MECHANISMS OF ACTION OF ROSIGLITAZONE IN THE PROTECTION OF PANCREATIC BETA CELLS FROM FREE FATTY ACID INDUCED DAMAGE

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Abstract

A wealth of recent evidence implicates increasing obesity levels in the rising global epidemic of type 2 diabetes. At a cellular level, high fatty acid concentrations contribute to increasing β-cell dysfunction and eventual β-cell failure. We have shown that the thiazolidinedione (TZD) rosiglitazone has direct effects on pancreatic β-cell function. The aim of this study was to determine the cell signalling molecules mediating these effects, and to test the hypothesis that rosiglitazone can protect pancreatic β-cells from the detrimental effects of free fatty acids. Utilising the mouse β-cell line MIN6, MTT assays showed that at 5 mM glucose concentrations, addition of 0.4 mM palmitate for 72 hours resulted in a 60% loss of MIN6 cell viability (P<0.01). However, the presence of 10 µM rosiglitazone completely abolished the effect of palmitate. To determine the cell signalling pathways involved, MIN6 cells were stimulated with 10 µM rosiglitazone for 2 hours and whole cell extracts analysed. Western blot analysis indicated that total levels of the stress activated p38 MAPK were reduced by over 50% (P<0.05) in the presence of rosiglitazone. Rosiglitazone stimulated phosphorylation of ACC (P<0.01), an effect inhibited by compound C, indicating that rosiglitazone activates AMPK in MIN6 cells over 2 hours. Finally, casein kinase 2 (CK2) activity assays indicated that rosiglitazone stimulates a 3-fold increase in CK2 activity (P<0.01). This activation was inhibited by compound C, indicating that CK2 lies downstream of AMPK in the signalling pathway stimulated by rosiglitazone. In conclusion, this study has shown that rosiglitazone modulates p38, AMPK and CK2 signalling in MIN6 cells, and can protect MIN6 cells from the detrimental effects of palmitate. Our results indicate that early rosiglitazone administration in obese patients at risk of Type 2 diabetes could well protect and preserve β-cell mass and function.
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Abbreviations

ACC – acetyl co-A carboxylase
AICAR – 5-aminoimidazole-4-carboxamide riboside
AMPK – adenosine monophosphate activated protein kinase
ANOVA – analysis of variance
ATP – adenosine triphosphate
Bcl2 – B-cell lymphoma 2
BMI – body mass index
BSA – bovine serum albumin
CK2 – casein kinase 2
CPT1 – carnitine palmitoyl transferase 1
DAG – diacylglycerol
DAPI – 4’,6-diamidino-2-phenylindole
DGAT – diacylglycerol acyltransferase
DMEM – Dulbecco’s modified Eagle’s medium
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
DPP-IV – dipeptidyl peptidase IV
DTT – dithiothreitol
ECL – enhanced chemiluminescence
EDTA – ethylenediaminetetraacetic acid
EGTA – ethylene glycol tetraacetic acid
eNOS – endothelial nitric oxide synthase
ER – endoplasmic reticulum
FBS – foetal bovine serum
FFA – free fatty acids
FOXA2 – forkhead homeobox A2
FOXO1 – forkhead homeobox O1
GAPDH – glyceraldehyde-3-phosphate dehydrogenase
GLP-1 – glucagon-like peptide 1
GLUT2 – glucose transporter 2
GPR40 – G protein coupled receptor 40
GSIS – glucose-stimulated insulin secretion
HbA1C – glycated haemoglobin
HNF – hepatocyte nuclear factor
HOMA – homeostatic model assessment
HRP – horseradish peroxidase
HUVEC – human umbilical vein endothelial cells
IFNγ – interferon gamma
IL-1β – interleukin 1β
IL-6 – interleukin 6
iNOS – inducible nitric oxide synthase
IRS – insulin receptor substrate
JNK – c-Jun N-terminal kinase
LPS - lipopolysaccharide
MafA - musculoaponeurotic fibrosarcoma oncogene homolog A
MAPK – mitogen activated protein kinase
MODY – maturity onset diabetes of the young
mRNA – messenger ribonucleic acid
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH – nicotinamide adenine dinucleotide
NEFA – non-esterified fatty acids
NFκB – nuclear factor kappa B
NP-40 – Nonidet P-40
PBS – phosphate buffered saline
PDX1 – pancreatic/duodenal homeobox 1
PERK – protein kinase-like endoplasmic reticulum kinase
PI3K – phosphatidylinositol-3-kinase
PKB – protein kinase B
PML - promyelocytic leukemia
PPARγ – peroxisome proliferator activated receptor gamma
PPRE – peroxisome proliferator activated receptor response element
PTEN - phosphatase and tensin homolog
RNA – ribonucleic acid
ROS – reactive oxygen species
RT-PCR – reverse transcription polymerase chain reaction
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM – standard error of the mean
SGLT - sodium-glucose cotransporter
SREBP-1 – sterol regulatory element binding protein-1
TAG – triacylglycerol
TBB – 4,5,6,7-tetrabromobenzotriazole
TEM – transmission electron microscopy
TNFα – tumour necrosis factor alpha
TZD - thiazolidinedione
UCP2 – uncoupling protein 2
VEGF – vascular endothelial growth factor
1. Introduction

1.1 The pancreas

The pancreas is a multi-functioning organ made up primarily of two parts, the exocrine pancreas which secretes digestive enzymes into the duodenum and the endocrine pancreas which produces and secretes hormones. The endocrine pancreas is made up of the islets of Langerhans, which are clusters of cells that can be divided into five types: α-cells, β-cells, δ-cells, PP-cells and ε-cells (Elayat et al., 1995; Andralojc et al., 2009).

The α-cells produce glucagon, a hormone which is secreted in response to low blood glucose levels, and signals to target tissues to break down glycogen stores and stimulate gluconeogenesis to maintain normal blood glucose (Matsuda et al., 2002). The β-cells produce insulin, which is secreted in response to increased blood glucose levels and increases glucose uptake from the blood into target tissues where it can be utilised, stored as glycogen or converted to fatty acids. Insulin also inhibits gluconeogenesis in the liver. As the brain can only utilise glucose for energy, it is vital that blood glucose is tightly regulated. The δ-cells secrete somatostatin, which has a wide range of functions throughout the body, mediated by 5 different receptor subtypes (Portela-Gomes et al., 2000). The PP-cells secrete pancreatic polypeptide, and the ε-cells secrete ghrelin (Andralojc et al., 2009).

1.2 Diabetes mellitus

Diabetes mellitus is a disorder referring to either a total or relative lack of insulin, which results in an inability to regulate blood glucose levels. Less glucose is taken up from the blood and glucose and fatty acids are released into the blood stream as a result of increased lypolysis in adipocytes as the body is unable to utilise the glucose ingested from meals, and the lack of insulin signalling is misinterpreted as a starvation signal.
(Souza et al., 1998). As a result, blood glucose is poorly controlled and the large fluctuations can be deadly. In the short term both hyperglycaemia, which can result in ketoacidosis, and severe hypoglycaemia can lead to coma or even death. In the longer term, chronic hyperglycaemia can result in complications later in life. It is therefore vital to help patients with diabetes to maintain good glucose homeostasis. There are many complications that can arise in diabetic patients such as neuropathy, nephropathy and retinopathy, and improving glycaemic control can help prevent or delay the onset of these complications (Molyneaux et al., 1998; Meeme and Kasozi, 2009).

Diabetes is estimated to affect over 171 million people worldwide, a figure which is predicted to rise to 366 million by 2030 (Wild et al., 2004). Individuals with impaired glucose tolerance have an increased risk of developing type 2 diabetes, in addition to complications in the absence of diabetes. In the UK, treating diabetes and its complications costs the NHS £9 billion a year, which equates to 10% of its budget (The NHS Information Centre, 2010). The cost of diabetes is not just to the individual, but to the economy, particularly if current trends of increasing prevalence continue as predicted.

Diabetes mellitus is divided into two major types: type 1 and type 2. Type 1 diabetes, sometimes referred to as insulin-dependent diabetes mellitus, usually presents in children or young adults, and arises from a complete loss of β-cells in the pancreas. Type 2 diabetes is also referred to as non insulin-dependent diabetes or maturity onset diabetes, although these terms can be misleading due to the increasing frequency of type 2 diabetes occurring in younger people, and the fact that many people with type 2 diabetes will eventually require regular insulin injections. In type 2 diabetes the β-cells are still present, although this may be at a reduced mass, but they are unable to secrete sufficient insulin to lower blood glucose to within the normal range.
1.3 Type 1 diabetes

Type 1 diabetes is an autoimmune disease where the β-cells of the pancreas are specifically targeted and destroyed by T cells, thus leaving the pancreas unable to produce or secrete insulin. The cause of type 1 diabetes is unclear, although genetics appear to play an important role, and more recently viral infection has been implicated (Richardson et al., 2009). Symptoms develop quickly, and treatment invariably involves administration of exogenous insulin.

Patients with type 1 diabetes currently require multiple injections of insulin every day, to enable the body to utilise glucose from the food consumed. Alternatives to insulin injections have been explored with limited success, such as inhaled insulin (Garg et al., 2009) and gene therapy (Oh et al., 2006).

Insulin pumps, where a constant supply of insulin is administered subcutaneously, and a controlled bolus of insulin delivered before a meal, can reduce the need for several injections daily, and can be useful in helping patients gain better control of their blood glucose levels (Thrailkill et al., 2011). Attempts are being made to link insulin pumps to continuous blood glucose monitoring, so the pump releases insulin in response to blood glucose, forming a closed-loop system and effectively making an artificial pancreas (Bruttomesso et al., 2009).

One of the most promising treatments for type 1 diabetes is islet transplantation, where islets are isolated from donor pancreases and transplanted into the hepatic portal vein of the patient. To date, islet transplants have had limited success. In trials, few recipients have remained off insulin within 5 years (Bellin et al., 2008; Girman and Saudek, 2011). Drawbacks that need to be addressed are the shortage of available islets, and the poor survival rate of functioning islets post-transplantation. As with all organ transplants, a shortage of donors is a major problem. With islet transplantation, it is only the β-cells that are required, as opposed to a complete complex organ; therefore the
potential for using stem cells as a source for new β-cells is being explored (Neshati et al., 2010; Wang et al., 2011). Once transplanted, islets face assault from immune-suppressive drugs, which impair normal β-cell function, including insulin secretion, as well as inducing apoptosis (Johnson et al., 2009). It has been found that inhibition of p38 mitogen activated protein kinase (MAPK) can enhance human islet graft survival in mice (Matsuda et al., 2005). This suggests the p38 pathway as a possible mechanism through which islet transplants in humans are failing.

Ensuring long term survival of transplanted islets and preservation of normal β-cell function, along with overcoming the issue of limited sources of β-cells, could prove to be a major advance in curing type 1 diabetes.

1.4 Type 2 diabetes

Type 2 diabetes is a metabolic disorder characterised by β-cell dysfunction and insulin resistance. The onset of symptoms in type 2 diabetes is typically slower than in type 1 diabetes, and as a result diagnosis can take much longer. It has been estimated that the prevalence of undiagnosed diabetes in 2001 in the UK was 6.7% in men and 6.0% in women, and the prevalence of impaired fasting glucose was approximately 20% (Thomas et al., 2005). Early diagnosis and intervention is important as it can prevent further deterioration of β-cell mass and function and the occurrence of complications that arise from poorly regulated blood glucose (Uusitupa, 1996).

Type 2 diabetes occurs as a result of a total or relative lack of insulin due to β-cell dysfunction. Insulin resistance is a common feature in individuals with obesity, although in most cases this is compensated for by increased insulin secretion, maintaining normal blood glucose. When the β-cells of the pancreas can no longer secrete sufficient insulin, type 2 diabetes ensues (Shimabukuro et al., 1998b; Weyer et al., 1999; Prentki and Nolan, 2006).
Insulin resistance often arises as a result of increased circulating free fatty acids (FFA) or cytokines, such as tumour necrosis factor α (TNF-α), which block the normal signal transduction of insulin in target tissues such as the liver and skeletal muscle (Peraldi et al., 1997; Reynoso et al., 2003; Liu et al., 2007). It has been suggested that insulin resistance may also play a role in β-cell dysfunction. In the event of insulin resistance, plasma free fatty acid levels increase as a reduction in insulin signalling in adipocytes increases the rate of lipolysis and the secretion of fatty acids into the bloodstream (Jensen et al., 1989). The detrimental effects of hyperglycaemia and hyperlipidaemia on β-cells are well documented, and both have been shown to impair glucose-stimulated insulin secretion and increase apoptosis (Zhou and Grill, 1995; Lupi et al., 2002; Maedler et al., 2002; Dubois et al., 2004; Dubois et al., 2007). Besides an increase in circulating free fatty acids and glucose, obesity and insulin resistance also increase the levels of adipocyte derived factors such as TNF-α and resistin, which appear to contribute to β-cell dysfunction (Hotamisligil et al., 1995).

1.5 Insulin resistance

Insulin resistance can occur in various cell types, such as muscle and the liver, when insulin binding to the insulin receptor no longer elicits the usual response for that cell. It is usually caused by interference with the insulin signal transduction pathway, often following chronic exposure to elevated FFA or cytokines (Li et al., 2007; Liu et al., 2007).

In healthy cells, insulin binds to the insulin receptor, resulting in autophosphorylation of the receptor, and activation of one of the insulin receptor substrates (IRS-1 and IRS-2). This leads to activation of the phosphotidylinositol-3-kinase (PI3K) pathway. In muscle and liver cells this causes an increase in glucose uptake from the blood and glycogen synthesis. In the liver, this also decreases how
much glucose is given out by reducing glycogenolysis and gluconeogenesis. In adipocytes, glucose is taken up and stored as fat.

In obesity, plasma FFA and cytokines are frequently elevated. Saturated fatty acids are overwhelmingly implicated in causing insulin resistance, whereas unsaturated fatty acids have been found to cause insulin resistance in some cell types *in vitro* (Liu *et al.*, 2007; Ragheb *et al.*, 2009), but there is some evidence to suggest that plasma levels of unsaturated fatty acids correlate positively with insulin sensitivity. There is a strong correlation between muscle triglyceride content and insulin resistance (Morino *et al.*, 2005), although it is unclear which is the causative factor. *In vitro* studies have shown that fat accumulation within myocytes can interfere with normal insulin signalling, whereas *in vivo* insulin resistance may cause an increase in FFA uptake into muscle cells due to the reduced glucose transport.

FFA, cytokines and hydrogen peroxide have been shown to cause phosphorylation of the serine 307 residue on the insulin receptor, which blocks its activation by phosphorylation at the tyrosine residue (Bloch-Damti *et al.*, 2006; Li *et al.*, 2007). The equivalent serine 312 of IRS1 in humans has been shown to be hyperphosphorylated in the muscle of insulin resistant individuals (Morino *et al.*, 2005). In the whole organism this means that it requires much higher concentrations of insulin to activate the PI3K pathway and thus promote uptake of glucose from the blood. In most individuals with insulin resistance the β-cells can compensate for peripheral insulin resistance and react to the sustained high glucose concentration by secreting more insulin. In some people this process can go on indefinitely and in the long term may lead to increased β-cell mass to meet the increased demand for insulin.

Identifying patients in this phase of compensation and treating the insulin resistance could prevent many cases of type 2 diabetes and reduce the risk of
complications, although most do not present with any symptoms until the β-cells can no longer secrete sufficient insulin and they can no longer regulate blood glucose.

1.6 The β-cell

In the endocrine pancreas, β-cells are highly specialised cells that sense circulating glucose levels, and secrete insulin accordingly to maintain normal blood glucose levels. In type 1 diabetes, the β-cells are specifically targeted by the immune system and destroyed, whilst other islet cell types remain intact, causing a total inability to produce or secrete insulin. In type 2 diabetes, β-cells are still present but are unable to secrete sufficient insulin.

Glucose is taken up into the β-cell via the glucose transporter GLUT2, and through oxidative phosphorylation, ATP is produced. The increase in ATP in the cell results in closure of the ATP-dependent K⁺ channel and membrane depolarisation. This causes voltage gated Ca²⁺ channels to open, which leads to an increase in insulin granule docking at the membrane and subsequent insulin secretion.

Although GLUT2 is required for glucose to be taken up into the β-cells, it has been shown that GLUT2 is not rate limiting in GSIS, as overexpression of GLUT2 in MIN6 cells does not enhance GSIS, whereas glucokinase activity closely relates to the glucose sensitivity of the cell (Ishihara et al., 1995). Glucokinase has a high K_m, meaning that the level of glucose-6-phosphate is closely linked to the extracellular glucose concentration.

In a state of insulin resistance, the β-cell is normally able to compensate and secrete more insulin. However, some of the factors that cause insulin resistance, such as increased free fatty acids and cytokines, are also detrimental to β-cell function and can result in β-cell death. Chronic exposure to free fatty acids results in a decrease in GSIS,
Glucose is transported into the β-cell by GLUT2, where it undergoes glycolysis to produce pyruvate, which is decarboxylated by pyruvate dehydrogenase to form acetyl co-A. Acetyl co-A then enters the tricarboxylic acid cycle, producing NADH, which then undergoes oxidative phosphorylation, leading to an increase in the ATP/ADP ratio. This causes the ATP-dependent K\(^+\) channel to close, and an accumulation of potassium ions outside the cell. The membrane depolarisation results in the opening of the voltage gated Ca\(^{2+}\) channel, and an influx of calcium ions into the β-cell. This causes the insulin containing granules to dock with the cell membrane, and insulin is secreted from the β-cell.

**Figure 1.1 Glucose stimulated insulin secretion in the pancreatic β-cell**

Glucose is transported into the β-cell by GLUT2, where it undergoes glycolysis to produce pyruvate, which is decarboxylated by pyruvate dehydrogenase to form acetyl co-A. Acetyl co-A then enters the tricarboxylic acid cycle, producing NADH, which then undergoes oxidative phosphorylation, leading to an increase in the ATP/ADP ratio. This causes the ATP-dependent K\(^+\) channel to close, and an accumulation of potassium ions outside the cell. The membrane depolarisation results in the opening of the voltage gated Ca\(^{2+}\) channel, and an influx of calcium ions into the β-cell. This causes the insulin containing granules to dock with the cell membrane, and insulin is secreted from the β-cell.
possibly by increasing uncoupling protein 2 (UCP2) levels, resulting in reduced glucose disposal, exacerbating the problem further.

The term glucolipotoxicity is used to describe the combined effects of chronic hyperglycaemia and hyperlipidaemia on the β-cell (El-Assaad et al., 2003). Glucose and free fatty acids alone may have detrimental effects on the β-cell, but together they have been shown to act synergistically, with high glucose concentrations enhancing free fatty acid cytotoxicity. This is highly relevant to the study of type 2 diabetes, as in patients with elevated circulating free fatty acids, such as in obesity, failure to maintain normal glucose concentrations may result in increased β-cell death, and a further decrease in glucose homeostasis.

β-cell dysfunction ultimately marks the onset of type 2 diabetes, so the treatment of this disease needs to be aimed at either early intervention in those with insulin resistance, to prevent β-cell failure, or treatments which protect the β-cell from damage. Sulfonylureas, which target insulin secretion directly, can help patients regulate their blood glucose, but cannot ultimately protect against β-cell failure (Smith et al., 2004).

A large network of transcription factors are important in β-cell development and function. Mutations in some of these genes have been found to cause diabetes. The Maturity Onset Diabetes of the Young (MODY) class of monogenic diabetes includes several hepatocyte nuclear factors (HNFs) and pancreatic/duodenal homeobox 1 (PDX1), as well as glucokinase. All these genes have a role in GSIS, and a mutation in any of them causes diabetes (Winckler et al., 2007). Exposure of β-cells to cytokines or free fatty acids in vitro can lead to downregulation of some of these genes. The direct effects of free fatty acids on β-cell transcription factors could impair insulin secretion, and may play a role in β-cell dysfunction.

MafA is a transcription factor that binds to the RIPE3b/C1-A2 site of the insulin promoter, and has functions in regulating GSIS. Palmitate has been shown to decrease
MafA expression, whereas inhibiting p38 with SB203580 increases the stability of MafA protein (Kondo et al., 2009).

Preceding the onset of type 2 diabetes, the increased demand for insulin is met through either increasing the secretory capacity of the β-cells, or by increasing β-cell mass. Post mortem examination of insulin resistant individuals who had not developed diabetes shows an increase in islet mass and it is believed to be caused by an increase in β-cell proliferation. Islets deficient in IRS2 have a reduced β-cell and α-cell mass (Cantley et al., 2007), and are less able to cope with the increased demand associated with peripheral insulin resistance, suggesting that insulin may be an important growth factor mediating β-cell compensation. Sustained hyperinsulinaemia, as in insulin resistance, may have a feedback effect on the β-cell, resulting in β-cell expansion and enabling a longer term solution to compensate for insulin resistance.

It has been suggested that β-cells have a limited number of replications, and that increased proliferation in response to insulin resistance leads to premature cell senescence, and ultimately the onset of diabetes (Sone and Kagawa, 2005).

### 1.7 The obesity epidemic

A growing tendency for calorie dense foods and inactive lifestyles has resulted in a global obesity epidemic. Once afflicting the rich, being overweight or obese is becoming increasingly common, including in developing countries, where obesity and malnutrition can exist in the same community. In 2005, 23.2% of the world’s adult population was overweight (937 million people) and 9.8% was obese (396 million people). It is predicted that in 2030, the number of overweight adults will rise to 1.35 billion, and the number of obese adults will rise to 573 million (Kelly et al., 2008).

Although genetic factors can influence the onset of type 2 diabetes, obesity remains the biggest preventable cause, and the increased prevalence of childhood
obesity is being blamed for the emergence of type 2 diabetes developing in adolescents and even children. Abdominal circumference is potentially a more accurate predictor for diabetes risk than body mass index (BMI) alone, as it is widely accepted that visceral fat storage is more detrimental to health than subcutaneous fat storage (Kissebah et al., 1982; Enzi et al., 1988). Adipocytes have evolved as an efficient energy store in the form of lipids within the body, for times of starvation. When fat accumulates in other cell types it can impair their normal function.

In ob/ob mice, a mouse model of obesity-induced diabetes where the gene encoding leptin is mutated, overexpressing adiponectin caused significant weight gain compared with non-transgenic ob/ob mice (Kim et al., 2007c). This was due to expansion of fat mass through an increased number of adipocytes that were smaller in size, and a reduction in triglycerides stored in the liver and muscle, compared with ob/ob mice that did not overexpress adiponectin. Overexpression of adiponectin also resulted in improved insulin sensitivity and reductions in fasting serum glucose, insulin and triglyceride levels, preventing the diabetic phenotype observed in normal ob/ob mice. This study highlights that it is not simply excess body weight that is the risk factor for developing diabetes, but that where fat is stored in the body plays an important role.

In obese individuals, higher circulating free fatty acids and cytokines interfere with the normal signal transduction of insulin in its target tissues, leading to insulin resistance (Hotamisligil et al., 1995). In addition to the increased demand for insulin production and secretion, the β-cells of the pancreas must contend with the direct detrimental effects of obesity. Chronic exposure to elevated free fatty acids leads to a decrease in insulin processing, and impaired glucose stimulated insulin secretion as well as apoptosis (Joseph et al., 2004; Lupi et al., 2004).
1.8 Saturated versus unsaturated fat

Saturated and unsaturated fats in the human diet have differing effects, with mono-unsaturated and poly-unsaturated fats not only being less harmful but may also prove beneficial (Paniagua et al., 2007). It has long been known that those living in Mediterranean countries had a longer lifespan compared with those living in northern Europe. This has been attributed to the ratio of unsaturated to saturated fat present in the diet. Besides the fresh vegetables which make up a large portion of each meal, the Mediterranean diet is based on fish, as opposed to meats likely to have higher saturated fat content, and much of the added fat comes from olive oil, high in mono-unsaturated fatty acids.

Acute exposure to free fatty acids, both *in vitro* and *in vivo* has been shown to enhance glucose-stimulated insulin secretion. There have been several studies showing GPR40, a G protein coupled receptor which responds to medium and long chain fatty acids, to be the mechanism through which this occurs (Briscoe et al., 2003; Itoh et al., 2003; Fujiwara et al., 2005). The evidence for GPR40 exerting the longer term effects of free fatty acids is limited, with the uptake of fatty acids rather than action at a cell surface receptor more probable, although the involvement of an as yet unidentified fatty acid receptor cannot be ruled out.

*In vitro* experiments have consistently shown that chronic exposure to saturated fatty acids, such as palmitate, leads to impaired cell function and apoptosis. In the pancreatic β-cell, palmitate has been shown to decrease insulin processing and reduce glucose-stimulated insulin secretion, as well as impacting on crucial β-cell transcription factors, and inducing apoptosis (Lupi et al., 2002; Oprescu et al., 2007; Meidute Abaraviciene et al., 2008; Sun et al., 2008).

The effect of unsaturated fatty acids is subject to debate. While some studies have found that they are also detrimental to cells, others suggest a role in protecting
against the damage caused by saturated fatty acids. Unsaturated fatty acids have been implicated in inducing insulin resistance, ER stress and apoptosis, as well as impaired glucose-stimulated insulin secretion in β-cells (Frigerio et al., 2006; Martinez et al., 2008). However, in a variety of cell types unsaturated free fatty acids can protect against saturated fatty acids (Welters et al., 2004b; Coll et al., 2008).

The difference in toxicity between saturated fatty acids and unsaturated fatty acids is thought to be due to the relative ease with which unsaturated fatty acids can be stored as triacylglycerol, compared with saturated fatty acids (Cnop et al., 2001). Palmitate is favoured to form diacylglycerol, reactive oxygen species, or ceramide, which can induce apoptosis, and rarely forms triacylglycerol (Shimabukuro et al., 1998b). Palmitate is also favoured above oleate to undergo β-oxidation, a by-product of which is acylcarnitines and implicated in insulin resistance (Gavino et al., 2003). Although storage of fat in non-adipocytes is not ideal, it may be less harmful than alternative metabolic fates for fatty acids.

