Protection of Pancreatic Beta Cells
by Rosiglitazone - Mechanisms and Pathways

Tomader Faroug Mohammed Ali

August 2011

Submitted in Part Fulfilment of Requirements for the
Degree of Doctor of Philosophy
School of Pharmacy and Biomolecular Sciences
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ABSTRACT

The thiazolidinedione Rosiglitazone can protect pancreatic beta cells from the detrimental effects of free fatty acids. The aim of this study was to determine the differential effects of saturated fatty acids on beta cells and to identify the mechanisms through which Rosiglitazone can protect beta cell viability. Utilising the mouse beta cell line MIN6, transmission electron microscopy (TEM) was used to determine the effects of palmitate and Rosiglitazone on lipid uptake and cell ultra-structure. H/PI staining and MTT assays were used to determine cell viability. Confocal microscopy was used to determine the intracellular localisation of the transcription factor NFκB. Real time RT-PCR was used to determine changes in gene expression. Western blotting was used to determine the expression and intracellular localization of key beta cell target proteins. 72 hour exposure to palmitate resulted in a 50% loss of MIN6 cell viability, which was prevented by the presence of Rosiglitazone. This was supported by HPI staining, indicating an increase in apoptosis in response to palmitate, which was prevented by Rosiglitazone. TEM confirmed that palmitate has detrimental effects on beta cell ultra-structure. Rosiglitazone protected MIN6 cell ultra-structure from palmitate induced damage. Palmitate induced translocation of NFκB from the cytoplasm to the nucleus and reduced Bcl2 mRNA levels, with both of these effects being prevented by Rosiglitazone. Rosiglitazone reduced expression of UCP2 in a PPARγ-dependent manner, whilst activating phosphorylation of the mTORC1 substrate S6K. At a cell signalling level, Rosiglitazone was also shown to down regulate the stress activated p38 signalling cascade, whilst activating both the AMPK and CK2 signalling molecules. In conclusion, the work presented in this thesis has shown that the mechanisms through which Rosiglitazone protects pancreatic beta cell viability from the detrimental effects of free fatty acids are far more complex than just the simple activation of PPARγ. In fact, this study has shown that the protective actions of Rosiglitazone occur through the coordinated modification of multiple signalling pathways in pancreatic beta cells.
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ABBREVIATIONS

AMPK......................................Adenosine Mono-phosphate activated Protein Kinase
APS......................................................Ammonium persulphate
Bcl2........................................................B-cell lymphoma 2
BSA........................................................Bovine Serum Albumin
DMSO.......................................................Dimethyl sulfoxide
EDTA......................................................Diaminoethanetetraacetic acid
EGTA.....................................................Ethylene glycol tetraacetic acid
FBS........................................................Foetal Bovine Serum
GLUT2..................................................Solute carrier family 2 (facilitated glucose transporter), member 2
GSIS.......................................................Glucose stimulated insulin secretion
KCl..........................................................Potassium chloride
ml..........................................................Milli-litres
mGAPDH.............................................Mitrochondrial glycerol 3-phosphate dehydrogenase
MTT.....................................................(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl........................................................Sodium Chloride
PBS......................................................Physiological Phosphate Buffered Saline
PDX-1....................................................Pancreatic duodenal homeobox-1
PPARγ.................................................Peroxisome proliferator-activated receptor(gamma subunit)
Rpm........................................................Revolutions per minute
SDS........................................................Sodium dodecyl sulphate
TEMED.......................................................N’-tetramethyl-ethane-1,2-diamine
μl............................................................Micro-litres
μM........................................................Micro-molar
CHAPTER 1

Introduction

Part I: General Introduction

Diabetes

Diabetes mellitus is a chronic metabolic disease caused by the inability to maintain controlled glucose levels due to either insulin resistance or insulin deficiency (Cerielo, Motz et al. 1997; Lo, Parham et al. 2010). Diabetes mellitus mainly occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces, manifesting as hyperglycaemia. Insulin is a polypeptide hormone secreted by the beta cells of the pancreas in response to high blood glucose levels (Bansal and Wang 2008). It strongly stimulates the storage of glucose as glycogen in muscle and as fat in adipose tissue and works to maintain normo-glycaemia. In the UK alone, there are 2.6 million people suffer with the disease (Diabetes UK 2010). Diabetes is a progressive disease with growing prevalence and globally, 346 million people worldwide currently with diabetes (World Health Organization 2011).

The main symptom of diabetes is hyperglycaemia or raised blood glucose levels, a common effect of uncontrolled diabetes leading to serious damage to many of the body's systems especially the nerves and blood vessels (Adeghate 2007). Hyperglycaemia occurs when the body does not have enough insulin or has a glucose disposal malfunction (Birrer 2001). Classic symptoms include great thirst (polydipsia), dry mouth and frequent urination (polyuria). Other signs include tiredness, blurred vision, weight loss, muscle wasting, frequent infections or slow-healing sores, itchiness around genitalia and regular episodes of thrush for women (NHS Choices 2011). Diabetes is also an independent risk factor for coronary heart disease, stroke, peripheral arterial disease and heart failure, which are the main causes of death in these patients (Triches, Schaan et al. 2009). Ketoacidosis, which is a severe metabolic derangement, could
also occur in the absence of insulin (Sampson, Brennan et al. 2007) and eighty percent of people with type 2 diabetes suffer from the complications of cardiovascular atherosclerosis (Imrie, Abbas et al. 2009) There are different types of diabetes. Type 1 diabetes mellitus (T1DM) is characterised by insulin deficiency, sudden onset, severe hyperglycaemia, rapid progression to ketoacidosis and death unless treated with insulin (Hsueh, Jackson et al. 2001). Type 2 Diabetes Mellitus (T2DM) affects about 5% of adults worldwide and this prevalence is rising rapidly (International Diabetes Federation 2010). Despite the effective oral glucose-lowering agents used most patients will experience a gradual loss of glycaemic control irrespective of underlying levels of insulin resistance due to progressive failure and loss of beta cells (Standl 2007).

**Obesity**

Obesity is a medical condition in which excess body fat accumulates to extents where adverse effects on health are observed, leading to increased mortality (Haslam and James 2005). Obesity is commonly caused by a combination of excessive dietary calories, lack of physical activity and genetic susceptibility (Adams and Murphy 2000; Ness-Abramof and Apovian 2009) and is a major risk factor for cardiovascular diseases and cancer (World Health Organization (WHO) 2010). The primary treatment is dieting and an increase in physical exercise (Apovian 2010) with beneficial effects on glycaemic control, triglyceride levels, and high-density lipoprotein cholesterol levels (Wylie-Rosett and Davis 2009). If modifying diet and exercise fails, anti-obesity drugs may be taken to reduce appetite or inhibit fat absorption (Pagotto, Vanuzzo et al. 2008). Obesity is fast becoming the developed world's biggest health problem with over 9,000 deaths a year in England alone (NHS Choices 2009). The Department of Health (DoH) estimates the resultant cost to the NHS to be £4.2 billion annually and this is predicted to rise to £50 billion by 2050 if left unchecked (Department of Health 2011). Campaigns have been set up by the government to tackle diabetes management on a national basis. This includes campaigns such as Change4Life which is a recent movement aiming to
help the public and especially the younger generation to eat better, move more and live longer (International Diabetes Federation 2010). Interestingly obesity was once considered a problem only in the developed world with access to fast foods, processed food and sugars, in combination with the modern sedentary lifestyle, but the increased prevalence now does not seem to fit only this criteria and the occurrence of obesity is increasing even in the developing world (Guize, Jaffiol et al. 2008; Blackburn, Wollner et al. 2009; World Health Organization (WHO) 2010) and the occurrence is increasing globally (Walter, Kunst et al. 2009).

**Obesity: Genes or Lack of Exercise?**

There has been a lot of controversy on the topic of obesity being related to a genetic make-up of the affected individuals or whether it is due to lack of exercise. Obesity has been attributed to a combination of environmental factors alongside multiple genes of small effect and although there has been considerable advancement in characterizing genes in several pathways contributing to metabolic disease, understanding the relationships between these genes and their synergistic effects upon obesity if any, lags behind (Fawcett, Jarvis et al. 2010). In the literature, there is great emphasis on the important roles genes play in the regulation of processes that impact the development and the progression of diabetes mellitus; or example, availability of CD36, a membrane receptor with a wide variety of functions including the regulation of energy metabolism, fat storage, and adipocyte differentiation has recently been found to be associated with body weight variability (Bokor, Legry et al. 2010). No single factor can completely explain obesity thus the role of exercise cannot be underestimated.

**Exercise**

Daily activities should be encouraged earlier among children as an early intervention for their positive influences on parameters involved in metabolic syndrome and diabetes (Venables and Jeukendrup 2009). The effects of physical activity on overall quality of life are well established
in the general population and have been analyzed on various dimensions of the Health-Related Quality of Life (HRQL); a questionnaire with a scale assessing and including physical and social functioning, subjective well-being, emotion and mood, self-esteem and self-perception, cognitive performance and sleep quality and how it affects disease states (Zanuso, Balducci et al. 2009). Even for pregnant women exercise is an important component of their healthy lifestyle and is highly recommended (DeMaio and Magann 2009). In diabetic subjects treated with insulin or insulin secretagogues, exercise may lead to complications as too much insulin causes hypoglycaemia and not enough insulin can cause hyperglycaemia and possibly ketoacidosis, both complications occurring several hours after exercise (Pugliese, Zanuso et al. 2009). This highlights the importance of blood glucose self-monitoring before, during and after physical exercise due to the change in glucose metabolism for diabetic individuals.

**Glucose Metabolism**

Glucose metabolism is defined as the process by which glucose found in many foods is processed by the body and used to produce energy in the form of ATP. Once consumed, glucose is absorbed by the intestines into the blood and extra glucose is stored in the muscles and liver as glycogen. When needed, glycogen is hydrolyzed to glucose and released into the blood (Cooper 2000). Glucose is an essential body fuel regulated by many hormones including insulin ‘the hormone of plenty’ as it is released when there is plenty of glucose (Dean and McEntyre 2004).

Gluco-regulatory hormones include insulin and amylin (derived from pancreatic beta cells), glucagon (derived from the pancreatic α-cells), glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic peptide (GIP) (derived from the L-cells of the intestine) as well as adrenaline, cortisol, and growth hormone (Stephen, Aronoff et al. 2009). These hormones are designed to maintain circulating glucose concentrations in a relatively narrow range. Insulin stimulates muscle and fat cells (removing glucose from the blood), adipose tissue (aiding in
storing glucose as fat for long-term energy reserve), as well as stimulating cells to use glucose in protein synthesis (Dean and McEntyre 2004) (see Figure 1. Overview of Glucose Metabolism on page 18). Glucagon is the main hormone opposing the action of insulin, helping the body to switch to using other resources such as fat and protein instead of glucose. Insulin triggers the formation of glycogen via an anabolic or energy-requiring effect, while glucagon on the other hand triggers glycogen breakdown and that is via a catabolic energy releasing effect (see Figure 2. Anabolism and Catabolism of Glucose on page 18).

The process by which plasma glucose concentration is maintained is complex but briefly it is accomplished by maintaining the rate at which glucose enters the circulation and by the rate it is removed, however, the circulating blood glucose level itself is derived from three sources; intestinal absorption during the fed state, glycogenolysis (the breakdown of glycogen, the polymerized storage form of glucose) and gluco-neogenesis (glucose formation primarily from lactate and amino acids during the fasting state) (Stephen, Aronoff et al. 2009). In the fasting state, glucose leaves the circulation at a constant rate. The sole source of endogenous glucose production is the liver, hepatic gluco-neogenesis, which contributes substantially to the systemic glucose pool only during periods of extreme starvation. Although most tissues have the ability to hydrolyze glycogen, only the liver and kidneys contain glucose-6-phosphatase, the enzyme necessary for the release of glucose into the circulation. In the bi- hormonal model of glucose homeostasis, insulin is the key regulatory hormone of glucose disappearance, and glucagon is a major regulator of glucose appearance. Glucagon is produced in the pancreatic alpha cells and controls glycogenolysis and gluco-neogenesis. In the initial first 8–12 hours of fasting, glucagon facilitates glycogenolysis; however longer fasting durations require gluco-neogenesis by the liver involvement (Stephen, Aronoff et al. 2009).
Figure 1. Overview of Glucose Metabolism

Glucose is used for many purposes in the body and thus is metabolised and converted into energy via pyruvate and the tricarboxylic acid (TCA) cycle. Glucose can also be converted to fat for long-term storage or glycogen for short-term storage. Some amino acids may also be synthesized directly from pyruvate contributing towards protein synthesis. Image from (Dean and McEntyre 2004).

Figure 2. Anabolism and Catabolism of Glucose

Glucose metabolism involves two processes; one which produces energy known as catabolic (in orange) and one which consumes energy known as anabolic (shown in green). Image from (Dean and McEntyre 2004).
**Glycolysis**

Glycolysis is usually considered as a paradigm metabolic pathway by which glucose is consumed in most organisms thus the involved pathways in catalysis are very similar (Sugden and Holness 2011). Glycolysis is a central pathway for the catabolism of carbohydrates in which six-carbon sugars are split to three-carbon compounds with subsequent release of energy used to transform ADP to ATP and it can proceed under either aerobic (with oxygen) or anaerobic (without oxygen) conditions. This pathway converts glucose to two pyruvate molecules with a net equation:

\[
\text{Glucose} + 2\text{ADP} + 2\text{NAD}^+ \rightarrow 2\text{Pyruvate} + 2\text{ATP} + 2\text{NADH}
\]

**The Pancreas**

**Growth and Development**

Understanding the details of pancreatic endocrine cell development is imperative if the correct and effective therapies are to be developed. Scientifically, clarification is a major challenge as the processes involved are rather complex and unravelling the complexity aids significantly in the ability to treat the different types of diabetes effectively (Scharffmann, Duvillie et al. 2008).

**Structure**

The pancreas is a compound gland responsible for the discharge of digestive enzymes into the gut and secretion of the hormones insulin and glucagon, which are essential in carbohydrate metabolism, into the bloodstream (Robert and Utiger 2011). In humans the pancreas weighs approximately 80 grams and is located in the upper abdomen, lying adjacent to the duodenum with the body and tail extending across the midline near the spleen. In adults, most of the pancreatic tissue is dedicated to exocrine function, where digestive enzymes are secreted into the duodenum, via the pancreatic ducts by acinar cells (see Figure 3. Structures of the Pancreas on page 20).
Figure 3. Structures of the Pancreas

The Islets of Langerhans are cell clusters in the pancreas secreting hormones such as insulin and glucagon into the capillary network directly which also is joined to the pancreatic duct. The acinar cells produce digestive enzymes instead of hormones and the enzymes are secreted into small ducts which then feed into the pancreatic duct. Image from (Robert and Utiger 2011).
The biology of the pancreas is exquisitely complex and involves both endocrine and exocrine functions that are regulated by an integrated array of neural and hormonal processes (Chandra and Liddle 2009). The primary function of the endocrine pancreas is the secretion of insulin and other polypeptide hormones essential for glucose, amino acids, and triglycerides cellular storage or mobilization. In the exocrine pancreas hormones are secreted into the blood but digestive enzymes along with the alkaline pancreatic juice are secreted into the small intestine through the exocrine ductal system, mostly in response to secretin and cholecystokinin which are hormones of the small intestine. Other pancreatic enzymes include digestive enzymes such as trypsin, chymotrypsin, pancreatic lipase, and pancreatic amylase that are produced and secreted by the acinar cells of the exocrine pancreas (Maton, Hopkins et al. 1993).

The pancreas is made up of approximately a million cell clusters called Islets of Langerhans, located between the clusters of acinar cells and named after the 19th century German pathologist Paul Langerhans and there are four main cell types within each islet, classified by their secretions (Hellman, Gylfe et al. 2007). The cells are mainly alpha (α) cells secrete glucagon (increasing glucose in the blood), beta (β) cells secrete insulin, (decreasing glucose in the blood), delta (δ) cells secrete somatostatin (regulating the α and β cells) and the (F or PP) polypeptide producing cells (Constanzo 2010). Beta cells account to only 1-2% of the pancreatic tissue and weigh about 1 gram (Robert and Utiger 2011). Cells that make up the islets arise from both endodermal and neuroectodermal precursor cells, but the islets as a whole originate from endocrine progenitors within the pancreatic ductal epithelium (Greiner, Kesavan et al. 2009).

75% of the cells in each islet are insulin-producing beta cells, found clustered centrally in the islet. Alpha, delta, and F (or PP) cells make up the remainder of the islets and are located at the
periphery (Katsuta, Akashi et al. 2010). There are also numerous nerve endings around the islets, predominantly involuntary or autonomic nerves that monitor and control internal organs (Robert and Utiger 2011). Thus islet function may be regulated by signals initiated by autonomic nerves, circulating metabolites such as glucose, amino acids and ketone bodies, circulating hormones, or local/paracrine hormones.

**Insulin Receptor**

Some of the most important drugs for diabetes treatment rely on the binding and activation of crucial receptors in the pancreas, including the nuclear hormone receptor (NHR) peroxisome proliferator-activated receptor-γ (PPARγ) which is the most common and is of particular significance in relation to type 2 diabetes (Chuang, Cha et al. 2008). The NHR superfamily consists of forty nine members and thus they are one of the largest transcription factor families in the mammalian genome, PPARγ being one of the members. This superfamily is key to glucose regulation as they have potent serum glucose lowering effects (Chuang, Cha et al. 2008). The most widely prescribed oral drugs for treating type 2 diabetes, such as the thiazolidinediones, exert their actions, in part, by binding and activating PPARγ (Lehmann, Moore et al. 1995), (both drug and receptor will be covered in more detail; see Rosiglitazone section on page 36 and PPAR section on page 44).

Disruption of the insulin receptors in mouse beta cells leads to a phenotype that mimics human type 2 diabetes (Folli, Okada et al. 2011). The insulin receptor family and the continuously expanding family of insulin-like peptides (such as IR, IGF-1R and IRR) have identical gene structure (apart from the lack of exon 11 in the IGF-1R gene). The insulin receptor is a transmembrane receptor belonging to the large class of tyrosine kinase receptors and is activated by insulin. The receptor is made up of four subunits; two alpha and two beta subunits encoded by the one gene INSR. The two beta subunits going through the cell membrane linked via disulphide bonds. These receptors therefore exist as dimmers linked with disulphide links.
where each of the monomers are cleaved into an N-terminal α-chain and a C-terminal, membrane spanning, β-chain. The insulin receptor itself is present in two isoforms; IR-A and IR-B and they differ in the absence or presence, respectively, of the 12 amino acids coded for by exon 11; these residues lie near the C-terminus of the receptor α-chain (Ward and Lawrence 2009). The insulin receptor substrate (IRS) proteins integrate the activated membrane-bound insulin receptor (IR) kinases to downstream adapter proteins and enzymes. IRS1, IRS2 and IRS4 are expressed in humans. Processes such as carbohydrate and lipid metabolism are initiated through IRS1 and IRS2 (White 2003) and the IRS proteins are targeted by binding of the specific N-terminal phosphotyrosine binding (PTB) domain to the phosphorylated NPXY-motif in the cytoplasmic juxtamembrane region of the insulin or IGF1 receptors. The C termini of IRS1 and IRS2 are poorly conserved, but they both contain over twenty recognizable tyrosine phosphorylation motifs in similar positions that can bind signalling molecules (Cheng, Tseng et al. 2010). It is known from studies, as indicated by the literature, that insulin uptake and degradation is a feature of all insulin sensitive tissues whereby at physiological concentrations, uptake is mediated primarily by the insulin receptor with a smaller contribution from nonspecific processes. This is reversed at higher insulin concentrations where non-receptor processes have greater importance (Duckworth, Bennett et al. 1998).

