ***stock 2***;

Step3: *Dissolve 0.72g $CaCl_2$ into 50 ml distilled water to make* ***stock 3***;

Step4: *Add 1.23 g $MgSO_4.7H_2O$ to 50 ml of distilled water to make* ***stock 4***;

Step5: *Add 0.35 g $NaHCO_3$ to 10ml of distilled water to make* ***stock 5***;

Step6: *Combine the solutions in following in order: 10.0 ml stock 1; 1.0 ml stock 2; 1.0 ml stock 3; 86.0 ml distilled H_2O; 1.0 ml stock4 to make a* ***Hank's Premix***;

Step7: *9.9 ml Hank's Premix and 0.1 ml stock 5 to make a final working solution.*