In the instances of unsaturated fat protecting against saturated fat, it is proposed that saturated fatty acids are more likely to be incorporated into triacylglycerol in the presence of unsaturated fatty acids than saturated fat alone, allowing inert storage of fat and a reduction in saturated fatty acids available for ceramide production or β-oxidation (Listenberger et al., 2003). However, some studies have found that the protective effects of unsaturated fatty acids are not dependent on incorporation of saturated fatty acids into triacylglycerol. Treatment of BRIN-BD11 β-cells with palmitate led to activation of the PERK-dependent ER stress pathway, which was prevented by the addition of the mono-unsaturated fatty acid palmitoleate (Diakogiannaki et al., 2008). The antagonistic effect of palmitoleate on the ER stress response to agents other than saturated fatty acids implies a broader role for unsaturated fatty acids in the protection of cell viability.
Figure 1.2 The different fates of palmitate and oleate once taken up by the β-cell

This diagram illustrates the difference in storage and metabolism of palmitate and oleate in the β-cell. While the monounsaturated fatty acid oleate is favoured for storage as triacylglycerol, the saturated fatty acid palmitate has a tendency to form diacylglycerol, ceramide or reactive oxygen species. It is also favoured above oleate to undergo β-oxidation in the mitochondria. Transport of fatty acyl Co-A into the mitochondria by CPT1 is inhibited by ACC. An increase in glucose concentration leads to an inhibition of CPT1, suggesting how glucose can interfere with fatty acid oxidation.
Palmitate and oleate are two of the most common fatty acids used for *in vitro* studies investigating differences in the effects of saturated and unsaturated fat as they make up a large portion of the free fatty acids found in our diet.

1.9 Palmitate

Palmitate is one of the most common saturated fatty acids found in human diet. It has a 16 carbon chain, and is the first fatty acid made in lipogenesis. *In vitro* studies show palmitate treatment leads to insulin resistance in numerous cell types (Reynoso *et al.*, 2003; Coll *et al.*, 2008; Ragheb *et al.*, 2009). It is thought that an increase in phosphorylation of the serine residue of insulin receptor substrate 1 in response to palmitate inhibits phosphorylation on the tyrosine residue, which is required for normal insulin signal transduction. Inhibition of protein kinase C was found to prevent the palmitate-induced reduction of tyrosine phosphorylation (Reynoso *et al.*, 2003).

Palmitate has also been shown to increase levels of phosphorylated p38 and JNK in a variety of cell types, leading to insulin resistance, ER stress (Guo *et al.*, 2007) and apoptosis. There is also evidence for activation of NFκB (Saitoh *et al.*, 2008), caspase 3, and decreases in the level of the anti-apoptotic protein Bcl-2 (Lupi *et al.*, 2002) as potential mechanisms mediating palmitate-induced apoptosis.

In β-cells, palmitate can impair glucose-stimulated insulin secretion and induce apoptosis. The effect of chronic palmitate treatment on apoptosis has been associated with activation of p38 and caspase 3, which was blocked by the presence of rosiglitazone (Abaraviciene *et al.*, 2008).

The use of the CPT1 inhibitor etomoxir was found to augment β-cell apoptosis in response to palmitate by blocking β-oxidation whilst inhibiting the formation of long chain fatty acyl-coenzyme A from palmitate was found to inhibit β-cell death (El-Assaad *et al.*, 2003). Blocking fatty acyl-coA synthesis with triascin C prevented the
fatty acid induced decrease in Bcl2 expression in isolated islets (Shimabukuro et al., 1998a). It has also been found that blocking the formation of ceramide upon treatment with palmitate can protect the cells from apoptosis (Shimabukuro et al., 1998b). This suggests that it is the intermediates formed in fatty acid metabolism that are cytotoxic, as opposed to the fatty acids themselves. This could also be the mechanism through which elevated glucose concentrations enhance palmitate-induced apoptosis, as high glucose inhibits AMPK, which leads to a decrease in fatty acid oxidation.

There is no clear mechanism through which chronic palmitate treatment impairs insulin secretion, as different studies have found different pathways to be crucial in exerting these effects. Exposure of pancreatic islets to palmitate caused an upregulation of UCP2 expression, increased basal insulin secretion and decreased glucose-stimulated insulin secretion, all of which were blocked by the addition of rosiglitazone (Tian et al., 2006). In islets from mice where UCP2 was knocked out, there was enhanced GSIS, and they were protected from the effects of palmitate (Joseph et al., 2004). However, more recently, the effect of palmitate on insulin secretion has been linked to the dissociation of Ca^{2+} channels from insulin secretory granules (Hoppa et al., 2009). Palmitate has also been shown to decrease expression of IRS2, and inhibit GSIS in isolated islets, although this was not observed in islets isolated from SREBP-1-null mice (Kato et al., 2008).

A wide range of mechanisms have been implicated in exerting the effects of palmitate on β-cells, and blocking these pathways may play a vital role in the preventing the onset of type 2 diabetes in obese individuals.

1.10 Oleate

Oleate is a common mono-unsaturated fatty acid with 18 carbons and a double bond between C9 and C10. It is found in olive oil, and makes up a large part of dietary fat intake in those following a Mediterranean diet. While most studies investigating the
Palmitic acid

![Structure of Palmitic Acid]

Oleic acid

![Structure of Oleic Acid]

Figure 1.3 Structure of palmitic acid and oleic acid

Palmitic acid is a 16 carbon chain, with all carbons saturated. Oleic acid is an 18 carbon chain with a cis-double bond between carbons 9 and 10.
direct effects of oleate on pancreatic β-cells have focused on cell viability, the molecular mechanisms remain unclear. In a variety of cell types it has been shown that oleate can activate p38 (Liu et al., 2007; Ragheb et al., 2009) and can cause ER stress (Kharroubi et al., 2004; Su et al., 2009). While some studies have found that oleate can cause insulin resistance, others have shown that oleate can prevent palmitate-induced insulin resistance in skeletal muscle cells (Coll et al., 2008).

In NIT-1 cells, a mouse β-cell line, knockdown of GPR40 using siRNA partially blocked the ability of oleate to protect against palmitate-induced apoptosis (Zhang et al., 2007). In Chinese Hamster Ovary cells, treatment with oleate led to an increase in triglyceride accumulation, whereas palmitate treatment did not and led to apoptosis. When cells were treated with both palmitate and oleate, palmitate was channelled into triglyceride stores and reduced apoptosis (Listenberger et al., 2003). Impairing triglyceride synthesis in these cells increased apoptosis in response to oleate, comparable to that seen with palmitate, suggesting that inert storage of fatty acids is responsible for the differences seen in toxicity.

Although there is much evidence to support that channelling of saturated fatty acids into inert lipid storage by oleate can protect the cell, oleate, and another mono-unsaturated fatty acid palmitoleate, have been found to prevent apoptosis in BRIN-BD11 cells in response to serum withdrawal and cytokines (Welters et al., 2004b). This suggests a much more general anti-apoptotic role for unsaturated fatty acids.

1.11 Lifestyle intervention in the treatment of type 2 diabetes

In many cases, type 2 diabetes can be managed by diet and exercise and individuals who are overweight will often benefit from losing weight, as this facilitates improvements in insulin resistance and blood glucose regulation, as well as reducing the risk of diabetes complications. As the β-cells still produce and secrete some insulin in type 2 diabetes,
improvements in insulin resistance may reduce insulin demand sufficiently enough for the β-cells to cope. In addition to improvements in insulin sensitivity in response to weight loss, β-cell function is also improved (Solomon et al., 2010).

A variety of diets have been claimed to be beneficial to people with type 2 diabetes, with the focus on preventing large fluctuations of blood glucose. Typically, patients are advised to refrain from eating foods with a large sugar content, favouring the consumption of complex carbohydrates, which take longer to be broken down into glucose, thereby preventing rapid increases in post-prandial blood glucose concentrations. Glycaemic Index refers to the speed at which carbohydrates are broken down into its constituent sugars. Foods with a high Glycaemic Index are broken down quicker than foods with a low Glycaemic Index, and thus cause a rapid increase in blood glucose.

Adherence to a Mediterranean diet has been associated with improved glycaemic control in patients with type 2 diabetes, with reductions reported in both postprandial glucose and HbA1C (Esposito et al., 2009b), and increased adiponectin concentrations (Mantzoros et al., 2006). It has also been associated with delaying the need for treatment with anti-diabetic drugs (Esposito et al., 2009a). A diet high in mono-unsaturated fat, such as from olive oil, may be more beneficial than a diet high in poly-unsaturated fat, with respect to blood pressure (Thomsen et al., 1995) and atherosclerosis risk (Madigan et al., 2000), which could be important in managing the risk of diabetic complications.

In type 2 diabetic patients, exercise has been shown to improve insulin sensitivity and glycaemic control, and also many parameters indicative of diabetic complications, such as triglycerides, cholesterol and inflammatory markers. In addition to facilitating weight loss, which can itself improve the diabetic condition, exercise provides other benefits in the treatment of diabetes. Muscle contraction activates AMPK
and results in increased translocation of GLUT4 to the membrane, leading to increased uptake of glucose. This occurs independently of PI3K, and therefore does not rely on normal insulin signalling (Lund et al., 1995). Type 2 diabetic patients do need to be wary of the increased risk of hypoglycaemia following exercise (Younk et al., 2011).

Through adjustments in diet, and with the incorporation of exercise, some patients with type 2 diabetes do not require any further interventions to control blood glucose, other than monitoring for complications associated with diabetes. However, many type 2 diabetic patients will require drug treatment or insulin therapy to control their blood glucose, when lifestyle changes alone are not sufficient to maintain normal glucose homeostasis.

**1.12 Pharmacological treatment of type 2 diabetes**

There are several different classes of drugs used in the treatment of type 2 diabetes, for patients who are unable to control blood glucose with diet and exercise alone.

Insulin secretagogues, such as sulfonylureas, act directly on the β-cell to increase insulin secretion (Gorus et al., 1988). Drugs such as glibenclamide act directly on the ATP-dependent K⁺ channel, causing closure and membrane depolarisation. This leads to the opening of voltage-gated Ca²⁺ channels, and the subsequent influx of calcium ions induces insulin secretion (Schmid-Antomarchi et al., 1987). However, sulfonylureas have been shown to induce β-cell apoptosis *in vitro* (Sawada et al., 2008), as well as impaired insulin secretory response, and may explain the relatively poor long term success rates of sulfonylureas in type 2 diabetic patients (Remedi and Nichols, 2008). As they induce insulin secretion independently of glucose concentration, there is an increased risk of hypoglycaemia (Fonseca et al., 2011).

Inhibitors of α-glucosidase, which target the enzymes that break down oligo- and disaccharides into monosaccharides in the villi of the small intestine, slow the rate
at which monosaccharides are absorbed into the blood, and allow some undigested carbohydrate to be excreted (Dimitriadis et al., 1985). This prevents rapid increases in blood glucose, and there is little risk of hypoglycaemia when used alone. However, side effects include bloating and flatulence, causing many patients to be dissatisfied with this treatment.

Insulin sensitizers act on normal insulin target tissues to improve the response to insulin, and thus reduce the demand on β-cells for insulin secretion. The most commonly prescribed insulin sensitizer is metformin, a biguanide. Early biguanides, such as phenformin and buformin have since been withdrawn due to increased incidences of lactic acidosis. Metformin is currently the only biguanide still prescribed for treatment of diabetes, and the risk of lactic acidosis is much lower than with other biguanides (Cavallo-Perin et al., 1989). It improves insulin resistance to allow an increase in glucose uptake and decrease glucose output from the liver (Perriello et al., 1994; Abbasi et al., 1998). It is secreted in the urine unmetabolised, therefore it is well tolerated in combination with other drugs. When it is prescribed in accordance with stringent guidelines, and patients monitored for signs of impaired renal function which can lead to accumulation of the drug in tissues, metformin has a good safety profile. It is therefore a popular choice in the treatment of type 2 diabetes.

Thiazolidinediones also improve insulin sensitivity, acting mainly on adipocytes, but are thought to also improve insulin sensitivity in the liver and skeletal muscle (Stumvoll, 2003). Although they are not intended to act directly on the β-cells, in vivo they can be associated with improved β-cell function (Gastaldelli et al., 2007), which is likely due to the reduction in circulating glucose concentrations, as chronic elevated glucose is known to be detrimental to β-cell viability and function. However, more recently in vitro investigations on isolated islets and β-cell lines highlight a potential for direct effects in β-cell for some of these insulin sensitizers (Lupi et al.,
2004; Richardson et al., 2006). Some of the side effects associated with thiazolidinediones are weight gain, oedema, headache, and upper respiratory tract infection.

Incretin-based therapy has recently been developed in the treatment of type 2 diabetes. Glucagon-like peptide 1 (GLP-1) exerts its anti-diabetic effects through multiple mechanisms, such as enhancing insulin secretion, decreasing glucagon secretion, increasing satiety and slowing gastric emptying (Perfetti and Merkel, 2000). As GLP-1 has a very short half-life, therapy involves either incretin mimetics such as Exenatide, which has the same effects as GLP-1 but has a longer half-life (Egan et al., 2002), or dipeptidyl peptidase-IV (DPP-IV) inhibitors, which slow the rate at which GLP-1 is broken down (Mari et al., 2005). Exenatide treatment has been associated with some weight loss, as well as side effects of nausea and possible hypoglycaemia. DPP-IV inhibitors have few side effects and do not appear to cause any changes in weight.

Other types of drugs are also being developed with a view to treating type 2 diabetes through novel mechanisms. Drugs that inhibit glucose renal reabsorption by acting on sodium-glucose co-transporters (SGLT), specifically SGLT2, can lower blood glucose levels by increasing the amount of glucose excreted in the urine (Kakinuma et al., 2010). Side effects of these drugs that have been noted in trials to date include nausea, diarrhoea, constipation and hypoglycaemia, and may also be associated with some weight loss, however, larger trials are needed to assess the safety of these drugs for the treatment of type 2 diabetes.

1.12.1 Metformin

Metformin is commonly prescribed to patients with type 2 diabetes and acts as an insulin sensitizer. It is commonly prescribed in patients alone or in combination with a sulfonylurea, often when sulfonylurea therapy alone has failed to achieve sufficient
blood glucose control. In the liver, it decreases gluconeogenesis and glycogenolysis. It can also act directly on the muscle to increase the uptake of glucose from the blood. It is thought that the beneficial effects of metformin occur through the inhibition if complex 1 in the mitochondrial respiratory chain, and through activation of AMPK (Turner et al., 2008).

In pancreatic β-cells, there is some evidence to suggest metformin may have direct beneficial effects. It has been shown to increase nuclear levels of PDX1 and FOXA2 under high glucose conditions (Richardson et al., 2006). Some studies have shown metformin is able to protect β-cells from the detrimental effects of free fatty acids. In islets isolated from type 2 diabetic subjects, those treated with 15 µM metformin had increased insulin content, improved glucose-stimulated insulin secretion and reduced rates of apoptosis compared with untreated islets (Marchetti et al., 2004b). However, in other studies it has been found to impair glucose-stimulated insulin secretion and induce apoptosis in β-cells through its activation of AMPK (Kefas et al., 2004), although the concentration used was much higher than physiological levels, and higher than the concentration used to show beneficial effects. Plasma concentrations of metformin have been shown to reach up to 40 µM, however, it is argued that metformin accumulates in tissues at concentrations of up to 200 µM. The studies finding negative effects of metformin on β-cells tended to use concentrations of between 0.5 mM and 2 mM, which exceeds the highest concentration found accumulated in tissues.

1.12.2 Rosiglitazone

Rosiglitazone, a thiazolidinedione, is a peroxisome proliferator activated receptor gamma (PPARγ) agonist used in the treatment of type 2 diabetes (Furnsinn and Waldhausl, 2002). It is often prescribed once other treatment options have failed. Through its actions on PPARγ, rosiglitazone improves insulin sensitivity. Targeting
tissues of glucose utilisation and storage, it increases insulin-dependent glucose uptake, decreases glucose output from the liver and alters adipocyte metabolism (Stumvoll, 2003).

PPARs are a family of nuclear hormone receptors, which are involved in the regulation of genes containing peroxisome proliferator response elements (PPRE). PPARγ is abundantly expressed in adipocytes, but is also expressed in β-cells (Welters et al., 2004a), although little is known about its function in β-cells. In adipocytes PPARγ is known to regulate genes involved in adipocyte differentiation, lipid storage and glucose metabolism. Through activation of PPARγ, rosiglitazone stimulates the differentiation of adipocytes (Reyes and Lazalde, 2007), causing the accumulation of a larger number of small adipocytes, which are more responsive to insulin than larger adipocytes. Its effects on adipocytes lead to improved fat storage and glucose utilisation, a reduction in the secretion of adipokines, and an increase in adiponectin. The resulting reduction in circulating free fatty acids and adipocyte derived cytokines can also improve insulin sensitivity in other tissues, such as the liver and muscle.

Improved fat storage in adipocytes, along with increased adiponectin secretion, may also result in decreasing the amount of lipid stored in other tissues, such as the liver, which in turn will lead to improved insulin sensitivity. Rosiglitazone treatment in a diabetic rat model increased subcutaneous adiposity, whilst decreasing fat in the muscle, liver and pancreas, reduced plasma FFA and improved insulin sensitivity (Kim et al., 2007b). This was associated with increased expression of perilipin, which is an important protein in lipid droplet formation, in subcutaneous adipose tissue. Overexpression of perilipin in INS-1 cells ameliorated palmitate toxicity (Borg et al., 2009), therefore it may be hypothesised that agents that increase expression of perilipin in the β-cells may protect against free fatty acid-induced damage by promoting the formation of lipid droplets. Rosiglitazone is able to retain insulin sensitivity in the
presence of TNF-α (Hernandez et al., 2004) and interleukin (IL)-6 (Lagathu et al., 2003) and restore insulin-dependent glucose uptake in adipocytes.

It is known that upon treatment with rosiglitazone in vivo PPARγ lowers circulating free fatty acids. It was initially suggested that improvements of β-cell function and increased insulin sensitivity in skeletal muscle and the liver in response to rosiglitazone were an indirect effect, and resulted from decreases in FFA and adipokines, increased adiponectin, and improved glycaemic control. In vivo administration of rosiglitazone is able to improve β-cell function in Type 2 diabetic patients (Fonseca et al., 2000; Ovalle and Bell, 2002, 2004; Pfutzner et al., 2006; Gastaldelli et al., 2007). Studies utilising techniques and measurements such as the homeostatic model assessment (HOMA) and proinsulin:immunoreactive insulin ratios show that rosiglitazone treatment is able to improve β-cell function compared to placebo or treatment with metformin or sulphonylurea (Zinman, 2001). However, there is also evidence of rosiglitazone exerting direct effects on tissues other than adipose tissue which can lead to improved insulin sensitivity, and improved β-cell function.

Studies on isolated tissue and cell lines from liver, skeletal muscle and pancreatic β-cells show that rosiglitazone can have direct effects, in the absence of adipocyte-derived factors. The presence of free fatty acids undoubtedly has detrimental effects on these cell types and impairs insulin sensitivity and secretion, and improvements in lipid storage as a result of more differentiated adipocytes are likely to play an important role in preserving β-cell function in type 2 diabetes. However, increasing numbers of studies have shown that rosiglitazone is able to protect isolated islets and β-cell lines from the detrimental effects of direct incubation with free fatty acids such as palmitate (Lupi et al., 2004; Tian et al., 2006; Abaraviciene et al., 2008; Meidute Abaraviciene et al., 2008; Vandewalle et al., 2008). Rosiglitazone is able to reverse many of the detrimental effects of palmitate in the regulation of multiple genes,
such as PPARγ, insulin and uncoupling protein (UCP)-2 (Tian et al., 2006) and restores glucose-stimulated insulin secretion (Lupi et al., 2004). In MIN6 β-cells, rosiglitazone promotes nuclear levels of the transcription factors PDX1 and FOXA2, and increases activity of the PDX1 gene promoter; effects which occur in the presence of the PPARγ antagonist GW9662 (Richardson et al., 2006). In human islets, exposure to free fatty acids resulted in an increase in triglyceride content, an effect prevented by co-incubation with rosiglitazone (Vandewalle et al., 2008). Studies on isolated skeletal muscle tissue also show direct effects of rosiglitazone, in the absence of adipocyte-derived factors, although these appear to vary depending on the type of tissue and rosiglitazone concentrations used (Al-Khalili et al., 2005; Lessard et al., 2006).

Rosiglitazone prevented apoptosis of primary dorsal root ganglion neurons in response to nerve growth factor withdrawal, and PC12 nerve cells from β-amyloid in a PPARγ-dependent manner. These effects were associated with an upregulation of the anti-apoptotic protein Bcl2 (Fuenzalida et al., 2007).

Recently, studies investigating the direct effects of rosiglitazone on pancreatic β-cells, skeletal muscle and hepatocytes have suggested that potentially beneficial effects can occur independently of PPARγ. It is not yet clear the mechanisms involved in exerting the effects of rosiglitazone on β-cells. In muscle cells, it was found that rosiglitazone treatment led to activation of AMPK, and restored AMPK signalling in the skeletal muscle of insulin resistant obese Zucker rats (Lessard et al., 2006). Treatment of Type 2 diabetes with rosiglitazone leads to decreased gluconeogenesis in the liver (Gastaldelli et al., 2006). Given that activation of AMPK in the liver can lead to reduced gluconeogenesis, this may be a key alternative pathway in mediating the effects of rosiglitazone in cell types other than adipocytes. Rosiglitazone has been shown to block activation of p38 MAPK in response to TNF-α in brown adipocytes (Hernandez et al., 2004), and in response to palmitate in β-cells (Abaraviciene et al., 2008). The p38
pathway may therefore be a key target for rosiglitazone in the protection of cells from obesity-associated factors.

A meta-analysis looking at trials investigating treatment with rosiglitazone found an increased risk of myocardial infarction in patients taking rosiglitazone compared with those taking a placebo (Nissen and Wolski, 2007). Although there appears to be an increased risk of heart failure in patients treated with rosiglitazone, there is no increased risk of death from cardiovascular events, or increased risk of death from any cause. In the RECORD trial there was an increased number of hospitalisations as a result of cardiac events between the rosiglitazone treatment group and the group treated with a combination of metformin and a sulfonylurea, but there was no increased risk of cardiovascular mortality, although the low frequency of total cardiac events may have reduced the statistical power of this study (Home et al., 2009). Another study has found that a combination of rosiglitazone treatment and following an exercise programme reduced markers associated with increased risk of heart failure, such as body weight and cholesterol, although this was a small trial and was not long enough to determine the subsequent risk of heart failure or death (Kadoglou et al., 2009).

It is likely then that the glucose lowering potential of rosiglitazone in the treatment of type 2 diabetes is a result of both direct and secondary effects on a variety of tissues, not just adipocytes. There is also increasing evidence that rosiglitazone does not exert these effects solely through its agonistic action on PPARγ.

1.13 PPARγ

PPARγ belongs to the superfamily of nuclear hormone receptors. PPARγ is most abundantly expressed in adipocytes, but is also present in other tissues such as the pancreas, liver and skeletal muscle. It has been found to be expressed in human and rat islets (Dubois et al., 2000; Parton et al., 2004), as well as pancreatic β-cell lines (Xu et
al., 2006). In adipocytes it is involved in regulating the transcription of genes involved in fat metabolism. Treatment of both 3T3-L1 and human adipocytes with the PPARγ agonist pioglitazone increased the expression of IRS2 (Smith et al., 2001). Mice that lack expression of leptin (ob/ob) are the most obese of mouse models, with dyslipidaemia, hyperglycaemia and hyperinsulinaemia. The use of a PPARγ agonist reversed these traits, as did transgenically expressing adiponectin to achieve levels comparable to treatment with PPARγ agonist (Kim et al., 2007c). Increased levels of adiponectin caused expansion of adipose tissue, improving triglyceride storage, and reducing the amount of lipid stored ectopically in liver and muscle. In PC12 nerve cells, overexpression of PPARγ prevented, whereas dominant negative PPARγ potentiated, damage from hydrogen peroxide. This correlated with increased or decreased expression of the anti-apoptotic protein Bcl2 respectively (Fuenzalida et al., 2007).

The role of PPARγ in β-cells is less clear. Some studies have found a reduction in insulin expression in response to activation of PPARγ, whereas others have found improved islet function by reducing endoplasmic reticulum stress (Evans-Molina et al., 2009), and enhanced GSIS, likely due to increased expression of GLUT2 (Xu et al., 2006). In INS-1E cells pre-treated with a PPARγ agonist for 48 hours, there was enhanced insulin secretion in response to high glucose, with no significant effect on basal insulin secretion or on insulin content (Santini et al., 2004). In human islets, treatment with palmitate led to increased triglyceride content, reduced insulin content and reduced viability, all of which were prevented by rosiglitazone in a PPARγ-dependent mechanism. The palmitate-induced increase in iNOS gene expression was significantly blocked by rosiglitazone, an effect which was abolished by the addition of the PPARγ antagonist GW9662 (Vandewalle et al., 2008). In human islets, expression of a dominant negative PPARγ blocked the protective effect of rosiglitazone against human islet amyloid polypeptide-induced apoptosis (Lin et al., 2005). In addition, the
protection of RINm5F β-cells from cytokine-induced toxicity with the TZDs troglitazone and citaglitazone was prevented by GW9662, and NFκB activation was prevented by both PPARγ ligands, and by overexpression of PPARγ (Kim et al., 2007a).

It remains unclear as to whether rosiglitazone acts as a PPARγ agonist in β-cells, as in BRIN-BD11 cells transfected with a PPRE plasmid, rosiglitazone alone at concentrations known to affect PPARγ in other cells had no effect. However, when cells were co-transfected with a PPARγ expression vector, rosiglitazone increased activity of the PPRE, suggesting that the endogenous PPARγ concentration was insufficient for rosiglitazone to act as an agonist (Welters et al., 2004a).

1.14 AMPK

AMPK is a key fuel sensing molecule and is recognised as a master regulator of cellular energy consumption. It is activated by a reduction in the ATP:AMP ratio, causing a switch from ATP consuming cellular events to ATP producing mechanisms. In addition to activation by changes in ATP levels, AMPK can also be activated by phosphorylation in response to other stimuli (Winder, 2001).