The Beta Cell

Origin and Function
In the very early stages of development, the pancreas arises from a flat sheet of cells (endoderm) which becomes specified during gastrulation (Stoffers and Oliver-Krasinski 2008). As embryogenesis proceeds with the expression of pancreatic hormones and digestive enzymes, acinar cell clusters that empty into the ductal network leading into the duodenum also form. These are interspersed with endocrine cells that delaminate from the epithelium in turn forming islet clusters (Jørgensen, Ahnfelt-Rønne et al. 2007).
There are various factors regulating islet development including (Yamaoka and Itakura 1999):

- Cell adhesion molecules; N-CAM & cadherins
- Hormones; insulin, the GH family, PTHrP, TRH & gastrin
- Differentiation factors; Shh, activin, follistatin & TGF alpha
- Growth factors; the EGF family, HGF, IGF-I/II, PDGF, FGF, VEGF & NGF
- Transcriptional factors; PDX1, Isl1, Pax4/6, Nkx2.2, Nkx6.1, BETA2 & HNF

The transcription factors in pancreatic development are critical in determining the development of the pancreatic beta cell (see Figure 4. Transcription Factor Profile During the Stages of Differentiation on page 25). Transcription factors play different roles in maintaining the differentiated phenotype of the beta cell as well as defining mechanisms that allow the beta cell to adapt to changing metabolic demands that occur throughout life (Bernardo, Hay et al. 2008).

**Growth, Preservation and Regenerative Therapy**

It is well established that beta cell deficiency is a pathophysiologic component of diabetes mellitus and a primary cause of islet dysfunction which is in turn a prerequisite for diabetes advancement (individuals with insulin resistance do not develop hyperglycemia unless beta cell compensation fails) (Ritzel 2009). The balance between beta cell replication and neogenesis still remains a huge topic of debate as the relative importance of both processes alone and in relation to each other remains vague (Ritzel 2009). Islet loss due to the limited regenerative capacity of islet cells is largely due to increased beta cell apoptosis and that is limited further by the vulnerability of the replicating beta cell to apoptotic processes. It therefore follows that grasping the mechanisms regulating normal beta cell adaptation to such changes will aid in finding new therapeutic targets particularly for the preservation and/or regeneration of beta cell mass. All beta cells contribute equally to islet maintenance thus therapeutic approaches need to address both processes of islet turnover/regeneration and cellular loss equally (Ritzel 2009).
**Figure 4. Transcription Factor Profile During the Stages of Differentiation**

During beta cell formation, numerous transcription factors are expressed at each stage of development and are colour coded in the figure above based on their source; gut endoderm (purple), pancreatic endoderm progenitor (blue), early endocrine progenitor (black), endocrine progenitor (orange), beta cell (red). Image from (Mimeault and Batra 2008).
**Pre-diabetes**
As can be seen from the progression of the diabetic disease state, beta cell preservation and increase in mass, along with stem cell research, are the current direction for treatment and the hope for future improved interventions. The choice of treatment, however, does not start with the diagnosis of diabetes, which brings rise to the question; what about pre-diabetes? The treatment approach is twofold: glycaemic control and control of cardiovascular risk factors, mainly hypertension and hyperlipidemia. For low risk patients, the initial proactive approach is intensive lifestyle modification but when this approach fails, pharmacological approaches are used and medication such as Metformin is recommended. If high-risk remains despite these changes then thiazolidinediones are recommended (Sharma and Garber 2009). Although prediabetes refers to individuals with impaired fasting glucose and/or impaired glucose tolerance who are at increased risk for type 2 diabetes mellitus, these patients manifest distinct metabolic abnormalities (Abdul-Ghani and DeFronzo 2009).

**Types of Diabetes**

**Type 1 Diabetes Mellitus (T1DM)**
Type 1 diabetes is caused by an immune-mediated destruction leading to insulin deficiency, impaired intermediary metabolism and elevated blood glucose concentrations (Pechhold, Koczwara et al. 2009). The beta cells of the pancreas are responsible for insulin production thus their total destruction resulting in type 1 diabetes is hugely problematic as beta cell maintenance, growth and regenerative repair is thought to occur predominately, if not exclusively, through the replication of existing beta cells (Brennand and Melton 2009). Profound metabolic changes occur in individuals with type 1 diabetes mellitus during insulin deprivation including an increase in basal energy expenditure, a reduction in mitochondrial function and additionally protein metabolism is significantly affected during insulin deprivation (Hebert and Nair 2010). The coexistence of insulin resistance, obesity, diabetes
mellitus, hypertension and dyslipidaemia is defined as the metabolic syndrome but interestingly, recent clinical features of insulin resistance are actually also present in many patients with type 1 diabetes such as decreased insulin sensitivity and that was previously linked only with obesity and diabetes type 2 (Uruska and Araszkiewicz 2009).

While at autoimmune diabetes onset a limited number of beta cells persist, the regenerative potential and regulation of the beta cells has remained largely unexplored. The data suggests that timely control of the destructive immune response after disease manifestation could allow spontaneous regeneration of sufficient beta cell mass to restore normal glucose homeostasis (Pechhold, Koczwara et al. 2009). Type 1 diabetes development is also in part dictated by the failure of regulatory T-cells to maintain peripheral tolerance. Therapies aimed at correcting the declining function of regulatory T-cells have been the subject of intense investigation, with the discussion mainly focusing on the potential of regulatory T-cells to suppress auto-reactive responses and preventing type 1 diabetes induced by viral infection while maintaining protective antiviral immunity (Richer and Horwitz 2009). Other markers of autoimmune beta cell destruction include the circulating CD4 T-cells specific for peptide epitopes of proinsulin and other auto-antigens but the roles of some are more investigated than others. Recent studies have shown that CD8 T-cells of a diabetic patient for instance, after re-challenge with proinsulin peptides, secrete interferon-gamma (IFNγ) and granzyme B which are markers of their effector capacity while on the other hand, CD8 T-cells of the same patient in a "cross-talk" with proinsulin-specific CD4 T-cells suppressed beta cell proliferation (Batarelo and Durinovic-Belló 2008).

As insulin therapy is the only option apart from islet transplants, youths with type 1 diabetes mellitus may decide to use insulin pump therapy as opposed to being injected however limited information describes glycaemic control with insulin pump therapy in this age category.
(Nelson, Genthe et al. 2009). In individuals with type 1 diabetes and poor glycaemic control, disordered lipid and lipoprotein metabolism is common and is characterized with increased concentrations of triglycerides and triglyceride-rich lipoproteins (Pantelinac 2009). Adequate insulin treatment enables good diabetes control and the dual prevention of the lipid and lipoprotein disorders and diabetic complications.

**Type 2 Diabetes Mellitus (T2DM)**

Formally known as maturity onset diabetes, type 2 diabetes currently affects a large percentage of the world population and almost 4% of the UK population (Wyness 2009). Approximately 80%-90% of patients are overweight (Brunton 2009) with increased cardiovascular disease risks amongst other complications (Ji-Wom, Ji-Hun et al. 2009). There is a steady increase in prevalence which is predicted to continue rising (Lammert 2008; Ji-Wom, Ji-Hun et al. 2009). Type 2 diabetes is thought to be related to middle aged overweight individuals but this trend is changing as in recent years diagnosis is increasingly including children and adolescents (Phillips and Phillips 2009). Having one or more parents with type 2 diabetes gives offspring up to an 80% chance of developing the disease (Paradis, Godin et al. 2009). Type 2 diabetes mellitus denotes a complex and heterogeneous group of metabolic disorders including hyperglycemia, impaired insulin action and/or insulin secretion (Lin and Sun 2010) and is the cause of many cerebrovascular and peripheral vascular diseases as well as myocardial infarctions, coronary atheromas and possible mortality as it is characterized by beta cell failure in the setting of insulin resistance. In early stages of the disease pancreatic beta cells show an increase in mass and function as a natural response to adapt to the occurring insulin resistance (Madan, Chen et al. 2010). The fact that type 2 diabetes is generally associated with nutrient excess, hyperglycemia and elevated free fatty acids negatively impacts further on beta cell function distressing the already vulnerable beta cell. This happens by numerous mechanisms, including the generation of reactive oxygen species, alterations in metabolic pathways,
increases in intracellular calcium and the activation of endoplasmic reticulum stress to name just a few (Araki, Oyadomari et al. 2003).

Regardless of the mechanism, processes which adversely affect beta cells, for instance by impairing insulin secretion or decreasing insulin gene expression, ultimately end in apoptosis (Chang-Chen, Mullur et al. 2008). Glycaemic control is the primary focus in terms of treatment, however it is extremely important to factor in clinical management of patients for a more comprehensive approach aiming initially at minimizing morbidity (Brunton 2009). Lifestyle interventions, especially tight glycaemic control, can prevent and/or delay the development of chronic complications (Sliva 2004).

Medical intervention includes anti-diabetic agents such as sulfonylureas, meglitinides, thiazolidinediones and insulin. There have been some associations with weight gain for some of those treatments, with the exception of Metformin, which is seen as weight-neutral or weight-sparing (Brunton 2009). In severe hypoglycaemia, insulin may be required initially to achieve normo-glycaemia and is then phased out with Metformin phased in later (Davis, Forges et al. 2009). The main aim of this combination approach of pharmacologic and non-pharmacologic therapy is to achieve and maintain normo-glycaemia, weight reduction and increase physical activity (Stone 2008).

Thiazolidinediones have also been very successful as a medical intervention and until recently, the addition of low-dose Rosiglitazone to insulin therapy is an effective and well-tolerated treatment option for patients who continue to have poor glycaemic control despite administration of exogenous insulin as mono-therapy (Hollander, Dahong et al. 2007). Insulin however is most likely to be required again later in the natural history and progression of the disease (Phillips and Phillips 2009). New research explores new interventions such as the
incretin-based therapies like glucagon-like peptide-1 (GLP-1) receptor agonists and the dipeptidyl peptidase-4 (DPP-4) inhibitors favourable for their effects on weight; weight-reducing and weight-neutral respectively (Ji-Wom, Ji-Hun et al. 2009). The development of an ideal therapeutic programme is complicated due to the intricate nature of the pathways involved and the amount of cross-talk and negative feedback mechanisms controlling these events (Lee, Demeterco et al. 2009). The mechanisms responsible for beta cell function loss are multi-factorial and may involve toxicity because of elevated glucose and/or lipid levels, increased secretory demand because of insulin resistance, amyloid deposition and altered levels of cytokines (Standl 2007). Research still highlights the importance of prevention and early intervention as a preferred mechanism for at least delaying the onset of the disease (Matthews and Levy 2009).

Regardless of the cause of diabetes (genes, obesity, lifestyle, diet and exercise) the aetiology of the disease depends heavily on the physiology of the pancreas and ultimately the beta cell.

**Part II: Targets and Stimuli Utilized to Investigate the Effects of the Free Fatty Acid Palmitate and the Thiazoladinedione Rosiglitazone on Pancreatic Beta Cells**

**Free Fatty Acids**

**Palmitate**

Palmitate is a term for salts or esters of palmitic acid. Palmitic acid \((\text{CH}_3\text{CH}_2\text{OH})_{14}\text{COOH}\) or hexadecanoic acid is one of the most common saturated 16-carbon fatty acids found in animals and plants (Beare-Rogers, Dieffenbacher et al. 2001; The Merck Index 2009). Palmitic acid was discovered by Edmond Frémy in 1840 in saponified palm oil. It is more formally known as retinyl palmitate and it is also the first product of lipogenesis (fatty acid synthesis). Retinyl palmitate also named retinol palmitate or vitamin A palmitate is the ester of retinol (vitamin A) and palmitic acid, with formula \(\text{C}_{36}\text{H}_{60}\text{O}_{2}\) (see Figure 5. Free Fatty Acids on page 31).
Figure 5. Free Fatty Acids

Molecular formulas for palmitic acid, retinyl palmitate, palmitate, oleic acid and oleate. Images from (Beare-Rogers, Dieffenbacher et al. 2001; The Medical Dictionary 2007; Lomberk 2009; The Merck Index 2009).
Palmitate is also responsible for the alteration of multiple cellular processes including transcription, translation, enzymatic activities, cellular signalling, ion homeostasis, membrane the pancreas, lipid metabolism has a huge impact during normal digestive processes and disease states such as pancreatitis, diabetes and cancer (Lomberk 2009). During the development of diabetes, lipotoxicity causes many metabolic changes during the metabolic syndrome, fuelled by the imbalance of diet and exercise (Tovar and Torres 2010).

In a normal cycle of metabolism, palmitate negatively feeds back on Acetyl-CoA Carboxylase (ACC), a downstream target of AMPK, which is responsible for converting acetyl-ACP to malonyl-ACP on the growing acyl chain, in turn preventing further palmitate generation (Genome 2011). AMPK forms the energy sensing apparatus in the cell regulating cellular metabolism. Studies have shown that following a 24 hour stimulation palmitate caused activation of AMPK and a resultant suppression of glucose-stimulated insulin secretion (GSIS) was observed, however that did not affect the response of insulin secretion to combined stimuli of glucose and palmitate (Wang, Zhou et al. 2007).

There is a wealth of literature reporting the high incidence of beta cell death and apoptosis in pancreatic beta cells (Abaraviciene, Lundquist et al. 2008; Lupi and Del Prato 2008) and much to support the notion that Rosiglitazone can exert protective effects and preserve beta cell function from the detrimental effects of palmitate (Lupi, Del Guerra et al. 2003; Abaraviciene, Lundquist et al. 2008; Han, Kang et al. 2008). We wanted to examine this further in our Min6 model and elucidate the specific pathways and gene targets involved. The literature highlights the reduction in beta cell function, insulin resistance and ultimately beta cell apoptosis and death (Welters, Diakogiannaki et al. 2006; Lopes, Oliveira et al. 2008; Pipeleers, Chintinne et al. 2008).
fluidity and cell survival (Kadotani, Tsuchiya et al. 2009). Long-term exposure to high levels of non-esterified fatty acids (NEFA) alone (lipotoxicity) or in combination with elevated glucose (gluco-lipotoxicity) both affect the release of glucagon and somatostatin, which may aggravate hyperglycaemia in individuals with type 2 diabetes (Collins, Salehi et al. 2008). Glucagon regulates hepatic glucose production (Maida, Lamont et al. 2011) and pancreatic somatostatin is a hormone that has no major direct effect on glucose metabolism but is a potent paracrine inhibitor of both insulin and glucagon secretion (Braun, Ramracheya et al. 2009).

Pancreatic beta cells metabolise both lipid and glucose nutrients, but the over-exposure of beta cells to fatty acids such as palmitate in states of obesity causes the over-stimulation or exhaustion of the beta cells (Standl 2007). Studies have shown that MIN6 beta cells pre-treated with palmitate were depleted of intracellular insulin content consistent with the down-regulation of expression of the beta cell specific transcription factor pancreatic/duodenum homeobox protein 1 (PDX-1) levels in both the MIN6 cell lines and isolated islets tested (Standl 2007). Prolonged exposure to excessive fatty acid levels have been shown to impair insulin gene transcription in isolated rat islets (Lupi, Dotta et al. 2002). Observed alterations in the expression and binding activity of key beta cell regulatory factors included the reduction in nuclear localization of PDX-1, and blocked stimulation of MafA mRNA and protein expression by glucose (Hagman, Hays et al. 2005). MafA is a transcription factor which specifically binds to the insulin enhancer element (RIPE3b) and activates insulin gene expression (GeneCards 2011). Following 24 hour palmitate exposure studies have shown an observed inhibition of glucose stimulated and a decrease in insulin stores (Ritz-Laser, Meda et al. 1999).
Research has also demonstrated that palmitate had effects on the imbalance of pro- and anti-apoptotic proteins and a down-regulation of mRNA levels of the anti-apoptotic protein Bcl-2 (Landau, Forti et al. 2006). Apoptotic balance is closely related to the inflammatory and stress pathways and the activation of proteins such as the inflammatory nuclear factor kappa-B (NFκB) (see NFκB section on page 50) and the stress activated p39 mitogen activated Kinase (p38 MAPK) (see p38 section on page 51) respectively. Elevated plasma free fatty acids contribute to the pathogenesis of insulin resistance and endothelial dysfunction and many coronary complications that follow through the p38 MAPK-dependent pathway mechanism and reduced inhibitor of nuclear factor-kappaB (IκB) levels independently (Chai and Liu 2007).

Another palmitate effect was on the Akt family, also known as protein kinase B (PKB), which play pivotal roles in cell signalling and are involved in survival pathways by inhibiting apoptotic processes (Song, Ouyang et al. 2005). In humans Akt1 codes for enzymes that are members of the serine/threonine-specific protein kinase family and Akt1 particularly is key in protein signalling and cellular/tissue growth pathways. In relation to diabetes, Akt2 is a vital signalling molecule in the insulin signalling pathway, as it induces glucose transport (Song, Ouyang et al. 2005).

Akt is phosphorylated by its activating kinases such as phosphoinositide dependent kinase 1 (PDPK1) (at threonine 308) and mTORC2 (at serine 473), the latter being involved in growth and proliferation (Cai, Wang et al. 2008). INS-1 beta cells, with or without expression of dominant-negative mutant of Akt, cultured with palmitate for 24 hours showed a decrease in cell number and a dose-dependent increase in phosphorylation of Ser$^{473}$ in Akt/PKB, along with an increase in NFκB, suggesting that the Akt/PKB signalling is involved in palmitate-induced cell death and survival in these cells (Higa, Shimabukuro et al. 2005).
**Oleate**

Different fatty acids have different cytotoxic potentials. The long chain saturated molecules, such as palmitate, seem to be the most potent but contrastingly monounsaturates such as oleate and polyunsaturates seem to be comparatively well endured and in some cases are actively cyto-protective; the cyto-protective effects are thought to be receptor mediated involving the antagonistic actions on the effector arm of the endoplasmic reticulum stress pathway (Morgan 2009). Oleate is a term for salts or esters of oleic acid. Oleic acid is a mono-unsaturated omega-9 fatty acid containing the ion C17H33COO⁻ or the group C17H33COO⁻, found in various animal and vegetable sources. Oleic acid is the most abundant fatty acid in human adipose tissue (Kokatnur, Oalman et al. 1979). ‘Oleic’ refers to the source and is derived from oil or olive. Adipose tissue produces tumour necrosis factor-alpha (TNFα) which has an inhibitory effect on insulin and oleic acid pre-treatment has reversed this (Vassiliou, Gonzalez et al. 2009). The reversal of these negative inflammatory effects, along with highlighting the differential effects of monounsaturated and polyunsaturated fatty acids, may prove beneficial in therapeutic approaches for the treatment of obesity and diabetes.

**Cellular Responses to Palmitate and Oleate**

*In vitro* beta cells respond differentially to long-chain saturated and mono-unsaturated fatty acids such as palmitate and oleate respectively. This suggests that these molecules may regulate cell functionality by different mechanisms, particularly as saturated fatty acids exert detrimental effects on beta cells, while the mono-unsaturated fatty acids seem well tolerated and even cyto-protective in many cases (Morgan, Dhayal et al. 2008).

Recent studies found that in beta cells oleate was more protective than palmitate; however many cellular pathways were differentially regulated; pathways include lipid, cholesterol, fatty acid, and triglyceride metabolism, IκB kinase and NFκB kinase inflammatory cascades. Co-
incubation of beta cells with both fatty acids showed that oleate decreased the palmitate-induced damage and completely blocked the palmitate-induced PI3K Interacting Protein 1 (PIK3IP1) activation (Das, Mondal et al. 2010). PI3K Interacting Protein 1 (PIK3IP1) is a transmembrane protein which binds to the p110 catalytic subunit of PI3K inhibiting PI3K, the regulator of cell proliferation, migration and survival in most cell types (He, Zhu et al. 2008).

Other studies on INS-1 cells show that palmitate induced endoplasmic reticulum (ER) stress by altering the distribution of ER chaperones and altering the ER morphology while oleate treatment did not have any detrimental effects (Karaskov, Scott et al. 2006). The differences observed in the effects between the saturated and mono-unsaturated fatty acids were related to the ability of unsaturated molecules to preferentially stabilise mitochondria (Hardy, El-Assad et al. 2003). This in turn minimised the release of cytochrome c and the release and activation of caspases (Ostrander, Sparagna et al. 2001). In previous studies, unsaturated fatty acids attenuated mitochondrial cytochrome c release in beta cells (Maedler, Spinas et al. 2001). Studies have also established that saturated and monounsaturated fats have similar plasma trafficking and clearance however physical inactivity still influences the partitioning of saturated fats toward storage, likely leading to an accumulation of palmitate, particularly in muscle fat (Bergouignan, Trudel et al. 2009).

**Rosiglitazone**

Rosiglitazone is a thiazolidinedione (TZD), (see Figure 6 on page 37), prescribed initially (see Rosiglitazone and The Press section on page 40) as an anti-hyperglycaemic agent for treatment of type 2 diabetes. Rosiglitazone was marketed as Avandia by the pharmaceutical company GlaxoSmithKline (InPharm 2010) and it was approved in the United States, by the Food and Drug Administration (FDA) on May 28, 1999 (Goldberg, Kendall et al. 2005).
**Figure 6. Rosiglitazone**

Table of the full chemical name, formula and structure of Rosiglitazone (Lin, Han *et al.* 2011).
Rosiglitazone is a potent thiazolidinedione (TZD) administered orally as an anti-diabetic agent and it differs structurally from Pioglitazone and Troglitazone (other approved thiazolidinediones) with a greater PPARγ binding affinity (See Peroxisome Proliferator-Activated Receptor (PPAR) section on page 44) and anti-hyperglycaemic potency in pre-clinical models (Derosa 2010).

**Physiological Effects of Rosiglitazone**

Rosiglitazone is available both as a standalone preparation or in combination with Metformin (Avandamet) or with glimepiride (Avandaryl), all approved by the FDA. The National Institute for Clinical Excellence (NICE) guidance states that patients should be offered Rosiglitazone combination therapy as an alternative to injected insulin if (National Institute for Clinical Excellence 2010):

- they are unable to take Metformin and sulphonylurea as a combination therapy
- their blood glucose remains high despite adequate trial of this combination treatment
- the combination of Rosiglitazone and Metformin is preferred to the combination of Rosiglitazone and a sulphonylurea - particularly for obese patients.

The manufacturer's Summary of Product Characteristics states that Rosiglitazone is (GlaxoSmithKline Pharma 2010):

- not recommended for use in combination with insulin
- adverse events include fluid retention, anaemia, and weight gain
- contraindicated in patients with cardiac failure, hepatic impairment and severe renal insufficiency
Rosiglitazone Effects on Beta Cells

In type 2 diabetes there has been a growing body of evidence that TZDs rejuvenate beta cells and improve their function (Smith 2002). Further evidence of this TZD-induced preservation is documented by the findings of several recent trials (Lupi, Del Guerra et al. 2003) and the effects of Rosiglitazone are being investigated in vivo and in vitro. More recently the effects of Rosiglitazone and Metformin on pancreatic beta cell gene expression were studied (Richardson, Campbell et al. 2006). Our group published data which indicated both drugs had direct effects on beta cell gene expression, suggesting a previously unrecognised role in the direct regulation of pancreatic beta cell function (Campbell and Macfarlane 2002). It was observed that over a time course of 24 hours Rosiglitazone was found to promote nuclear accumulation of the insulin promoter factor-1 (IPF1) and forkhead homeobox A2 (FOXA2), independent of glucose concentration, and the increase in IPF1 gene promoter activity was unaffected by the presence of the peroxisome proliferator activated receptor gamma (PPARγ) antagonist GW9662 (Richardson, Campbell et al. 2006). Rosiglitazone has also been shown to inhibit the early stages of glucolipotoxicity-induced beta cell apoptosis, indicating again direct effects on beta cells rather than simply an indirect effect of improving insulin sensitivity.

In type 1 diabetes, the pathogenesis of post-transplant diabetes mellitus (PTDM) is thought to be partly related to the direct toxic effect of the immunosuppressants such as Rapamycin and cyclosporine (CsA). Recently, studies had shown direct protective Rosiglitazone effects on beta cell dysfunction and death in a study undertaken to clarify the effects of CsA-induced beta cell dysfunction and death (Ji-Wom, Ji-Hun et al. 2009). The deterioration in glucose tolerance caused by the CsA administration was significantly improved by co-treatment with Rosiglitazone and there was a marked protection against the relative volume and absolute beta cell mass reduction, against the induction of beta cell death and the previously increased expression of endoplasmic reticulum (ER) stress markers (Ji-Wom, Ji-Hun et al. 2009)
Rosiglitazone Effects on the Periphery

In addition to the effects of Rosiglitazone on a molecular level, on a whole organ and body level, Rosiglitazone has been shown to exert additive effects in the deterrence of obesity (Kuda, Jelenik et al. 2009). Rosiglitazone also reduced adipocyte hypertrophy, low-grade adipose tissue inflammation as well as dyslipidaemia and insulin resistance in parallel with adiponectin induction, hepatic lipogenesis suppression and a decrease in muscle ceramide concentration (Kuda, Jelenik et al. 2009). This highlights the three main effects of Rosiglitazone on the periphery:

1-Insulin Sensitivity: Rosiglitazone has been shown to improve peripheral insulin sensitivity (Schindler, Rieger et al. 2009). There is also a noticeable improvement in glucose tolerance which was in part attributed to the synergistic stimulatory effects on muscle glycogen synthesis (Kuda, Jelenik et al. 2009).

2-Glucose Liver Output: Rosiglitazone effectively reduced liver fat by approximately 30-50% and in turn sensitized the liver to insulin which inhibits the endogenous and exogenous insulin required to inhibit hepatic glucose production (Yki-Järvinen 2009).

3-Adipocyte Metabolism: Adipocytes play a distinguished role in the pathogenesis of metabolic disease, diabetes and insulin resistance via the transcription factors of the different PPAR family isoforms, which play diverse roles in lipid regulation and glucose homeostasis (Christodoulides and Vidal-Puig 2010).

Rosiglitazone and the Press

In September 2010, BBC Panorama raised publicly the ongoing issue of the safety of Rosiglitazone branded as (Avandia®) by GlaxoSmithKline (GSK). It asked the question as to why Rosiglitazone remains widely prescribed as a therapy for type 2 diabetes in spite of all the concerns over its cardiovascular safety (BBC News Health 2010).
According to a BBC Health report, an expert panel at the Medicines and Health Regulatory Authority (MHRA) voted unanimously in July earlier in the year for the withdrawal of the drug from the UK market (BBC Panorama Report on Rosiglitazone). There have been links to heart attacks, strokes, myocardial infarctions and possible incidence of mortality (Wertz, Chang et al. 2010). This has been argued for the past few years with both supportive and challenging trials including large scale trials such as Action to Control Cardiovascular Risk in Diabetes (ACCORD) and Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycemia in Diabetes (RECORD) to name a few (MacDonald, Petrie et al. 2011).

Some debate arose from the fact that one group of researchers at the Food and Drug Administration accused their colleagues of ‘stacking the deck’ of a crucial advisory panel in favour of the drug (Harper 2010). Following the BBC programme GSK said: ‘We have carried out an extensive research programme, involving more than 50,000 patients to analyse the safety and benefits of Avandia. We have shared the results from our continuing research programme with regulatory authorities including the MHRA’ (GSK Pharma 2010). That said, the FDA stated that the overall “benefits of Rosiglitazone no longer outweigh its risks” thus recommended its suspension from the European market (Cohen 2010). Consequently, in the 27 member nations of the European Union, the EMA has actually finally suspended the marketing authorization for all Rosiglitazone-containing medicines (Avandia®, Avandamet® and Avaglim®) and all physicians in Europe have been advised to transition patients onto alternative treatment options (Hofland 2010).

The suspension by the EMA will remain in place unless there is convincing data provided to identify a group of patients to whom this drug will have benefits that outweigh its risks. The general overview in the literature and the resultant action to suspend Rosiglitazone from the drug market is thought provoking from a research point of view. Evidence and literature
indicate that this drug has many beneficial effects on the pancreatic beta cell both short and long term, as will be discussed later and there has been much evidence to support positive Rosiglitazone effects on beta cell growth and proliferation, which highlights the flexibility of use of this drug (Bell 2003). That stated, it perhaps opens the avenues of exploring its use as an agent for protecting beta cells and increasing their growth and proliferation in a type 1 diabetes setting prior to islet transplants, since its use systemically is deemed to result in cardiovascular complications.

**Comparison to Other Thiazolidinediones**

Rosiglitazone (Rosi), Troglitazone (Tro) and Pioglitazone (Pio) are all anti-diabetic thiazolidinediones functioning as ligands for PPARγ (Liao, Wang et al. 2010) (See Peroxisome Proliferator-Activated Receptor (PPAR) section 44). Troglitazone in the year 2000 was withdrawn from the pharmaceutical market due to its negative and toxic effects on the liver, thought to be mediated via mitochondrial dysfunction (Liao, Wang et al. 2010). Pioglitazone is slightly different as it displays a high affinity for PPARγ (1) and PPARγ (2) isoforms that are predominately expressed in adipose tissue (Kuda, Jelenik et al. 2009). Many parameters investigated following the use of Pioglitazone such as fasting glucose, insulin parameters and beta cell function were all enhanced (the efficacy comparative to third-generation sulphonylureas, Metformin and dipeptidyl peptidase-4 inhibitors) (Derosa 2010). There was also a reduction in blood pressure, vascular risk, lipid profiles and inflammatory markers independent of its glycaemic effect with an interestingly reduced risk of hospitalization for acute myocardial infarction when compared to Rosiglitazone (Derosa 2010).

The general recommendation for glycaemic management was always initially the adoption of a healthy lifestyle then followed by Metformin and sulphonylurea treatment, if tolerated, and then a TZD such as Rosiglitazone or Pioglitazone alone or in combination with insulin. This is
in accordance with the NICE guidelines as Pioglitazone for instance can be used in conjunction with insulin therapy but not as triple therapy (National Institute for Clinical Excellence 2010). In the years 2007 and 2008, data with regards to the TZDs demonstrated a potential increased risk of myocardial infarction with Rosiglitazone and increased risk of heart failure, peripheral fractures and macular oedema with both Pioglitazone and Rosiglitazone (Phillips and Twigg 2010). Thus it is imperative to decide on a patient to patient basis what the best option for therapy is based on each individual’s medical history.

**Comparison to the Biguanide Metformin**

It is well established now that in type 2 diabetes there is an insulin-resistant state as beta cells are unable to produce sufficient insulin to overcome insulin resistance in the muscles and liver, which is continuously exacerbated by increased circulating free fatty acids (Bell 2003). When mono-therapy fails, combination therapy with a sulfonylurea drug and Metformin is usually the next step. Metformin belongs to the biguanide class of anti-hyperglycaemic drugs acting mainly by slowing the release of glucose from the liver, in turn having positive effects on insulin sensitivity (International Diabetes Federation 2011). The two oral anti-hyperglycaemic drugs Rosiglitazone (Avandia by GlaxsoSmith Kline) and Metformin (Glucophage by Bristol-Myers Squibb) both share the overall net effect of improving insulin-sensitive glucose uptake but the direct effects on the pancreatic beta cells are still being investigated.

Our group set to investigate the direct effects of these agents on beta cell gene expression using reporter gene analysis to examine the effects of both drugs on the activity of the proinsulin and insulin promoter factor 1 (IPF1) gene promoters in the glucose-responsive mouse beta cell line MIN6 (Campbell and Macfarlane 2002). The published data indicated that over a time course of 24 hours Rosiglitazone promoted nuclear accumulation of IPF1 and the transcription factor forkhead homeobox A2 (FOXA2), independently of glucose concentration, and the increase in
IPF1 gene promoter activity was unaffected by the presence of the PPARγ antagonist GW9662 (Richardson, Campbell et al. 2006). No effect of either Rosiglitazone or Metformin was observed on proinsulin promoter activity, however Metformin stimulated IPF1 nuclear accumulation and DNA binding activity in a time-dependent manner with maximal effects observed after 2 hours (Richardson, Campbell et al. 2006). This is a fundamental study in the delineation of the distinct and direct effects of both agents, highlighting that they might play a previously unrecognised role in the direct regulation of pancreatic beta cell function.

**Combination Therapy: Rosiglitazone and Metformin**

Many studies have shown that both Rosiglitazone and Metformin provoke significant improvements in all measures tested on individuals with type 2 diabetes such as body mass index (BMI), glycaemic control, fasting and post-prandial plasma glucose, insulin levels and homeostasis model assessment [HOMA] index as well as systolic and diastolic blood pressure (Derosa, Fogari et al. 2007). The aim of a 12-month, multi-centric double-blinded randomized controlled parallel-group trial, where all patients started treatment with Metformin, then were randomized for self-administration of either Pioglitazone or Rosiglitazone for 12 months, found that the combination of thiazolidinediones and Metformin was associated with a slight but significant improvement in the long-term blood pressure control and an improvement in the anti-inflammatory state, both of which are related to a similar reduction in insulin-resistance (Medina-Gomez, Gray et al. 2007).

**Peroxisome Proliferator-Activated Receptor (PPAR)**

**Isoforms**

PPARs are members of the superfamily of ligand-activated nuclear transcription factors (Smith 2002). There are three types of PPARs have been identified: alpha, gamma and delta, each with different tissue expression as follows:
- **α (alpha)** - expressed in liver, kidney, heart, muscle, and adipose tissue,
- **γ (gamma)** - although transcribed by the same gene, this PPAR exists in three forms:
  - γ1 - expressed in virtually all tissues, including heart, muscle, colon, kidney, pancreas and spleen
  - γ2 - expressed mainly in adipose tissue
  - γ3 - expressed in macrophages, large intestine and adipose tissue
- **δ (delta)** - expressed in many tissues but markedly in brain, adipose tissue and skin

**Structure**

PPARγ is the molecular target for the thiazolidinediones (TZDs) which sensitize tissues to the actions of insulin and lower glucose and fatty acid levels in patients with type 2 diabetes. Thus, PPARγ agonists may also have utility in the treatment of other disease states like inflammation and cancer (Kliewer and Willson 1998). PPARγ binds to deoxyribonucleic acid (DNA) as a heterodimer with the retinoid X receptors (RXRs), receptors for the vitamin A metabolite 9-cis-retinoic acid (9cRA) (Kliewer, Forman et al. 1994). The three RXR subtypes bind to DNA and activate transcription in response to 9cRA as homodimers. The nuclear receptor PPARγ/RXRα heterodimer regulates glucose and lipid homeostasis and is the target for anti-diabetic drugs such as the TZDs. The PPARγ/RXRα heterodimer is asymmetric and the heterodimer interface is composed of conserved motifs in PPARγ and RXRα (Gampe, Montana et al. 2000). The effects of hormones on PPARγ, RXR and other nuclear receptors are mediated through the ligand-binding domain (LBD) (Weatherman, Fletterick et al. 1999). In addition to its role in hormone binding the LBD also contains dimerization and transactivation functions including the transcriptional activation function 2 (AF-2). Upon hormone binding the LBD undergoes a conformational change most notably in the AF-2 domain resulting in the displacement of co-repressor proteins that inhibit transcription and the recruitment of co-activator proteins (family members involved in transcriptional activation) (Freedman 1999).
The understanding at the molecular level of how nuclear receptor ligands exert their effects has been dramatically enhanced by the elucidation of the crystal structures of the apo- and/or ligand-bound LBDs of several nuclear receptors, which provide a framework for the rational design of high-affinity ligands targeted against the PPARγ/RXRα complex for use in the treatment of type 2 diabetes and other diseases (Xu, Lambert et al. 1999).

**Investigated Target Proteins Modulated by Palmitate and Rosiglitazone**

**5' AMP-activated protein/AMP-dependent kinase (AMPK)**

AMPK is an enzyme that plays a role in cellular energy homeostasis and is expressed in a number of tissues including the liver, brain, and skeletal muscle (Winder and Hardie 1999). Effects are muscle glucose uptake, cholesterol synthesis inhibition, skeletal muscle fatty acid oxidation stimulation, modulation of insulin secretion by pancreatic beta cells, lipogenesis (fatty acid synthesis and subsequent triglyceride synthesis), inhibition of adipocyte lipogenesis and lipolysis (breakdown of fat stored in fat cells) and stimulation of hepatic fatty acid oxidation and ketogenesis (ketone body production following fatty acid breakdown). Glucose has marked effects on the pancreatic beta cell on both oxidative metabolism and total intracellular adenosine triphosphate (ATP) which is the major energy currency molecule of the cell (Winder and Thomson 2007). These changes are reflected in clear increases in the free concentration of ATP. Such changes are implicated in the regulation of insulin secretion (high glucose closes ATP-sensitive K⁺ channels) and triggers membrane depolarisation and calcium influx leading to insulin exocytosis. Thus on theoretical ground alone AMPK seems to be an attractive candidate in regulating the secretory activity of these fuel-sensing cells (Winder and Thomson 2007). In the non-pharmacologic context, activation of AMPK occurs in response to exercise which is of significant benefit for individuals with type 2 diabetes and it therefore follows that future therapy targets will, in part, focus on agents that can exert these beneficial effects on AMPK activity (Misra and Chakrabarti 2007). The signalling cascades initiated by
the activation of AMPK exert effects on glucose and lipid metabolism, gene expression and protein synthesis (Ferreira, Xu et al. 2005) which are important for regulating metabolic events in the liver, skeletal muscle, heart, adipose tissue, and pancreas. The uptake of glucose increases dramatically in response to stress and exercise and is stimulated by insulin-induced recruitment of glucose transporters to the plasma membrane, primarily GLUT4 (Ferreira, Xu et al. 2005). There are several known physiologic targets for AMPK (see Figure 7. Role of AMPK in Metabolism Regulation in Response Nutrient-or Exercise-induced Stress on page 48) and several pathways whose flux is affected by AMPK activation (The Medical Biochemistry Page 2007). The upstream AMPK activating kinase (LKB1) activity is essential for the regulation of gluconeogenic flux and thus glucose homeostasis (Hardie 2003).