ACC is a rate-limiting factor in the production of malonyl co-A, a precursor for fatty acid synthesis which also inhibits fatty acid oxidation. Inactivation of ACC by AMPK leads to a decrease in fatty acid synthesis and an increase in fatty acid oxidation. Malonyl Co-A inhibits carnitine palmitoyl transferase 1 (CPT1), which transports fatty acids into the mitochondria and is rate-limiting in fatty acid oxidation (Cohen et al., 1998). In Chinese hamster ovary cells, palmitate-induced caspase activation and cell death were reduced by the addition of the AMPK activator 5-aminomidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR), suggesting that increased β-oxidation can reduced fatty acid toxicity (Borradaile et al., 2006)
In multiple cell types, rosiglitazone has been shown to activate AMPK (Lessard et al., 2006), and it is thought that this is a key alternative pathway to PPARγ through which rosiglitazone exerts its anti-diabetic effects. In muscle cells, activation of AMPK by AICAR promotes GLUT4 translocation to the plasma membrane, resulting in an increase in glucose uptake (Kurth-Kraczek et al., 1999).

The potential targeting of AMPK as a therapeutic target in type 2 diabetes is currently subject to debate. Activating AMPK in muscle and liver leads to increased glucose uptake and decreased hepatic glucose output. Different studies have shown stark contrasts in the effect of activating AMPK in β-cells, with some suggesting increases in insulin secretion (Sun et al., 2008; Zhang et al., 2009), while others showed increased apoptosis (Kefas et al., 2004). In INS-1 cells overexpressing SREBP-1c, where increased lipogenesis impairs insulin secretion, the addition of AICAR was able to partially preserve glucose stimulated insulin secretion (Yamashita et al., 2004). While many studies have investigated the effects of targeting AMPK in different tissues, few have looked at the implications in human diabetic patients. Intravenous administration of AICAR in patients with type 2 diabetes indeed showed a decrease in hepatic glucose output, resulting in lower plasma glucose (Boon et al., 2008). There was also a decrease in circulating NEFA due to suppression of lipolysis in adipose tissue, and increased fatty acid oxidation in the liver.

Recently, it has been shown that rosiglitazone causes an increase in insulin secretion at both basal and high glucose concentrations by targeting the ATP-dependent K⁺ channel in a PI3K dependant manner (Chang et al., 2009). Phosphorylation of both AMPK and the inwardly rectifying K⁺ channel subunit (Kir6.2) were observed in response to rosiglitazone, and blocked by the addition of PI3K inhibitor LY294002. The effect of rosiglitazone on the Kir6.2 was abolished by compound C.
Rosiglitazone has been found to improve endothelial function by increasing NO bioavailability (Boyle et al., 2008). In human aortic endothelial cells, rosiglitazone increased AMPK, ACC and eNOS phosphorylation. In human umbilical vein endothelial cells, AICAR and a constitutively active form of AMPK were able to prevent palmitate-induced NKκB activation (Cacicedo et al., 2004). This suggests that activation of AMPK may play an important role in preventing apoptosis.

1.15 p38 MAPK

P38 belongs to the group of mitogen activated protein kinases (MAPK), and has been implicated in apoptosis in a variety of cell types, including pancreatic β-cells. It is activated by a wide range of stimuli, such as cytokines (Schrader et al., 2007), UV radiation (Pandey et al., 1996), and free fatty acids (Abaraviciene et al., 2008). Activation of p38 in T cells has been implicated in the destruction of β-cells in a mouse model of type 1 diabetes (Medicherla et al., 2006).

In cultured islets and β-cells, activation of p38 in response to immunosuppressive drugs, such as mycophenolic acid, has been shown to lead to apoptosis (Kim et al., 2008b). It may be suggested that inhibition of p38 signalling in β-cells may prevent the loss of β-cell mass observed following islet transplantation. Inhibition of p38 with SB203580 has also been linked with improving islet revascularisation (Johansson et al., 2006). P38 has been found to be important in determining the effect of VEGF on angiogenesis, with inhibition of p38 causing an decrease in vascular permeability and increasing the oxygenation of islet cells (Johansson et al., 2006). In human islets, SB203580 failed to suppress increases in IL-1β mRNA in response to lipopolysaccharide (LPS) or LPS with TNF-α and IFNγ. However, the presence of SB203580 blocked increases in IL-1β protein levels and its
release into the media, suggesting a role for p38 in post-transcriptional regulation of cytokines (Matsuda et al., 2005).

Activation of p38 in tissues such as adipose and muscle in response to free fatty acids and cytokines can cause insulin resistance by increasing inhibitory serine phosphorylation of the insulin receptor, thus blocking insulin signal transduction. Agents that block phosphorylation of p38 also lead to the cells retaining sensitivity to insulin (Hernandez et al., 2004; Li et al., 2007). In skeletal muscle, increased p38 phosphorylation was observed alongside other markers of insulin resistance and decreased insulin-induced glucose uptake (Archuleta et al., 2009). Inhibition of p38 partially improved insulin signalling through restoration of IRS1 levels, but not IRS2.

P38 has been implicated in cell senescence, and it has been suggested that senescence of beta cells following enhanced replication in response to high fat diet may play a role in the development of diet-induced type 2 diabetes (Sone and Kagawa, 2005).

1.16 Akt/PKB

Akt forms part of the PI3K pathway and is a downstream mediator in the insulin signalling pathway. It is present in a wide variety of cell types, and is implicated in cell proliferation and also in the protection against cell death induced by different agents (Lin et al., 2005; Storling et al., 2005).

The importance of insulin signalling in β-cells is becoming clear, as disruptions to the insulin receptor or IRS2 affect β-cell mass and insulin secretion. Akt, along with IRS2, has been implicated in the increase in β-cell proliferation and mass arising from peripheral insulin resistance (Jetton et al., 2005; Rafacho et al., 2008). In pancreatic β-cells, Akt phosphorylates FOXO1, excluding it from the nucleus so it can no longer bind to the PDX1 promoter, where it blocks FOXA2 mediated PDX1 expression. Akt
also phosphorylates and inhibits GSK3β, which can enhance β-cell survival and proliferation (Mussmann et al., 2007). The PI3K/Akt pathway also plays a role in insulin synthesis, as mutations in IRS1 impair insulin synthesis.

In mice lacking the leptin receptor, there was an initial increase in β-cell mass, followed by a decline at 7 weeks of age. This correlated with a decrease in Akt phosphorylation and increased markers of ER stress. In MIN6 cells, it was found that inhibition of insulin signalling with LY294002 induced ER stress (Matsuda et al., 2008).

Expression of a constitutively active form of Akt in isolated islets prevented the impairment of glucose-stimulated insulin secretion in response to palmitate (Kato et al., 2008). In the β-cell line INS832/13, expression of a constitutively active Akt protected the cells from apoptosis in response to high glucose, palmitate or both, whereas the dominant-negative Akt blocked the protective effect of GLP-1 from these conditions (Buteau et al., 2004).

In pancreatic islets, resistin blocked the activation of Akt in response to insulin and impaired the β-cell response to glucose, with increased basal insulin secretion and decreased glucose-stimulated insulin secretion (Nakata et al., 2007). The use of transgenic mice expressing a kinase-dead version of Akt resulted in defective insulin secretion due to impaired insulin exocytosis (Bernal-Mizrachi et al., 2004). It was found that in vivo these animals had impaired adaptation to high fat diet-insulin resistance, which was caused by decreased insulin secretion, with no effect observed on β-cell mass.

In a broad number of cell types, Akt plays an important role in cell survival and proliferation. In β-cells, it also has a role in glucose responsiveness and insulin secretion, making Akt a potential target for preserving β-cell function.
1.17 CK2

Casein kinase 2 (CK2) is a pleiotropic protein that has been implicated in cell survival, proliferation and tumourogenesis (Meggio and Pinna, 2003), although its role and regulation in β-cells is not fully understood. Its proliferative and anti-apoptotic effects appear to occur via multiple pathways. CK2 activation can lead to direct phosphorylation of Akt (Di Maira et al., 2005; Guerra, 2006), which has been implicated in cell survival. CK2 can also indirectly activate Akt by phosphorylation and inactivation of the tumour suppressor PTEN (phosphatase and tensin homolog) (Al-Khoury et al., 2005), a negative regulator of the PI3K/Akt pathway. CK2 promotes the degradation of promyelocytic leukemia (PML), a tumour suppressor, by phosphorylation (Scaglioni et al., 2006; Scaglioni et al., 2008). Increases in CK2 activity lead to increased expression of anti-apoptotic factors via degradation of inhibitor of κB (IκB) and subsequent activation of nuclear factor-kappa B (NFκB) (Romieu-Mourez et al., 2002). CK2 can also phosphorylate caspase targets close to their caspase recognition sites (McDonnell et al., 2008), preventing caspase-mediated cleavage, which again leads to decreased apoptosis.

Although these effects of CK2 have not been studied in pancreatic β-cells, it may be hypothesised that agents increasing CK2 activity may play a role in preventing free fatty acid or cytokine-induced apoptosis.

1.18 MIN6 cell line

MIN6 cells are among many cell lines used to model the pancreatic islet. MIN6 cells have been found to have a realistic insulin secretion pattern and respond to glucose similarly to normal β-cells, and they can be clustered to form pseudoislets, which enhances the insulin secretory response compared with single cells and can be used to model normal islets (Hauge-Evans et al., 1999).
Figure 1.4 Targets of CK2

CK2 modulates multiple pathways within the cell. Here, we highlight the targets of CK2 that affect apoptosis and cell proliferation. CK2 has inhibitory actions on PTEN (phosphatase and tensin homolog), promyelocytic leukemia (PML), and inhibitor of κB (IκB), which has subsequent inhibitory action on nuclear factor-kappa B (NFκB). CK2 inhibition of PTEN can lead to activation of phosphatidylinositol-3-kinase (PI3K). CK2 can also directly and indirectly activate Akt, which can prevent apoptosis through inhibition of Bcl-XI/Bcl-2-associated death promoter homolog (BAD) and caspase-9.
The experiments in this study were carried out on MIN6 monolayers to test the direct effects of free fatty acids and anti-diabetic drugs on the signalling events within the cell in the absence of other contributing factors. This may help to determine which effects seen \textit{in vivo} are caused by the drugs on pancreatic β-cells directly, rather than due to changes in blood glucose, lipid levels and cytokines.

\textbf{1.19 Aims}

The aim of this study was to investigate the effects of the saturated free fatty acid palmitate, and the mono-unsaturated free fatty acid oleate on MIN6 cell viability, and on key signalling pathways in the cell. We also aimed to determine if the anti-diabetic drugs metformin and rosiglitazone could offer protection against free fatty acid-induced damage, and if so, to begin to define the mechanisms involved.

In chapter 3 we investigated the different effects of palmitate and oleate on MIN6 cell viability, and whether metformin and rosiglitazone could protect against any detrimental effects observed. We also compared lipid storage in MIN6 cells upon treatment with palmitate and oleate using transmission electron microscopy and oil red O staining.

In chapter 4, we investigated the effects of palmitate, oleate and rosiglitazone on the key signalling pathways identified earlier; PPARγ, p38, AMPK, CK2 and Akt.

Chapter 5 investigated the effect of palmitate on the key β-cell transcription factors PDX1 and FOXA2, and whether rosiglitazone was able to prevent any of these effects. The direct effect of rosiglitazone on the expression of two proteins important in glucose uptake and sensing by the β-cell, glucokinase and GLUT2, was also determined.
2. Methods

2.1 Cell culture

MIN6 cells, a beta cell line derived from transgenic mice expressing the SV40 large T antigen under the control of the rat proinsulin gene promoter (Miyazaki et al., 1990), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 5 mM glucose, supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. All of the present studies were performed using cells between passage numbers 27 and 32.

2.2 Preparation of drug solutions

Metformin stocks were prepared at a concentration of 15 mM in DMEM containing 5 mM glucose. Rosiglitazone stocks were prepared at a concentration of 10 mM in DMSO. The p38 inhibitor SB203580 stocks were prepared at a concentration of 20 mM in DMSO. Drug solutions were diluted in DMEM containing 5 mM glucose to the final concentration immediately prior to addition to the cells. The final concentration of DMSO in the medium was 0.1% (v/v).

2.3 Free fatty acid stocks conjugated to bovine serum albumin

100 mM palmitic acid or oleic acid was prepared in water and ethanol at a ratio of 1:1 and dissolved at 50°C. 500µl of free fatty acid stock solution was added per 9.5 ml of 10% BSA (w/v), to a final concentration of 5 mM, and stirred at 37°C for 1 hour. Once cooled to 20°C, the solution was filter sterilised using a 0.45 µm cellulose filter. Before use, free fatty acid stocks were heated to 50°C for 15 minutes, and then maintained at 37°C prior to addition to cells. The approximate molar ratio of free fatty acid to BSA was 3.5:1. The final concentration of ethanol in the medium was 0.2% (v/v), and the final concentration of BSA was 0.75% (w/v).
2.4 Free fatty acid stocks without BSA

Palmitic acid or oleic acid was dissolved in ethanol at a concentration of 40 mM immediately prior to addition to cells. The final concentration of ethanol in the medium was 1% (v/v).

2.5 Plasmids

The PPRE plasmid contained 4 copies of a PPAR response element upstream of the firefly luciferase gene (Welters et al., 2004a). The pGL3 control plasmid contained the firefly luciferase gene with no upstream promoter region. Plasmid DNA was prepared using an endotoxin-free Maxiprep kit (Qiagen, Crawley, UK).

2.6 Transfecting cells and luciferase assay

To investigate if rosiglitazone was able to act as a PPARγ agonist in MIN6 cells, a plasmid containing a PPRE upstream of the firefly luciferase gene was transfected into cells. MIN6 cells were transfected with 2 µg plasmid DNA using Lipofectamine (Invitrogen) in 1ml serum-free Opti-MEM. 5 hours after transfection, 1 ml of DMEM media containing 20% (v/v) FBS was added to each well. 48 hours after transfection cells were incubated in DMEM containing 5 mM glucose alone or with the addition of 10 µM rosiglitazone for 24 hours. Cells were then harvested into 100 mM KH₂PO₄ with 1M DTT, freeze-thawed 3 times to lyse cells and centrifuged at 13000 rpm for 30 seconds to remove cell debris. To measure the luciferase activity of samples, 20 µl of cell extract was added to 350 µl of buffer A, pH 7.8 (15mM MgSO₄, 30mM glycylglycine, 2mM Na₂ATP) containing 0.45 mM Coenzyme A and 2.56mM Triton X-100. To this, 150µl of buffer G (30 mM glycylglycine) containing 0.5 mM luciferin (Sigma) was injected and the luminescence read at 560 nm using a Berthold Luma LB9501. Protein concentrations were measured using Bio-Rad Protein Assay Kit.
**Figure 2.1 PPRE and pGL3 plasmids**

The PPRE plasmid contains four copies of a PPAR response element, upstream of the firefly luciferase gene. The control pGL3 plasmid contains the firefly luciferase gene but lacks a promoter region.
reagent and titrated against known concentrations of bovine serum albumin, to obtain the relative luciferase activity.

### 2.7 Protein extraction

Following treatment, cells were washed with PBS, harvested and centrifuged at 13000 rpm for 3 minutes. Cells were resuspended in 400 μl buffer A (containing 10 mM KCl, 10 mM Hepes pH 7.9, 0.1 mM EDTA pH 8.0, 0.1 mM EGTA pH 8.0, 1 mM DTT and 1x protease inhibitor cocktail) and incubated on ice for 15 minutes. For whole cell extracts, Triton X-100 was added to a final concentration of 0.625% (v/v) then followed by a further 30 minute incubation on ice. Samples were then centrifuged for 60 seconds at 13000 rpm and the supernatant retained. For nuclear and cytoplasmic fractionation, 0.625% (v/v) NP-40 was added, vortexed for 30 seconds and centrifuged at 13000 rpm for 45 seconds. The supernatant was retained as the cytoplasmic fraction. The pellet, containing the nuclear proteins, was resuspended in 50 μl buffer C (containing 0.4 M NaCl, 20 mM Hepes pH 7.9, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 1 mM DTT, 5% (v/v) glycerol and 1x protease inhibitor cocktail (Roche, UK)), incubated at 4°C shaking for 1 hour, then centrifuged at 13000 rpm at 4°C for 30 seconds. The supernatant containing the nuclear proteins was retained and snap frozen in liquid nitrogen.

### 2.8 Western blotting

Protein concentrations were measured using Bio-Rad Protein Assay Kit reagent and titrated against known concentrations of bovine serum albumin. 20 μg of extract was added per well for whole cell and cytoplasmic extracts. 5 μg of extract was added per well for nuclear extracts. Samples were fractionated by 10% SDS-PAGE (7% for ACC Western blots) and transferred to polyvinylidene fluoride membrane, pore size 0.45 μM
(Millipore, Billerica, MA) using the Bio-Rad Semi Dry Transblot. The membrane was then placed in 10% (w/v) non-fat dried milk in wash buffer (0.5M NaCl, 10mM Tris-HCl, 0.05% Tween 20) for 1 hour at room temperature with agitation. The membrane was rinsed with wash buffer, then incubated 3 times for 5 minutes in wash buffer with agitation before overnight incubation in primary antibody at 4°C. Primary antibodies were used at the concentrations described in the table below. Following overnight incubation, membranes were rinsed twice with wash buffer, then incubated 3 times in wash buffer (1x 15 minutes then 2 x 5 minutes) with agitation. Membranes were then incubated in secondary antibody for 1 hour at room temperature. Secondary HRP-conjugated antibodies were purchased from Amersham (Buckinghamshire, UK) and used at a concentration of 1:5000. For developing, the membrane was drained on blotting paper and placed on Saran wrap. 1ml of ECL plus (GE Healthcare, UK) detection solution (1ml solution A and 25µl solution B, mixed immediately prior to use) was added to the membrane and left for 5 minutes. The membrane was then covered with Saran wrap and excess solution drained using blotting paper. ECL Hyperfilm (GE Healthcare, UK) was exposed to the membrane, and developed using the Compact X4-Automatic X-ray Film Processor (Xograph Imaging Systems, UK).

**Table 2.1 Primary antibody information**

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Purchased from</th>
<th>Concentration</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>p38</td>
<td>Sigma (Dorset, UK)</td>
<td>1:5000</td>
<td>Anti rabbit</td>
</tr>
<tr>
<td>Phospho p38</td>
<td>Cell Signaling Biotechnology (Beverly, MA)</td>
<td>1:1000</td>
<td>Anti rabbit</td>
</tr>
<tr>
<td>Akt</td>
<td>Cell Signaling Biotechnology (Beverly, MA)</td>
<td>1:1000</td>
<td>Anti rabbit</td>
</tr>
<tr>
<td>Phospho Akt Thr 308</td>
<td>Cell Signaling Biotechnology (Beverly, MA)</td>
<td>1:1000</td>
<td>Anti rabbit</td>
</tr>
<tr>
<td>Phospho Akt Ser 473</td>
<td>Cell Signaling Biotechnology (Beverly, MA)</td>
<td>1:1000</td>
<td>Anti rabbit</td>
</tr>
<tr>
<td>ACC</td>
<td>AbCam (Cambridge, UK)</td>
<td>1:2000</td>
<td>Anti rabbit</td>
</tr>
</tbody>
</table>
## 2.9 Preparation of cDNA

RNA was extracted using the Sigma GenElute mammalian total RNA kit according to manufacturer’s instructions. DNA was removed using Sigma DNase at 37°C for 30 minutes. DNase stop solution was added and incubated at 65°C for 10 minutes. Samples were checked for DNA contamination and integrity on a 1% (w/v) agarose gel. RNA concentration was measured, along with DNA and protein contaminants, by measuring absorbance at 260nm and 280nm using the Eppendorf biophotometer (AG-Model No.6131022336). RNA was reverse transcribed using the Invitrogen Cloned AMV First Strand Synthesis Kit.

## 2.10 Real time reverse transcription PCR

cDNA was amplified using the Bio-Rad iQ SYBR Green Supermix. Each primer was added to a final concentration of 1 µM. Primer sequences and conditions are described in the table below. For each run, an initial denaturing step of 95°C for 5 minutes and final extension of 72°C for 2 minutes was included. Samples were run on the Qiagen Rotor-Gene Q real time PCR machine with fluorescence detected at the end of the extension step in each cycle. Following amplification, a melt curve analysis was performed, with fluorescence detection after increases in temperature in 1°C increments.
for 10 seconds, between 60°C and 95°C. Following each run, samples were analysed on a 1% agarose gel to verify product size. Results were analysed using the Pfaffl method (Pfaffl, 2001). The PCR efficiency was calculated for each primer set, using 5 concentrations in 10-fold dilution series to create a standard curve, which was performed for every PCR experiment run. All serial dilutions and samples were run in duplicate in each experiment. Results are expressed as the relative changes in target gene expression compared with untreated samples.

**Table 2.2 Primer sequences and conditions**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Denature</th>
<th>Anneal</th>
<th>Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Sense: 5’-GTG AAG GGC TCA TGA CCA CAG TCC AT- 3’ Anti-sense: 5’-TGT CAG ATC CAC GAC GGA CAC AT- 3’</td>
<td>95°C for 30 seconds</td>
<td>55°C for 30 seconds</td>
<td>72°C for 30 seconds</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Sense: 5’-TTA GCA ACT GGG TCT GCA AT- 3’ Anti-sense: 5’-GTT GTA GTC CTA CAC TCA TG- 3’</td>
<td>95°C for 30 seconds</td>
<td>60°C for 30 seconds</td>
<td>72°C for 45 seconds</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>Sense: 5’-AAG GGA ACA ACA TCG TAG GA- 3’ Anti-sense: 5’-CAT TGG CGG TCT TCA TAG TA- 3’</td>
<td>95°C for 30 seconds</td>
<td>58°C for 30 seconds</td>
<td>72°C for 45 seconds</td>
</tr>
</tbody>
</table>

**2.11 CK2 assay**

CK2 enzyme activity was assayed by measuring the incorporation of $^{32}$P from [$\gamma^{32}$P]ATP into 1 mM of the synthetic peptide substrate “CK2-tide“ [RRRADDSDDDDDD] (Jovanovic et al., 2007). Briefly, samples were suspended to a final volume of 25 μl in standard assay buffer (50 mM HEPES, pH 7.0, 50 mM NaF, 1 mM EGTA, 1 mM EDTA, 0.2 % (v/v) Tween-20, 10% (v/v) glycerol) and incubated at 30°C for 30 minutes, followed by termination by spotting a 15 μl aliquot onto a 1 cm² piece of P-81 phosphocellulose paper and washing 3 x 5 minutes in 1% phosphoric acid.
Samples were then air-dried and incorporation of labelled phosphate was quantified using a Packard Instant Imager (Packard, Nijmegen, Netherlands).

2.12 Preparation of oil red O stain

Oil red O stock stain was made up at a concentration of 0.5% (w/v) in isopropanol, and heated gently to dissolve. Working solutions of oil red O stain were made immediately prior to use. Oil red O stock stain was mixed with deionised water at a ratio of 3:2, left to stand for 10 minutes and filtered through Whatmann paper.

2.13 Oil red O staining

MIN6 cells were seeded at a density of 100,000 cells per well in 2-well chamber slides. Cells were treated with palmitate or oleate either dissolved in ethanol or conjugated to BSA for 24 hours, with or without the addition of rosiglitazone. Following treatment, cells were washed 3 times in PBS and fixed with 3.7% formalin for 10 minutes at room temperature. Oil red O stain solution was added to the cells, and incubated for 2 hours at 37°C. Cells were washed 5 times with PBS to ensure removal of excess oil red O stain. The chambers were removed from the slides and cells were mounted using Vectashield with DAPI.

Untreated MIN6 cells do not have much lipid stored, so as a positive control for staining, cells were treated with either oleate or palmitate that was dissolved in ethanol, not conjugated to BSA, to allow rapid uptake into the cells (Figure 2.2). Ethanol was present at a final concentration of 1%. These cells had greatly increased uptake of lipid, with the effect more pronounced in the oleate treated cells compared with the palmitate treated cells.
A.

B.

Figure 2.2 High levels of oil red O staining with ethanol-dissolved oleate

MIN6 cells were cultured in DMEM containing 5 mM glucose alone (A) or with the addition of 0.4 mM oleate dissolved in ethanol for 24 hours (B), fixed with 3.7% formalin, and stained with oil red O. Treatment with oleate dissolved in ethanol was used as a positive control in all oil red O staining experiments, due to the high levels of staining.
2.14 MTT viability assay

MIN6 cells were seeded in 6-well plates at a density of 2 x 10^5 cells per well. MTT powder (Calbiochem, Darmstadt, Germany) was dissolved in PBS at a concentration of 0.5 mg/ml. Following 72 hours treatment, media was aspirated from the cells, washed with PBS, and 1ml MTT solution added per well. Cells were then incubated at 37ºC for 1 hour. MTT solution was removed from the cells, and 1 ml DMSO added to dissolve the MTT crystals. 200 µl of each sample was added to 2 wells of a 96-well plate, and absorbance measured at 540 nm on the Ascent Multiskan plate reader. The measurement for DMSO alone was subtracted from all other readings, and the mean value obtained. All readings were then divided by the mean value for untreated to express all values relative to untreated cells.

2.15 H/PI staining

MIN6 cells were seeded in 6-well plates at a density of 2 x 10^5 cells per well. H/PI solution (0.5 mg/ml Hoechst 33258 and 50 µg/ml propidium iodide in cell culture media) was made up immediately prior to use and shielded from light. Following 72 hours treatment, media was aspirated from the cells and 400 µl H/PI solution added to each well. The plates were kept dark for 5 minutes incubation before visualisation on the Zeiss Axiovert 25 CFI microscope.