**Uncoupling Protein 2 (UCP2)**

In terms of cellular energy, other proteins along with AMPK play important roles such as the uncoupling protein family (UCPs). These mitochondrial membrane transporters located in the mitochondria are involved in the control of energy conversion and there is evidence, both experimental and genetic, suggesting that their dysfunctions are linked with metabolic syndrome and obesity (Villarroya, Iglesias et al. 2007). Increasing UCP2 expression in beta cells impairs insulin secretion (Chan, De Leo et al. 2001) and islets isolated from UCP2 knockout animal models are protected from lipotoxicity (Joseph, Koshkin et al. 2002 ). Prolonged exposure of pancreatic beta cells to fatty acids increased basal insulin secretion but inhibited GSIS and islets cultured in elevated palmitate for 48hours showed an increased basal and decreased GSIS where the mRNA levels of UCP2 were increased by almost four-fold (Tian, Li et al. 2006).
Figure 7. Role of AMPK in Metabolism Regulation in Response Nutrient-or Exercise-induced Stress

Several of the known physiologic targets for AMPK are included as well as several pathways whose flux is affected by AMPK activation. Arrows indicate positive effects of AMPK, whereas, T-lines indicate inhibitory effects. Image from (The Medical Biochemistry Page 2007).
Casein Kinase II CK2

Protein kinase CK2 is a ubiquitous and highly pleiotropic protein kinase (Bortolato, Cozza et al. 2008). It is a stress-activated serine/threonine protein kinase that is oncogenic and frequently over expressed in human tumours of multiple histological origins (Scaglioni, Yung et al. 2008). This protein kinase targets an immense assortment of substrates, positioned in a number of cellular compartments. Therefore it follows that as a signalling protein, CK2 could be targeted in response to various stress stimuli such as heat shock, UV irradiation, hypoxia, DNA damage and viral infections (Filhol and Cochet 2009). CK2 is a cytosolic serine/threonine kinase that is also involved in pathways such as the cell cycle, neoplasia, and virus infection signalling and in spite of this constitutive activity with hundreds of target proteins affected, only a few are metabolic enzymes (Meggio and Pinna 2003). CK2 overexpression as a result of gene amplification has been reported to be an adverse prognostic factor in non-small cell lung cancer thus CK2 inhibitors present valuable anti-cancer therapy options (Scaglioni, Yung et al. 2008). Potent and selective CK2 inhibitors are also being investigated (Sliva 2004; Bortolato, Cozza et al. 2008). As well as cancer; implications have also been made regarding CK2 activity in terms of ageing. This was attributed to increases in SIRT1 levels, that were shown to improve cellular survival in response to stimuli such as stress and DNA damage through deacetylating a number of substrates including pro-apoptotic protein p53 (Kang, Jung et al. 2009).

Glucose Transporter 2 (GLUT2)

Carrying the official name of solute carrier family 2 (facilitated glucose transporter) member 2, this transmembrane carrier protein enables passive glucose movement across cell membranes (NCBI Gene 2011). It is an integral plasma membrane glycoprotein of the liver, pancreatic islet beta cells, the intestine and the kidney epithelium mediating facilitated bi-directional glucose transport in and out the cell due to its low affinity and high capacity and due to its low affinity
for glucose (NCBI Gene 2011). It has thus been suggested as a glucose sensor. Sugars and hormones in particular regulate GLUT2 gene expression, especially as GLUT2 also functions as a membrane receptor of sugar where the cellular activities dependent on glucose in the intestine and possibly those in the liver and pancreas are governed by its location on the cell surface (Thorens 2001). GLUT2 detects the presence of extracellular sugar and signals the modulation of cell functions including beta cell insulin secretion, renal re-absorption, and intestinal absorption independently of glucose metabolism (Leturque, Brot-Laroche et al. 2009). Glucose also acts as a key signal in pancreatic beta cells for regulating insulin secretion because GLUT2 is expressed in liver and beta cells and is responsible for sensing glucose levels in the blood (Im, Kim et al. 2006). Physiological studies performed with GLUT2-/‐ knockout mice have also indicated that this transporter is essential for pancreatic beta cell glucose sensing (Thorens 2001).

**Nuclear factor kappa-light-chain-enhancer of activated B (NFkB)**

NFκB is a transcription factor, a member of the Rel family of proteins that function as DNA-binding transcription factors, with their activity regulated at the level of sub-cellular localization (Kumar 2004). NFκB is composed of a heterodimer of p65 and p50 subunits in most cell types and when bound to its inhibitor, IκB, NFκB is sequestered in the cytoplasm (Hiroaki, Hiroaki et al. 1999). Upon the phosphorylation of IκB by IκB kinase (IκBK), IκB is degraded allowing NFκB to move to the nucleus (Higa, Shimabukuro et al. 2005). Human studies have shown that Rosiglitazone exerts some anti-inflammatory effects (Derosa, Fogari et al. 2007) by decreasing the levels of NFκB (Akama, Albanese et al. 1998). In diabetic rats the up-regulation of NFκB in their liver tissues is thought to play a role in the hepatic fibrogenesis (Wang, Chen et al. 2010). Data from recent studies indicate that NFκB blockade with pyrrolidine dithiocarbamate (PDTC), an NFκB inhibitor, directly alleviated oxidative stress and improved mitochondrial structural integrity possibly via the down-regulation of increased oxygen-free radicals (Mariappan, Elks et al. 2010). The NFκB pathway has been implicated in
diabetic nephropathy (Kuhad and Chopra 2009) as well as diabetic retinopathy where NFκB was associated particularly in the early inflammatory stages (Kowluru, Koppolu et al. 2003; Chan and Kowluru 2007; Kim, Kim et al. 2010). Pancreatic beta cells from transgenic mice expressing a non-degradable form of IκBα (RIP-mlκBα mice) were more susceptible to killing by the pro-inflammatory cytokines tumour necrosis factor (TNF-α) plus interferon alpha (IFN-γ) but were more resistant to anti-inflammatory cytokine IL-1beta than normal beta cells (Kim, Millet et al. 2007). This also implicated the p38 mitogen-activated protein kinase highlighting the value of p38 since it is thought to be accountable for some of the pro-apoptotic function in pancreatic beta cells and that it may contribute to the pathogenesis of autoimmune type 1 diabetes (Johansson, Sandvik et al. 2006) as well as type 2 diabetes (Saldeen, Lee et al. 2001).

p38 mitogen-activated protein kinases (p38 MAPK)

The p38 mitogen-activated protein kinases are kinases belonging to the mitogen-activated protein (MAP) kinase family and they respond to stress stimuli (cytokines, ultraviolet irradiation, heat shock, and osmotic shock) as well as being involved in cell differentiation and apoptosis (Chai and Liu 2007). MAPKs are a family of serine/threonine kinases of three main subgroups; ERK (Extracellular signal–Regulated Kinase), p38 MAPK and JNK (c-Jun N-terminal Kinases) which participate in signalling cascades controlling cellular responses to cytokines and stress (Chai and Liu 2007). In type 2 diabetes many of the detrimental effects on beta cells due to the combination of hyperglycaemia and increase in circulating lipids are mediated by reactive oxygen species (ROS) and nitrogen free radicals (RNS) and these molecules have been associated with the regulation of cellular function along with the p38 pathway (Lopes, Oliveira et al. 2008). Hindering p38 activation in MIN6 cells and isolated mouse islets has enhanced the stability of the Mammalian MafA/RIPE3b1 (MafA) in cells under high and low levels of hyperglycaemia. MafA is a central glucose-responsive transcription factor regulating the function, maturation and survival of beta cells thus it follows that its increased expression may result in improved GSIS and beta cell function (Kondo, El
Khattabi et al. 2009). In islet transplantation, in the early post-transplantation phase, re-vascularisation is vital and thus the involvement of the vascular endothelial growth factor (VEGF) secreted from the beta cells in response to their subjected hypoxia during culture is important. Research has provided evidence that p38 MAPK activity played a role here too whereby its inhibition enhanced VEGF-induced angiogenesis and abrogated VEGF-induced vascular permeability at the same time (Johansson, Sandvik et al. 2006).

**B-cell lymphoma 2 (Bcl2)**

Bcl2 and the proteins they produce encode a family of mammalian genes that govern apoptosis by controlling mitochondrial outer membrane permeabilization (MOMP) but they can be either pro-apoptotic (Bax, BAD, Bak and Bok) or anti-apoptotic (Bcl2 proper, Bcl-xL, and Bcl-w) (Lupi, Dotta et al. 2002). Immunolocalization studies revealed that Bcl2 is an integral inner mitochondrial membrane protein and its over-expression obstructs apoptotic death making it therefore a unique protein localized to mitochondria that interferes with programmed cell death independent of promoting cell division (Hockenbery, Nuñez et al. 1990). Electron microscopy on human beta cells specified positive healthy morphological appearances that correlate with the presence of good basal levels of the pro-apoptotic protein Bcl2 in beta cells (Landau, Forti et al. 2006). Investigations into the effects of FFAs (48hr pre-culture FFA treatment) on isolated human islet function and survival found that in comparison with control islets, FFA-treated islets had a significantly increased triglyceride (TG) and insulin content and glucose-stimulated insulin release accompanied by a significant reduction in glucose utilization and oxidation (Lupi, Dotta et al. 2002).

**Bcl2 associated X protein (Bax)**

Apoptosis is the process of programmed cellular self-destruction and death and genes such as Bcl2 and Bax are known to hinder and encourage apoptosis respectively. Studies have
demonstrated that apoptosis induced in pancreatic beta cell lines involves the apoptotic pathways through the Bcl2 and Bax genes (Mizuno, Yoshitomi et al. 1998). In most apoptotic insulin producing beta cells there were always detectable levels of Bcl2 and Bax and in the isolated islets it was noted that following 4 weeks in culture with free fatty acids both the insulin content and Bcl2 staining were decreased (Hanke 2001). These markers of apoptosis are also key in pointing out the pattern of vulnerability of beta cells. This was following studies into the life cycle of the beta cells in the islets that noted a biphasic apoptotic activity in the endocrine pancreas during the lifetime of rats (13 time points between day E19 and 18 months) (Hanke 2000). This in situ data is important for understanding the increased age-related vulnerability of islet cells and for further studies of isolated and cultivated rat islets aiming at protecting the already vulnerable beta cell in a diabetes setting.

**Serine/threonine p70S6 Kinase (S6K)/mTOR**

The serine/threonine p70S6 kinase targets the substrate S6 ribosomal protein. Phosphorylation of S6 induces protein synthesis in a signalling pathway that includes mTOR (the mammalian target of rapamycin) which can be activated in distinct ways (Zamzami, Brenner et al. 1998). S6K is thought to control protein translation (Ferrari and Thomas 1994) and glucose-induced pro-insulin biosynthesis has been shown to be mediated via ATP modulation of the mTOR/p70S6K pathway in MIN6 cells and islets (Saxena, Houk et al. 2003). The mammalian target of rapamycin complex 1 (mTORC1) pathway has integrating inputs from nutrients and growth factors required for cell growth. The phosphorylation levels of ribosomal protein S6 and eukaryotic initiation factor 4E binding protein 1, by the downstream effectors for mTORC1, were up-regulated in transgenic beta cells from transgenic mice that over-express Rheb in beta cells (Hamada, Hara et al. 2009). In terms of islet transplants, progressive decline in islet function is a major obstacle. Rapamycin is an immunosuppressant used as an agent to treat islet transplant recipients but it has been known to have detrimental effects on islet growth.
and proliferation and in turn function (Aronovitz, Josefson et al. 2008). Data suggested that these effects were mediated, in part, through the mTOR signalling pathway via possible downstream targets such as S6K (Aronovitz, Josefson et al. 2008).

**Hypoxia**

We are also going to discuss in this thesis hypoxia. Hypoxia, or low oxygen levels, is a rate-limiting step in pancreatic islet transplants as they often fail due to this subjected hypoxia and this is particularly crucial in the initial phase immediately following transplantation (Ko, Ryu et al. 2008; Kugelmeier, Nett et al. 2008; Maillard, Sanchez-Dominguez et al. 2008). Hypoxia along with many cellular inflammatory and apoptotic events all contribute to a poor islet yield post-transplantationally (Lau, Henriksnäs et al. 2009). Limiting this hypoxia might therefore limit the damage, especially by targeting the hypoxia-induced cellular pathways that cause apoptosis. Low revascularization and impaired oxygenation seems to prevail in intra-portal transplanted islets. This is problematic as clinically, islet transplants have clearly demonstrated exceptional glycaemic control and often insulin independence, thus offering patients with type 1 diabetes an improved therapeutic option (Emamaullee and Shapiro 2007). Hypoxia has a marked effect on grafted islets non-functionality and the mechanisms causing this still remain unknown, however some of it is attributed to the involvement of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) signalling in beta cells (Ko, Ryu et al. 2008). Pancreatic islets face hypoxia during transplantation mainly because they are separated from their vascular system and here the hypoxia-inducible factor 1 (HIF-1), particularly the alpha subunit (HIF-1α), is vital, with studies showing it was up-regulated to mediate this metabolic adaptation (Moritz, Meier et al. 2002). Vascular endothelial growth factor (VEGF) has also been shown to promote the revascularization of transplanted islets, thereby reducing the initial number required to prevent diabetes (Stagner, Mokshagundama et al. 2004). Clarification of the roles of both of these targets in beta cell responses to hypoxia therefore becomes imperative.
**Hypoxia-Inducible Factor 1 (HIF-1)**

Hypoxia activates the hypoxia-inducible factor heterodimeric transcription factor consisting of an oxygen-dependent HIF-1α subunit and a constitutive partner HIF-1β (ARNT) causing its stabilization and its translocation from the cytoplasm to the nucleus (Moritz, Meier et al. 2002). In other cell types, such as human breast cancer cells, human liver cells and kidney cells, it seems that HIF-1α has positive effects, as hypoxia induces target genes including VEGF, PGK-1 and GLUT-1 by stabilizing HIF-1α and the latter would otherwise be degraded by a ubiquination-dependent proteasome under normoxic conditions (Moritz, Meier et al. 2002). In beta cells increased HIF-1α in vitro has been associated with apoptosis and HIF-1 suppression showed an opposite effect in islet transplants, where levels peaked at post-transplant day 3 (POD3) and diminished by POD14 (Miao, Ostrowski et al. 2006). HIF-1 also has an imperative function in beta cell role through the regulation of ARNT expression, a gene important for beta cell function, and studies indicated that through this mechanism, HIF-1α could be a potential therapeutic target for treating beta cell dysfunction (Thangarajah, Vial et al. 2010). In terms of beta cell differentiation, recent evidence suggests that hypoxia controls beta cell differentiation and this is mediated by HIF-1 (Heinis, Simon et al. 2010). Recently, studies have indicated that repressed HIF1α expression, promoted development of endocrine progenitors and induced beta cell differentiation by changes in oxygen tension (Miao, Ostrowski et al. 2006). The significant effects caused by HIF-1 make HIF-1 a very interesting therapeutic target.

**Vascular Endothelial Growth Factor (VEGF)**

VEGF is an endothelial cell mitogen that has an essential role in both vasculogenesis and angiogenesis (Ouchi, Shibata et al. 2005). VEGF has many isoforms and VEGF–A seems to be closely associated to beta cell revascularization (in particular VEGF121, VEGF145 and VEGF165 which are all secreted, the latter being the most abundant and biologically active) (Cross,
Richards et al. 2007). VEGF$_{189}$ was also detected in human islets, however this isoform is mainly cell attached and membrane anchored unless sequestered or cleaved (Kampf, Mattsson et al. 2006). The islets of Langerhans naturally have a rich oxygen supply and hold an intense vascular structure where VEGF-A plays an indispensable role in the formation of this structure. Animal studies utilizing the beta cell-specific VEGF-A-deficient mouse (RIP-Cre:Vegf(fl/fl)) revealed that deficiency in the normal vascular structure is paralleled with abnormal insulin secretion and concluded that the islet vascular system is fundamental for normal insulin secretion (Watada 2010). As VEGF is linked to HIF-1 and both to hypoxia, tackling these angles is of great interest when exploring therapeutic options in terms of islet grafts and transplants for individuals with type 1 diabetes. This is because of the critical post-transplantation stage where the optimum and prolonged function and survival of the pancreatic beta cell is of utmost importance. This includes the need for a fast and sufficient revascularization (Kampf, Mattsson et al. 2006). Understanding islet biology is important. It was found recently that in cultured islets treated with exogenous VEGF, a resultant dilation of the blood vessels was observed (Olsson, Maxhuni et al. 2006). Smaller islets have a higher capacity to stimulate re-growth of blood vessels following transplantation and interestingly transplanted islets have increased concentrations of VEGF as a programmed defence and compensation mechanism (Johansson, Sandvik et al. 2006). However, there is still not enough VEGF released to overcome the hypoxic challenge thus there is still a place for therapeutic agents that aid in the enhancement of this process. Studies have shown that Rapamycin, an effective inhibitor of mTOR, inhibited the mTOR pathway and downstream signalling target molecules such as S6K1 and partially Akt by decreasing the phosphorylation of both targets (Xue, Nagy et al. 2009). Rapamycin had potently inhibited early and mid stages of VEGF-A$_{164}$-driven angiogenesis and this is not only essential for growth and proliferation but also for angiogenesis, which include processes such as mother vessel formation and increased vascular permeability.
Programmed Cell Death 4 (PDCD4)

PDCD4 is a gene encoding a protein localized to the nucleus in most proliferating cells with an expression level modulated by cytokines in natural killer and T cells (GeneCards 2011). It functions as a tumour suppressor and inhibits tumour promoter-induced neoplastic transformation by inhibiting the helicase activity of eIF4A and cap-dependent translation via RNA binding. The gene product may play a role in apoptosis but the specific role has not yet been confirmed (GeneCards 2011). PDCD4 is a translation factor that inhibits cap-dependent translation by binding to eIF4A, (a protein complex that aids in ribosome recruitment to mRNA) making it a primary target as well as a limiting step for controlling protein translation (Gingras, Raught et al. 1999; Hilliard, Hilliard et al. 2006). Studies by our collaborators have established a method to instigate pancreatic duct cell proliferation and found that a brief temporary sixty second occlusion of the main pancreatic duct resulted in ductal cell proliferation (Ferris, Woodroof et al. 2001). The same was observed when firmly tying a cellophane strip around the head of the pancreas for longer periods of time. Interestingly both methods stimulated a biphasic increase in ductal cell proliferation (maximum proliferation at 3 and 14 days post operation) which lead to the conclusion that signals which commence proliferation transpire rapidly at the beginning of each procedure.

Considering all the targets and agents discussed thus far, the aim of current research is to protect the vulnerable beta cell in all the settings, whether it be type 1 and type 2 diabetes mellitus, or islet transplants. Specific signalling pathway targeting, such as the neogenic and developmental pathways, as well as the proliferative and survival pathways, is imperative to the protection and promotion of beta cell function and we aim to decipher these events in the current project.
AIMS

- To establish the extent of the detrimental effect(s) of the saturated free fatty acid palmitate on our utilized pancreatic MIN6 beta cell model; specifically beta cell viability and target gene regulation.

- To decipher whether the anti-hyperglycaemic agent Rosiglitazone can protect pancreatic beta cells from palmitate-induced decrease in viability and alteration in beta cell gene regulation.

- To identify the specific gene targets modulated by both palmitate and Rosiglitazone in our MIN6 beta cell model.

- To investigate the cell signalling mechanisms involved and the pathways implicated in the regulation of the above mentioned processes in our MIN6 beta cell model.
CHAPTER 2

Materials and Methods

1. Cell Culture

1.1 Cell Line
The insulinoma MIN6 cell line is a beta cell line derived from transgenic mice expressing the SV40 large T antigen under the control of the rat pro-insulin gene promoter (Miyazaki, Araki et al. 1990), (see Figure 8. Cell Morphology of MIN6 Beta Cells on page 60). They were cultured in appropriate complete media (5mmol/l glucose DMEM - Dulbecco's Modified Eagle's Medium - with the addition of 10% heat-inactivated foetal calf serum, & 100U/ml penicillin and 100mg/ml streptomycin). All present studies were performed using cells passage numbers 27 to 34.

1.2 Cryo-preservation and Resurrection of Cells
For long-term storage, beta cells were kept in liquid nitrogen. For freezing, a T75 cell culture flask of confluence 80-90% was washed in Phosphate Buffered Saline (PBS) then incubated in 2ml trypsin of 25% trypsin-75% PBS (0.05% w/v trypsin/0.5mM EDTA) for 5 minutes at 37°C to allow cells to detach. To arrest the trypsin reaction, 8ml of appropriate complete media was added and cells were centrifuged at a Relative Centrifugal Force (RCF) of 500 (500xg) for 5 minutes. MIN6 cells were re-suspended in 1.5ml freezing buffer (90% appropriate complete media + 10% Dimethyl sulfoxide - DMSO). Cell suspensions were transferred into cryo-vials (Nalgene, UK) then stored in polystyrene containers at -80°C overnight. This allows slow freezing of the cells. Cryo-vials were then returned for storage in liquid nitrogen.
Figure 8. Cell Morphology of MIN6 Beta Cells

Figure represents MIN6 mouse pancreatic beta cells. Image captured on the light microscope [Nikon Eclipse microscope - ELWD 0.3/OD75 (Nikon Instruments, UK)] on x100 magnification. Cells grow as 2D monolayer in groups.
1.3 Preparation of Rosiglitazone

All Rosiglitazone stock solutions were made to 1000x in DMSO and volumes were used accordingly to a final concentration of 10µM unless stated otherwise.

1.4 Preparation of Palmitate/Bovine Serum Albumin (BSA) Solution

Palmitic acid stock solutions (100mM) were prepared by dissolving Sodium Palmitate (Sigma [catalogue No.P9767], UK) in 10ml ethanol (1:1 vol:vol) at 50°C. 10% BSA solutions were prepared by dissolving BSA (Sigma [catalogue No. A7030]) in sterile water (wt:vol) at 50°C. Aliquots of palmitate stock solutions (5mM palmitate/10% BSA stock solutions were prepared by adding 500µl 100mM palmitate to 9.5ml 10% BSA) were complexed with fatty-acid free BSA by stirring for 1 hour at 37°C. Aliquots were then cooled to room temperature and filter sterilise using 0.45µM filter ready for storage at -20°C for 3-4 weeks. Prior to stimulations aliquots were heated for 15 minutes at 50°C then kept at room temperature.

1.5 Cellular Stimulations

MIN6 pancreatic beta cells were stimulated with various stimuli for durations between 1-72 hours as indicated.

1.6 Hoechst/Propidium Iodide (H/PI) Staining

MIN6 pancreatic beta cells were cultured in 6-well cell culture plates and stimulated with various stimuli for durations between 1-72 hours as indicated. A mixture of both dyes was added to the cells at a final concentration of 10µg/ml. A volume of 300µl of the H/PI mix was added to each well in the absence of light and the plates were covered with aluminium foil prior transferring to the fluorescence microscopy. Cells were viewed under the broad band fluorescent filter setting (excitation wavelength 365nm and emission 397nm) using the Nikon Eclipse microscope - ELWD 0.3/OD75 (Nikon Instruments, UK).
1.7 The 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) Assay

To measure cell viability the MTT assay was used. MIN6 cells were cultured in 5mM glucose DMEM 6-well plates at a seeding density of 2x10^5 cells per well and treated with serum free media 24 hours before stimulations for 72 hours. Palmitate was added to a final concentration of 0.4mM. Rosiglitazone was added to a final concentration of 10μM. To determine the role of individual signalling pathways, the following cell signalling inhibitors were used: AMPK inhibitor compound C (CC, 20μM); PPARγ inhibitor GW9662 (30μM); p38 inhibitor SB203580 (20μM); Casein kinase 2 inhibitor (TBB) (20μM).

At the end of the time course, media containing stimulants was aspirated and 1ml MTT solution (0.05g/ml PBS) was added per well on a 6-well plate and left in the incubator for an hour to allow for purple coloured crystals to form should stimulations result in apoptotic death. This solution was then aspirated and 1ml DMSO was added to each well. Purple crystals, if present, were dissolved by repeated pipetting in and out of the well. 200μl of the resultant mixed solution was added per well in a 96-well plate for assaying in a plate reader (Thermo Multiskan Ascent Type 354 Microplate Photometer) at a wavelength of 540nm. All readings per treatment were in duplicate in the 6-well plate and each of the wells was read in duplicate in the 96-well plate.

1.8 Cellular Counts

For cellular viability counts, MIN6 cells were cultured in 5mM glucose DMEM 6-well plates at a seeding density of 2x10^5 cells per well and treated with serum free media 24 hours prior to stimulations. Palmitate was added to a final concentration of 0.4mM. Rosiglitazone was added to a final concentration of 10μM. At the end of each time course, medium was aspirated and cells in each well were treated with 400μl trypsin for 2 minutes at 37°C to allow cells to detach. 600μl of medium was then added and cells were centrifuged to obtain a cellular pellet.
The pellet was then re-suspended in 1ml DMEM medium then 100µl of this suspension was added to 900µl medium (1 in 10 dilution). 11µl of the diluted suspension was then added to each of the 2 sides of the glass haemocytometer (Marienfield Neubauer Improved Bright-line 0.100mm/0.0025mm²) with each count representing an average of four counts (total time for the procedure was 7 days in total (Day 1 seeding, Day2 medium change, Day 3 medium change to serum free medium, Days 4-7 are the 24, 48, 72 hour stimulations).

For Rosiglitazone treatment versus pre-treatment studies, in order to determine the effect of Rosiglitazone treatment/co-incubation versus pre-treatment on beta cell viability, cell counts were carried out daily. MIN6 cells were cultured in 5mM glucose DMEM in 6-well plates at a seeding density of 2x10⁵ cells per well. There were two groups; one group was not pre-treated with Rosiglitazone for 72 hours prior to cellular counts and the other group was pre-treated with Rosiglitazone for 72 hours prior to cellular counts. Following the 72 hours, medium was aspirated from each well and cells were either cultured in 5 mM glucose DMEM alone or with the addition of Rosiglitazone for 6 days (216 hours) giving a total study time course of 9 days. All reading were carried out in duplicate.

2. Preparation of Whole Cell, Nuclear and Cytoplasmic Extracts

For all extracts, cells were washed once in PBS then PBS added; 1.5ml per 10cm dish or 2ml per 75ml flask, and cells were harvested by scraping. Cells were then centrifuged at 13000rpm - revolution per minutes - for 3 minutes. Pellets were re-suspended in 200µl Buffer A (10mM HEPES pH 7.9; 10mM KCL; 0.1mM EDTA pH8; 0.1mM EGTA pH8; 1mM DTT; 1 x protease inhibitor cocktail (Roche, UK) and incubated on ice for 15 minutes. For whole cell extracts, 25µl of 10% (v/v) triton X-100 was added for cell extracts from T75 flasks. Samples were incubated for 30 minutes on ice and were then centrifuged for 60 seconds at 13000rpm.
The supernatant was the whole cell extract which was then removed and snap frozen in liquid nitrogen in preparation for storage at -80°C. For cytoplasmic extracts, 25µl of 10% (v/v) NP-40 was added to the cells in T75 flasks. Samples were then vortexed for 30 seconds then centrifuged at 13000rpm for 45 seconds. The supernatant was the cytoplasmic extract. It was removed and snap frozen for liquid nitrogen. For nuclear extracts, the pellet remaining after the removal of the cytoplasmic supernatant removal was then re-suspended in 50µl Buffer C (20mM HEPES pH 7.9; 400mM NaCl; 1mM EDTA pH8; 1mM EGTA pH8; 1mM DTT; 5% glycerol; 1 x protease inhibitor cocktail) and incubated for 1 hour at 4°C with vigorous shaking. Following this, the supernatant was removed and snap frozen in liquid nitrogen in preparation for storage at -80°C.

3. Western Blotting

Changes in protein were detected using western blotting techniques as previously described (Meidute-Abaraviciene, Lundquist et al. 2008). Between 10-30µg of protein (cytoplasmic, nuclear and whole cell extracts) were loaded per sample following a Bradford Assay as indicated. All antibodies were purchased from AbCam (Cambridge, UK) unless stated otherwise. The p38 antibody was from Sigma (Dorset, UK); phospho p38 antibody was from Cell Signalling Technology (Beverly, MA); GAPDH antibody was from Santa Cruz Biotechnology (Santa Cruz, CA) and α-Tubulin and β-actin antibodies were from SIGMA (UK). The ECL™ secondary HPR- conjugated anti-mouse and anti-rabbit antibodies were both from GE Healthcare Limited (UK). Membranes were stripped and re-probed with housekeeping genes for accuracy using Restore™ Plus Western Blot Stripping Buffer (Thermo Scientific, UK).
3.1 Bradford Protein Assay

A stock solution of Bio-Rad protein Assay dye in sterile water was prepared (4:1 water: dye). 1ml of this stock solution was added to 3μl of cell extract for assaying and mixed by inversion. Absorbance was determined by spectrophotometry (eppendorf BioPhotometer No: 6131 22336) at 595nm. Protein concentration was calculated using a standard curve by titration against known concentrations of bovine serum albumin (BSA) (see Figure 9. Example of Bradford Protein Assay to Determine Protein Concentrations on page 66).

3.2 SDS-PAGE

For all SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) analysis of cell extracts, sample volumes were made up to 10µl with sterile water. 10ul of SDS sample buffer (20% sodium dodecyl sulphate (SDS); 0.1% bromophenol blue; 1.25m sucrose;1M Tris-HCl pH 6.8; 10% β-mecaptoethanol) was then added to make a total sample volume of 20µl. Proteins were separated by running on an SDS-PAGE gel (10% gel unless otherwise stated); resolving gel (30% acrylamide/Bis mix; 1.5M Tris-HCl pH8.8; 10% SDS; 10% APS; TEMED) and stacking gel (30% acrylamide/Bis mix; 1.5M Tris-HCl pH6.8; 10% SDS; 10% APS; TEMED). Samples were run for 60 minutes at 150V in SDS running buffer (3g/l Tris base; 14.4g/l glycine; 1g/l SDS).

3.3 Transfer

Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (MILLIPORE Immunoblot TM Transfer Membranes, pore size 0.45µm) using the TRANS BLOT® semi-dry blotting apparatus (Bio-Rad, UK). 3mm filter paper (Bio-Rad, UK) were soaked in 1x transfer buffer (3g/l Tris base; 14.4g/l glycine), placed on the blotting apparatus and air bubbles were excluded. Transfer was performed at 50mV for 30 minutes per gel (or 50 minutes for 2 gels). This stage was followed by blocking. Membranes were placed in 10% (v/v) milk solution
Figure 9. Example of Bradford Protein Assay to Determine Protein Concentrations

A known volume of sample is titrated against a standard curve; known volume of Bovine Serum Albumin (BSA 1µg/µl) in 1ml Bio-Rad Protein Assay Kit reagent. This example shows 3µl of treated sample and 30µg of protein used for western blotting SDS-PAGE.

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<th>R² = 0.9966</th>
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<td>3.00</td>
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<td>3.00</td>
<td>8.55</td>
<td>3.51</td>
<td>6.49</td>
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(10g dried milk in 100ml 1 x wash buffer) for 60 minutes with gentle agitation. Milk concentrations and blocking periods were adjusted as required. Membranes were then washed (3 x 5 minutes each) in 1 x wash buffer before placing in primary antibody. Membranes were incubated with rotation overnight at 4°C. The following day, membranes were washed 3 times, 5 minutes each, in 1 x wash buffer. Secondary antibodies were then added (1:1000 sometimes with the addition of 1% milk). Secondary antibodies were the ECL™ secondary Hourseradish Peroxidase (HPR)-conjugated anti-mouse and anti-rabbit antibodies (GE Healthcare Limited, UK). Membranes were incubated with rotation for 1 hour at room temperature then washed quickly, then once for 15 minutes and then for a final 5 minutes in 1 x wash buffer.

3.4 Developing
Each membrane was drained on blotting paper and placed on a bubble free piece of Saran Wrap (Dow, UK). 1.25ml (25µl Solution A + 1ml solution B) of ECL Plus Western Blot Detection System Kit (GE Healthcare, UK) was added per membrane. Membranes were left for 2-3 minutes in solution then covered with another piece of saran wrap. The film was then developed using developer Compact X4-Automatic X-ray Film Processor (Xograph Imaging Systems, UK).

4. Polymerase Chain Reaction (PCR)
RNA was prepared from harvested MIN6 cells following stimulations as indicated; extracted and quantified. This was followed by reverse transcription prior to performing PCRs.

4.1 RNA Extraction
DNA was extracted from cells using the Gene Elute™ Mammalian Total RNA Miniprep Kit (SIGMA) as per manufacturer’s instructions. RNA was eluted to a final volume of 50µl using the Elution Solution™ and stored at -80C.
4.2 RNA DNase Treatment and Quantification

RNA was treated with DNase to remove genomic DNA. 20µl of extracted RNA was incubated with 1µl RQI RNase-free DNase solution (Promega, UK) in a water bath for 10 minutes at 37°C. 1µl RQI RNase-free DNase Stop Solution was then added to the mixture and was then incubated in the water bath for 5 minutes at 65°C. Treated RNA quality was assessed by running 3µl on a 1% w/v agarose gel as per section 3.5. Quantification was carried out by adding 2µl of the DNase treated RNA to 50µl TE Buffer pH 8.0. Samples were assessed in Eppendorph UVettes and absorbance was measured in the Eppendorph Bio-photometer (AG-Model No. 6131022336) at 260nm. RNA quality was determined by running 2µl samples on a 1% w/v agarose gel and visualizing in the FlourChem machine (AlphaInnotech MultiImage TM Light Cabinet) under the UV Gels setting.

4.3 Reverse Transcription

cDNA was generated by reverse transcription using the Cloned AMV First-strand Synthesis Kit (Invitrogen). 4µl of DNase treated RNA was added to 1µl Oligo DT Primer, 2µl 10mM dNTP Mix and 5µl DEPC treated water to make a total volume of 12µl. An extra PCR tube was made by replacing 4µl of DNase treated RNA with sterile waster to act as a no template control (NTC). Following brief centrifugation, the RNA and primer were denatured by incubation (5 minutes at 65°C). Two mixes were made, both to a total volume of 8µl; one mix contained (4µl 5x DNA Synthesis Buffer; 1µl 0.1M DTT; 1µl RNase Out; 1µl AMV RT and 1µl DEPC treated water) while the other had no AMV RT (no enzyme control). The 8µl mix was added to the 12µl sample, total sample volume of 20µl which was then incubated for 60 minutes at 50°C. The cDNA samples were then ready for PCR.
4.4 Real Time PCR

For each cDNA sample made a total volume of 25µl reaction was added to each well of the 96-well plate; 23µl reaction supermix (12.5µl SYBR iQ™ SYBR® Green supermix, 2.5µl forward primer, 2.5µl reverse primer and 5.5µl sterile water) and 2µl cDNA template. Reactions were run on the (Thermal iCycler iQ™ Real-Time PCR Detection System Catalogue No: 170-8740) on the conditions stated on section 4.6 below. Target genes were UCP-2, GLUT2 and Bcl2. GAPDH was the housekeeping gene. PCR for UCP2 as previous (Tian, Li et al. 2006), for Bcl2 as previous (Karakas, Maurer et al. 1998) and for GLUT2 as previous (Palumbo, Levi et al. 2006). GAPDH was co-amplified and used as the endogenous control. Results were analysed according to the Pfaffl method (Hunt 2010). For amplification curve example see Figure 10. Example of a Real-Time qPCR Amplification/Cycle Graph on page 70 and for melt curve example see Figure 11. Example of Real-Time PCR Melt Curve on page 70.

4.5 Semi-quantitative PCR

For the PCR amplification 3µl of reverse transcription mix or the appropriate amount of cDNA was added to the PCR mix (5µl 10x dNTP; 5µl 10x PCR Buffer; 1.5µl 50mM MgCl₂; 50pmol 3’ primer; 50pmol 5’ primer; 0.5µl Taq (Platinum ® Taq polymerase, [Invitrogen, UK]); 25µl sterile water). Semi-quantitative PCR conditions were determined for gene products expressed in MIN6 cells. Target genes were VEGF and PDCD4. Housekeeping gene used was β-actin.

4.6 Conditions

Conditions for each gene tested were as detailed below. Housekeeping genes were analysed on the same 96-well plate and run at the same experimental time. GAPDH was not used in the VEGF and PDCD4 investigations as GAPDH changes with the different conditions that were tested and would thus not be the most suitable housekeeping gene to use.
Figure 10. Example of a Real-Time qPCR Amplification/Cycle Graph

Each of the coloured single traces is a representative for each single well. During the PCR, at each cycle, a single data point is plotted. At the end of the reaction, the readings are analysed and C\textsubscript{T} values are calculated. There are 3 groups of traces; all representing samples from MIN6 beta cells treated with palmitate and Rosiglitazone. At the earliest cycles (~C\textsubscript{10}-12) detection was for the housekeeping gene GAPDH, (~C\textsubscript{17}-19) detection was for the target gene UCP-2 and (C\textsubscript{27}-29) detection was for the no template controls (NTC).

Figure 11. Example of Real-Time PCR Melt Curve

Each of the coloured single traces is a representative for each single well. During the PCR, at each cycle, a single data point is plotted. At the end of the reaction, each trace peak represents the product obtained by the reaction for each sample. The traces represent samples from MIN6 beta cells treated with palmitate and Rosiglitazone (melt curve data set complementary to the sample analysed and displayed in Figure 14).
UCP2

_Ucp2 (mouse forward)_

GCATTGGCCTCTACGACTCT

_Ucp2 (mouse reverse)_

GGAAGCGGACCTTTACC

For Real Time qPCR conditions were as follows:

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<tr>
<th>Step</th>
<th>Cycle</th>
<th>Temp (°C)</th>
<th>Time</th>
</tr>
</thead>
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<td>1</td>
<td>95</td>
<td>3 min</td>
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<td>Annealing</td>
<td>2</td>
<td>95</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>58.7-64.7</td>
<td>1 min</td>
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<tr>
<td>Extension</td>
<td>3</td>
<td>55</td>
<td>1 min</td>
</tr>
<tr>
<td>Melt Curve</td>
<td>4</td>
<td>55</td>
<td>10 sec</td>
</tr>
</tbody>
</table>

UCP2
Required a temperature of 64°C thus was loaded on Row B on the 96-well plate (64.3°C)

GAPDH
Required a temperature of 60°C thus was loaded on Row F on the 96-well plate (59.9°C)
**GLUT2**

*Glut2 (mouse forward)*

CGGTGGGACTTGTGCTGCTGG

*Glut2 (mouse reverse)*

CTCTGAAGACGCCAGGAATTCCAT

For qPCR conditions were as follows:

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<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturing</td>
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<td>10 seconds</td>
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<td>Extension</td>
<td>64°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Melt Curve</td>
<td>55°C</td>
<td>10 seconds</td>
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</tbody>
</table>

**GLUT2**

Required a temperature of 64°C thus was loaded on Row C on the 96-well plate (64.3°C)

**GAPDH**

Required a temperature of 60°C thus was loaded on Row F on the 96-well plate (58.8°C)
Bcl2

*Bcl2 (mouse forward)*

AAACAGAGGCCGCATGCTG

*Bcl2 (mouse reverse)*

CCGCTACCGCCGCGACTTC

For qPCR conditions were as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycle</th>
<th>Temp 1</th>
<th>Temp 2</th>
<th>Time 1</th>
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<td>Annealing</td>
<td>2</td>
<td>94°C</td>
<td>60 - 70°C</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>(Temperature Gradient)</td>
</tr>
<tr>
<td>Extension</td>
<td>3</td>
<td>72°C</td>
<td></td>
<td>7 minute</td>
</tr>
<tr>
<td>Melt Curve</td>
<td>4</td>
<td>55°C</td>
<td></td>
<td>10 seconds</td>
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</table>

Bcl2

Required a temperature of 64°C thus was loaded on Row E on the 96-well plate (63.9°C)

GAPDH

Required a temperature of 60°C thus was loaded on Row H on the 96-well plate (60.0°C)
**VEGF**

*VEGF (mouse forward)*

CGAAGTGGTGAAAGTTCATGGATG

*VEGF (mouse reverse)*

TTCTGTATCAGTCTTTCTGCGT

Denaturing Cycle 1: (x1) 94°C 1 minute

Annealing Cycle 2: (x40) 60°C 1 minute

Extension Cycle 72°C 1 minute

Final Extension Cycle 3: (x1) 72°C 10 minutes

PCR performed at an annealing temperature of 60°C loaded onto PCR micro tubes
## PDCD4

*PDCD4 (Rat forward)*

AGATGAAGAGTGGTGTGCCCGTGTT

*PDCD4 (Rat reverse)*

ACTGGCCCACCAACTGTGGTGCTCT

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<td>Final Extension</td>
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PCR performed at an annealing temperature of 60°C loaded onto PCR micro tubes
4.7 Agarose Gel Electrophoresis

DNA and RNA samples were prepared for agarose gel electrophoresis by making up to a final volume of 10µl with sterile water followed by the addition of 2µl agarose loading dye (4g/10ml sucrose; 2.5mg/10ml bromophenol blue; made up in TE Buffer). Samples were run on a 1% w/v agarose gel (1g/100ml agarose; 3µl/100ml ethidium bromide [10mg/ml]) in TBE buffer (10.8g/l Tris-base;5.5g/l Boric acid;2mM EDTA pH 8.0) for 30 minutes (or longer if required) at 100V. Gels were visualized and images captured on the FlourChem machine (AlphaInnotech MultiImage TM Light Cabinet) under the UV Gels setting.