2.16 Transmission Electron Microscopy (TEM)

MIN6 cells were cultured in DMEM containing 5 mM glucose and treated with various stimuli as indicated for 24 hours. Following treatment, cells were fixed in 2.5% (v/v) glutaraldehyde in 0.1M Na Cacodylate/HCl buffer, pH 7.4 for a 3 hours at room temperature and then overnight at 4 ºC. After buffer rinsing, cells were post-fixed in 1% (w/v) osmium tetroxide (OsO4) for 2 hours at room temperature. The cells were then
thoroughly rinsed in distilled water, dehydrated in 50%, 75% and 3 X absolute ethanol for 20 minutes each, passed through propylene oxide (PO; 2 X 20min) and infiltrated in 50:50 PO/Taab Low Viscosity (TLV) resin overnight. After daily changes of resin for 48 hours, the samples were polymerised for 16 hours at 60 °C. Thin sections were cut with an LKB Ultracut ultramicrotome, post-stained in 2% (w/v, aqueous) uranyl acetate for 90 minutes followed by lead citrate for 15 minutes. Stained sections were examined in a Hitachi 7100 transmission electron microscope at 100 kV and images acquired with a Gatan Ultrascan 1000 CCD camera.

**2.17 Statistics and densitometry**

Statistical analysis was performed using one-way ANOVA, followed by a paired student t-test to determine Statistical significance between two means, using SPSS statistical analysis software (IBM, Portsmouth, UK).

For quantification of western blots, densitometry was performed using the FluoroChem Alpha Innotech MultiImage Light Cabinet.
3. Differential effects of palmitate and oleate on MIN6 β-cells

3.1 Introduction

There is much evidence to suggest that treatment of cells with saturated fatty acids is detrimental to cell function and can induce apoptosis in a variety of cell types (Maedler et al., 2001; Chai and Liu, 2007; Lai et al., 2008; Henique et al., 2010; Soumura et al., 2010). Many mechanisms have been implicated in the mediation of cell death, including ER stress, caspase activity and cytokine production. In pancreatic β-cells, chronic exposure to saturated free fatty acids decreases insulin production (Lupi et al., 2004), impairs glucose-stimulated insulin secretion (Joseph et al., 2004), and increases the rate of apoptosis (Laybutt et al., 2007).

The effect of unsaturated fatty acids is less clear. Some studies have found unsaturated fatty acids can also cause apoptosis, but often to a lesser extent than with saturated fatty acids, and some of those that looked at the mechanisms found that distinct pathways were involved in exerting the effects of saturated and unsaturated fatty acids (Roche et al., 1999; Cunha et al., 2008; Yuan et al., 2010). In contrast, many studies found that not only do unsaturated fatty acids have no detrimental effects on β-cells alone, but that they can protect against the detrimental effects of saturated fatty acids (Maedler et al., 2003; Welters et al., 2004b).

There is conflicting evidence as to what happens once fatty acids are taken up by the cell and what makes them so damaging. It has been found that increasing fatty acid oxidation can protect the cell, whereas blocking β-oxidation of palmitate by inhibiting its uptake into the mitochondria enhanced palmitate-induced apoptosis (El-Assaad et al., 2003). However, promoting the storage of fatty acids as triglycerides can also prevent the detrimental effects (Listenberger et al., 2003). Overall, the evidence
suggests that it is the intermediates formed in the metabolism of saturated fatty acids that are harmful to the β-cell (Choi et al., 2011). Therefore strategies preventing apoptosis in these cells should be directed at promoting lipid storage, increasing fatty acid oxidation, or blocking the pathways activated by saturated fatty acids that cause apoptosis.

As β-cell dysfunction ultimately marks the distinction between insulin resistance with compensatory increased insulin secretion, and type 2 diabetes, finding ways that can protect against β-cell apoptosis and impaired insulin secretion is key to delaying or preventing the onset of type 2 diabetes. As studies have found that the anti-diabetic drugs rosiglitazone and metformin can improve markers of β-cell function in human studies, and both can have direct effects on MIN6 cells in vitro, we aimed to test if they can prevent β-cell apoptosis.

The aim of this study was to determine the effects of free fatty acids on MIN6 cells, whether saturated and mono-unsaturated fatty acids exert different effects, and to test if rosiglitazone, metformin, and the p38 inhibitor SB203580 can protect against any detrimental effects observed.

3.2 Results

3.2.1 Rosiglitazone protects MIN6 cells from the detrimental effect of palmitate on MIN6 cell viability

To determine if rosiglitazone can protect MIN6 cell viability against palmitate, MTT assays were carried out. MIN6 cells treated with 0.4 mM palmitate for 72 hours had a 50-60% decrease in cell viability as measured by MTT assay compared with untreated cells ($p<0.01$) (Figure 3.1). The addition of 10 µM rosiglitazone alone did not affect cell viability, but was able to prevent the decrease observed with palmitate ($p<0.01$).
Figure 3.1 MTT assay to determine the protective effect of rosiglitazone against palmitate-induced damage in MIN6 cells

MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 0.4 mM palmitate and/or 10 µM rosiglitazone for 72 hours. MTT assay was then performed to determine cell viability. Values are mean ± SEM, expressed as a percentage of control. Samples were analysed in duplicate and results obtained from at least 3 separate experiments. ** $p<0.01$. 
3.2.2 Metformin does not prevent the palmitate-induced decrease in MIN6 cell viability
To determine if metformin can protect MIN6 cell viability from palmitate-induced damage, MTT assays were performed. Treatment of MIN6 cells with 0.4 mM palmitate for 72 hours led to a 60% decrease in cell viability ($p<0.01$). The presence of 15 µM metformin alone had no effect on cell viability. Unlike rosiglitazone, metformin was unable to protect MIN6 cells from the detrimental effect of palmitate, with viability decreased by 60% compared with untreated cells ($p<0.01$) (Figure 3.2).

3.2.3 Oleate has no effect MIN6 cell viability, but can protect against palmitate-induced damage
To determine the effect of oleate on MIN6 cell viability, and if oleate has any impact on the detrimental effect of palmitate, MTT assays were carried out. Treatment of MIN6 cells with 0.4 mM palmitate decreased cell viability by 40% ($p<0.01$) (Figure 3.3). Treatment with 0.4 mM oleate alone had no significant effect on MIN6 cell viability. However, the cells treated with the combination of 0.4 mM oleate and 0.4 mM palmitate had significantly higher viability than treatment with palmitate alone ($p<0.05$).

3.2.4 Inhibition of p38 with SB203580 partially protected MIN6 cells from the detrimental effect of palmitate
To investigate the role of the p38 MAPK pathway in exerting the detrimental effect of palmitate on cell viability, MTT assays were performed utilising the specific p38 inhibitor SB203580. MIN6 cells treated with 0.4 mM palmitate had decreased viability compared with untreated cells ($p<0.01$) (Figure 3.4). The addition of 20 µM SB203580 alone for 72 hours did not significantly affect cell viability. The addition of SB203580 30 minutes prior to treatment with palmitate significantly blocked the decrease in cell viability compared with palmitate treatment alone ($p<0.05$). However, cell viability was
Figure 3.2 MTT assay to determine if metformin can protect against palmitate-induced damage

MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 0.4 mM palmitate and/or 15 µM metformin for 72 hours. MTT assay was then performed to determine cell viability. Values are mean ± SEM, expressed as a percentage of control. Samples were analysed in duplicate and results obtained from at least 3 separate experiments. ** p<0.01.
Figure 3.3 MTT assay to determine the effect of oleate on MIN6 cell viability

MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 0.4 mM palmitate and/or 0.4 mM oleate for 72 hours. MTT assay was then performed to determine cell viability. Values are mean ± SEM, expressed as a percentage of control. Samples were analysed in duplicate and results obtained from at least 3 separate experiments. * $p<0.05$, ** $p<0.01$. 
Figure 3.4 MTT assay to determine the effect of SB203580 on the detrimental effect of palmitate on MIN6 cell viability

MIN6 cells were cultured in DMEM containing 5 mM glucose alone, with the addition of 0.4 mM palmitate, or treated with 20 µM SB203580 for 30 minutes prior to the addition of 0.4 mM palmitate, and incubated for 72 hours. MTT assay was then performed to determine cell viability. Values are mean ± SEM, expressed as a percentage of control. Samples were analysed in duplicate and results obtained from at least 3 separate experiments. * $p<0.05$, ** $p<0.01$. 
still significantly reduced compared with untreated cells ($p<0.01$), showing that inhibition of p38 offers MIN6 cells a level of protection against palmitate, but this is not sufficient to fully protect the cells from palmitate induced damage. This suggests there are multiple mechanisms through which palmitate can exert its effects on MIN6 cells.

### 3.2.5 Effects of palmitate, oleate, rosiglitazone and metformin on cell death in MIN6 cells

To determine if the detrimental effect of palmitate on MIN6 cell viability is due to an increase in apoptosis or necrosis, H/PI staining was performed. Hoechst is a dye that appears blue when bound to DNA, it binds to chromatin within the cells. Healthy cells are dark blue, whereas apoptotic cells appear bright blue as the chromatin is condensed. Propidium iodide appears pink when bound to DNA, and healthy cells are impermeable to this dye, however, necrotic cells or cells that are undergoing late stage apoptosis appear pink.

In untreated MIN6 cells, most of the cells appear healthy, with few apoptotic cells and very few necrotic cells (Figure 3.5). Treatment of MIN6 cells with 0.4 mM palmitate for 72 hours increased the number of both apoptotic and necrotic cells compared with untreated. 72 hour treatment with 10 µM rosiglitazone alone appeared similar to untreated, with few apoptotic or necrotic cells. The presence of rosiglitazone was able to prevent the increase in cell death in response to palmitate, with cells appearing similar to untreated. MIN6 cells treated with 15 µM metformin alone for 72 hours had a similar number of apoptotic and necrotic cells as untreated. The addition of metformin to palmitate treatment was unable to prevent the increase in cell death, as there were more apoptotic and necrotic cells than untreated. Treatment with 0.4 mM oleate alone did not appear to have any effect on the number of apoptotic and necrotic cells compared with untreated MIN6 cells. The presence of oleate with palmitate appeared to reduce the
Figure 3.5 H/PI staining to determine the protection of MIN6 cells from apoptosis in response to palmitate

MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 0.4 mM palmitate, 0.4 mM oleate, 10 µM rosiglitazone and/or 15 µM metformin for 72 hours as indicated. Cells were then incubated for 5 minutes in H/PI solution before visualisation on the Zeiss Axiovert 25 CFI microscope. Results are representative of at least 3 separate experiments.
number of apoptotic and necrotic cells compared with palmitate treatment alone, suggesting that oleate protects MIN6 cells from palmitate-induced cell death.

3.2.6 Transmission electron microscopy of MIN6 cells treated with palmitate, oleate, and rosiglitazone

To examine the effects of palmitate and oleate on MIN6 cell ultrastructure, and lipid storage within the cell, transmission electron microscopy was performed. For each treatment, 10 images were analysed. Figure 3.6 shows the untreated MIN6 cell. In MIN6 cells treated with 0.4 mM palmitate for 24 hours there appears to be some damage to the cell ultrastructure, with the organelles within the cell appearing less defined than untreated cells (Figure 3.7). There was no observable lipid storage in the cells following palmitate treatment. MIN6 cells treated with 10 µM rosiglitazone for 24 hours appeared similar to untreated cells (Figure 3.8). Cells treated with both palmitate and rosiglitazone for 24 hours appeared healthier than those treated with palmitate alone (Figure 3.9). No storage of lipid within the cell could be detected upon treatment with both palmitate and rosiglitazone. MIN6 cells treated with 0.4 mM oleate for 24 hours did not appear to be as healthy as untreated cells (Figure 3.10), but the damage was less than that observed with palmitate treatment. Following treatment with oleate, there was an accumulation of lipid droplets within the cytoplasm of the cells, which can be seen as large dark spots. The addition of rosiglitazone to oleate treatment did not prevent the accumulation of lipid droplets within the cells (Figure 3.11). Figure 3.12 shows all the above treatments to allow comparison.

3.2.7 Oleate, but not palmitate treatment, leads to an accumulation of triglyceride in MIN6 cells

To determine the effects of palmitate and oleate on lipid storage within the cell, and if rosiglitazone can modulate these effects, oil red O staining was performed.
Figure 3.6 Transmission electron microscopy of untreated MIN6 cells

MIN6 cells were cultured in DMEM containing 5 mM glucose with no additions for 24 hours, fixed and then analysed using transmission electron microscopy. Image is 1000x magnification. The untreated MIN6 cells appear healthy, with an intact nuclear envelope and organelle structure.
Figure 3.7 Transmission electron microscopy of MIN6 cells treated with palmitate

MIN6 cells were cultured in DMEM containing 5 mM glucose with the addition of 0.4 mM palmitate for 24 hours, fixed and then analysed using transmission electron microscopy. Image is 1000x magnification. The organelles within the cells appear much less defined than untreated cells, indicating degradation in response to palmitate treatment. There is no obvious sign of lipid storage within the cells treated with palmitate.
Figure 3.8 Transmission electron microscopy of MIN6 cells treated with rosiglitazone

MIN6 cells were cultured in DMEM containing 5 mM glucose with the addition of 10 µM rosiglitazone for 24 hours, fixed and then analysed using transmission electron microscopy. Image is 1000x magnification. The cells treated with rosiglitazone appear similar to untreated cells, with a healthy organelle structure.
Figure 3.9 Transmission electron microscopy of MIN6 cells treated with palmitate and rosiglitazone

MIN6 cells were cultured in DMEM containing 5 mM glucose with the addition of 0.4 mM palmitate and 10 µM rosiglitazone for 24 hours, fixed and then analysed using transmission electron microscopy. Image is 1000x magnification. The cells treated with both palmitate and rosiglitazone appear healthier than those treated with palmitate alone, although there is no indication of an increase in lipid stored within the cells.
MIN6 cells were cultured in DMEM containing 5 mM glucose with the addition of 0.4 mM oleate for 24 hours, fixed and then analysed using transmission electron microscopy. Image is 1000x magnification. Although the cells treated with oleate did not appear as healthy as untreated cells, the damage appeared to be less than with palmitate. In many of the oleate treated cells, there were large lipid droplets accumulated within the cytoplasm, which appear as dark circles.

Figure 3.10 Transmission electron microscopy of MIN6 cells treated with oleate
MIN6 cells were cultured in DMEM containing 5 mM glucose with the addition of 0.4 mM oleate for 24 hours, fixed and then analysed using transmission electron microscopy. Image is 1000x magnification. The addition of rosiglitazone did not prevent the accumulation of lipid droplets within the cells in response to oleate.
Figure 3.12 Transmission electron microscopy of MIN6 cells treated with palmitate, oleate and rosiglitazone

A comparison of TEM images of MIN6 cells treated with 0.4 mM palmitate, 0.4 mM oleate and/or 10 µM rosiglitazone for 24 hours. All images are 1000x magnification.

There is a clear difference in the effects of palmitate and oleate on the cells after 24 hours, with the appearance of large lipid droplets within the cytoplasm in response to oleate, but not with palmitate.
In untreated MIN6 cells, there does not appear to be any lipid storage within the cell, as determined by oil red O staining (Figure 3.13). MIN6 cells treated with 0.4 mM palmitate for 24 hours appeared similar to untreated cells, with no increase in lipid storage observed (Figure 3.14). Treatment of MIN6 cells with 10 µM rosiglitazone alone for 24 hours had no effect on triglyceride storage (Figure 3.15). The addition of rosiglitazone to palmitate treatment did not lead to an increase in lipid storage within the cells (Figure 3.16). Treatment of MIN6 cells with 0.4 mM oleate for 24 hours led to the accumulation of lipid within the cells (Figure 3.17). The addition of rosiglitazone did not appear to alter this effect, suggesting that rosiglitazone does not prevent the storage of oleate within the cell (Figure 3.18). Figure 3.19 shows oil red O staining for all the treatments described above, to allow for comparison.

3.3 Discussion

The TEM images show that treatment with palmitate for 24 hours appears to cause damage to the cell infrastructure, with the organelles being less well defined. This was not seen to the same extent with oleate treatment, and MTT viability assay and H/PI staining showed no significant detrimental effect of oleate on MIN6 cells. Cell viability was reduced by approximately 50% upon 72 hour treatment with palmitate, an effect that was inhibited by the presence of rosiglitazone. Using H/PI staining to differentiate between healthy cells and apoptotic or necrotic cells, treatment with palmitate for 72 hours appeared to increase the number of apoptotic and necrotic cells compared with the untreated cells, which had few dead cells. Rosiglitazone treatment for 72 hours had no effect on cell viability as determined by MTT assay, and H/PI staining was similar to untreated. The addition of rosiglitazone was able to prevent the loss of cell viability seen with palmitate treatment as measured by MTT assay, and there appeared to be
**Figure 3.13 Oil red O staining in untreated MIN6 cells**

MIN6 cells were cultured in DMEM containing 5 mM glucose with no additions for 24 hours, fixed in 3.7 % formalin and stained with oil red O for 3 hours and then analysed using a light microscope. Image is representative of at least 3 separate experiments. In the untreated cells there does not appear to be any staining within the cells.
**Figure 3.14 Oil red O staining in MIN6 cells treated with palmitate**

MIN6 cells were cultured in DMEM containing 5 mM glucose with the addition of 0.4 mM palmitate for 24 hours, fixed in 3.7 % formalin and stained with oil red O for 3 hours and then analysed using a light microscope. Image is representative of at least 3 separate experiments. There does not appear to be much lipid within the cells treated with palmitate, and they appear similar to untreated cells.
MIN6 cells were cultured in DMEM containing 5 mM glucose with the addition of 10 μM rosiglitazone for 24 hours, fixed in 3.7 % formalin and stained with oil red O for 3 hours and then analysed using a light microscope. Image is representative of at least 3 separate experiments. The cells treated with rosiglitazone appear similar to untreated cells, with no lipid staining within the cells.
Figure 3.16 Oil red O staining in MIN6 cells treated with palmitate and rosiglitazone

MIN6 cells were cultured in DMEM containing 5 mM glucose with the addition of 0.4 mM palmitate and 10 µM rosiglitazone for 24 hours, fixed in 3.7 % formalin and stained with oil red O for 3 hours and then analysed using a light microscope. Image is representative of at least 3 separate experiments. The cells treated with palmitate and rosiglitazone appear similar to untreated cells and cells treated with palmitate alone, indicating that rosiglitazone does not promote the storage of lipid in MIN6 cells.
Figure 3.17 Oil red O staining in MIN6 cells treated with oleate

MIN6 cells were cultured in DMEM containing 5 mM glucose with the addition of 0.4 mM oleate for 24 hours, fixed in 3.7 % formalin and stained with oil red O for 3 hours and then analysed using a light microscope. Image is representative of at least 3 separate experiments. The cells treated with oleate appear to have a large accumulation of lipid contained within the cytoplasm of the cells as indicated by increase in staining.
MIN6 cells were cultured in DMEM containing 5 mM glucose with the addition of 0.4 mM oleate and 10 µM rosiglitazone for 24 hours, fixed in 3.7 % formalin and stained with oil red O for 3 hours and then analysed using a light microscope. Image is representative of at least 3 separate experiments. The cells appear similar to the cells treated with oleate alone, with an increase in lipid staining within the cytoplasm of the cells, indicating that rosiglitazone does not prevent the accumulation of lipid within the cells in response to oleate.
Figure 3.19 Oil red O staining in MIN6 cells treated with palmitate, oleate and rosiglitazone

A comparison of oil red O staining in MIN6 cells treated with 0.4 mM palmitate, 0.4 mM oleate and/or 10 µM rosiglitazone for 24 hours. The difference between the effects of palmitate and oleate on lipid accumulation within the cells can clearly be seen.
fewer apoptotic cells as seen with the H/PI staining compared with palmitate treatment alone.

Treatment of MIN6 cells with metformin alone had no effect on cell viability as determined by MTT assay. In contrast to the results with rosiglitazone, metformin was unable to protect against the detrimental effects of palmitate. Treatment of β-cells with metformin has previously been shown to have positive effects, increasing the nuclear levels of PDX1 in MIN6 cells (Richardson et al., 2006). There is some evidence to suggest that metformin may be able to protect β-cells from free fatty acid induced damage (El-Assaad et al., 2003; Marchetti et al., 2004b), but it has also been found to impair insulin secretion, potentially via activation of AMPK, albeit at a much higher concentration than used in these experiments (Leclerc et al., 2004).

As p38 has previously been implicated in apoptosis in a number of cell types, including pancreatic β-cells, we tried to block the effect of palmitate on cell viability using the p38 inhibitor SB203580. The inhibitor alone did not affect cell viability, but offered significant protection from the effects of palmitate, although it was not sufficient to fully protect against the decrease in cell viability caused by palmitate. As the inhibitor alone did not increase cell viability, this suggests that the detrimental effects of palmitate are partially dependent on p38 activity, as palmitate and SB203580 are not merely exerting opposing effects on the MIN6 cells. However, as inhibiting p38 was not sufficient to completely block the detrimental effect of palmitate on cell viability, there must be additional mechanisms modified by palmitate in MIN6 cells that result in decreased viability.

Treatment of MIN6 cells with oleate did not significantly affect viability as measured by MTT assay and H/PI staining indicated there was no increase in cell death in response to oleate. However, the addition of palmitate and oleate to MIN6 cells significantly prevented the decrease in cell viability observed with palmitate alone.
TEM imaging and oil red O staining showed that treatment of MIN6 cells with oleate for 24 hours led to the accumulation of lipid droplets within the cells, whereas palmitate treatment did not result in any obvious storage of lipid. This could explain the differences in toxicity of these two fatty acids, as oleate is sequestered within the cell but palmitate is much more metabolically active. There is some evidence to suggest that the presence of unsaturated fatty acids induces the incorporation of saturated fatty acids into triacylglycerol within the cell, which is considered as relatively neutral lipid storage (Listenberger et al., 2003). On their own, saturated fatty acids are more likely to be stored as diacylglycerol, which is more harmful to the cells, or undergo desaturation before storage as triglyceride (Coll et al., 2008). Alternatively, palmitate forms ceramide or undergoes β-oxidation in the mitochondria, which has by-products that are detrimental to the cell (Maedler et al., 2001; Maedler et al., 2003).

Rosiglitazone treatment did not induce the storage of palmitate as lipid droplets within the cell, as TEM and oil red O staining appeared similar to untreated, suggesting that the protective effect of rosiglitazone in preventing palmitate-induced apoptosis is not mediated through promoting storage of palmitate as triacylglycerol.

These data suggest that palmitate causes apoptosis in MIN6 cells through a mechanism that partially relies on p38 activity, but this is not the sole pathway. Rosiglitazone was able to prevent this increase in apoptosis, but metformin was not. As neither rosiglitazone nor SB203580 alone caused an increase in cell viability, this suggests that the effect on cell viability as measured by the MTT assay occurred by directly protecting the cells from palmitate induced apoptosis rather than by increasing cell proliferation.

In the following chapter, we will begin to determine the signalling pathways regulating these events in more detail.
4. Effects of palmitate and rosiglitazone on signalling pathways in the β-cell

4.1 Introduction

The effects of rosiglitazone, acting via PPARγ, in adipocytes are well established. PPARγ is involved in regulating genes promoting adipocyte differentiation (Tontonoz et al., 1994). PPARγ has been shown to be expressed in β-cells, although its role is not yet established. Recent studies investigating the direct effects of rosiglitazone on pancreatic β-cells suggest rosiglitazone is not acting solely through PPARγ (Richardson et al., 2006; Vandewalle et al., 2008), and in some cell types may not be acting as a PPARγ agonist at all (Brunmair et al., 2001; Welters et al., 2004a). Rosiglitazone treatment of MIN6 cells increased expression of the important β-cell transcription factors PDX1 and FOXA2 (Richardson et al., 2006). This effect on PDX1 was found to occur in the presence of the PPARγ antagonist GW9662. When BRIN-BD11 β-cells were transfected with a plasmid containing a PPRE upstream of the luciferase gene it was found that rosiglitazone alone, at concentrations within the normal therapeutic range, did not increase luciferase activity (Welters et al., 2004a). Co-transfection with a PPARγ expression vector enabled an increase in activity of the PPRE upon treatment with rosiglitazone, suggesting that PPARγ may need to be more highly expressed in these cells for rosiglitazone to act as a PPARγ agonist. The ability of rosiglitazone to act as a PPARγ agonist in MIN6 cells has not yet been investigated.

It has been shown that both palmitate and oleate can activate p38 in a variety of cell types, but this has not been tested in MIN6 cells. In chapter 3 it was shown that an inhibitor of p38 was able to partially protect MIN6 cells from the detrimental effect of palmitate on cell viability (figure 3.4). It is therefore likely that palmitate may be able to
directly act on p38, and this may be an important event in exerting the different effects of palmitate and oleate on MIN6 cell viability. Activation of p38 in response to free fatty acids or cytokines has been shown to induce insulin resistance or result in apoptosis. In brown adipocytes, rosiglitazone was able to prevent the activation of p38 induced by TNF-α (Hernandez et al., 2004), and in β-cells rosiglitazone was able to prevent the activation of p38 caused by palmitate but not by a cytokine mixture in islets (Abaraviciene et al., 2008). Although rosiglitazone has been shown to prevent activation of p38, the effect of rosiglitazone alone is unclear, but preventing activation of the p38 signalling pathway may protect cells from damage. In this chapter, we aim to determine the direct effect of rosiglitazone on p38 in MIN6 cells.

AMPK is an important energy sensor in many cell types, and activation leads to an increase in energy conserving pathways and a reduction in energy consuming pathways. In muscle cells, AMPK activation through ATP depletion from exercise, or from other agents that act on AMPK, there is an increase in fatty acid oxidation, to provide energy for the cell (Merrill et al., 1997; Vavvas et al., 1997). Although there seems to be conflicting evidence as to whether activation of AMPK per se is beneficial or detrimental to pancreatic β-cells (Kefas et al., 2004; Sun et al., 2008), in the presence of chronic free fatty acid exposure an increase in β-oxidation may avert the production of more harmful fatty acid-derived agents, such as ceramide and diacylglycerol, as well as providing the cell with additional sources of energy. Rosiglitazone has been shown to activate AMPK in other cell types, although this has not yet been investigated in MIN6 cells. In this chapter, we will investigate the effect of rosiglitazone on the AMPK pathway.