4.8 Efficiency Calculation

To be able to compare fold change detected the assumption is that both target and reference or housekeeping gene have the same efficiency during the PCR reaction. This is not reliable and thus the efficiency for both genes has to be determined by running a standard curve using the cDNA template for the gene in question. Efficiency of 100% equates to 2 and to compare the target and reference genes they have to be within 10% of this value i.e. between ranges of 1.85 - 2.05. Efficiency (E) is calculated using the following equation where the (s) in (-1/s) is the slope of the standard curve obtained; 

\[ E = 10^{(-1/s)} - 1. \]

5. Microscopy

5.1 Light and Fluorescence

Cells were grown as described in 1.1 (Cell Culture) in 6-well cell culture plates or T75 cell culture flasks. Images were captured using the Nikon Eclipse microscope - ELWD 0.3/OD75 (Nikon Instruments, UK). This was used for both the light microscopy and the H/PI Fluorescence staining/ imaging. Inverted images were captured for the confocal microscopy as per section 4.2.
5.2 Confocal Microscopy

MIN6 cells cultured in 5mM glucose DMEM media (+10% FBS+Pen/Strep) were seeded in 2-chamber slides and stimulated for 24 hours. Cells were then washed with Phosphate Buffered Saline (PBS) and fixed with 37% formalin for 1 hour at room temperature, then washed with PBS and left at room temperature with gentle agitation in blocking buffer (0.7% glycerol, 0.2% Tween-20 and 2% BSA in PBS) for 15 minutes. Blocking buffer was discarded and primary antibodies diluted in blocking buffer were added. Incubation was overnight at 4°C with gentle agitation. NFκB antibody was from AbCam (UK) and the Bcl-2 antibody was from Santa Cruz (USA).

Primary antibody was removed by washing in blocking buffer. The secondary antibodies [FITC-conjugated for NFκB (AbCam) and TRITC-conjugated for Bcl-2 (SIGMA)] were diluted in blocking buffer and cells were incubated for an hour at room temperature with gentle agitation in the dark. Washing in wash buffer (0.7% glycerol, 0.4% Tween-20 and 2% BSA in PBS) followed for 1 hour with gentle agitation. The chambers were then removed from the chamber slides and cells were mounted in vectashield with DAPI (Vector Laboratories, CA 94010) and finally affixed on the cover slips for examination. Visualisation was via confocal microscopy, the machine model used was the Leica TCS SP5 and analysis was via the Leica Application Suite Advanced Fluorescence Software (LAS AF).

5.3 Transmission Electron Microscopy (TEM)

MIN6 pancreatic beta cells cultured in 5mM glucose DMEM media were fixed in 2.5% (v/v) glutaraldehyde in 0.1M Na Cacodylate/HCl buffer, pH 7.4 for 2 hours at room temperature and then overnight at 4°C. 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 min 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 48hr, the samples were polymerised for 16h at 60°C. Thin sections were cut with an LKB Ultracut ultramicrotome, post-stained in 2% (w/v, aqueous) uranyl acetate for 90min followed by lead citrate for 15min. Stained sections were examined in a Hitachi 7100 transmission electron microscope at 100kV and images acquired with a Gatan Ultrascan 1000 CCD camera.

6. Data Analysis
Statistical analysis was carried out using a paired Student t-test. Probability values less than 0.05 were considered as significant and marked with a single *, values less than 0.01 were marked with a double **.

For relative quantification of Western blots and semi-quantitative PCR results, gel images were subject to densitometry scanning and images were analysed by the Analysis Tools function on the FluoroChem machine (FluoroChem Alpha Innotech MultiImage™ Light Cabinet).
CHAPTER 3

Free Fatty Acids: Effects on Beta Cell Viability and Ultra-structure

1. Introduction

Currently there is much interest in the link between obesity and type 2 diabetes; both being worldwide epidemics. The interest is fuelled by the multi-factorial nature of the condition, as it encompasses a whole range of aspects; environmental factors such as cultural differences, behaviour and attitude, as well as socio-economic factors, which all exert their effects on diet and exercise, the main contributors to this type of diabetes mellitus. This is of course in addition to the participating genetic factors (Dean and McEntyre 2004). Increase in dietary intake, when not balanced with exercise, overloads the body with surplus energy. Food is eventually broken down into sugars such as glucose and the excess is then stored as glycogen. Saturated fats such as palmitate contribute greatly to this overload as discussed previously (see section Palmitate, p39). Saturated fats found in butter, lard and ghee, fatty meats and meat products such as sausages and pies, full fat milk, coconut oil, palm oil, cream, cheese and ice cream and such like foods are harmful. The new generation of fast food in combination with fast pace of modern life makes savoury snacks, such as crisps, biscuits, cakes, pastries, sweets and chocolate a very quick and easily accessible alternative to healthy snacks such as fruits and vegetables.

Rosiglitazone, is an anti-hyperglycaemic agent known for its positive effects on the periphery (adipose tissue and liver) and the resultant overall improvement in insulin sensitivity (see section Rosiglitazone, p45). In this study we wanted to establish the extent of fatty acid-induced damage and the effects of Rosiglitazone, if any, on this and consequently the possible mechanisms and pathways implicated in our MIN6 pancreatic beta cell model.
2. Results

2.1 Effects of Palmitate and Rosiglitazone on Beta Cell Viability

In order to determine the effects of palmitate and Rosiglitazone on beta cell viability 72 hour studies were performed. That time point was chosen to try and mimic the effects of palmitate long term. It was the maximum experimental time whereby the cellular responses were not due to medium and nutrition deprivation and cell death as the total time for the procedure was seven days in total (see Cellular Counts, section 1.8 on page 62). Shorter time courses of 24 and 48 hour time points were used (data not shown) but that did not produce an optimum time point. Stable consistent cell growth essential for the correct interpretation of stimuli effects was not achieved at these time points. Stimulations with palmitate and Rosiglitazone were carried out using them either alone or in combination. All stimuli concentrations were kept as physiological as possible, aimed at mimicking the internal physiological environment of an individual with type 2 diabetes. Elevated circulating free fatty acid levels are in the range of 0.2-0.6mM and Rosiglitazone 10µM is equal to the levels in the blood following a therapeutic dose of 8mg a day. Cells cultured in serum free media were used as a negative control.

To determine the effect of palmitate and Rosiglitazone on beta cell viability, MTT viability assay was used. At 72 hours, palmitate induced a significant decrease in the MIN6 cell viability (p<0.01) when compared to untreated controls (see Figure 12. MTT Viability Assays to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage in MIN6 Beta Cells on page 81). Rosiglitazone alone had no effect cell viability. Interestingly the co-incubation of palmitate and Rosiglitazone resulted in a percentage viability value similar to that of the untreated controls. Rosiglitazone therefore significantly protected MIN6 beta cells from palmitate-induced damage and the resultant reduction in cell viability (p<0.001).
Figure 12. MTT Viability Assays to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage in MIN6 Beta Cells

MIN6 cells were cultured in 5mM glucose DMEM alone or with the addition of 0.4mM palmitate and/or 10μM 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 were obtained from at least 3 separate experiments with error bars representing standard deviation *p<0.05 & **p<0.01.
To determine the possible mechanism for the above reported cell death; was it apoptosis or necrosis? H/PI staining was used to distinguish between apoptotic cells (bright blue colour) and necrotic cells (bright pink colour) when compared to normal healthy cells (dark blue colour) (see Figure 13. Hoechst 33342/Propidium Iodide (H/PI) Staining for Apoptosis and Necrosis to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage in MIN6 Beta Cells on page 83). In the Palmitate treated group in (PANEL B) there was a distinctive area of bright blue stained cells indicating apoptosis when compared to the untreated controls. The Rosiglitazone treated group (PANEL C) showed a pattern similar to the untreated controls (PANEL A), indicating Rosiglitazone alone had no effect. Interestingly, in the group co-incubated with palmitate and Rosiglitazone (PANEL D), there was an absence of the bright blue stained apoptotic cells. It seems that the addition of Rosiglitazone protected MIN6 beta cells from palmitate-induced apoptosis. The lack of apoptosis and resultant cellular death mirrored the MTT cell viability data (see Figure 12 on page 81).

Having confirmed that Rosiglitazone protects against the palmitate-induced damage to beta cell viability, we next investigated possible mechanisms involved. Using MTT viability assays we aimed to explore the possible role of AMPK in this protection. The AMPK inhibitor Compound C was used. The same pattern with the controls (untreated, palmitate, Rosiglitazone) was observed as previously and so were the protective effects of Rosiglitazone from the palmitate-induced damage (see Figure 14. MTT Viability Assays to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage and the Involvement of the AMPK Pathway in MIN6 Cells on page 84). In the presence of the AMPK inhibitor Compound C, Rosiglitazone was unable to prevent the detrimental effects of palmitate on MIN6 cell viability (p<0.001) as Compound C completely blocked 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.
Figure 13. Hoechst 33342/Propidium Iodide (H/PI) Staining for Apoptosis and Necrosis to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage in MIN6 Beta Cells

MIN6 cells were cultured in 5mM glucose DMEM alone or with the addition of 0.4mM palmitate and/or 10μM Rosiglitazone for 72 hours. Cells were then incubated for 5 minutes in H/PI solution before visualization on the Zeiss Axiovert 25 CFI microscope. Dark blue indicates viable cells, light blue indicates apoptotic cells and pink indicates necrotic cell. Results were obtained from at least 3 separate experiments.
MIN6 cells were cultured in 5mM glucose DMEM alone or with the addition of 0.4mM palmitate, 10μM Rosiglitazone and 20μM of the AMPK inhibitor Compound C 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 were obtained from at least 3 separate experiments with error bars representing standard deviation **p<0.01.
We next investigated the potential involvement of the p38 stress activated pathway in our model of cell viability. This pathway has been implicated in apoptotic death in many cell lines (see p38 section on page 51). The p38 inhibitor SB203580 was used. Again, the same pattern with the controls (untreated, palmitate, Rosiglitazone) was observed as previously and so were the protective effects of Rosiglitazone from the palmitate induced damage (see Figure 15. MTT Viability Assays to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage and the Involvement of the Stress Activated p38 MAPK Pathway in MIN6 Beta Cells on page 86).

The p38 inhibitor SB203580 had no effect on the ability of Rosiglitazone to protect MIN6 cells from the detrimental effects of palmitate (p<0.001). However, some of our published work using western blots carried out in our laboratory has shown that following densitometric analysis Rosiglitazone had rapidly down-regulated levels of the stress activated protein kinase p38 total protein, with a ~50% decrease occurring after 2 hours, potentially providing a level of beta cell protection (p<0.005) (Ali, Landy et al. 2008) (see Figure 16. The Modulation of p38 Protein by Rosiglitazone Treatment on page 87).

Finally, we investigated what other targets could be involved in this Rosiglitazone activated protection of pancreatic beta cells. CK2 as discussed previously (see CK2 section, p58) is an enzyme involved in cellular growth. The role of CK2 was investigated utilizing the inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB). The same pattern with the controls (untreated, palmitate, Rosiglitazone) was observed as previously and so were the protective effects of Rosiglitazone from the palmitate-induced (see Figure 17. MTT Viability Assays to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage and the Involvement of the Casein Kinase 2 (CK2) Pathway in MIN6 Beta Cells on page 88).
Figure 15. MTT Viability Assays to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage and the Involvement of the Stress Activated p38 MAPK Pathway in MIN6 Beta Cells

MIN6 cells were cultured in 5mM glucose DMEM alone or with the addition of 0.4mM palmitate, 10μM Rosiglitazone and 20μM of the p38 inhibitor (SB203580) 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 were obtained from at least 3 separate experiments with error bars representing standard deviation **p<0.01.
Figure 16. The Modulation of p38 Protein by Rosiglitazone Treatment

Western blot analysis of total p38 protein following treatment of MIN6 cells with 10μM Rosiglitazone for 2 hours. PANEL A; Rosiglitazone caused a significant decrease in total p38 levels, approximately 50%. PANEL B; is a densitometry analysis representation. Values are mean ± SEM, expressed as a percentage of control. Samples were analysed in duplicate and results were obtained from at least 3 separate experiments with error bars representing standard deviation *p<0.05. Figure adapted from (Ali, Landy et al. 2008).
Figure 17. MTT Viability Assays to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage and the Involvement of the Casein Kinase 2 (CK2) Pathway in MIN6 Beta Cells

MIN6 cells were cultured in 5mM glucose DMEM alone or with the addition of 0.4mM palmitate, 10μM Rosiglitazone and 20μM of the Casein Kinase 2 (CK2) inhibitor (TBB) 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 were obtained from at least 3 separate experiments with error bars representing standard deviation *p<0.05.
However, we found that 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). 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.

The data thus far has proved valuable in showing the significant effects of Rosiglitazone on beta cell viability. We have reported that the cell viability protected from the palmitate-induced damage was not just due to loss of beta cell numbers but was through apoptosis, a mechanistic programmed cell death process. As apoptosis is a controlled process governed by internal cellular mechanisms, our data suggests that Rosiglitazone protects pancreatic beta cells through effects on beta cell internal cellular processes. As the precise internal processes within the beta cell rely on cellular organelles, we next wanted to decipher exactly what the beta cell looks like following treatment with each of our stimuli. We wanted to visualize the internal integrity of the beta cell and examine what effects our stimuli might have.

2.2 Cellular Ultra-structure Studies – Transmission Electron Microscopy (TEM)

2.2(a) Rosiglitazone Protects MIN6 Cell Organelles and Ultra-structure from Palmitate-induced Damage

To determine if Rosiglitazone protects MIN6 cell organelles and ultra-structure from palmitate-induced damage, MIN6 cells were stimulated for 24, 48 and 72 hours with 0.4mM palmitate or 10µM Rosiglitazone either alone or in combination. Visualization following Transmission electron microscopy followed. Examination with TEM demonstrated a trend consistent throughout all the time points studied. MIN6 cells treated with palmitate alone showed degradation in cell organelle structure in comparison to untreated controls.
Interestingly, co-incubation with Rosiglitazone protected from damage. This was evident in the TEM images at both the (2000x) and (5000x) as will be discussed below.

At 24 hours, a clear structure of the cells can be seen with visible distinguishable cell organelles (see Figure 18. Transmission Electron Microscopy (TEM) Imaging of Healthy Untreated MIN6 Beta Cell Ultra-structure on page 91). There is a collection of mature and immature secretory insulin vesicles and granules (Arrows A and B respectively). The cell nucleus is well rounded with an intact nuclear envelope Arrows C and D respectively). The golgi apparatus (Arrow E) present and the mitochondrion (Arrow F) that can be seen are of a the regular healthy morphology expected for these organelles. This gives an overall picture of how a healthy pancreatic beta cell should look like.

In the presence of palmitate, some signs of stress appear within the cell (see Figure 19. Transmission Electron Microscopy (TEM) Imaging Showing Changes in MIN6 Beta Cell Ultra-structure following Palmitate Treatment on page 92). The mitochondria become swollen and more rounded than oblong (Arrow A). There does not seem to be many insulin-containing vesicles or granules and they are mostly empty with no insulin to secrete (Arrow B). Although there is a sign of a golgi apparatus (Arrow C) is rather small with less layers or strata. The nucleus and nuclear pore however are still intact (Arrow D and E respectively). At 24 hours in the presence of Rosiglitazone the cellular structure looks similar to that of the untreated control (see Figure 20. Transmission Electron Microscopy (TEM) Imaging Showing MIN6 Beta Cells following Rosiglitazone Treatment on page 93). All the organelles are clearly defined and with the correct morphology.
Figure 18. Transmission Electron Microscopy (TEM) Imaging of Healthy Untreated MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM alone for 24 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ulracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Arrow A shows an example of mature secretory insulin granule, Arrow B shows an example of an immature secretory insulin granule, Arrow C shows nucleus, Arrow D shows an intact nuclear envelope Arrow E shows golgi apparatus and Arrow F shows a mitochondrion. Bar = 2µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Figure 19. Transmission Electron Microscopy (TEM) Imaging Showing Changes in MIN6 Beta Cell Ultra-structure following Palmitate Treatment

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate for 24 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Arrow A shows an example of a stressed and rounded mitochondrion, Arrow B shows an example of an empty secretory insulin granule, Arrow C shows an example of golgi apparatus, Arrow D shows the nucleus and Arrow E shows an intact nuclear envelope. Bar = 2µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Figure 20. Transmission Electron Microscopy (TEM) Imaging Showing MIN6 Beta Cells following Rosiglitazone Treatment

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 10μM Rosiglitazone for 24 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Bar = 2μm and images were representative of fields taken at random obtained from at least 3 separate experiments.
At 24 hours in the presence of both Palmitate and Rosiglitazone (see Figure 21. Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure on page 95), there is an interesting and evident protection of the cellular ultra-structure by Rosiglitazone from the palmitate-induced stress seen in the palmitate treated sample. The organelles such as the mitochondria are oblong and not rounded with stress, the insulin granules and vesicles contain insulin and present in both the mature and immature form. There is a clear presence of golgi apparatus and the nucleus is rounded with an intact nuclear pore. The cell has a consistent ribosomal granulation all similar morphologies to that seen in the untreated control group. The comparison of the palmitate sample with the palmitate and Rosiglitazone treated sample highlights the clearly visual evidence of the protective effects of Rosiglitazone from the palmitate-induced damage and the protection from the detrimental effects of this free fatty acid on cellular ultra-structure (see Figure 22. Comparative Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure on page 96). The same trend in this time point is seen in all the samples at the higher magnification of 5000x (See figures 23-26 on pages 97-100) and the organelle and structural damage is even clearer, particularly in Figure 27. Comparative Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure on page 101), which compares the palmitate sample with the palmitate and Rosiglitazone co-incubated sample. If the palmitate-induced damage to cellular ultra-structure we have observed thus far is progressive then it could help explain the palmitate-induced decrease in cell viability we have reported. It would also be interesting to see if Rosiglitazone can still protect cellular ultra-structure from the palmitate-induced damage. We therefore investigated the effect of palmitate and Rosiglitazone on cellular ultra-structure on the longer time course of 48 hours.
Figure 21. Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate and 10µM Rosiglitazone for 24 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Bar = 2µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate alone (PANEL A) or in combination with 10μM Rosiglitazone (PANEL B) for 24 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Bar = 2μm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Untreated MIN6 Cells – 24hr

Figure 23. Transmission Electron Microscopy (TEM) Imaging of Healthy Untreated MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM alone for 24 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Figure 24. Transmission Electron Microscopy (TEM) Imaging Showing Changes in MIN6 Beta Cell Ultra-structure Following Palmitate Treatment

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate for 24 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Rosiglitazone Treated MIN6 Cells – 24hr

Figure 25. Transmission Electron Microscopy (TEM) Imaging Showing MIN6 Beta Cells following Rosiglitazone Treatment

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 10μM Rosiglitazone for 24 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascain 1000 CCD camera (5000x magnification). Bar = 0.5μm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Figure 26. Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate and 10μM Rosiglitazone for 24 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5μm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Figure 27. Comparative Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate alone (PANEL A) or in combination with 10µM Rosiglitazone (PANEL B) for 24 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
At 48 hours, the same pattern is seen again. In the untreated control sample (see Figure 28. Transmission Electron Microscopy (TEM) Imaging of Healthy Untreated MIN6 Beta Cell Ultra-structure on page 104) the cellular ultra-structure is similar to that seen at 24 hours. All the organelles exhibit their usual characteristics with normal morphology and no indication of stress is present.