Although the role of CK2 has not previously been examined in β-cells, it is ubiquitously expressed and is frequently implicated in proliferation and the inhibition of apoptosis (Scaglioni et al., 2008). This may make CK2 a desirable target in the
prevention of β-cell apoptosis in response to free fatty acids and cytokines. In other cell
types CK2 has been shown to activate Akt, either directly or indirectly (Di Maira et al.,
2005; Chao et al., 2007). In β-cells, Akt activation through insulin signalling causes
upregulation of PDX1 expression by phosphorylating FOXO1, leading to its exclusion
from the nucleus (Meur et al., 2011). Along with the more general role of Akt in
proliferation and cell survival, this makes Akt a potential target for protection of the β-
cell. In this chapter, we will investigate the effects of palmitate and rosiglitazone on
CK2 and Akt in MIN6 cells, to determine their role, if any, in palmitate-mediated
apoptosis.

4.2 Results

4.2.1 Rosiglitazone increases activity of a PPAR response element in MIN6 cells

To determine if rosiglitazone can act as a PPARγ agonist in MIN6 cells, cells were
transfected either with a plasmid containing a PPARγ response element upstream of the
luciferase gene, or with a control plasmid containing the luciferase gene with no
promoter region. In MIN6 cells transfected with the PPRE plasmid, treatment with 10
µM rosiglitazone for 24 hours led to a 50% increase in relative luciferase activity
compared with untreated cells (P<0.05) (Figure 4.1). No effect of rosiglitazone was
observed in cells transfected with the control pGL3 plasmid.

4.2.2 Palmitate increases phosphorylation of p38 in a time-dependent manner

In chapter 3, we have shown that an inhibitor of p38, SB203580, partially prevents the
detrimental effects of palmitate on MIN6 cell viability (Figure 3.4). To examine the
effect of palmitate treatment on p38 phosphorylation, western blotting was carried out
for phospho p38 and total p38 (Figure 4.2). Treatment of MIN6 cells with 0.4 mM
palmitate increased phosphorylation of p38 within 6 hours and was maintained over 72
Figure 4.1 Luciferase assay to determine the effect of rosiglitazone on the PPRE promoter activity in MIN6 cells

MIN6 cells transfected with either the pGL3 or PPRE plasmid were cultured for 24 hours in DMEM post-transfection, and then either maintained in DMEM containing 5 mM glucose with no additions or with the addition of rosiglitazone for 24 hours. Cells were then harvested and assayed for luciferase activity and protein content. The luciferase activity was then adjusted for protein content. Values are expressed as means ± SEM relative luciferase activity, relative to untreated for each plasmid. Samples were analysed in triplicate and results were obtained from at least 3 separate experiments. ** p<0.01.
hours, with a maximum 2.5 fold increase after 24 hours (P<0.01).

4.2.3 Oleate has no effect on p38 phosphorylation in MIN6 cells over a 72 hour timecourse

To investigate if oleate treatment had any effect on p38 activation, western blotting was performed for phospho p38 and total p38. MIN6 cells treated with 0.4 mM oleate had no change in the levels of total or phosphorylated p38 between 1 hour and 72 hours (Figure 4.3). This highlights a stark difference between the effects of saturated and unsaturated fatty acids in MIN6 cells, as in chapter 3 we have shown that inhibition of the p38 pathway partially protected MIN6 cells from palmitate-induced damage (Figure 3.4).

4.2.4 Rosiglitazone decreases levels of total and phosphorylated p38 in MIN6 cells

To determine the effect of rosiglitazone treatment on p38 in MIN6 cells, western blotting was carried out for phosphorylated p38, total p38 and GAPDH. MIN6 cells treated with 10 µM rosiglitazone had decreased levels of phosphorylated p38 within 1 hour and maintained over 72 hours (P<0.01), with a maximal decrease of approximately 45% (Figure 4.4). Levels of total p38 were also decreased by up to 37% in response to rosiglitazone treatment within 1 hour and maintained over 72 hours (P<0.01) (Figure 4.5).

4.2.5 Rosiglitazone prevents palmitate-induced increases in levels of phosphorylated p38

To determine if rosiglitazone can prevent palmitate-mediated increases in p38 activation in MIN6 cells, western blotting was carried out for phospho p38 and GAPDH. In MIN6 cells treated with 0.4 mM palmitate for 48 hours there was an approximate 70%
A. MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 0.4 mM palmitate for between 1 hour and 72 hours as indicated. Cells were then harvested and protein extracted, and western blotting was carried out for phosphorilated and total p38. B; densitometric analysis of 3 separate experiments, where values are mean relative density ± SEM. * p<0.05, ** p<0.01 relative to untreated control.

Figure 4.2 Western blot to determine the effect of palmitate on p38 phosphorylation in MIN6 cells
Figure 4.3 Western blot to determine the effect of oleate on p38 phosphorylation in MIN6 cells

A; MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 0.4 mM oleate for between 1 hour and 72 hours as indicated. Cells were then harvested and protein extracted, and western blotting was carried out for phosphorylated and total p38. B; densitometric analysis of 3 separate experiments, where values are mean relative density ± SEM.
Figure 4.4 Western blot to determine the effect of rosiglitazone on phosphorylated p38 in MIN6 cells

A; MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 10 µM rosiglitazone for between 1 hour and 72 hours as indicated. Cells were then harvested and protein extracted, and western blotting was carried out for phosphorylated p38 and GAPDH. B; densitometric analysis of at least 3 separate experiments, where values are mean relative density ± SEM. ** p<0.01 relative to untreated control.
Figure 4.5 Western blot to determine the effect of rosiglitazone on total p38 protein levels in MIN6 cells

A; MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 10 µM rosiglitazone for between 1 hour and 72 hours as indicated. Cells were then harvested and protein extracted, and western blotting was carried out for total p38 and GAPDH. B; densitometric analysis of at least 3 separate experiments, where values are mean relative density ± SEM. * p<0.05, ** p<0.01 relative to untreated control.
Figure 4.6 Western blot to determine the effect of rosiglitazone on the levels of p38 phosphorylation in response to palmitate in MIN6 cells

A; MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 0.4 mM palmitate, 10 µM rosiglitazone, or palmitate and rosiglitazone together for 48 hours. Cells were then harvested and protein extracted, and western blotting was carried out for phosphorylated p38 and GAPDH. B; densitometric analysis of 3 separate experiments, where values are mean relative density ± SEM. * p<0.05, ** p<0.01.
increase in levels of phosphorylated p38 compared with untreated cells (P<0.01). The addition of 10 µM rosiglitazone alone decreased phosphorylated p38. In cells treated with both palmitate and rosiglitazone, there was a significant reduction in the levels of phosphorylated p38 compared with palmitate alone (P<0.05) (Figure 4.6). As inhibition of p38 with SB203580 partially prevented the detrimental effect of palmitate on cell viability as measured by MTT assay, the prevention of palmitate-induced increases in p38 activation is likely to contribute to the protection of MIN6 cells from palmitate with rosiglitazone.

4.2.6 Rosiglitazone increases phosphorylation of ACC

To investigate the effect of rosiglitazone on the AMPK pathway in MIN6 cells, western blotting was carried out for phospho ACC and total ACC, as this is a known indicator of AMPK activity. In MIN6 cells treated with 10 µM rosiglitazone for 2 hours there was an 80% increase in phosphorylation of ACC compared with untreated controls (P<0.05) (Figure 4.7). This increase was blocked by the presence of the AMPK inhibitor compound C. The addition of 1 mM AICAR, an AMPK activator, provoked a similar increase in ACC phosphorylation to rosiglitazone, suggesting that rosiglitazone activates AMPK in MIN6 cells.

4.2.7 Rosiglitazone increases CK2 enzyme activity in an AMPK dependent manner

To determine the effect of rosiglitazone on CK2 in MIN6 cells, a CK2 enzyme activity assay was performed. In MIN6 cells treated with 10 µM rosiglitazone for 2 hours there was a 3 fold increase in CK2 activity compared with untreated cells (P<0.01) (Figure 4.8). Although the CK2 inhibitor TBB reduced CK2 activity alone, it was unable to prevent the rosiglitazone-induced increase. Treatment of MIN6 cells with 20 µM compound C alone increased CK2 activity by approximately 25%, but it was able to
Figure 4.7 Western blot to determine the effect of rosiglitazone on the levels of phosphorylated ACC in MIN6 cells

A; MIN6 cells were cultured in DMEM containing 5 mM glucose alone, with the addition of 10 μM rosiglitazone, with 20 μM compound C 30 minutes prior to addition of rosiglitazone, or with 1 mM AICAR for 2 hours. Cells were then harvested and protein extracted, and western blotting was carried out for phosphorylated and total ACC. B; densitometric analysis of 3 separate experiments, where values are mean relative density ± SEM. * p<0.05 relative to untreated control.
Figure 4.8 CK2 assay to determine the effect of rosiglitazone on CK2 activity

MIN6 cells were cultured in DMEM containing 5 mM glucose alone, with the addition of 10 µM rosiglitazone, 20 µM 4,5,6,7-tetrabromobenzotriazole (TBB), a specific inhibitor of CK2 activity, with TBB 30 minutes prior to the addition of rosiglitazone, 20 µM compound C, or with compound C 30 minutes prior to addition of rosiglitazone. Following 2 hours treatment, whole cell extracts were prepared by sonication and CK2 activity analysed by measurement of incorporation of $^{32}$P into 1 mM of the synthetic peptide substrate “CK2-tide” [RRRADDSDDDDD] (CPM). Samples were analysed in duplicate and data obtained from at least 3 separate experiments. Values represent mean ± SEM. ** $p<0.01$ compared with untreated, # $p<0.01$ compared with rosiglitazone alone.
MIN6 cells were cultured in DMEM containing 5 mM glucose alone, with the addition of 10 µM rosiglitazone, 0.4 mM palmitate, or both rosiglitazone and palmitate for 2 hours. Whole cell extracts were prepared by sonication and CK2 activity analysed by measurement of incorporation of $^{32}$P into 1 mM of the synthetic peptide substrate “CK2-tide” [RRRADDSDDDDD] (CPM). Samples were analysed in duplicate and data obtained from at least 3 separate experiments. Values represent mean ± SEM. ** $p<0.01$. 

**Figure 4.9 CK2 assay to determine the effect of palmitate on CK2 activity**
significantly block the effect of rosiglitazone (P<0.01), suggesting that the increase in CK2 activity observed with rosiglitazone is dependent on the ability of rosiglitazone to activate AMPK. The presence of 0.4 mM palmitate alone did not alter CK2 activity, nor did it affect the increase in activity observed with rosiglitazone (Figure 4.9).

4.2.8 Palmitate has no effect on Akt phosphorylation in MIN6 cells

To investigate the effect of palmitate on Akt in MIN6 cells, western blotting was performed for phospho Akt, total Akt and α-tubulin. MIN6 cells were treated with 0.4 mM palmitate for between 1 hour and 72 hours and western blotting carried out for Akt phosphorylated at the serine 473 residue, total Akt and α-tubulin. The levels of Akt phosphorylation did not alter upon treatment with palmitate at any of the time points examined (Figure 4.10).

4.2.9 Rosiglitazone increases phosphorylation of Akt in MIN6 cells

To determine the effect of rosiglitazone on Akt activation in MIN6 cells, western blotting was carried out for phospho Akt, total Akt and α-tubulin. In MIN6 cells treated with 10 µM rosiglitazone there was an increase in the phosphorylation of Akt at the serine 473 residue within 48 hours (P<0.05) and maintained over 72 hours (P<0.01), with a maximal increase of 75% (Figure 4.11). At the earlier time points, there was no significant effect of rosiglitazone on Akt phosphorylation.

4.3 Discussion

In MIN6 cells transfected with the PPRE plasmid, there was a 50% increase in activity in response to rosiglitazone. This was a relatively modest increase in activity compared with activation in other cell types in response to PPARγ agonists (Davies et al., 2001), and perhaps supports the suggestion that PPARγ is not the primary target for rosiglitazone in β-cells.
**Figure 4.10 Western blot to determine the effect of palmitate on Akt phosphorylation in MIN6 cells**

MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 0.4 mM palmitate for between 1 hour and 72 hours as indicated. Cells were then harvested and protein extracted, and western blotting was carried out for phospho Akt, total Akt and α-tubulin.
Figure 4.11 Western blot to determine the effect of rosiglitazone on Akt phosphorylation in MIN6 cells

A; MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of rosiglitazone for between 1 hour and 72 hours. Cells were then harvested and protein extracted, and western blotting was carried out for phospho Akt, total Akt and α-tubulin. B; densitometric analysis of at least 3 separate experiments, where values are mean relative density ± SEM. * $p < 0.05$, ** $p < 0.01$ relative to untreated control.
Although palmitate treatment caused an increase in phosphorylation of p38 in MIN6 cells, the use of the p38 inhibitor SB203580 did not fully protect the cells from the detrimental effects of palmitate on cell viability. This was perhaps surprising, as p38 has been implicated in apoptosis in many cells types, but demonstrates the complex effects of palmitate on MIN6 cells. However, blocking the activation of p38 caused by palmitate and cytokines in insulin target tissues has been shown to ameliorate insulin resistance (Hernandez et al., 2004). The importance of insulin signalling in β-cells is becoming clear (Haber et al., 2003), regulating expression of PDX1 and cell survival and proliferation. Although it has not been fully investigated, activation of p38 in β-cells by saturated free fatty acids may lead to defective insulin signalling, and subsequent β-cell dysfunction.

Oleate had no effect on p38 in MIN6 cells, indicating that the effect of the saturated fatty acid palmitate was specific. Although oleate has been shown to activate p38 in other cell types (Liu et al., 2007), these studies also found oleate to be detrimental to the cells. In MIN6 cells, oleate had no significant effect on cell viability. These results further suggest that activation of p38 in MIN6 cells is an important target in exerting the detrimental effect of palmitate on cell viability.

Rosiglitazone was able to rapidly decrease levels of total and phosphorylated p38 in MIN6 cells, and was able to impair the increase in phosphorylated p38 upon treatment with palmitate. As activation of p38 by palmitate plays a role in exerting its detrimental effects, inhibition by rosiglitazone could play a vital part in the protection against palmitate, both in preventing apoptosis and a potential role for maintaining normal insulin signalling within the cell.

Activation of AMPK causes phosphorylation and subsequent inactivation of ACC. Within 2 hours, rosiglitazone increased phosphorylation of ACC at a level similar to AICAR. ACC-dependent production of malonyl Co-A has been shown to inhibit
CPT1, which then results in a decrease in fatty acid oxidation in the mitochondria. The inhibition of ACC in response to rosiglitazone in MIN6 cells is likely to cause an increase in palmitate oxidation, which may contribute to the protective effect of rosiglitazone, as there are fewer fatty acyl moieties.

We have shown for the first time that CK2 is a target for rosiglitazone. The 3-fold increase in CK2 activity is likely to offer the β-cells some protection from apoptosis, as well as a potential role in increasing proliferation. The addition of the AMPK inhibitor compound C significantly prevented the increase in CK2 activity in response to rosiglitazone treatment, without any effect on CK2 activity on its own, suggesting that the effect of rosiglitazone on CK2 is at least partially dependent on AMPK. Palmitate did not affect CK2 activity, showing that palmitate and rosiglitazone do not have directly opposing effects on signalling pathways in the β-cell, but that mechanisms through which rosiglitazone can act interfere with the detrimental effects of palmitate.

Rosiglitazone caused increased phosphorylation of Akt within 48 hours. In other cell types, CK2 has been shown to directly phosphorylate Akt, although the time delay between increased CK2 activity and Akt phosphorylation in response to rosiglitazone treatment suggests that CK2 is unlikely to directly phosphorylate Akt in this instance. Indirect phosphorylation of Akt in response to increased CK2 activity cannot be ruled out, with a decrease in PTEN possibly acting as an intermediate. Testing for the dependence of CK2 activity on the activation of Akt was not possible in this case, given the inability of the CK2 inhibitor TBB to prevent the increase in CK2 activity in response to rosiglitazone.

Rosiglitazone appears to be acting via multiple pathways in MIN6 cells, inhibiting p38, activating AMPK, CK2 and Akt, in addition to its agonistic actions on PPARγ. It
seems likely that a complex combination of these effects is what enables rosiglitazone to protect the cells from palmitate induced damage.

In the following chapter, we extend these studies to examine the effects of palmitate and rosiglitazone on β-cell gene expression.
5. Effects of palmitate and rosiglitazone on β-cell gene expression

5.1 Introduction

The previous chapters have established that rosiglitazone can protect MIN6 cells from palmitate-induced damage, and highlighted several signalling pathways that may be responsible for exerting this effect, such as p38, AMPK, and CK2. The aim of this chapter was to investigate the effects of palmitate and rosiglitazone on crucial β-cell transcription factors and other key targets within the β-cell. In chapter 3, it was found that palmitate is detrimental to MIN6 cell viability and induced apoptosis, an effect prevented by rosiglitazone. Here, we aim to determine the effects of palmitate on genes indicative of normal β-cell function.

It has previously been shown that rosiglitazone treatment leads to increased levels of PDX1 and FOXA2 in the nucleus of MIN6 cells (Richardson et al., 2006). Both transcription factors are fundamental to the development of the pancreas, as well as having roles in transcriptional regulation in adult β-cells. PDX1 has been shown to regulate insulin gene expression in response to elevated glucose concentrations, as well as genes involved in glucose sensing in the β-cell. Reduction of PDX1 activity is detrimental to the normal functioning of β-cells. FOXA2 is one of a number of transcription factors which can regulate PDX1 expression (Wang et al., 2002), which competes with FOXO1, a negative regulator of PDX1 expression, to bind to the PDX1 promoter (Lee et al., 2002).

Most studies investigating rosiglitazone and glucose stimulated insulin secretion found no significant effect. However, one study that did find a direct effect in INS1 cells found that rosiglitazone treatment enhanced both basal and glucose stimulated insulin
secretion, increased insulin content, as well as increasing the expression of GLUT2 and glucokinase (Kim et al., 2008a). All of these effects were abolished by the addition of the PPARγ antagonist GW9662. There is more evidence to suggest a role for rosiglitazone in preventing the detrimental effect of free fatty acids on insulin secretion in β-cells, as opposed to having a direct effect on secretion, with UCP-2 being the target most frequently implicated (Vandewalle et al., 2008).

5.2 Results

5.2.1 Palmitate decreases PDX1 protein levels
To investigate the effect of palmitate treatment on PDX1 protein levels in MIN6 cells, western blotting was performed for PDX1 and GAPDH over a time course of between 1 hour and 72 hours. Six hours treatment with palmitate significantly decreased the levels of PDX1 protein in the nucleus of MIN6 cells, by approximately 55% (P<0.05), which was sustained over 72 hours (P<0.05) (Figure 5.1). Although the levels of PDX1 appeared to be decreased following 1 hour treatment with palmitate, this was not statistically significant.

5.2.2 Rosiglitazone increases nuclear levels of PDX1 protein, and prevents the decrease observed with palmitate
To determine the effect of rosiglitazone on PDX1 in MIN6 cells, and whether it can prevent palmitate-mediated decreases in protein levels within the nucleus, western blotting was carried out for PDX1 and GAPDH. Following 48 hours treatment with palmitate, PDX1 levels in the nucleus decreased by approximately 40% (P<0.01) (Figure 5.2). Following treatment with rosiglitazone for 48 hours, PDX1 protein levels within the nucleus approximately doubled (P<0.05). Rosiglitazone was also able to prevent the decrease in PDX1 levels in response to palmitate (P<0.01).
Figure 5.1 Western blot analysis to determine the effect of palmitate on levels of PDX1 in the nucleus of MIN6 cells

MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 0.4 mM palmitate for between 1 hour and 72 hours. Cells were then harvested and nuclear protein extracted, and western blotting was carried out for PDX1 and GAPDH. Densitometry was performed on results from at least 3 separate experiments, where values are mean relative density ± SEM. * $p<0.05$ relative to untreated control.
Figure 5.2 Western blot analysis to determine the effect of palmitate and rosiglitazone on nuclear levels of PDX1 in MIN6 cells

MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 0.4 mM palmitate, 10 µM rosiglitazone or both palmitate and rosiglitazone for 48 hours. Cells were then harvested and nuclear protein extracted, and western blotting was carried out for PDX1 and GAPDH. Densitometry was performed on results from at least 3 separate experiments, where values are mean relative density ± SEM. * p<0.05, ** p<0.01 relative to untreated control.
Figure 5.3 Western blot analysis to determine the effect of palmitate on nuclear levels of FOXA2 in MIN6 cells

MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 0.4 mM palmitate for between 1 hour and 72 hours. Cells were then harvested and nuclear protein extracted, and western blotting was carried out for FOXA2 and GAPDH. Densitometry was performed on results from at least 3 separate experiments, where values are mean relative density ± SEM.
5.2.3 Palmitate has no effect on FOXA2 protein levels

To determine the effect of palmitate treatment on nuclear FOXA2 protein levels in MIN6 cells, western blotting was carried out for FOXA2 and GAPDH in nuclear extracts from MIN6 cells treated with palmitate for between 1 hour and 72 hours. Palmitate treatment had no effect on nuclear levels of FOXA2 protein in MIN6 cells within 72 hours (Figure 5.3).

5.2.4 Rosiglitazone has no effect on glucokinase mRNA levels.

To determine if rosiglitazone has any effect on glucokinase expression in MIN6 cells, real time RT-PCR was performed. Treatment with rosiglitazone for 48 hours did not result in any significant difference in glucokinase mRNA levels compared with untreated cells (Figure 5.4).

5.2.5 Rosiglitazone decreases GLUT2 mRNA levels whereas palmitate has no effect.

To determine the effects of palmitate and rosiglitazone on GLUT2 in MIN6 cells, real time RT-PCR was performed. Following 48 hours treatment with palmitate, no effect was observed on GLUT2 mRNA (Figure 5.5). Treatment of MIN6 cells with rosiglitazone for 48 hours resulted in approximately 30% decrease in GLUT2 mRNA compared with untreated cells (P<0.05).

5.2.6 Rosiglitazone decreases levels of GLUT2 protein.

To determine if the effect of rosiglitazone on GLUT2 mRNA resulted in a decrease in GLUT2 protein within the cell, and the time scale involved, western blotting was performed. Treatment of MIN6 cells with rosiglitazone for between 24 and 96 hours had no significant effect on GLUT2 protein levels (Figure 5.6), however, after 120
MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 10 µM rosiglitazone for 48 hours. Cells were then harvested, mRNA extracted, reverse transcribed and RT-PCR performed for glucokinase and GAPDH. Results were analysed using the Pfaffl method. Results indicate mean fold change ± SEM.
Figure 5.5 Real time RT-PCR to determine the effect of palmitate and rosiglitazone on GLUT2 in MIN6 cells

MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 0.4 mM palmitate, 10 µM rosiglitazone or DMEM containing 25 mM glucose for 48 hours. Cells were then harvested, mRNA extracted, reverse transcribed and RT-PCR performed for GLUT2 and GAPDH. Results were analysed using the Pfaffl method. Results indicate mean fold change ± SEM. * p<0.05 relative to untreated control.
Figure 5.6 Western blot to determine the effect of rosiglitazone on GLUT2 protein levels in MIN6 cells

A. MIN6 cells were cultured in DMEM containing 5 mM glucose alone or with the addition of 10 µM rosiglitazone for between 24 hours and 120 hours. Cells were then harvested and protein extracted, and western blotting was carried out for GLUT2 and GAPDH. B. Densitometric analysis of results from at least 3 separate experiments, where values are mean relative density ± SEM. ** p<0.01 relative to untreated control.
hours treatment with rosiglitazone, GLUT2 protein levels were decreased by approximately 35% compared with untreated cells (P<0.01).

5.3 Discussion

In MIN6 cells treated with palmitate, there was a 55% decrease in PDX1 protein levels in the nucleus within 6 hours. This is likely to contribute to the detrimental effect of palmitate on MIN6 cells, and possibly affect insulin production. Rosiglitazone was able to protect the observed decrease in PDX1 at 48 hours, highlighting another mechanism through which this drug can protect against the detrimental effects of saturated fatty acids.

Although palmitate decreased levels of PDX1 in the nucleus, there was no effect on FOXA2, another important β-cell transcription factor, indicating that the observed effects on PDX1 are not dependent on alterations of FOXA2 localisation within the cell.

Using real time RT-PCR, it was found that treatment of MIN6 cells with rosiglitazone for 48 hours decreased the levels of GLUT2 mRNA by approximately 30%, whereas palmitate treatment had no effect. This decrease in GLUT2 mRNA did not result in a significant decrease in GLUT2 protein levels until 120 hours. This delay is probably due to the abundance of GLUT2 within the β-cell. The significance of this decrease in GLUT2 in response to rosiglitazone in MIN6 cells remains to be fully established, although it may reduce the rate at which glucose is taken up into the cell at high glucose concentrations. This may prove beneficial in lipotoxic conditions, where the combination of high glucose and high levels of free fatty acids is more detrimental to the β-cell than either condition alone. It is unlikely to have a major impact on glucose stimulated insulin secretion, as GLUT2 is not considered rate-limiting in this process and the level to which it is decreased in response to rosiglitazone is rather modest.
That rosiglitazone had no observed effect on glucokinase mRNA levels suggests that rosiglitazone alone is unlikely to affect glucose-stimulated insulin secretion. However, palmitate has previously been shown to increase levels of UCP-2, which is known to result in decreased glucose stimulated insulin secretion, an effect prevented by rosiglitazone (Tian et al., 2006). This highlights a role for rosiglitazone in protecting against the detrimental effects of saturated fatty acids on insulin secretion rather than directly affecting GSIS.
6. General Discussion

The aim of this study was to determine the different effects of the saturated fatty acid palmitate and the mono-unsaturated fatty acid oleate on MIN6 cells and whether the anti-diabetic drugs metformin and rosiglitazone could prevent any detrimental effects. We have shown that not only were there stark differences between the effects of palmitate and oleate on the cell, but that oleate was able to protect MIN6 cells from palmitate-induced apoptosis. There is evidence to suggest that both metformin and rosiglitazone can exert positive effects on pancreatic β-cells with both increasing nuclear levels of the transcription factor PDX1 in MIN6 cells, albeit through different mechanisms (Richardson et al., 2006). However, here we have shown that rosiglitazone was able to protect against the detrimental effects of palmitate in MIN6 cells, whereas metformin did not. We also investigated the possible mechanisms through which rosiglitazone was able to protect MIN6 cells from palmitate-induced damage, with p38, AMPK, CK2 and Akt pathways all implicated.