At 48 hours in the presence of palmitate sample (see Figure 29. Transmission Electron Microscopy (TEM) Imaging Showing Changes in MIN6 Beta Cell Ultra-structure Following Palmitate Treatment on page 105), we begin to see the indications of stress by this saturated free fatty acid. The organelles are increasingly mostly indistinguishable, the cellular ribosomal granulation is decreased, there are no visible insulin secreting granules. Other organelles such as mitochondria and golgi apparatus are either stressed and swollen and rounded, cannot be seen or extremely few in number in comparison to the untreated controls.

At 48 hours in the presence of Rosiglitazone the cellular structure looks similar to that of the untreated control (see Figure 30. Transmission Electron Microscopy (TEM) Imaging Showing MIN6 Beta Cells Following Rosiglitazone Treatment on page 106), All the organelles are clearly defined and with the correct morphology. In the presence of both Palmitate and Rosiglitazone (see Figure 31. Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure on page 107), there is again evident protection of the cellular ultra-structure by Rosiglitazone from the palmitate-induced stress seen. All organelle morphology is similar to that seen in the untreated control group.

The comparison between the palmitate treated sample with the palmitate and Rosiglitazone treated sample highlights the clearly visual evidence of the protective effects of Rosiglitazone
from the palmitate-induced damage (See Figure 32. Comparative Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure on page 108).

The same trend in this time point is seen in all the samples at the higher magnification of 5000x (See figures 36-39 on pages 109-112) and the organelle and structural damage is even clearer particularly in Figure 37. Comparative Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure on page 113) which compares the palmitate sample with the palmitate and Rosiglitazone co-incubated sample.

We have demonstrated successfully that the internal ultra-structure of the beta cell has been compromised by exposure to the unsaturated free fatty acid palmitate and that Rosiglitazone co-incubation had protected from this damage even at 48 hours. We have shown that these effects occur in a time-dependant manner following incubation with palmitate and that the protection by Rosiglitazone also seems to occur in a time-dependant manner.

The time scales by which we have previously examined beta cell death and the effects on viability and apoptosis were on an extended time course of 72 hours. It is not unreasonable to suggest this has an impact on cell viability and thus this could complement our previous viability data should the trend observed with the TEM investigations continues. As the time course for our viability data was at 72 hours, we then repeated the investigation with the longer time course of 72 hours.
Figure 28. Transmission Electron Microscopy (TEM) Imaging of Healthy Untreated MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM alone for 48 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Bar = 2µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate for 48 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Bar = 2µm and images were representative of fields taken at random obtained from at least 3 separate experiments.

Figure 29. Transmission Electron Microscopy (TEM) Imaging Showing Changes in MIN6 Beta Cell Ultra-structure Following Palmitate Treatment
MIN6 cells were cultured in 5mM glucose DMEM with the addition of 10μM Rosiglitazone for 48 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascian 1000 CCD camera (2000x magnification). Bar = 2μm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Figure 31. Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate and 10μM Rosiglitazone for 48 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Bar = 2μm and images were representative of fields taken at random obtained from at least 3 separate experiments.
MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate alone (PANEL A) or in combination with 10μM Rosiglitazone (PANEL B) for 48 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Bar = 2μm and images were representative of fields taken at random obtained from at least 3 separate experiments.
UNTREATED MIN6 CELLS – 48HR

Figure 33. Transmission Electron Microscopy (TEM) Imaging of Healthy Untreated MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM alone for 48 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Figure 34. Transmission Electron Microscopy (TEM) Imaging Showing Changes in MIN6 Beta Cell Ultra-structure Following Palmitate Treatment

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate for 48 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Rosiglitazone Treated MIN6 Cells – 48hr

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 10μM Rosiglitazone for 48 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5μm and images were representative of fields taken at random obtained from at least 3 separate experiments.

Figure 35. Transmission Electron Microscopy (TEM) Imaging Showing MIN6 Beta Cells Following Rosiglitazone Treatment
Figure 36. Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate and 10μM Rosiglitazone for 48 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5μm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Figure 37. Comparative Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate alone (PANEL A) or in combination with 10μM Rosiglitazone (PANEL B) for 48 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5μm and images were representative of fields taken at random obtained from at least 3 separate experiments.
At 72 hours, the same pattern prevailed. In the untreated control sample (see Figure 38. Transmission Electron Microscopy (TEM) Imaging of Healthy Untreated MIN6 Beta Cell Ultra-structure on page 115), the cellular ultra-structure is similar to that seen at 24 and 48 hours. All the organelles exhibit their usual characteristics with normal morphology and no indication of stress present.

At 72 hours in the presence of palmitate (see Figure 39. Transmission Electron Microscopy (TEM) Imaging Showing Changes in MIN6 Beta Cell Ultra-structure Following Palmitate Treatment on page 116), the indications of stress by this saturated free fatty acid are at their most damaging thus far. The organelles are certainly indistinguishable, the cellular ribosomal granulation is decreased and there are no visible insulin secreting granules. Other organelles such as mitochondria and golgi apparatus cannot be seen and there is a lot of debris in comparison to the untreated controls.

At 72 hours in the presence of Rosiglitazone the cellular structure looks similar to that of the untreated control (see Figure 40. Transmission Electron Microscopy (TEM) Imaging Showing MIN6 Beta Cells Following Rosiglitazone Treatment on page 117). All the organelles are clearly defined and with the correct morphology. At 72 hours in the presence of both Palmitate and Rosiglitazone (see Figure 41. Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure on page 118), there is again evident protection of the cellular ultra-structure by Rosiglitazone from the palmitate-induced stress seen in the palmitate treated sample. All organelle morphology is similar to that seen in the untreated control group.
Untreated MIN6 Cells – 72hr

Figure 38. Transmission Electron Microscopy (TEM) Imaging of Healthy Untreated MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM alone for 72 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Bar = 2µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate for 72 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Bar = 2µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Rosiglitazone Treated MIN6 Cells – 72hr

Figure 40. Transmission Electron Microscopy (TEM) Imaging Showing MIN6 Beta Cells Following Rosiglitazone Treatment

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 10µM Rosiglitazone for 72 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Bar = 2µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Figure 41. Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate and 10μM Rosiglitazone for 72 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Bar = 2μm and images were representative of fields taken at random obtained from at least 3 separate experiments.
The comparison between the palmitate treated sample with the palmitate and Rosiglitazone treated sample highlights the clearly visual evidence of the protective effects of Rosiglitazone from the palmitate-induced damage (see Figure 42. Comparative Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure on page 120). The same trend in this time point is seen in all the samples at the higher magnification of 5000x (see figures 46-49 on pages 121-124) and the organelle and structural damage is even clearer particularly in Figure 47. Comparative Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure on page 125), which compares the palmitate sample with the palmitate and Rosiglitazone co-incubated sample. As seen above, throughout the time courses studied the palmitate-induced damage on MIN6 beta cells occurred over an extended time course. At 24 hours, the palmitate treatment had already started to have a negative effect on cellular ultrastructure. At 48 hours this negative effect was more pronounced and by 72 hours the detrimental effects of palmitate had completely destroyed the internal cellular structure. Treatment with Rosiglitazone alone does not affect beta cell structure at all and the cellular morphology and structure is the same as untreated controls throughout all the time courses studied. The protective effects of Rosiglitazone have also occurred in a time in-dependant manner. At 24 hours, the Rosiglitazone treatment started to have a positive effect and protected against palmitate-induced damage. At 48 hours this was still the case and by 72 hours Rosiglitazone protected the beta cells from the completely detrimental effects of palmitate that had destroyed the internal cellular structure entirely. Figure 48, on page 126, comparing the palmitate treated samples, at high magnifications, merely highlights the extent of the time-dependant palmitate damage.
Figure 42. Comparative Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate alone (PANEL A) or in combination with 10µM Rosiglitazone (PANEL B) for 72 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (2000x magnification). Bar = 2µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Untreated MIN6 Cells – 72hr

Figure 43. Transmission Electron Microscopy (TEM) Imaging of Healthy Untreated MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM alone for 48 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate for 24 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Rosiglitazone Treated MIN6 Cells – 72hr

**Figure 45. Transmission Electron Microscopy (TEM) Imaging Showing MIN6 Beta Cells Following Rosiglitazone Treatment**

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 10µM Rosiglitazone for 72 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Figure 46. Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate and 10μM Rosiglitazone for 72 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5μm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Figure 47. Comparative Transmission Electron Microscopy (TEM) Imaging to Determine the Protective Effects of Rosiglitazone Against Palmitate-induced Damage in MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate alone (PANEL A) or in combination with 10µM Rosiglitazone (PANEL B) for 72 hours. Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
Figure 48. Comparative Transmission Electron Microscopy (TEM) Imaging to Determine the Time-dependant Palmitate-induced Damage on MIN6 Beta Cell Ultra-structure

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM palmitate alone at 24hrs (PANEL A), 48hrs (PANEL B) and 72 hours (PANEL C). Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5µm and images were representative of fields taken at random obtained from at least 3 separate experiments.
MIN6 cells were cultured in 5mM glucose DMEM with the addition of 0.4mM Palmitate and 10μM Rosiglitazone at 24hrs (PANEL A), 48hrs (PANEL B) and 72 hours (PANEL C). Cells were fixed and stained from the cellular pellets cut with an LKB Ultracut ultramicrotome. Sections were examined in a Hitachi 7100 transmission electron microscope at 100kV. Images were acquired with a Gatan Ultrascan 1000 CCD camera (5000x magnification). Bar = 0.5μm and images were representative of fields taken at random obtained from at least 3 separate experiments.
3. Discussion

In the literature, it has been documented that lipid accumulation causes cellular dysfunction, with many associations to compromised endoplasmic reticulum morphology and integrity (Karaskov, Scott et al. 2006). It has been suggested that this lipid overload is the reason for the structural impairment and it is also a cellular response to these conditions (Borradaile, Han et al. 2006). Palmitate, a long chain saturated molecule, is cytotoxic to beta cells and it is associated with induction of apoptosis via the stimulation of many stress activated kinases and through the induction of cytokines and caspase activation (Welters, Diakogiannaki et al. 2006). The mechanisms through which the damage eventually occurs are thought to, in part, be due to the resultant decrease in protein synthesis causing an accumulation of unfolded proteins in the endoplasmic reticulum, triggering apoptosis (Morgan 2009). Palmitate damage on a molecular level is clearly visible in our observed TEM images (Corrons, Camihort et al. 1996). Functional organelles such as mitochondria, endoplasmic reticulum, ribosomes and golgi apparatus are vital for controlling cell function and responsible for the control of many processes from protein translation to apoptosis (Corrons, Camihort et al. 1996). They were destroyed following palmitate treatment, as seen from the images acquired, and this is of course in addition to the obvious damage to the insulin secretory granules. Palmitate caused a reduction in insulin granule number, both mature and immature granules, in a time dependant manner (having in turn a direct effect on insulin secretion, central for beta cell function).

The data from our study make Rosiglitazone a desirable agent for the protection of pancreatic beta cells. There was a significant protection of MIN6 beta cells from the detrimental effects of palmitate in terms of cellular viability and organelle structural damage. Treatment with Rosiglitazone therefore ensures the protection and longevity of beta cells as well as the regulation of many cellular processes dependant on functional organelles.
CHAPTER 4

Rosiglitazone, Cellular Energy and Stress

1. Introduction

As seen thus far, agents such as Rosiglitazone are desirable for diabetes therapy as evidence from our studies shows beta cell protection in many ways. We have seen a significant protection of MIN6 beta cells from the detrimental effects of palmitate, in terms of cellular viability and organelle ultra-structural damage. Therefore, such agents ensure the regulation of many cellular processes dependant on functional organelles and in turn the protection and longevity of beta cells. It therefore follows that it is important to identify the key pathways involved in these processes. Oxidative stress and free radicals have long been implicated in the pathogenesis of diabetes (Chan and Kowluru 2007) and closely associated with beta cell dysfunction and induced apoptosis (Lopes, Oliveira et al. 2008). As discussed previously, in terms of cellular energy, other proteins along with AMPK play important roles. Many genes and proteins are involved in redox and energy balance including the uncoupling protein family (UCPs) (Chan, Saleh et al. 2004) and the glucose transporter 2 (GLUT2) (Thorens 2001). The uncoupling protein family are mitochondrial transporters located in the inner mitochondrial membrane and are involved in the control of energy conversion. There is evidence, both experimental and genetic, suggesting that their dysfunctions are linked with metabolic syndrome and obesity (Villarroya, Iglesias et al. 2007). There is evidence to suggest that UCP2 isoform expression is increased in rodent islets after high-fat feeding (Briaud, Kelpe et al. 2002) or when islets were exposed to fatty acids in vitro (Patanè, Anello et al. 2002). It has been established that increasing UCP2 expression in beta cells impairs insulin secretion (Chan, De Leo et al. 2001). We therefore wanted to investigate what changes, if any, in UCP2 expression might occur in our MIN6 model following stimulations with palmitate and Rosiglitazone either alone or in combination.
2. Results

2.1 Effects of Palmitate and Rosiglitazone on UCP2 mRNA Expression Levels

In order to determine effects of palmitate and Rosiglitazone on UCP2 mRNA expression levels real time PCR was carried out on MIN6 cells stimulated with palmitate and Rosiglitazone either alone or in combination at 72 hours. In MIN6 cells treated with palmitate UCP2 mRNA levels were elevated significantly in comparison to untreated controls (~4 fold), an effect abolished by co-incubation with Rosiglitazone (p<0.01) (see Figure 50. Real-Time PCR Analysis of UCP2 mRNA Expression Levels for MIN6 Beta Cells Stimulated with Palmitate and Rosiglitazone, Either Alone or in Combination, for 72hours Error! Bookmark not defined.). Rosiglitazone alone had no effect. It has been previously suggested that free fatty acid induced beta cell defects are in part dependent on UCP2 expression (Joseph, Koshkin et al. 2004). Our data shows that Rosiglitazone protects from this palmitate-induced increase in UCP2 making such agents desirable for the treatment of type 2 diabetes.

We next wanted to investigate the role of the p38 stress activated pathway in our model and see if it had any effect on the protective Rosiglitazone mode of action observed above from the palmitate-induced increase in UCP2 mRNA expression. As previously discussed, (see p38 section, p62), the p38MAPK pathway is implicated in beta cell apoptosis and has been associated with palmitate-induced apoptosis. Following real time PCR analysis of MIN6 cells stimulated for 72 hours we have shown that Rosiglitazone treatment alone, as previously, had shown no significant change in the UCP2 expression levels when compared to untreated controls indicating it had no effect (see Figure 51. Real-Time PCR Analysis of UCP2 mRNA Expression Levels for MIN6 Beta Cells Stimulated with Rosiglitazone and the p38 Inhibitor (SB203580), Either Alone and in Co-incubation, for 72hours on page 132). The addition of the p38 inhibitor (SB203580) alone however decreased UCP2 mRNA levels below levels of untreated controls (~0.8 fold), an effect overcome by Rosiglitazone (p<0.05).
MIN6 cells were cultured in 5mM glucose DMEM in the presence or absence of 0.5mM palmitate, 10μM Rosiglitazone either alone and in co-incubation. PANEL A is representative of total UCP2 mRNA fold changes following Pfaffl method analysis of C_T values and PANEL B represents changes in raw C_T values (delta C_T, ΔC_T). Values are expressed in ΔC_T values relative to untreated controls and data represent at least 3 separate experiments with error bars representing standard deviation, **p<0.01.

Figure 50. Real-Time PCR Analysis of UCP2 mRNA Expression Levels for MIN6 Beta Cells Stimulated with Palmitate and Rosiglitazone, Either Alone or in Combination, for 72hours
MIN6 cells were cultured in 5mM glucose DMEM in the presence or absence of 10µM Rosiglitazone and 20µM of the p38 inhibitor (SB203580) either alone and in co-incubation. PANEL A is representative of total UCP2 mRNA fold changes following Pfaffl method analysis of CT values and PANEL B represents changes in raw CT values (delta CT, ΔCT). Values are expressed in ΔCT values relative to untreated controls and data represent at least 3 separate experiments with error bars representing standard deviation, **p<0.05.
Albeit a small fold difference (~1.0 fold) the p38 inhibitor SB203580 addition had no effect on the ability of Rosiglitazone to block the SB203580-induced decrease in UCP2 mRNA levels. In Chapter 3, we mentioned that upon using western blotting techniques, followed by densitometric analysis, our laboratory had shown that Rosiglitazone rapidly down-regulated total protein levels of the stress activated protein kinase p38 (see Figure 16 on page 87), with a ~50% decrease occurring after 2 hours, potentially providing a level of beta cell protection (p<0.005) (Ali, Landy et al. 2008). We therefore wanted to investigate whether the Rosiglitazone decrease in the p38 stress-activated MAP kinase is linked to the effects Rosiglitazone elicits on UCP2 mRNA expression. Following qPCR data analysis, we have observed that on the co-incubation of Rosiglitazone with the p38 inhibitor (SB203580), Rosiglitazone blocked the inhibitor-induced decrease in UCP2 mRNA significantly (p<0.01) (see Figure 51 on page 132). So far, we have shown however that palmitate increases UCP2 mRNA that Rosiglitazone protects from this palmitate-induced increase in UCP2 mRNA. We have shown that Rosiglitazone decreases p38 total protein and we have seen that Rosiglitazone was able to block the p38-induced decrease in UCP2 mRNA and restore UCP2 mRNA levels to those of untreated controls. Then, is there a link between the palmitate-induced increase in UCP2 mRNA and p38? Would the levels of UCP2 mRNA be modified in the presence of palmitate and p38 in co-incubation?

MIN6 beta cells were therefore treated with palmitate, Rosiglitazone and the p38 inhibitor (SB203580) either alone or in combination for 72 hours). Following qPCR data analysis, as seen previously, MIN6 cells treated for 72 hours with palmitate showed significantly elevated UCP2 mRNA levels similar to untreated controls (~1.5 fold) (see Figure 52. Real-Time PCR analysis of UCP2 mRNA expression levels for MIN6 cells stimulated with Palmitate, Rosiglitazone, p38 Inhibitor SB203580, Either Alone or in Combination, as indicated for 72hours on page 134), an effect abolished by co-incubation with Rosiglitazone (p<0.05).
Figure 52. Real-Time PCR analysis of UCP2 mRNA expression levels for MIN6 cells stimulated with Palmitate, Rosiglitazone, p38 Inhibitor SB203580, Either Alone or in Combination, as indicated for 72 hours.