6.1 The effect of saturated and unsaturated fatty acids

Using MTT assays to assess cell viability, we showed that treatment of MIN6 cells with palmitate for 72 hours led to a decrease in the number of viable cells compared with control MIN6 cells, whereas treatment with oleate did not significantly affect cell viability. We also showed that oleate was able to prevent the decrease in cell viability caused by palmitate. H/PI staining suggested that the decrease in the number of viable MIN6 cells in response to treatment with palmitate was due to increased apoptosis, and that oleate was able to prevent this palmitate-induced apoptosis.

Transmission electron microscopy and oil red O imaging highlighted how oleate treatment led to the appearance of large lipid droplets within the cell, but palmitate treatment had no such effect. It has previously been shown that incorporating saturated
fatty acids into triglyceride within the cell by the addition of unsaturated fatty acids reduces the toxic effect of saturated fatty acids (Listenberger et al., 2003). However, in BRIN-BD11 cells it was found that treatment with palmitate alone led to an increase in triglyceride content of the cell, and that co-incubation with methyl-palmitoleate, an analogue of the monounsaturated free fatty acid palmitoleate that is unable to be incorporated into TAG, was still able to protect the cells from the cytotoxic effects of palmitate (Diakogiannaki et al., 2007).

We have shown that while palmitate increased phosphorylation of p38, oleate treatment had no effect on p38 activation in MIN6 cells. The use of the p38 inhibitor SB203580 partially blocked the detrimental effect of palmitate on cell viability in MIN6 cells, however, it was not sufficient to fully protect the MIN6 cells as cell viability was statistically significantly lower than untreated cells. This suggests that palmitate-induced activation of the p38 pathway plays a role in the subsequent increase in apoptosis, but that other mechanisms are also involved in the damage caused by palmitate. Other potential mechanisms through which palmitate could be causing apoptosis in MIN6 cells could be the production of reactive oxygen species, ceramide and diacylglycerol, or it can undergo β-oxidation, which has harmful by-products such as acyl carnitines.

Although the mechanism through which oleate could protect MIN6 cells from palmitate-induced apoptosis was not established in this study, TEM imaging and oil red O staining have shown that oleate treatment led to the formation of large lipid droplets in the cells, whereas this was not the case upon treatment with palmitate. Further studies would need to be done to establish whether the addition of oleate induces the incorporation of palmitate into lipid droplets in MIN6 cells. Some previous studies have shown that unsaturated fatty acids protect against saturated fatty acids by promoting storage of the saturated fatty acids within the cells (Listenberger et al., 2003), whereas
other studies have found that inhibiting the formation of triacylglycerol did not prevent unsaturated fatty acids from protecting against saturated fatty acid-induced damage (Diakogiannaki et al., 2007).

Evidence suggests that a diet low in saturated fat and higher in unsaturated fat can have many health benefits over a diet high in unsaturated fat (Esposito et al., 2009b). It is now becoming clear that on a cellular level, unsaturated fatty acids are not only less toxic but can also be protective, so it is perhaps not surprising that studies have shown that a Mediterranean diet often has an improved health outcome than a diet that is low in both saturated and unsaturated fat (Paniagua et al., 2007; Salas-Salvado et al., 2011).

6.2 Metformin

Although treatment of β-cells with metformin has previously been found to have beneficial effects, it was unable to prevent palmitate-mediated apoptosis in these experiments. In other studies using metformin at a concentration of 15 µM it has been found to reduce the rate of apoptosis and increase insulin content in islets isolated from type 2 diabetic patients (Marchetti et al., 2004a). In studies using metformin at concentrations between 0.5 mM and 2 mM it has been shown to activate AMPK and induce apoptosis in MIN6 β-cells and primary rat β-cells (Kefas et al., 2004). Although these concentrations are much higher than the estimated highest plasma concentrations of 40 µM, there is evidence that metformin can accumulate in tissues (Wilcock and Bailey, 1994). It is argued that using much higher concentrations in short term experiments mirrors this accumulation that occurs after long term treatment. However, human trials show that metformin is well tolerated in vivo and, as long as patients are monitored for kidney function, it has a good safety profile making it a popular choice in the treatment of type 2 diabetes (Garber et al., 1997).
6.3 Rosiglitazone

Since the completion of this work, rosiglitazone was withdrawn from the European market in September 2010 (EMA, 2010), as a result of data implicating an increased risk of cardiovascular disorders in patients receiving rosiglitazone treatment (Nissen and Wolski, 2007). The use of rosiglitazone in the USA has been significantly restricted (FDA, 2010). Although rosiglitazone may no longer be a viable long term treatment option for patients with type 2 diabetes, effects of rosiglitazone on insulin sensitisation, and the direct beneficial effects on pancreatic β-cells should not be ignored. It is important to determine the mechanisms through which rosiglitazone exerts these positive effects in order to identify future targets for anti-diabetic drugs. It may also be possible to identify groups of patients for which the benefits of taking rosiglitazone outweigh the associated cardiovascular risks.

In this study, we have shown that the addition of rosiglitazone to MIN6 cells was able to prevent palmitate-induced apoptosis, as determined by H/PI staining, and preserve the number of viable cells. TEM imaging showed cells treated with palmitate in the presence of rosiglitazone appeared healthier than those treated with palmitate alone, but there was no evidence of lipid storage within the cell. Oil red O staining also showed no sign of lipid storage within the cells following treatment with palmitate, with or without the presence of rosiglitazone.

Although the mechanisms for the protective effect of rosiglitazone against palmitate-mediated damage are not definitive, it appears that a down-regulation of p38 plays a role. A rapid decrease in the levels of p38 protein was observed in MIN6 cells in response to rosiglitazone. We have also shown that inhibiting p38 with the specific inhibitor SB203580 partially blocked the effect of palmitate on MIN6 cell viability, so it is likely that rosiglitazone can prevent palmitate-mediated damage through this pathway. However, as p38 is not the only pro-apoptotic pathway activated by palmitate
in this instance, the ability of rosiglitazone to completely prevent cell death suggests multiple mechanisms of protection. As the addition of rosiglitazone did not appear to affect the storage of palmitate within the cell, this indicates that palmitate is still metabolically active within the cell, rather than being stored as inert lipid droplets. The other potential mechanisms for the protective effect of rosiglitazone include AMPK, Akt and CK2, all of which we have found to be activated by rosiglitazone.

In addition to protecting β-cells from the detrimental effects of free fatty acids, there may be a role for rosiglitazone in enhancing β-cell proliferation. Although there did not appear to be a significant increase in cell viability in response to 72 hours treatment with rosiglitazone compared with untreated cells as measured by MTT assay, effects on proliferation may take longer to show any significant effect. Treatment of MIN6 cells with rosiglitazone for 2 hours resulted in an increase in CK2 enzyme activity. As well as being implicated in protecting cells from apoptosis, increased CK2 activity is also linked to an increase in proliferation (Lebrin et al., 2001). Akt is also linked to cell proliferation (Jetton et al., 2005; Rafacho et al., 2008), and in MIN6 cells we have shown activation of Akt in response to rosiglitazone occurred within 48 hours, so significant effects on proliferation may take longer than 72 hours to present themselves.

**6.4 p38 MAPK**

Palmitate was found to activate p38 in MIN6 cells, and inhibition of p38 was able to partially protect MIN6 cells from palmitate-induced damage. We have shown that rosiglitazone can rapidly down regulate p38 in MIN6 cells, which could be vital in the protection of β-cells from palmitate. However, as rosiglitazone was able to offer full protection against palmitate, whereas specifically inhibiting the p38 pathway only
partially protected the cells, there must be additional mechanisms through which rosiglitazone exerts its protective effects on MIN6 cells.

Although insulin resistance was not investigated in this study, it can occur in β-cells and may lead to dysfunction and alterations in the expression of certain genes important for either glucose sensing or in the production of insulin (Cantley et al., 2007). The ability of rosiglitazone to down-regulate p38 may also be able to preserve normal β-cell functioning in the presence of palmitate, as well as preventing apoptosis. It is important that anti-diabetic drugs can not only preserve β-cell mass but that they can also maintain a normal β-cell function.

It is worth looking into the potential of drugs that can inhibit p38 in the treatment of type 2 diabetes. The activation of p38 is implicated in both apoptosis and fatty acid-induced insulin resistance in insulin target tissues, which are ideal targets in for anti-diabetic drugs.

6.5 AMPK

AMPK is a major regulator of energy consumption and its activation increases fatty acid oxidation. In the event of excess fatty acid exposure, AMPK may increase fatty acid oxidation, preventing the formation of harmful fatty acid moieties such as ceramide and diacylglycerol (Yamashita et al., 2004; Cacicedo et al., 2011). However, it is difficult to determine if activation of AMPK by rosiglitazone is important in exerting protection against palmitate, as inhibiting AMPK activity for 72 hours would likely be detrimental to cell viability on its own. The only viable method of testing would be to block the rosiglitazone-dependent activation of AMPK, by determining the mechanism through which rosiglitazone increases AMPK activity. In human umbilical vein endothelial cells, activation of AMPK by AICAR prevented palmitate-induced NFκB activation (Cacicedo et al., 2004). In MIN6 cells, palmitate has been shown to induce NFκB
nuclear localisation, suggesting a potential mechanism through which rosiglitazone-induced AMPK activation may prevent palmitate-induced apoptosis. Although it was not investigated in this study, it has previously been shown that rosiglitazone can prevent the detrimental effects of palmitate on glucose-stimulated insulin secretion in pancreatic β-cells (Tian et al., 2006; Meidute Abaraviciene et al., 2008). Using overexpression of SREBP-1c in INS-1 cells as a model of lipotoxicity, it has been shown that treatment of the cells with AICAR was able to rescue glucose-stimulated insulin secretion (Yamashita et al., 2004). The ability of rosiglitazone to activate AMPK in MIN6 cells is a potential mechanism through which it can preserve β-cells function in the presence of free fatty acids.

6.6 CK2

Although the role of CK2 in pancreatic β-cells is not yet clear, it has a general role in proliferation and protection against apoptosis. Palmitate treatment did not affect CK2 activity, so this is not the mechanism through which palmitate causes apoptosis in MIN6 cells. However, activation of CK2 by rosiglitazone may have effects downstream that can prevent palmitate-mediated apoptosis. In fibroblast cells it has been shown that CK2 can phosphorylate caspase targets, preventing caspase-mediated cleavage and subsequent apoptosis (McDonnell et al., 2008). As caspase-3 activity has previously been found to be important in palmitate-mediated apoptosis in β-cells (Abaraviciene et al., 2008), this could be a mechanism through which activation of CK2 by rosiglitazone can protect against palmitate.

CK2 has been shown to activate Akt, both directly (Di Maira et al., 2005), and indirectly through degradation of PTEN (Al-Khoury et al., 2005), which negatively regulates PI3K. Due to the differences in timescale between CK2 activation and Akt phosphorylation it is likely that phosphorylation of Akt in response to rosiglitazone does
not occur through direct phosphorylation by CK2. This may give rise to longer term proliferative effects of rosiglitazone in β-cells.

We have shown that rosiglitazone-induced CK2 activation in MIN6 cells was dependent on AMPK activity, as inhibition of AMPK with compound C prevented the increase in CK2 activity in response to rosiglitazone. As compound C alone had no effect on CK2 activity, it is not merely opposing effects of rosiglitazone and compound C. The increase in CK2 activity observed with rosiglitazone was unaffected by the presence of the CK2 inhibitor TBB. This suggests that rosiglitazone alters CK2 activity through an alternative site or by impacting on the affinity of TBB for CK2. CK2 is known to act as a chaperone with other proteins (Miyata and Nishida, 2004). It may be that activation of AMPK by rosiglitazone results in binding of the two kinases, which is able to overcome suppression of CK2 activity by TBB.

6.7 Akt

Akt has a broad role in many cell types in promoting proliferation and preventing apoptosis (Storling et al., 2005; Olsen et al., 2007). However, in these experiments, phosphorylation of Akt only occurred following 48 hours treatment with rosiglitazone. It is likely that the anti-apoptotic effect of rosiglitazone in MIN6 cells is exerted through pathways which are modulated before 48 hours, as the detrimental effect of palmitate is evident by 24 hours by TEM imaging, and is prevented by rosiglitazone at this time point. Treatment of MIN6 cells with palmitate had no effect on Akt. Rosiglitazone alone had no effect on cell viability within 72 hours, so the increase in Akt phosphorylation is unlikely to affect cell proliferation within the timescales tested here. However, as there does not appear to be one clear mechanism for the protective effect of rosiglitazone, the contribution of Akt cannot be discounted.
Other roles of Akt in the β-cell include insulin production and secretion (Bernal-Mizrachi et al., 2004). Although not tested here, activation of Akt by rosiglitazone may preserve GSIS in the presence of palmitate. The implications of activation of Akt in type 2 diabetic patients should be considered. In insulin resistant rats, β-cell growth and compensation are dependent on Akt (Jetton et al., 2005). It may be that in humans taking rosiglitazone, activation of Akt could preserve or enhance β-cell mass. If agents that target activation of Akt in pancreatic β-cells are prescribed to obese insulin resistant individuals, it may prevent the deterioration of islet mass that leads to the development of type 2 diabetes.

6.8 PPARγ

Rosiglitazone is an established PPARγ agonist, through which it can exert its insulin sensitising effects in insulin target tissues (Jiang et al., 2002; Diaz-Delfin et al., 2007). However, little is known about the role of PPARγ in pancreatic β-cells and many of the direct effects of rosiglitazone on β-cells in vitro have been shown to occur through mechanisms independent of PPARγ (Richardson et al., 2006; Vandewalle et al., 2008).

In this study, we have shown that rosiglitazone can act as a PPARγ agonist in MIN6 cells. It has previously been shown that overexpression of PPARγ in primary rat islets increased expression of genes responsible for fatty acid oxidation. At maximal activation of PPARγ 1, there was a decrease in glucose oxidation, cellular ATP content, and glucose-stimulated insulin secretion (Parton et al., 2004). It is yet to be established whether rosiglitazone treatment would have these effects in β-cells, as PPARγ may not be sufficiently abundant and the observed agonistic action of rosiglitazone on PPARγ was modest. Previous studies suggest that PPARγ is responsible for exerting some of the direct effects of rosiglitazone on β-cells (Lupi et al., 2004; Lin et al., 2005),
although it is clear that not all of the effects of rosiglitazone in β-cells are dependent on PPARγ and here we have highlighted multiple alternative pathways.

6.9 GLUT2

We have shown that rosiglitazone treatment in MIN6 cells decreased both mRNA and protein levels of GLUT2 by approximately 30%. The effects on mRNA were evident after 48 hours, although the protein levels were unaffected until 120 hours treatment with rosiglitazone. The time delay between the effects on mRNA and protein probably reflects the relative abundance of GLUT2 in the β-cell.

It has previously been shown that overexpression of FOXA2 in INS-1 β-cells can reduce expression of GLUT2 and glucokinase (Wang et al., 2002). As rosiglitazone increases nuclear levels of FOXA2 in MIN6 cells (Richardson et al., 2006), this is a potential mechanism for the observed effect on GLUT2, although here we have shown that rosiglitazone had no effect on glucokinase mRNA expression in MIN6 cells. Alternatively, in hepatocytes, activation of AMPK led to decreased HNF4α and subsequently reduced GLUT2 expression (Leclerc et al., 2001). As we have shown, rosiglitazone activated AMPK in MIN6 cells, which could be a potential mechanism through which rosiglitazone decreased GLUT2 expression. However, the effects of rosiglitazone on HNF4α remain unclear.

It is a natural assumption that decreasing GLUT2 levels would negatively impact on GSIS, however the evidence suggests that other factors are more important for GSIS than GLUT2. Overexpression of GLUT2 does not enhance GSIS in MIN6 cells (Ishihara et al., 1995). As glucokinase appears to be the rate-limiting factor in GSIS, a 30% decrease in GLUT2 protein levels is unlikely to have a large effect on GSIS, and there was no difference observed in the levels of glucokinase mRNA upon treatment with 10 µM rosiglitazone for 48 hours.
Figure 6.1 Proposed mechanisms through which rosiglitazone can protect MIN6 cells from palmitate-induced apoptosis

In this study, we have identified multiple targets of rosiglitazone (in blue). This diagram shows the mechanisms of how they may be preventing palmitate-induced damage. Green indicates possible mechanisms of palmitate-induced damage. Black lines indicate a positive effect and red lines indicate an inhibitory effect.
Although it is counter-intuitive that a reduction in GLUT2 expression may be beneficial to the β-cell, the delicate balance between glucose and fatty acid oxidation needs to be addressed. High glucose concentrations in combination with elevated free fatty acids appear to act synergistically to enhance β-cell death more than either on its own (El-Assaad et al., 2003), as fatty acids can interfere with normal glucose metabolism. Previously, it has been found that treatment of β-cells with rosiglitazone has increased expression of GLUT2 (Kim et al., 2008a). It has also been found that 100 µM rosiglitazone increased GLUT2 mRNA and protein, although at this concentration insulin secretion was reduced, suggesting that GLUT2 expression does not directly relate to GSIS (Blumentrath et al., 2001). In this study, lower concentrations of rosiglitazone moderately decreased GLUT2 protein levels. Although the effect of decreasing GLUT2 expression in MIN6 cells is unclear, it may be beneficial in the event of glucolipotoxicity. The combined detrimental effect of chronically elevated glucose and free fatty acid levels is well established, and slowing the rate at which glucose is taken up by the cell may help to reduce free fatty acid-induced cell death. Although it has previously shown that in vitro rosiglitazone can reduce GSIS (Blumentrath et al., 2001), there is much evidence to suggest that rosiglitazone can prevent the detrimental effect of free fatty acids on GSIS (Lupi et al., 2004; Tian et al., 2006), and also preserve β-cell function in vivo (Gastaldelli et al., 2007).

### 6.10 Wider implications

This study has shown that rosiglitazone is effective at protecting β-cells against palmitate-induced damage, and has highlighted several pathways that are potentially involved in offering this protection. Although rosiglitazone may no longer be a viable treatment option in type 2 diabetic patients, it is important to learn the mechanisms of how it can offer protection to the β-cells in order to identify targets for future drug
treatments. As we have shown, rosiglitazone directly affects multiple pathways associated with preventing apoptosis, as summarised in Figure 6.1. This suggests a potential role for rosiglitazone in promoting islet survival post-transplantation, with short term prescription of rosiglitazone unlikely to pose the same cardiovascular risk as identified in type 2 diabetic patients receiving long term rosiglitazone treatment.

Other thiazolidinediones, such as pioglitazone, have been found to improve β-cell function (Gastaldelli et al., 2007; Evans-Molina et al., 2009), and to protect against free fatty acid-induced damage (Saitoh et al., 2008). It is not clear if the pathways found to be affected by rosiglitazone in this study are effects that are specific to rosiglitazone or if they are common to other TZDs. Pioglitazone appears to have a more favourable safety profile than rosiglitazone, so if the beneficial effects of rosiglitazone are also a feature of other drugs in the same class, we can get a better understanding of how these drugs can be used, with fewer adverse effects (Erdmann et al., 2010).

By determining the mechanisms of how rosiglitazone can protect β-cells from free fatty acid-induced damage, we can highlight potential targets for the development of future anti-diabetic drugs, and possibly drugs that can be used as an early intervention to prevent the decline from obesity-related insulin resistance.

6.11 The obesity crisis

The current obesity crisis has led to a dramatic increase in the number of people being diagnosed with type 2 diabetes. Even after diagnosis of type 2 diabetes, it has been found that weight loss surgery can significantly reverse diabetes in some patients (Schauer et al., 2003).

Taking into account the cost to the NHS of treating diabetes and its complications, in addition to other obesity related conditions, as well as the cost of benefits for those unable to work as a result of their condition, if the current trend for
the increasing rate of obesity continues the cost will be unsustainable. Not all treatments will remain available and tough decisions will have to be made with regards to who can be treated, and how to discriminate between who will receive treatment and who will not. There is also the question of whether it is ethical to continue treating people who could have prevented their current situation, when treatments for other diseases are considered too expensive.

The simplest solution to preventing the rising prevalence of obesity is to educate people to consume fewer calories and take more exercise. However, most people are already aware that poor diet and a sedentary lifestyle are bad for their health, it is identifying the reasons people choose not to do something about it, and finding solutions that are easy to incorporate into everyday life that will provide the key to preventing obesity.

While continuing to educate the public on the importance of a healthy balanced diet and remaining physically active is vital to prevent the current obesity crisis spiralling further, there is still a need for pharmacological interventions to treat patients with type 2 diabetes. The focus should be on early intervention and detection, using diet and exercise in the first instance. However, identifying drugs that are useful in preventing the development from insulin resistance to type 2 diabetes is important, rather than just prescribing drugs to treat diabetes once all other interventions have failed.

6.12 Future directions

Following this study, the mechanism through which oleate can prevent palmitate-induced apoptosis in MIN6 cells remains to be fully elucidated, as current literature investigating these effects in β-cells remains conflicting.
We have identified multiple targets of rosiglitazone which may be important in the survival of the β-cell in response to free fatty acids, such as p38, AMPK, CK2 and Akt. It is not yet known if these effects are specific to rosiglitazone or if they are a feature of thiazolidinediones, so future experiments would need to be carried out to investigate if other drugs of the same class, such as pioglitazone, can also exert a protective effect on the β-cell.

We have established that treatment of MIN6 cells with rosiglitazone can activate Akt, which is often linked with preventing apoptosis and promoting cell proliferation. This only occurred after 48 hours, which suggests that activation of Akt by rosiglitazone is not required for preventing palmitate-induced apoptosis as the detrimental effects of palmitate can be seen at 24 hours by TEM imaging, and this is prevented by rosiglitazone. It is likely that the effect of activating Akt by rosiglitazone exerts effects later than 72 hours, which is the timescale used in the majority of the experiments in this study. It is yet to be determined if treatment of β-cells with rosiglitazone for longer than 72 hours has any effect on proliferation as a result of activating Akt.

In this study, we have shown that rosiglitazone modulates multiple pathways in MIN6 cells, and can prevent palmitate-mediated apoptosis. The next step is to establish if rosiglitazone can exert these effects in pancreatic islets, and in vivo, as this would be important to determine the mechanism through which rosiglitazone can have positive effects on β-cell function in type 2 diabetic patients.
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Publications

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Submitted papers:


**Published Abstracts:**


Selenium stimulates pancreatic beta-cell gene expression and enhances islet function

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Abstract The present study investigated the role of selenium in the regulation of pancreatic beta-cell function. Utilising the mouse beta-cell line Min6, we have shown that selenium specifically upregulates Ipf1 (insulin promoter factor 1) gene expression, activating the −2715 to −1960 section of the Ipf1 gene promoter. Selenium increased both Ipf1 and insulin mRNA levels in Min6 cells and stimulated increases in insulin content and insulin secretion in isolated primary rat islets of Langerhans. These data are the first to implicate selenium in the regulation of specific beta-cell target genes and suggest that selenium potentially promotes an overall improvement in islet function.

Keywords: Beta-cell; Islets of Langerhans; Transcription; Selenium; Ipf1

1. Introduction

The essential trace element selenium forms a key constituent of selenoproteins such as glutathione peroxidases [1] and acts as a critically important antioxidant in many different cell types [2]. Selenium has also been found to have insulin-mimetic activities in vitro and in vivo [3] and has been shown to stimulate glucose uptake in adipocytes in vitro [4], restore normoglycaemia in streptozotocin-diabetic rats [2] and regulate vital metabolic processes such as glycolysis and gluconeogenesis [5]. The mechanisms by which selenium exerts these effects are still relatively unclear, however evidence indicates that selenium may activate key molecules in the insulin signalling cascade [6].

It has been shown in other tissues that different chemical forms of selenium have differing potencies in their effects on cellular function [7]. To date, the specific effects of different selenium moieties on the regulation of pancreatic beta-cell function have not been determined. In the present study, we have utilised the glucose-responsive mouse beta-cell line Min6 to begin investigating the specific effects of selenium on expression of the key beta-cell transcription factor Ipf1 (insulin promoter factor 1) and on the regulation of insulin gene expression. In combination with our analysis of the effects of selenium on insulin content and secretion from isolated rat islets of Langerhans, the results of the present study indicate that selenium provokes significant changes in pancreatic beta-cell gene expression, contributing to overall improvements in islet function.

2. Materials and methods

2.1. Materials

Cell culture materials, LipsfectAMINE and OptiMEM were purchased from Invitrogen (Paisley, UK); insulin was from Penho (Newcastle, UK); protein assay reagent was from Bio-Rad (Hemel Hempstead, UK). All other chemicals and materials were purchased from Sigma–Aldrich (Dorset, UK).

2.2. Cell culture and transfection

Min6 cells were cultured in selenium-free Dulbecco’s modified Eagle’s medium (DMEM) containing 5 mM/l glucose. Construct pGL3-Ipf1 contains a 4531 bp fragment of the mouse Ipf1 promoter upstream of the luclerase reporter gene [8]. pGL3-Ipf1 contains a section of the Ipf1 promoter between −2715 and −1966 bp. Plasmid DNA was prepared using an Endotex-free Miniprep Kit (Qagen, Crawley, UK). Min6 cells were transfected as previously described [8]. Forty-eight hours post-transfection cells were incubated with either 30 or 100 or 50 mM sodium selenite (NaSeO\textsubscript{3}), 30 mM sodium selenite (NaSeO\textsubscript{3}) or 30 mM sodium selenite (NaSeO\textsubscript{3}) for 3 h. Lucifere assays were performed as previously described [8]. Thirty min/l was used throughout the present study, based on the recommended RDA and healthy circulatory concentration of selenium [8].

2.3. RT-PCR

RNA was isolated using the Gen Elute Mammalian Total RNA Kit (Sigma) according to manufacturer’s protocol. The following encompassing primers and PCR conditions were utilised. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GAPDH forward primer 5'-TCTTCACGACCAACTGGCTG-3' and 5'-GGGAGGTGAGCATGCAAGGGTCAT-3', 95°C for 5 min, then 25 cycles consisting of 95°C for 30 s, 55°C for 45 s, 67°C for 45 s, with a final step of 72°C for 5 min. For Ipf1, 5'-TCTTCACGACCAACTGGCTG-3' and 5'-GGGAGGTGAGCATGCAAGGGTCAT-3', 95°C for 5 min, then 35 cycles consisting of 95°C for 30 s, 58°C for 45 s, 72°C for 45 s, with a final step of 72°C
for 5 min. For insulin, 5'-cactgattacgaacagca-3' and 5'-gtggagagaagaacagt-3' for 30 s, 55°C for 45 s, 72°C for 45 s, with a final step of 72°C for 5 min.

2.4. Western blotting

Whole cell extracts were prepared, fractionated by SDS-PAGE and Western blotting was carried out as previously described [8]. The IF1 promoter was detected using a Clontech kit. A GPx antibody was used (Bio-tek, Calixi, UK).

2.5. Glutathione peroxidase cellular assay

GPx activity was measured using the glutathione peroxidase cellular assay kit according to the manufacturer's protocol (Sigma). Min6 cells seeded at equal densities were stimulated with 30 nM Na2SeO3 for 3 h. Whole cell extracts were prepared using a low peroxide detergent Triton X-100 and absorbance at 340 nm was monitored in a Hitachi U-2001 Spectrophotometer using the kinetic program: wavelength 340 nm; initial delay: 15 s; interval: 10 s; number of readings: 6 and the ΔA4 min was determined.

2.6. Rat islet insulin content and secretion

Idiots were isolated from 30 g male Wistar rats (Charles River, Margate, UK) by collagenase digestion of dissociated islets as previously described [8]. Islets were stained and incubated in CMRL medium containing 5 mM glucose supplemented with 10% FCS (PAA, Someren, UK). 100 units/ml penicillin, 100 µg/ml streptomycin and ITS-A (Invitrogen) in non-adherent flasks (Nunc, Hereford, UK) for 24 h at 37°C in a humidified 5% CO2 incubator. Following washing with Hank's balanced salt solution (PAA), 10 size-matched islets were stained and cultured in CMRL medium supplemented with 0.2% human serum albumin, 100 units/ml penicillin, 100 µg/ml streptomycin and with or without 30 nM Na2SeO3 for 3 h, with six repeats for each condition. Islets were cultured at 37°C in a humidified 5% CO2 incubator for 72 h. Supernatants and islets were collected and stored at –20°C for later insulin determination. Islets were lysed in PBS by 3 cycles of freezing and thawing followed by centrifugation at 14000 rpm for 5 min. Secreted insulin in the supernatant and insulin content in the lysates was determined using the Insulin RIA kit (Merckodia, Dagenham, Milton Keynes, UK). Total protein was determined using Bio-Rad protein assay dye reagent.

2.7. Statistics and densitometry

The data are expressed as means ± S.D. Data were compared using Student's t-test. Densitometry was performed using the TINA v2.09g software (Raytest, Straubenhardt, Germany). A P-value of less than 0.05 was considered significant. *P < 0.05, **P < 0.01.

3. Results

3.1. Na2SeO3 significantly increases GPx activity in Min6 beta-cells

Little is currently known about the specific effects of selenium on pancreatic beta-cell gene expression and insulin cell function. Min6 cells were stimulated for 3 h with 30 nM Na2SeO3 and whole cell extracts assayed for glutathione peroxidase (GPx) activity (Fig. 1). Thirty nM Na2SeO3 addition stimulated a significant 5-fold increase in GPx activity (*P < 0.05), indicating that Min6 beta-cells are significantly selenium-sensitive.

3.2. Selenium stimulates the Ifp1 gene promoter in Min6 beta-cells

To study the effect of selenium on Min6 cell gene expression, we initially utilized the inorganic species Na2SeO3. Min6 cells were transfected with construct pGL3-IPF1, which contains the full-length mouse Ifp1 gene promoter; pGL3-IPFβ, containing the −2715 to −1960 bp section of the Ifp1 gene promoter shown to direct beta-cell-specific activity of the Ifp1 promoter [8]; or with control construct pGL3, which lacks the promoter fragment. Upon stimulation of cells with 30 nM Na2SeO3 for 3 h, a 3.5-fold increase (*P < 0.01) in the activity of the full-length Ifp1 promoter was observed (Fig. 2A), and a 7-fold increase in the activity of the beta-cell specific section of the promoter (**P < 0.01). No effect was observed on the control construct lacking the promoter fragment, which remained unaffected by the presence or absence of selenium.

To determine if the oxidation state of selenium was critical to the observed effects, we next investigated the effect of Na2SeO4. Addition of 30 nM Na2SeO4 for 3 h resulted in a 5-fold increase (*P < 0.05) in the activity of the full-length Ifp1 promoter (Fig. 2B), and a 7-fold increase in the activity of the beta-cell specific section of the promoter (**P < 0.05). No effect was observed on the control construct pGL3. To establish that selenium, and not the carrier molecule sodium, was stimulating Ifp1 gene promoter activity, we latterly investigated the effects of Na2SeO4, an important control since it has a similar oxamion moiety as Na2SeO3 and the same counter cation (Na+), but lacks the selenium ion. No effect of Na2SeO4 was observed on either the full-length or beta-cell portion of the Ifp1 gene promoter (Fig. 2C).

3.3. Selenium stimulates endogenous Ifp1 mRNA levels in Min6 beta-cells

To investigate the effect of selenium on endogenous Ifp1 mRNA levels, Min6 cells were incubated 30 nM Na2SeO3 for 48 h and Ifp1 mRNA levels analysed by semi-quantitative RT-PCR. Addition of 30 nM Na2SeO3 resulted in a significant increase in Ifp1 mRNA levels (Fig. 3, panels A and B) (**P < 0.05). No effect was observed on the control housekeeping gene Gapdh. A significant increase in insulin mRNA levels (Fig. 3, panels C and D) (**P < 0.01) was also observed. IPF1 protein levels were also significantly increased (**P < 0.05) in the presence of Na2SeO3 (Fig. 4, panel A). No effect was observed on the levels of the control protein GAPDH, which remained unaffected by the presence or absence of selenium (Fig. 4, panel B).
in short term (2 h) incubation studies with freshly isolated rat islets (data not shown).

4. Discussion

The present study has shown that the essential trace element selenium regulates several important aspects of beta-cell and islet function. We have shown that selenium specifically upregulates *Ipf1* gene expression, activating the −2715 to −1960 section of the *Ipf1* gene promoter. In addition, selenium increased insulin mRNA levels in Min6 cells and stimulated increases in both insulin content and insulin secretion in isolated primary rat islets of Langerhans. These data are the first to implicate selenium in the regulation of specific target genes in pancreatic beta-cells and suggest that selenium not only stimulates pancreatic beta-cell gene expression, but potentially promotes an overall improvement in islet function.

Our data indicates that selenium activation of the *Ipf1* gene promoter is most marked in the region −2715 to −1960 upstream from the transcriptional start site. We have previously shown that this region is sufficient to drive beta-cell specific expression of the *Ipf1* gene [8]. The transcription factors responsible for the activation of the promoter by selenium remain to be identified. However, this section of the promoter is known to bind hepatocyte nuclear factor 1 alpha (HNF1a), forkhead homeobox A2 (FOXA2), v-maf musculoaponeurotic fibrosarcoma oncogene homologue A (MAFA), paired box 6 (PAX6) and the PIF1 protein itself [10]. Further work is required to fully define the specific combination of transcription factors responsible for mediating the effects of selenium in the current study.

Our results suggest that accessibility to selenium ions may determine the effects of dietary intake of selenium on pancreatic beta-cell function. The observed effects of Na$_2$SeO$_3$ in the present study were shown to be specifically due to both the presence and the oxidation state of the Se ion. Hence sodium selenite, which contains selenium in a different oxidation state (Na$_2$SeO$_3$), also stimulated the *Ipf1* gene promoter, but to a lesser extent than Na$_2$SeO$_4$. This observed difference in magnitude may reflect differences in the accessibility of the selenium ion between these two moieties. Whereas selenium is readily reduced to selenide (Se$^-$) and subsequently assimilated into selenoproteins, the reduction of selenite to selenide appears only to be catalysed by specific enzymes. These multi-component molybdo-enzymes catalyse the selective two electron reduction of selenite to selenide. Cellular metabolism of selenite in mammalian cells, including its intermediary reduction to the oxidative state +IV, has previously been implicated in the magnitude of response to selenium-containing compounds in vivo [11].

Using viologen dyes as an artificial electron donor in a columnar assay we have detected a low level of selenite reductase activity in pancreatic cell extracts. The rate of auto oxidation of the viologen dye was routinely determined to be ~20 nmol [MV]$^+$ min$^{-1}$ ml$^{-1}$. The selenate dependent rate of oxidation was determined to be approximately 10 fold greater than the endogenous rate (~20 nmol [MV]$^+$ min$^{-1}$ ml$^{-1}$), suggesting the presence of selenate reductase activity in the pancreatic extract. No selenate dependent re-oxidation of methyl viologen was observed in the absence of the pancreatic cell extract. Despite the oxidation state of the selenium

3.4 Selenium increases insulin content and secretion from isolated intact rat islets of Langerhans

We firstly examined the effects of selenium on insulin content and secretion from isolated rat islets of Langerhans. Islets in batches of ten were incubated at 30 nmol Na$_2$SeO$_3$ for 72 h. Insulin content and secretion from sets of six replicate samples were analysed by enzyme-linked immunosorbent assay (ELISA) (Fig. 5). Addition of 30 nmol Na$_2$SeO$_3$ for 72 h significantly increased both insulin content ($P < 0.05$ vs. control) and insulin secretion from the islets ($P < 0.05$ vs. control). No effect was observed on glucose-stimulated insulin secretion
Fig. 3. Sodium selenite (Na₂SeO₃) stimulates endogenous Ipf1 and insulin mRNA levels in Min6 beta-cells. Min6 cells were incubated in the presence or absence of 30 nmol/l Na₂SeO₃ for 48 h. RNA was prepared, 1 μg reverse transcribed and Ipf1 and Gapdh mRNA levels analysed by semi-quantitative RT-PCR (Panel A). Panel B represents densitometric analysis of 4 separate experiments, with Ipf1 expressed as a ratio to Gapdh. Panel C represents analysis of insulin mRNA level under the same conditions. Panel D represents densitometric analysis of four separate experiments, with insulin expressed as a ratio to Gapdh. Error bars represent standard deviation. *P < 0.05 compared with the unstimulated samples.

Fig. 4. Sodium selenite (Na₂SeO₃) stimulates Ipf1 protein levels in pancreatic beta-cells. Min6 cells were incubated in the presence or absence of 30 nmol/l Na₂SeO₃ for 48 h. Whole cell extracts were prepared and 5 μg of each sample was separated by SDS-PAGE and Western blot analysis performed utilising specific Ipf1 and GAPDH antibodies (Panel A). Panel B represents densitometric analysis of 3 separate experiments, with Ipf1 expressed as a ratio to GAPDH. Error bars represent standard deviation. *P < 0.05 compared with the unstimulated samples.

oxonation, the observed effects on Ipf1 gene expression were specific to the selenium ion, as replacement of selenium with either nitrogen or sulfur was unable to reproduce the stimulatory effect on Ipf1 gene expression.

Fig. 5. Sodium selenite (Na₂SeO₃) increases insulin content and secretion from isolated intact rat islets of Langerhans. Isolated rat islets of Langerhans were separated into batches of 10 islets and incubated with or without 30 nmol/l Na₂SeO₃ for 72 h. The graph represents insulin content (white bars) and secretion (black bars) corrected for total protein content. Data are expressed as relative fold increase in comparison to control islets. Bars represent means ± S.D. of three independent experiments each of which consisted of six replicates of each condition. *P < 0.05 compared with the control.

GPs has previously been implicated in the protection of pancreatic beta-cells from oxidative stress in a model of glucose toxicity [12], and increased GPs expression has been shown to positively modulate beta-cell function [13]. The 5-fold increase in GPs activity observed in the present study in response to selenium addition is comparable to that observed in other seleno-sensitive cell types, such as liver cells [14]. The precise role of selenoproteins such as GPs in the modulation of beta-cell gene expression remains to be more fully explored. Oxidative stress has been shown, in the long term, to
decrease pancreatic beta-cell function [15]. Pancreatic islets and in particular beta-cells have very low amounts of antioxidant enzymes and are therefore very sensitive to oxidative stress [16]. The beta-cell dysfunction generated by oxidative stress can largely be attributed to the down regulation of insulin gene transcription and insulin secretion. This occurs as a result of down regulation of IPF1, a beta-cell specific homeodomain transcription factor that plays a vital role in the transcriptional regulation of insulin production in response to glucose [8]. Oxidative stress has been shown to down regulate both Ipfl mRNA and protein levels and also decrease the binding activity of IPF1 to the insulin gene promoter, all of which contribute to the observed loss of insulin gene expression. The overall role of selenium in the regulation of beta-cell gene expression also remains to be fully clarified. It is thought that the insulin-mimetic effects of selenium may in part be due to the activation of tyrosine kinases integral to the insulin signalling cascade [6], but the activation of these signalling molecules has not been studied in pancreatic beta-cells thus far.

In the present study, selenium stimulated significant increases in both insulin content and insulin secretion in isolated islets of Langerhans. This is an interesting observation since many studies have reported potentially protective effects of selenium in animal models of type 2 diabetes [17] and in patients with the disease [18]. To date, the specific molecular mechanisms driving this protection have not been identified. Since increasing IPF1 protein levels drive the initiation of insulin gene transcription, allowing the replenishment of intracellular insulin content and increased insulin secretion, it seems reasonable to hypothesise that the reported positive effects of selenium on beta-cell function may be mediated through initial changes in Ipfl gene expression and IPF1 protein levels.

The current recommended dietary intake of selenium in humans is between 55 and 75 μg per day [19]; however, many areas of the globe, including the UK, have intakes well below this recommended level. Although the beneficial effects of selenium remain to be fully clarified [20], the results of the present study begin to shed some light on potential mechanisms, suggesting that selenium has a multitude of positive effects on beta-cell gene expression, contributing to overall improvements in islet function. Further study is now required to fully define the transcriptional targets and potential cell signalling mechanisms central to this process.

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References
Tumor Suppressor Pdcd4 Is a Major Transcript That Is Upregulated During In Vivo Pancreatic Islet Neogenesis and Is Expressed in Both Beta-Cell and Ductal Cell Lines

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Objectives: We wished to identify a major transcript that is upregulated during in vivo pancreatic islet neogenesis and examine the expression of the gene in beta and ductal cells.

Methods: Differential display polymerase chain reaction was used to identify upregulated transcripts after islet neogenesis was stimulated in the rat by brief occlusion of the main pancreatic duct. The expression of this major transcript, namely PDCD4 (programmed cell death gene 4), was measured in beta and ductal cells after stimulation with the insulinotropic hormone glucagon-like peptide 1, mitogenic insulin, the thiazolidinedione rosiglitazone, and by high glucose concentrations. The subcellular location of the protein was also examined.

Results: The expression of the Pdcd4 gene in pancreatic beta and ductal cells was found to be stimulated in a comparable manner by either glucagon-like peptide 1, insulin, and high glucose concentrations. However, intracellular localization of the PDCD4 protein was shown to be differentially regulated by these stimuli in beta and ductal cells. Furthermore, the thiazolidinedione rosiglitazone specifically upregulates Pdcd4 gene expression in beta cells in a time-dependent manner.

Conclusion: This is the first study showing Pdcd4 expression in pancreatic cells. Our data indicate that Pdcd4 expression may be integral in the function of the adult pancreas.

Key Words: Pdcd4, islets, neogenesis, GLP1, thiazolidinedione, beta cells

Accurate regulation of the process of islet neogenesis is not only fundamental during development, but is also essential for pancreatic function throughout the lifetime of an individual. In the adult pancreas, mature insulin-producing beta cells have a life span of approximately 50 days, after which they are replaced by the highly regulated process of neogenesis from progenitor ductal cells. We have previously shown that temporary occlusion of the main pancreatic duct results in a duct cell proliferative response, followed by an increase in total islet mass. During our investigations, we have used this model of islet neogenesis to try to identify the key genes that are differentially expressed in this process. One of these genes, and the focus of the current study, is Pdcd4 (programmed cell death gene 4), which has not previously been described in the pancreas.

Recent work on PDCD4 has focused on the tumor suppressor properties of this protein. However, the exact function, biomolecular interactions, and regulation of PDCD4 have yet to be clearly delineated. Although the protein shuttles between the nucleus and cytoplasm, no nuclear function has been attributed to the protein, and, although PDCD4 inhibits translation, the mode of action leading to the regulation of proliferation, differentiation, and apoptosis has yet to be found. Accumulating evidence that Pdcd4 is down regulated in many cancers has further highlighted the need to determine how the expression and activity of this potentially pivotal protein are controlled.

Stimuli that promote islet neogenesis and improvements in pancreatic function are much in demand, and the mechanisms through which these effects are achieved must be better understood. In vitro and in vivo, the insulinotropic hormone GLP1 (glucagon-like peptide 1) and its longer lasting analogue exendin 4 have been shown to stimulate the expression of key beta-cell genes (Glut2, glucokinase, insulin, and Pdk1), promoting not only islet growth and beta-cell survival, but beta-cell neogenesis and the differentiation of ductal cells into insulin secreting cells. In the present study, we examine the effects of GLP1 on Pdcd4 gene expression, comparing the effects with the mitogenic stimuli insulin and high glucose concentrations. In addition to examination of the effects of these agents on Pdcd4 gene expression, we also analyze effects on the intracellular localization of the PDCD4 protein, both in beta cells and in ductal cells.

For stimulation of islet neogenesis to be truly effective as a therapeutic option, newly formed islets must be protected. Recent studies have shown that the thiazolidinedione rosiglitazone can protect pancreatic beta-cell mass and function from the detrimental effects of free fatty acids. Here, we examine for the first time the effects of rosiglitazone, a commonly prescribed drug for patients with type 2 diabetes, on Pdcd4 gene expression in pancreatic beta cells. The data presented in the current study identifies PDCD4 as a major transcript upregulated in the process of islet neogenesis. Furthermore, we show that Pdcd4 gene expression and intracellular localization are highly regulated in pancreatic beta cells by a key range of stimuli.

MATERIALS AND METHODS

Stimulation of Islet Neogenesis

Twelve 12-week-old female Wistar rats, (weight, ~180 g), were anesthetized and kept under surgical anesthesia using 2% isoflurane in oxygen. The animals were subjected to a midline laparotomy, and the main pancreatic duct was partially occluded for 60 seconds. The pancreas was then released and gently relocated in the abdomen. Four animals were terminated at 3, 7,
and 14 days after occlusion. Three age- and sex-matched rats were terminated immediately after a sham operation and used as a control (n = 6). All pancreases were rapidly removed and snap frozen in liquid nitrogen.

**Diffential Display Polymerase Chain Reaction**

RNA was isolated from frozen, crushed pancreases using Trizol reagent and differential display polymerase chain reaction (PCR) performed using an RNAImage kit (GenHunter Corp, Nashville, Tenn) with the H-T}_{3}, a primer, both according to the manufacturer's protocol. Radiolabelled fragments were separated by polyacrylamide gel electrophoresis (6% gel, TBE buffer), with differentially expressed fragments excised, realigned by PCR and cloned into pGEM T Easy (Promega, Southampton, UK) and sequenced. A 506 bp fragment of Pdc4 was subcloned using gene specific primers (S' - AGA TGA AGA GTG GTG TGC CCG TGT T'3 and S'-TCT GTG GTG GAA ATG CGG GT-3'). Quantification of specific mRNA transcripts was performed using an RNase protection assay kit (Roche, Welwyn, UK) according to the manufacturer's protocols. Housekeeping gene β-actin (S'-TGT TAA CAA CCT GGG AGC ATG TGG-3' and S'-GAT CTT GAT CTT CAT GGT GCT AGG-3') was used as a control.

**Cell Culture**

Min6 cells, a beta-cell line derived from transgenic mice expressing the SV40 large T antigen under the control of the rat proinsulin gene promoter, were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 5 mmol/L of glucose, supplemented with 10% heat-inactivated foetal calf serum, 100 U/ml of penicillin, and 100 μg/ml streptomycin. ARIP cells, a rat pancreatic ductal cell line, were cultured in DMEM-F12 (Sigma, Poole, UK), supplemented with 10% foetal calf serum, 2 mmol/L of r-glutamine, 100 μg/ml of streptomycin and 100 μg/ml of penicillin. For expression studies, the full-length Pdc4 cDNA was cloned into the pCIS.1 V His-TOPO plasmid vector upstream of a 6× His tag, with cells transfected as previously described. Rosiglitazone was a kind gift from GlaxoSmithKline (Harlow, UK). All other chemicals and materials were purchased from Sigma-Aldrich (Dorset, UK).

**Western Blotting**

Nuclear and cytoplasmic extracts were prepared and western blotting performed as previously described. Protein concentrations were determined using Bio-Rad Protein Assay Kit reagent (Biorad, Hemel Hempstead, UK). Protein S staining was used to confirm equal loading and transfer. Anti-His-tag antibody was purchased from Abcam (Cambridge, UK), with secondary horseradish peroxidase-conjugated antibody purchased from Amersham (Buckinghamshire, UK).

**Real-Time PCR**

RNA was extracted from Min6 and ARIP cells using the Gene Elute Mammalian Total RNA Miniprep Kit (Sigma, Poole, UK). cDNA was generated using the Cloned AMV First-Strand Synthesis Kit (Invitrogen, Paisley, UK). cDNA was used for real-time PCR using a SYBR Green PCR Kit (Bio-Rad, Hemel Hempstead, UK) and specific primers for Pdc4 (S'- AGA TGA AGA GTG GTG TGC CCG TGT T'3 and S'-ACT GGC CCC CCA ACT GTG GTG CTC T T3'), Gapdh (S'- AAG GCC TCA TGA CCA CAG TCC AT-3 and S'-TGT CAG ATC CAC GAC GGA CAC AT-3'), and β-actin (S'- CGA AGT GGT GAA GTT CAT GGA GAT G) and S'-TCT GTG ATG AGT CTT TCG T-3'). Amplification conditions for 40 cycles were as follows: denaturation at 95°C for 30 seconds, annealing and extension at 64°C for 1 minute. Results were analyzed according to the Pfaffl method. For semi-quantitative PCR amplification of Pdc4, 25% of the reverse transcription mix was added to the PCR reaction (95°C for 5 minutes, then 29 cycles consisting of 95°C for 30 seconds, 60°C for 45 seconds, 72°C for 45 seconds, with a final step of 72°C for 5 minutes). For Gapdh, 95°C for 5 minutes, then 25 cycles consisting of 95°C for 30 seconds, 55°C for 45 seconds, 72°C for 60 seconds, with a final step of 72°C for 5 minutes.

**Statistics and Densitometry**

The data are expressed as means ± SD. Data was compared using Student t test. Densitometry was performed using the TINA v2.09 g software (Raytest, Straubenhardt, Germany). P < 0.05 was considered significant.

**RESULTS**

**Pdc4 Is Upregulated During Islet Neogenesis**

Differential display PCR was used to identify differentially expressed gene fragments from pancreata stimulated to undergo islet neogenesis. Amplification of transcripts was compared between control pancreata (n = 2) and pancreata harvested 7 days after occlusion (n = 2). Visual analysis showed a distinct difference in the intensity of several bands when comparing the control and occluded transcripts. The transcript exhibiting the largest upregulation was excised, cloned, and sequenced. This was found to be Pdc4. RNase protection assay was then used to assess the expression of Pdc4 during the expansion of beta-cell mass that occurs after brief occlusion of the main pancreatic duct (Fig. 1). Pdc4 expression was significantly increased after 3 days (P < 0.01 compared with day 0), with maximal expression...
observed 7 days after occlusion (P < 0.01 compared with day 0), compared with the housekeeping gene Gapdh.

**Pdc4** Gene Expression Is Stimulated by GLP1, Insulin, and High Glucose Concentrations in Both Pancreatic Beta Cells and Ductal Cells

Having identified the upregulation of Pdc4 during the process of in vivo islet neogenesis, we next examined the effects on Pdc4 gene expression by agents known to stimulate an increase in beta-cell mass and improve function. To determine the effects of the incretin hormone GLP1, insulin, and high glucose concentrations on beta-cell expression of Pdc4 mRNA, Min6 cells were stimulated for 24 hours in DMEM supplemented with 10 mM of GLP1, 10 mM of insulin or glucose to a final concentration of 16 mM/L, as indicated. Cells were harvested, RNA prepared, and Pdc4 gene expression analyzed by semi-quantitative reverse transcription PCR (Fig. 2A). Denstometric analysis confirmed that GLP1, insulin, and 16 mM/L of glucose all stimulated a significant increase in Pdc4 mRNA levels for 24 hours, compared with the housekeeping gene Gapdh (Fig. 2B). To determine the effects of these agents on Pdc4 gene expression in pancreatic ductal cells, the same experiment was performed using the ARIP pancreatitis ductal cell line (Fig. 2C). Denstometric analysis again confirmed that GLP1, insulin, and 16 mM/L of glucose all stimulated significant increases in Pdc4 mRNA levels for 24 hours in ductal cells, compared with the housekeeping gene Gapdh (Fig. 2D). These data suggest that Pdc4 mRNA expression is controlled by similar mechanisms in pancreatic beta cells and ductal cells in response to mitogenic stimuli.

**Intracellular Translocation of the PDCD4 Protein Is Cell-Type Specific**

The activity of the PDCD4 protein in nonpancreatic cell types has been shown to be regulated in part by intracellular translocation of the protein between the cytoplasm and the nucleus. To determine the effects of GLP1, insulin, and high glucose on the intracellular localization of the Pdc4 protein, Min6 and ARIP cells were transfected with the pCR3.1-Pdc4 plasmid, containing the full-length Pdc4 cDNA flanked by a 6x-His tag, or with the empty control vector pCR3.1. Twenty hours after transfection, cells were incubated in 5 mM/L glucose overnight, before stimulation with 100 mM/L of insulin, 10 mM/L of GLP1 (7-36) amide, or glucose to a final

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**FIGURE 2.** Insulin, GLP1, and 16 mM/L of glucose stimulate Pdc4 gene expression in Min6 beta cells and ARIP ductal cells. Min6 cells (A) or ARIP ductal cells (C) were incubated for 24 hours in 5 mM/L of glucose ( ), or 5 mM/L of glucose supplemented with 100 mM/L of insulin (Ins), 10 mM/L of GLP1 (7-36) amide (GLP1), or 16 mM/L of glucose (16 M/L) as indicated. RNA was prepared and semi-quantitative reverse transcription PCR performed with primers specific for Pdc4 and Gapdh as indicated. Min6 (B) and ARIP (D) represent densitometric analysis of 3 separate experiments, with values expressed as Pdc4 relative to Gapdh under each condition. *P < 0.05, **P < 0.01.

**FIGURE 3.** Intracellular translocation of Pdc4 differs between Min6 beta cells and ARIP ductal cells. Min6 beta cells (A) or ARIP ductal cells (B) were transfected with pCR3.1-Pdc4. Forty-eight hours after transfection, cells were incubated for 24 hours in 5 mM/L of glucose ( ), or 5 mM/L of glucose supplemented with 100 mM/L of insulin (Ins), 10 mM/L of GLP1 (7-36) amide (GLP1), or 16 mM/L of glucose (16 M/L) as indicated. Nuclear and cytoplasmic extracts were prepared. Ten micrometers of nuclear extract or 20 mug of cytoplasmic extract were analyzed by western blotting using a His-tag primary antibody and a secondary horseradish peroxidase-conjugated antibody. These results have been reproduced in 3 separate experiments.
concentration of 16 mmol/L for 24 hours. Nuclear and cytoplasmic extracts were prepared and analyzed by western blotting using a specific anti-His-tag antibody. This methodology was used because commercially available anti-PDCD4 antibodies showed high nonspecific cross-reactivity. The antibody used bound the His-tagged pdec4 fusion, but did not detect the native pdec4 protein. In Min6 beta cells, 24-hour incubation with insulin and GLP1 caused translocation of the PDCD4 protein from the nucleus to the cytoplasm (Fig. 3A). Incubation in 16 mmol/L of glucose had no effect on PDCD4, which remained in the nucleus, confirming that the observed response to insulin and GLP1 was specific and not due to a general improvement in the growth environment. In ARIP pancreatic ductal cells, 24-hour incubation with insulin, GLP1 and high glucose had no effect on the cellular localisation of the PDCD4 protein, which remained in the nucleus under all conditions tested. These data strongly suggest that the observed effects of insulin and GLP1 on the intracellular localisation of PDCD4 in Min6 cells are beta-cell specific and that regulation of these events is controlled differently in ARIP pancreatic ductal cells.

**Pdec4 Expression in Pancreatic Beta Cells Is Stimulated by Rosiglitazone in a Time-Dependent Manner**

Recent studies have shown that beta-cell mass and function can be enhanced and protected by the thiazolidinedione rosiglitazone. The role of agents, which improve beta-cell mass and function in the regulation of Pdec4 gene expression, has not previously been examined. To determine the effects of rosiglitazone on Pdec4 gene expression, Min6 beta cells were incubated in 5 mmol/L of glucose DMEM supplemented with 10 μmol/L of rosiglitazone, with cells harvested at 0, 24, 48, and 72 hours. Pdec4 gene expression was analyzed by real-time PCR (Fig. 4A) and compared with housekeeping gene Gapdh. After 24 hours, rosiglitazone stimulated a significant 2.5-fold increase in Pdec4 expression. Stimulation of Pdec4 expression was maintained at a significant level at both 48 and 72 hours. However, as statistical analysis between expression at 24, 48, and 72 hours was not found to be significant (although statistically different from the control). We wished to confirm that this lack of temporal Pdec4 expression after administration of rosiglitazone over the period analyzed was not simply a reflection of an overall improvement in beta-cell function. Consequently, as a positive control the same analysis was performed for the tumor associated antigen gene Vegfr (Fig. 4B), which is known to be stimulated by rosiglitazone in other specialised cell types. Vegfr expression was not significantly different after 24 hours but was significantly increased at 48 hours. By 72 hours, Vegfr expression levels had returned to control levels. This confirms that the sustained increase in Pdec4 expression is not due to general nonspecific upregulation of genes.

**DISCUSSION**

The results of the present study have shown for the first time that the tumor suppressor protein PDCD4 is highly regulated in pancreatic beta and ductal cells, and expression of Pdec4 is greatly increased in vivo after brief occlusion of the main pancreatic duct, a simulation which promotes an increase in islet mass.

We have previously found that gentle, brief occlusion of the main pancreatic duct results in a 2.5-fold increase in duct cell proliferation at 3 days after occlusion and an 80% increase in total islet mass at 56 days. During the characterization of this model, we investigated the possibility that pancritisis was promoted by the manipulation of the pancreas. However, it was found that serum amylase levels, although slightly raised in occluded animals, were not statistically different from the sham-operated control rats. Extensive microscopic analysis of all pancreatic sections from occluded rats showed that inflammatory cell infiltration was extremely rare, which along with the amylase data, indicated that gentle, brief occlusion of the main pancreatic duct does not instigate a significant inflammatory response. Furthermore, specific analysis around the occluded region did not show obvious cellular necrosis or apoptosis. Consequently, it is assumed that Pdec4 expression after brief occlusion of the main pancreatic duct is primarily associated with the process that leads to the expansion of beta-cell mass.

The possible involvement of Pdc4 in islet neogenesis adds to a growing, complex body of evidence implicating Pdc4 in the balance that exists between proliferation, differentiation, and apoptosis. For example, decreased Pdc4 expression is associated with impaired differentiation in nasopharyngeal carcinoma and in pancreatic cancer. Transgenic mice overexpressing Pdc4 in the demes have decreased proliferation and increased cellular differentiation. However, in these and many other studies, an overriding theme is that the relationship between Pdc4 expression/localization and differentiation is likely to be cell type and signaling pathway specific. PDCD4 has 2 nuclear translocation sequences and is known to shuttle between the nucleus and the cytoplasm. It is thought that these events are regulated in part through phosphorylation and phosphorylation sites for caspase kinase II, PKC, proline-directed protein kinase, PKB/Akt, and p70 S6 kinase have been identified. In the present study, Pdc4 expression was upregulated in vivo during the process of islet neogenesis.
is known to consist of expansion of the ductal cells before differentiation to a beta-cell phenotype. Consequently, we wished to examine whether Pdc4d is expression in ductal and/or beta cells and if expressed, whether mitogenic stimuli changed intracellular localisation of PDCD4.

In the current study, insulin and GLP1 promoted translocation of PDCD4 from the nucleus to the cytoplasm in Min6 beta cells, but not in ARIP ductal cells. As PDCD4 can modulate protein translation,6 it is tempting to hypothesise that this translocation is associated with this known cytoplasmic function of the protein and possibly with cellular maturation. However, in ductal cells, the PDCD4 protein remained localised to the nucleus in all of the stimuli tested. Although no clearly defined nuclear function has been attributed to the protein, PDCD4 was recently reported to interact with the HIF1A transcription factor Twist,22 a protein implicated in neural crest cell migration and differentiation.33 Hence, it is possible that interaction with Twist and the downstream transcription factor Y-box binding factor 1 may play a role in the nuclear activities of PDCD4. The role of Twist in islet neogenesis remains to be investigated.

Improvements in islet and beta-cell function, as well as beta-cell mass in vivo upon rosiglitazone administration have largely been attributed to overall improvements in glycemic control and circulating lipid levels in patients taking this drug. However, more recently, rosiglitazone has been shown to directly target pancreatic beta cells, stimulating a dose-dependent increase in the activity of the Ipf1 gene promoter, and upregulating nuclear levels of the key transcription factors Ipf1 and FoxA2.23 It is becoming increasingly clear that rather than acting solely through PPARy, many of the positive effects of rosiglitazone on pancreatic islet mass and function occur through the activation of multiple signaling pathways, including downregulation of the stress activated p38 pathway, and upregulation of AMPK, CK2 and SK6.34 In the current study, rosiglitazone stimulated a significant and prolonged increase in beta-cell Pdc4d gene expression. Hence, regulation of Pdc4d expression may represent a new avenue for investigation in the quest to improve beta-cell mass and function in patients with diabetes.

Overall, we believe this is the first documentation of the expression of Pdc4d in non-tumorous pancreata. Although we have not shown a direct cause-and-effect relationship with neogenesis, Pdc4d upregulation was found to be associated with the expansion of islet mass in vivo. The increase Pdc4d expression after the addition of GLP1, a promoter of islet growth and beta-cell survival,13,14 to Min6 and ARIP cells, together with the translocation of the protein to the cytoplasm only in Min6 cells, suggests that PDCD4 may have a different function in ductal and beta cells. This requires further investigation.

REFERENCES


Rosiglitazone Protects Pancreatic Beta-cells from Fatty Acid Induced Lipotoxicity - An Imaging Analysis

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1. Introduction

- In Type 2 diabetes, prolonged exposure to high glucose concentrations and free fatty acids is implicated in β-cell dysfunction.
- The thiazolidinediones (TZD) rosiglitazone is an oral anti-hyperglycaemic agent used in the treatment of Type 2 diabetes and is known to act as a peroxisome proliferator activated receptor (PPAR)-γ agonist.
- The aspecific actions of this TZD on PPARG are known to result in decreased hepatic glucose output, increased peripheral glucose uptake and alterations in adipocyte metabolism.
- In addition to improving islet function in vitro, rosiglitazone can directly impact on the β-cell in vivo, increasing nuclear levels of the transcription factors FOXA2 and PDX1.
- Loss of β-cell function in type 2 diabetes is increasingly associated with free fatty acid induced β-cell damage and a decrease in β-cell number and viability.
- The aims of this study were to determine: the differential detrimental effects of saturated and mono-unsaturated free fatty acids on β-cell ultra-structure; to determine whether rosiglitazone was able to directly protect the β-cell from these effects; and lastly to begin to identify the mechanisms involved starting with examination of the key transcription factor NFκB.

2. Methods

- Cell Culture: Min6 cells were cultured in 5% CO₂ in a humidified incubator. Stimuli were added to a final concentration of 0.4µM palmitate, 0.4µM oleate and 10µM rosiglitazone, either alone or in combination.
- TEM Microscopy: Min6 cells were treated with various stimuli as indicated for 24 and 72 hours and then fixed. Thin sections were cut with an LKB Ultratome ultramicrotome, post-stained and sections were examined with the a Hitachi 7100 transmission electron microscope.
- Confocal Microscopy: Min6 cells were seeded in 2-chamber slides and stimulated for 24 hours with various stimuli as indicated and fixed. A primary NFκB and secondary FITC-conjugated antibody (both AbCam UK) were used. Slides were mounted in vectashield with DAPI and examined with the Leica TCS SFI confocal microscope.
- Oil Red O Staining: Min6 cells were seeded in 2-well chamber slides. Following 24 hour treatment, cells were washed, fixed and working Oil Red O solution was added to the cells and incubated for 3 hours at 37°C. Cells were mounted with vectashield with DAPI and visualised with the Zeiss Axivert 250 confi microscope.

3. Results

- Figure 1: Palmitate-induced damage to cellular ultra-structure was prevented by rosiglitazone. Transmission electron microscopy imaging of Min6 cells treated with various stimuli as indicated. Cells were cultured for (A) 24 and (B) 72 hours in 0.4µM DMEM with or without rosiglitazone, either alone or in combination. Cells were fixed and analysed using transmission electron microscopy, both panels (A) and (B) were examined under a (5000x) magnification while panel (C) was examined under a (5000x) magnification. This data represents 3 separate experiments. For (A) and (B), Bar = 2µm. For (C) Bar = 0.5µm.

- Figure 2: Increased triglyceride accumulation in response to oleate but not palmitate in Min6 β-cells. Min6 cells were cultured in 5% DMEM glucose DMEM alone or with the addition of 0.4µM palmitate, 0.4µM oleate and/or 10µM rosiglitazone for 24 hours then stained with Oil Red O. Treatment with oleate led to an increase in triglyceride staining within the cells, whereas treatment with palmitate did not. This was unaffected by the presence of rosiglitazone.

- Figure 3: Palmitate damages but oleate accumulates in Min6 β-cells. Min6 cells were cultured in 5% DMEM glucose DMEM alone or with the addition of 0.4µM palmitate, 0.4µM oleate and/or 10µM rosiglitazone for 24 hours. Cells were then fixed and analysed using transmission electron microscopy at a magnification of 1000x. Upon treatment with oleate, triglyceride accumulation was observed in the form of droplets within the cell. This did not occur in response to treatment with palmitate. Bar = 2µm.

- Figure 4: Palmitate-induced NFκB nuclear localisation is prevented by rosiglitazone. Cells were cultured for 24 hours in 0.4µM palmitate alone or with the addition of 0.4µM palmitate and/or 10µM rosiglitazone. Cells were stained using the NFκB primary and FITC-conjugated secondary specific antibodies and visualised by confocal microscopy. Palmitate caused the transcription factor NFκB to be located in the nucleus. This was prevented by rosiglitazone. This data represents 3 separate experiments. Bar = 10µm.

4. Discussion

- Palmitate provoked total cellular ultra-structure damage. This effect was completely blocked by the presence of rosiglitazone.
- In contrast, oleate exposure resulted in cellular accumulation of triglycerides in the form of fat droplets.
- Palmitate promoted the nuclear localization of the transcription factor NFκB, which has been implicated in β-cell apoptosis. This effect was blocked by rosiglitazone, suggesting a novel mechanism through which rosiglitazone can protect against palmitate-induced β-cell death.
- In conclusion, we have demonstrated the different effects of the saturated fatty acid palmitate and the mono-unsaturated fatty acid oleate on Min6 cells and the potential for rosiglitazone to protect against palmitate.

References

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Rosiglitazone Down-regulates p38 Stress Activated Kinase and Protects Pancreatic Beta Cells from Lipotoxicity-induced Dysfunction

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1. Introduction

In Type 2 diabetes, prolonged exposure to high glucose concentrations and free fatty acids is implicated in β-cell dysfunction.

• The thiazolidinediones (TZD) rosiglitazone is an oral anti-hyperglycaemic agent used in the treatment of Type 2 diabetes and is known to act as a PPARγ agonist (1).

• The agonistic actions of this TZD on the nuclear hormone receptor PPARγ are known to result in decreased hepatic glucose output, increased peripheral glucose uptake and alterations in adipocyte metabolism (2).

• Rosiglitazone therapy has been shown to improve insulin function in vivo, although this effect was considered to be secondary to its glucose lowering properties.

• More recently it has been shown that rosiglitazone can directly impact on β-cell signaling, increasing nuclear levels of the transcription factors FOXA2 and PDX1 (3).

• The aim of this study was to determine if rosiglitazone is able to directly protect pancreatic β-cells from the detrimental effects of free fatty acids (palmitate), and to investigate the cell signaling mechanisms involved.

2. Methods

• Cell Culture: Min6 cells were cultured in ficoll/glucose DMEM with the addition of stimulants for 2 hours for western blot analysis, or 72 hours for cell viability assays. To determine the role of individual signaling pathways, the following cell signaling inhibitors were used: AMPK inhibitor compound C (CC, 20μM); p38 inhibitor SB203580 (20μM); GW9662 (30μM): Palmitate was added to a final concentration of 0.5mM. Rosiglitazone was added to a final concentration of 10μM.

• Luciferase Assay: Min6 cells were transfected with the PPRE plasmid, or the control construct pGL3. 48 hours after transfection, cells were incubated in ficoll/glucose DMEM with no additions (-) or 10μM rosiglitazone for 24 hours. Cells were harvested, lysed, and extracts assayed for luciferase activity and protein content. Luciferase activity was standardised against protein concentration.

• MTT Viability Assay: Using the Cell Growth Determination Kit (Sigma), cell extracts were analyzed on a Varian CARY 50 UV-Visible Spectrophotometer, with absorbance read at a wavelength of 540nm.

• Statistics and Denimometry: The data are expressed as mean ± S.D. Data was compared using Student’s t test. Denimometry was performed using the TINA v2.09g software (Kartox, Straubach, Germany). A p value of less than 0.05 was considered significant. *p<0.05, **p<0.01.

3. Results

Figure 1: MTT assays showed that 72 hour exposure to 0.5mM palmitate resulted in a 75% decrease in Min6 cell viability (P<0.01). However, the presence of 10μM rosiglitazone has then completely abolished the effect of palmitate.

Figure 2: pGL3 Min6 cells were incubated in ficoll/glucose DMEM alone (-) or in the presence of 0.4mM palmitate for between 1 hour and 72 hours, cytoplasmic extracts prepared and analysed by Western blotting using specific phospho-p38 or total p38 antibodies as indicated.

Figure 5: Real-Time PCRs of UCP2 mRNA expression levels

(a) palmitate stimulates a 4.5 fold increase in UCP2 mRNA levels (P<0.05), an effect blocked by rosiglitazone (P<0.01). (b) palmitate increases UCP2 mRNA levels (P<0.05), this increase is unaffected by SB203580.

References


4. Discussion

This is the first study to show that rosiglitazone modulates multiple signaling pathways in pancreatic β-cells and can protect β-cell viability from the detrimental effects of palmitate.

• Rosiglitazone rapidly down-regulates levels of the stress activated protein kinase p38, with a ~50% decrease occurring after 2 hours, potentially providing a level of β-cell protection.

• Rosiglitazone decreases CK2 activity within 2 hours, although this was blocked by compound C, at a time point and concentration also shown to block ACC phosphorylation, suggesting that AMPK activity lies upstream of CK2 in the signaling events being regulated by rosiglitazone.

• Compound C was able to completely block the protective effects of rosiglitazone on Min6 cell viability, indicating that the rosiglitazone-induced activation of AMPK is required for the protective effects against palmitate-induced lipotoxicity. The observation that TRB only partially blocks the protective effects of rosiglitazone supports our suggestion that AMPK is upstream of CK2 in this rosiglitazone-activated pathway.

• In conclusion, our data suggests that rosiglitazone can directly target and enhance β-cell function and that many of these effects occur independently of the activation of PPARγ. Further study is now required to fully delineate the transcriptional targets and gene expression changes regulating these events.
Rosiglitazone Modulates Multiple Signaling Pathways in Pancreatic Beta Cells and Protects Beta Cells from the Detrimental Effects of Free Fatty Acids

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1. Introduction

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The aim of this study was to determine if rosiglitazone is able to directly protect pancreatic β-cells from the detrimental effects of free fatty acids (palmitate), and to investigate the cell signaling mechanisms involved.

2. Methods

Cell Culture: Min6 cells were cultured in 5μM DME on with the addition of stimulants for 2 hours for western blot analysis, or 2 hours for cell viability assays. To determine the role of individual signaling pathways, the following cell signaling inhibitors were used: AMPK inhibitor compound C (CC, 20μM); p38 inhibitor SB203580 (20μM); Cason kinase 2 (CK2) inhibitor TBB (20μM). Palmitate was added to a final concentration of 5μM. Rosiglitazone was added to a final concentration of 1μM.

CK2 (CK2α/δε) activity assay: Assayed by measuring incorporation of 3P from 32P-ATP into 1 μM of the synthetic peptide substrate “CK2-alkyl” [READIDSDDID]. Incorporation of labelled phosphate was quantified using a Packard Instant Imager.

MTT Viability Assay: Using the Cell Growth Determination Kit (Sigma), cell extracts were analyzed on a Varian Cary 50 UV-Visible Spectrophotometer, with absorbance read at a wavelength of 540nm.

Statistics and Densitometry: The data are expressed as means ± S.D. Data was compared using Student’s t test. Densitometry was performed using the TINA v2.39g software (Raytest, Straubenhardt, Germany). P<0.05 was considered significant.

3. Results

Figure 1: MTT assays showed that 72 hour exposure to 0.5 mM palmitate resulted in a 70% loss of Min6 cell viability (P<0.01). However, the presence of 10 μM rosiglitazone completely abolished the effect of palmitate.

Figure 2: in the presence of the AMPK inhibitor compound C, rosiglitazone was unable to prevent the detrimental effects of palmitate on Min6 cell viability.

Figure 3: the p38 inhibitor SB203580 had no effect on the ability of rosiglitazone to protect Min6 cells from the detrimental effects of palmitate.

Figure 4: the presence of the CK2 inhibitor TBB was able to partially block the protective effect of rosiglitazone on Min6 cells, although cell viability was still significantly higher than treatment with palmitate alone (P<0.05).

This is the first study to show that rosiglitazone modulates multiple signaling pathways in pancreatic β-cells and can protect β-cell viability from the detrimental effects of palmitate.

Rosiglitazone rapidly down-regulates levels of the stress activated protein kinase p3K, with a ~50% decrease occurring after 2 hours, potentially providing a level of β-cell protection.

Rosiglitazone increases CK2 activity within 2 hours, although this was blocked by compound C, at a time point and concentration also shown to block ACC phosphorylation, suggesting that AMPK activity lies upstream of CK2 in the signaling events being regulated by rosiglitazone.

Compound C was able to completely block the protective effects of rosiglitazone on Min6 cell viability, indicating that the rosiglitazone-induced activation of AMPK is required for the protective effects against palmitate-induced lipotoxicity. The observation that TBB only partially blocks the protective effects of rosiglitazone supports our suggestion that AMPK is upstream of CK2 in this rosiglitazone-activated pathway.

In conclusion, our data suggests that rosiglitazone can directly target and enhance β-cell function and that many of these effects occur independently of the activation of PPARγ. Further study is now required to fully delineate the transcriptional targets and gene expression changes regulating these events.

References

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4. Discussion

Figure 5

Figure 6

Figure 1A: western blot analysis of total p38 protein following treatment of Min6 cells with 10μM rosiglitazone for 2 hours. Figure 1B: densitometry indicates over 50% decrease in total p38 levels (P<0.05). Figure 1C: phosphorylation of ACC was observed upon stimulation with rosiglitazone, an effect that was inhibited by compound C. Figure 1D: densitometry indicates that rosiglitazone activates AMPK in Min6 cells over 2 hours (P<0.05).

Figure 2A: western blot analysis of total p38 protein following treatment of Min6 cells with 10μM rosiglitazone for 2 hours. Figure 2B: densitometry indicates over 50% decrease in total p38 levels (P<0.05). Figure 2C: phosphorylation of ACC was observed upon stimulation with rosiglitazone, an effect that was inhibited by compound C. Figure 2D: densitometry indicates that rosiglitazone activates AMPK in Min6 cells over 2 hours (P<0.05).

Figure 3: the p38 inhibitor SB203580 had no effect on the ability of rosiglitazone to protect Min6 cells from the detrimental effects of palmitate.

Figure 4: the presence of the CK2 inhibitor TBB was able to partially block the protective effect of rosiglitazone on Min6 cells, although cell viability was still significantly higher than treatment with palmitate alone (P<0.05).

Figure 5A: western blot analysis of total p38 protein following treatment of Min6 cells with 10μM rosiglitazone for 2 hours. Figure 5B: densitometry indicates over 50% decrease in total p38 levels (P<0.05). Figure 5C: phosphorylation of ACC was observed upon stimulation with rosiglitazone, an effect that was inhibited by compound C. Figure 5D: densitometry indicates that rosiglitazone activates AMPK in Min6 cells over 2 hours (P<0.05).

Figure 6A: western blot analysis of total p38 protein following treatment of Min6 cells with 10μM rosiglitazone for 2 hours. Figure 6B: densitometry indicates over 50% decrease in total p38 levels (P<0.05). Figure 6C: phosphorylation of ACC was observed upon stimulation with rosiglitazone, an effect that was inhibited by compound C. Figure 6D: densitometry indicates that rosiglitazone activates AMPK in Min6 cells over 2 hours (P<0.05).
1. Introduction

Rosiglitazone is an oral anti-hyperglycaemic agent used in the treatment of Type 2 diabetes and is known to act as a PPARγ antagonist.

2. Methods

2.1 Culture: Min1 cells were cultured in 5mM glucose DMEM with the addition of stimulants for 2 hours for western blot analysis, or 72 hours for cell viability assays. To determine the role of individual signalling pathways, the following cell signalling inhibitors were used: AMPK inhibitor compound C (CC), SB203580 (SB), Rosiglitazone was added to a final concentration of 0.5μM. Rosiglitazone was added to a final concentration of 0.5μM.

3. Results

3.1 ACC Phosphorylation and CK2 Enzyme Activity

(a) Panel A: western blot showing phosphorylation of ACC upon stimulation with rosiglitazone, an effect that was inhibited by compound C (P<0.05). Panel B: densitometry values.

(b) Rosiglitazone stimulated a 3-fold increase in CK2 activity compared with untreated control over 2 hours (P<0.01).

4. Discussion

-MTT assays showed that addition of palmitate for 72 hours resulted in a 79% loss of Min1 cell viability (P<0.05). However, the presence of rosiglitazone completely abolished the detrimental effect of palmitate, preserving β-cell viability.

-Rosiglitazone increased phosphorylation of ACC (P<0.05) and CK2 activity (P<0.01) in Min1 cells within 2 hours. At a gene expression level, palmitate exposure significantly increased UCP2 mRNA levels (P<0.05), whereas treatment with rosiglitazone had no significant effect on UCP2 gene expression. However, addition of rosiglitazone in the presence of palmitate completely abolished the effect of palmitate on UCP2 expression (P<0.05).

The ability of rosiglitazone to block palmitate-induced increases in UCP2 gene expression was abolished by addition of the PPARγ antagonist GW6662, suggesting that the effects of rosiglitazone on UCP2 expression under these conditions are dependent on PPARγ activity.

The results of the present study indicate that rosiglitazone modulates multiple signalling pathways in pancreatic β-cells, protecting Min1 cells from the detrimental effects of palmitate and blocking palmitate-induced increases in UCP2 gene expression. This adds to a growing body of evidence that rosiglitazone can have direct protective effects on β-cells through both PPARγ-dependent and PPARγ-independent signalling pathways.

References


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