MIN6 cells were cultured in 5mM glucose DMEM in the presence or absence of 0.5mM Palmitate, 10μM Rosiglitazone and 20μM of the p38 inhibitor (SB203580) either alone and in co-incubation as indicated. PANEL A is representative of total UCP2 mRNA fold changes following Pfaffl method analysis of C_T values and PANEL B represents changes in raw C_T values (delta C_T, ΔC_T). Values are expressed in ΔC_T values relative to untreated controls and data represent at least 3 separate experiments with error bars representing standard deviation, *p<0.05.
Rosiglitazone alone showed UCP2 expression levels similar to untreated controls indicating it alone has no effect. The addition of SB203580 to palmitate had no significant effect on the ability of palmitate to increase UCP2 mRNA levels; both the palmitate alone and palmitate with SB203580 samples had a similar fold increase (~1.5 fold) in UCP2 mRNA levels when compared to untreated controls suggesting that the p38 pathway is not involved in the protective effects of Rosiglitazone from palmitate-induced increases in UCP2 mRNA levels. We have seen in the previous chapter that Rosiglitazone can protect beta cell viability from palmitate-induced damage through multiple signalling pathways. As inhibition of the p38MAPK pathway does not seem to affect palmitate-induced increases in UCP2; are other pathways involved? Is there a pathway that links Rosiglitazone, palmitate and the effects on UCP2? We therefore decided to investigate two major pathways; the AMPK fuel sensing pathway (blocking it with Compound C) and the PPAR-dependant pathway (blocking it with the PPARγ antagonist GW9992). Real time PCR was carried out on MIN6 cells stimulated with palmitate and Rosiglitazone either alone or in combination for 72 hours. As previous, in MIN6 cells treated for 72 hours with palmitate UCP2 mRNA levels were elevated significantly in comparison to untreated controls (~3 fold) (see Figure 53. Real-Time PCR Analysis of UCP2 mRNA Expression Levels for MIN6 Beta Cells Stimulated with Palmitate, Rosiglitazone, the AMPK Inhibitor Compound C and the PPARγ antagonist (GW9662), for 72 hours on page 136), an effect abolished by co-incubation with Rosiglitazone (P<0.01). A further two samples of the palmitate and Rosiglitazone co-incubated group were treated with either Compound C or GW9962. However, the protective effects of Rosiglitazone were significantly blocked by the PPARγ antagonist GW9662 (p<0.01) and the ability of Rosiglitazone to block palmitate-induced increases in UCP2 gene expression was partially abolished. This suggested that the protective effects of Rosiglitazone on decreasing the palmitate-induced UCP2 expression were PPARγ-dependent.
Figure 53. Real-Time PCR Analysis of UCP2 mRNA Expression Levels for MIN6 Beta Cells Stimulated with Palmitate, Rosiglitazone, the AMPK Inhibitor Compound C and the PPARγ antagonist (GW9662), for 72 hours

MIN6 cells were cultured in 5mM glucose DMEM in the presence or absence of 0.5mM Palmitate, 10μM Rosiglitazone, 20μM of the AMPK inhibitor compound C (CC) and 30μM of the PPARγ antagonist (GW9662) either alone and in co-incubation as indicated. PANEL A is representative of total UCP2 mRNA fold changes following Pfaffl method analysis of C_T values and PANEL B represents changes in raw C_T values (delta C_T, ΔC_T). Values are expressed in ΔC_T values relative to untreated controls and data represent at least 3 separate experiments with error bars representing standard deviation. **p<0.01.
2.2. Effects of Rosiglitazone on GLUT2 mRNA Expression Levels

As stated in Chapter 1, GLUT2 is a transmembrane carrier protein enabling passive glucose movement across cell membranes (NCBI Gene 2011). It is an integral plasma membrane glycoprotein of the liver, pancreatic islet beta cells, the intestine and the kidney epithelium, mediating facilitated bi-directional glucose transport in and out the cell due to its low affinity and high capacity and due to its low affinity for glucose (NCBI Gene 2011). Sugars and hormones in particular regulate GLUT2 gene expression, especially as GLUT2 also functions as a membrane receptor for glucose whereby the glucose-dependent cellular activities in the pancreas are governed by its location on the cell surface (Thorens 2001). Glucose also acts as a key signal in pancreatic beta cells for regulating insulin secretion, with GLUT2 being responsible for sensing glucose levels in the blood (Im, Kim et al. 2006). In order to determine the effects of Rosiglitazone on GLUT2 mRNA expression levels, real time PCR was carried out on MIN6 cells stimulated with Rosiglitazone alone for 24, 48 and 72 hours. Rosiglitazone significantly reduced GLUT2 mRNA levels in a time dependent manner (p<0.05), (see Figure 54. Real-Time PCR Analysis of GLUT2 mRNA Expression Levels for MIN6 Beta Cells Stimulated with Rosiglitazone for 24, 48 and 72 hours on page 138).

3. Discussion

The work presented in this chapter has shown that at a gene expression level, palmitate exposure significantly increased UCP2 mRNA levels (p<0.05), whereas treatment with Rosiglitazone alone had no significant effect on UCP2 gene expression. However, the 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 GW9662, 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 beta cells, protecting MIN6 cells from
Figure 54. Real-Time PCR Analysis of GLUT2 mRNA Expression Levels for MIN6 Beta Cells Stimulated with Rosiglitazone for 24, 48 and 72 hours

MIN6 cells were cultured in 5mM glucose DMEM in the presence or absence of 10μM Rosiglitazone alone. Figure is representative of total GLUT2 mRNA fold changes following Pfaffl method analysis of C_T values following changes in raw C_T values (delta C_T, ΔC_T). Values are expressed as a percentage of untreated controls and data represent at least 3 separate experiments with error bars representing standard deviation, **p<0.05.
the detrimental effects of palmitate and blocking palmitate-induced increases in UCP2 gene expression. In the literature, it has been documented that lipid accumulation causes cellular dysfunction with many associations with lipid overload compromising endoplasmic reticulum morphology and integrity (Borradaile, Han et al. 2006). Many processes occur in the endoplasmic reticulum (ER), such as oxidation involving reactive oxygen species (ROS) generation (Görlach, Klappa et al. 2006). To ensure cellular survival of ER stress, PERK [PKR (double-stranded-RNA-dependent protein kinase)-like ER kinase], an ER transmembrane kinase, is activated as part of the unfolded protein response. PERK is expressed greatly in beta cells and is fundamental in their development, differentiation and function (Herbert 2007). However, chronic activation of PERK can induce cell death, and its activation has been implicated in both Type 1 and Type 2 diabetes. It follows that proteins that affect or are affected by redox changes, such as UCP2, are vital for beta cell functionality. It has been shown from mouse studies that obesity may stimulate UCP2 deep within the cells. In this process, it essentially interferes with the ability of the beta cell to sense rising glucose levels, and some evidence even suggests that high levels of the uncoupling protein might precipitate progression from insulin resistance to diabetes (De Souza, Araujo et al. 2007). Previous work conducted on islets showed a relationship between Rosiglitazone, palmitate, insulin levels and the UCP2 levels (Tian, Li et al. 2006). It was established that prolonged exposure of pancreatic beta cells to fatty acids increases basal insulin secretion but inhibits glucose-stimulated insulin secretion, an effect blocked by Rosiglitazone without affecting cell viability (Tian, Li et al. 2006).

We have also shown intriguing data about the ability of Rosiglitazone to possibly modulate energy expenditure in the beta cell when looking at GLUT2. There is evidence in the literature that in beta cells, PPARγ and thiazolidinediones activate the genes encoding GLUT2 (Kim, Kim et al. 2000). Studies with diabetic or glucose-intolerant mice treated with the PPARγ
agonist GW99662, Pioglitazone and/or a control, indicated that isolated islets of Pioglitazone treated mice exhibited significantly improved glycaemic control, corresponding to increased serum insulin and enhanced glucose-stimulated insulin release and calcium (Ca$^{2+}$) responses from isolated islets showing improved islet function (Evans-Molina, Robbins et al. 2009). This was at least partially accredited to the significant up-regulation of the islet genes including GLUT2. However, on being prescribed Rosiglitazone, an individual would typically be a person with type 2 diabetes. There would be a case of excess circulating glucose. The treatment of type 2 diabetes with agents such as Rosiglitazone will usually endeavour to reduce glucose to combat the adverse effects that will have in a system where there is an existing malfunction in glucose regulation. As glucose uptake by tissues in a hyperglycaemic state is not much needed, we could speculate that less GLUT2 expression/activity would be needed. Thus Rosiglitazone reducing GLUT2 gene expression may not necessarily be seen as a disadvantage. Another reason might be to do with the nature of how GLUT2 works. GLUT2 provides facilitated glucose transport, needing energy in the form of ATP. This involves the AMPK fuel sensing pathway. In beta cells, both PPARγ and thiazolidinediones activate GLUT2 encoding genes (Kim, Kim et al. 2000). Some literature suggests that Rosiglitazone could stimulate the release and synthesis of insulin through the upregulation of GLUT2 (Hyo-Sup, Jung-Hyun et al. 2008). High doses of Rosiglitazone (100µM) were shown to increase GLUT2 mRNA, paralleled by an increase of GLUT2 protein after 24 hours of treatment (Blumentrath, Neye et al. 2001). In such studies however, non-physiological concentrations were used to highlight the effects Rosiglitazone might have on GLUT2 levels. We have carried on using physiological concentrations, as previously, to mimic what might be happening under physiological conditions in the beta cells in our investigations. We have revealed a significant time-dependant down-regulation in GLUT2 gene expression on exposure to Rosiglitazone. Our data shed light on another perspective, as discussed previously, that as Rosiglitazone down-regulates GLUT2, it not only reduces glucose uptake, which is harmful in a diabetes setting,
but it may also save the beta cell energy levels and perhaps help to prioritize where the energy and ATP is needed most, for instance for important processes such as insulin exocytosis. This not only highlights how much there is to investigate about the mechanism of actions of such drugs but what pathways are modulated in perhaps previously unobserved ways and how this may aid in the development of new therapeutic agents for the treatment of diabetes.

The beta cell is evidently highly specialized and well equipped to combat imbalances, such as energy imbalances that may disrupt its functionality. We have seen how the beta cell responds to stress stimuli such as palmitate and therapeutic agents such as Rosiglitazone that seem to reinstate balance within the cell and protect from the palmitate induced damage. This makes therapeutic agents like Rosiglitazone, that aid the beta cell in restoring balance within the cell, highly desirable. This gives rise to another interesting angle, that of cellular inflammation. Cellular inflammation has been implicated in much of the damage that occurs in many disease states and that is also true when looking at diabetes (Toborek, Lee et al. 2002; Wang, Lin et al. 2011). Stimuli such as saturated fatty acids have been found to cause inflammation in many cell types, for instance in endothelial cells (Toborek, Lee et al. 2002).

We have provided evidence with our data presented thus far, that cell viability and functionality can be affected in many ways and that Rosiglitazone can modulate the pathways involved through a multitude of signalling pathways and mechanisms. It therefore is not unreasonable to want to ask; what inflammatory processes could be triggered and what protein imbalances could occur under stress stimuli such as palmitate? Moreover, can Rosiglitazone protect the beta cell from such processes and modulate the signalling pathways implicated? We therefore set out to explore what potential pathways, and more specifically, what gene targets may be affected by our stimuli.
CHAPTER 5

Cellular Inflammation and Death

1. Introduction

As seen thus far from our study we have highlighted the involvement of pathways imperative for beta cell function and viability as well as the desire for agents such as Rosiglitazone for the treatment of diabetes. We have observed a significant protection by Rosiglitazone of MIN6 beta cells viability. Rosiglitazone had also ensured the preservation of organelles such as mitochondria, endoplasmic reticulum, ribosomes and golgi apparatus from palmitate-induced damage (Chapter 3). Many cellular processes are dependent on these functional organelles thus this is a great gain. We have also shown that Rosiglitazone modulated cellular energy levels involving ATP and genes such as GLUT2 and UCP2 (Chapter 4). The previous chapters also demonstrated the involvement of the AMPK fuel sensing pathway and down-stream targets such as ACC, as well as the pleiotropic CK2 and p38MAPK stress-activated pathway. The regulation of all the above pathways, processes, genes and proteins have an effect on cellular inflammation and death thus agents such as Rosiglitazone that modulate them are of interest, particularly if they favour beta protection, cell growth and rejuvenation.

Other proteins involved in cellular regulatory processes include NFκB. This transcription factor is a member of the Rel family of proteins that function as DNA-binding transcription factors with their activity regulated at the sub-cellular localization level (Kumar 2004). Human studies have shown that Rosiglitazone exerts some anti-inflammatory effects (Derosa, Fogari et al. 2007); in part by decreasing the levels of NFκB (Akama, Albanese et al. 1998). In other cells, such as adipocytes, saturated fatty acids such as palmitate have been found to increase cellular triglyceride accumulation generating reactive oxygen species (ROS) and increasing NFκB translocation when in both low (5 mmol/l) and high (25 mmol/l) glucose (Han, Kargi et
al. 2010). We thus wanted to investigate whether or not there was a similar involvement of NFκB in the effects we are seeing in our beta cell model.

2. Results

2.1 Modulation of the NFκB Pathway by Palmitate and Rosiglitazone in MIN6 Beta Cells

To determine the effect of Palmitate and Rosiglitazone on MIN6 cells, MIN6 cells were treated for 24 hours with 0.4 mM palmitate, there was an observed nuclear localization of NFκB from the cytoplasm (see Figure 55. Confocal Microscopy Imaging to Determine the Localisation of NFκB Following Palmitate and Rosiglitazone Stimulation of MIN6 Beta Cells, on page 144, Palmitate, PANEL C) when compared to all the other treatments groups (Untreated, Rosiglitazone, Pal + Rosi, PANEL C). There is less intense cytoplasmic staining in the palmitate treated sample (Palmitate, PANEL C) whereas it is more uniform in all the other samples, the untreated control (Untreated, PANEL C), the Rosiglitazone treated sample (Rosiglitazone, PANEL C) and the sample co-incubated with both (Pal + Rosi, PANEL C). Interestingly, in the samples co-incubated with both palmitate and Rosiglitazone, Rosiglitazone was able to prevent this palmitate-induced nuclear localization which is indicative of the activation of NFκB (Pal + Rosi, PANEL C). A clearer comparison can be seen between the palmitate and Palmitate with Rosiglitazone group in Figure 56 on page 145. In an attempt to confirm this NFκB nuclear localization by palmitate using a second technique; western blots were utilized. Whole cell, nuclear and cytoplasmic extracts were used. In MIN6 cells treated for 24 hours with 0.4 mM palmitate a decrease in the cytoplasmic levels of NFκB was evident in comparison to untreated controls, an effect blocked by Rosiglitazone addition (see Figure 57 on page 146). There was not enough sample number to carry out densitometric analysis however the trend does mirror that seen in the confocal microscopy.
Confocal microscopy imaging of MIN6 cells cultured for 24 hours in 5mM glucose DMEM with no additions (i.e. untreated controls) or with the addition of 0.4mM palmitate and 10μM Rosiglitazone either alone or in combination as indicated. Cells were stained using the NFκB primary and FITC-conjugated secondary specific antibodies. The data represents 3 separate experiments. Bar = 100μm.

Figure 55. Confocal Microscopy Imaging to Determine the Localisation of NFκB Following Palmitate and Rosiglitazone Stimulation of MIN6 Beta Cells
Figure 56. Confocal Microscopy Imaging to Highlighting the Localisation of NFκB Following Palmitate and Rosiglitazone Stimulations of MIN6 Beta Cells

Confocal microscopy imaging of MIN6 cells cultured for 24 hours in 5mM glucose DMEM with the addition of 0.4 mM palmitate in comparison with cells co-incubated with 0.4 mM palmitate and 10 μM Rosiglitazone. Cells were stained using the NFκB primary and FITC-conjugated secondary specific antibodies. The data represents 3 separate experiments.
Figure 57. Rosiglitazone Modulates NFκB Protein Expression in MIN6 Beta Cells

MIN6 cells were incubated in 5 mM glucose DMEM (Untreated controls – U), in the presence of 0.4 mM palmitate (P) or 10μM Rosiglitazone (R), either alone or in combination, as indicated. Whole cell, cytoplasmic and nuclear extracts were prepared and analyzed by western blotting using a specific NFκB, GAPDH and α-Tubulin specific antibodies as indicated. Protein sizes were marked against a Full Range Rainbow Marker; NFκB showing at ~75kDa, GAPDH at ~37kDa and α-Tubulin at ~50kDa. The data represents at least 3 separate experiments.
2.2 The Link between the p38 Pathway and MIN6 Beta Cell Viability

To investigate the link between the p38 pathway and MIN6 beta cell viability, MIN6 cells were cultured in 5mM glucose DMEM either alone (untreated controls) or in the presence of palmitate or Rosiglitazone either alone or in combination. The addition of 0.4 mM palmitate to MIN6 cells incubated in 5 mM glucose caused a significant decrease in cell viability when compared to untreated controls, an effect blocked by the addition of Rosiglitazone (see Figure 58. Inhibition of p38 Protects MIN6 Beta Cell Viability from Palmitate on page 148). The Rosiglitazone alone treated group had a cell viability level similar to untreated controls. So far, the trend remains as seen previously whereby Rosiglitazone protects beta cells from palmitate induced decrease in cell viability.

To investigate the involvement of the p38 pathway in cell viability we treated cells with 10 µM Rosiglitazone, 0.4 mM palmitate and 20 µM of the p38 inhibitor (SB203580) either alone or in combination as indicated (see Figure 58 on page 148). The palmitate and SB203580 co-incubated group had shown an increased cell viability when compared to palmitate alone (p<0.01) but viability was not as high as the untreated controls. The addition of Rosiglitazone to the palmitate and SB203580 group caused a significant increase in cell viability (p<0.01); levels comparable with untreated controls. This leads us to deduce that the inhibition of the p38 pathways protects MIN6 beta cell viability from palmitate.

2.3 The Modulation of the p38 Pathway by Palmitate and Rosiglitazone in MIN6 beta cells

Other pathways involved in cellular stress and apoptosis include the p38 mitogen-activated protein kinase (p38MAPK) pathway which we touched on briefly in Chapter 3. The p38 MAPKs are kinases belonging to the MAPK family and they respond to stress stimuli (cytokines, heat shock, and osmotic shock) as well as being involved in cell differentiation and apoptosis (Chai and Liu 2007). Our published work using western blots carried out in our group has shown that
Figure 58. Inhibition of p38 Protects MIN6 Beta Cell Viability from Palmitate

MIN6 cells were cultured in 5 mM glucose DMEM with the addition of 0.4 mM palmitate, 10 µM Rosiglitazone, and/or 20 µM SB203580 as indicated. After 72 hours, cells were harvested and MTT analysis performed to determine cell viability. Samples were analysed in duplicate and results obtained from at least 3 separate experiments. Values are expressed as a percentage of untreated control. Error bars represent standard deviation. **p<0.01.
Rosiglitazone rapidly down-regulated levels of the p38 total protein, ~50% decrease, occurring after 2 hours, potentially providing a level of beta cell protection (p<0.005) (Ali, Landy et al. 2008).

To determine the modulation of the p38 pathway by palmitate and Rosiglitazone more western blot analyses were carried out of cytoplasmic extracts from MIN6 cells cultured in 5 mM glucose DMEM in the presence of 0.4 mM palmitate. They indicated a significant up-regulation in phosphorylated p38 protein in ratio to total p38 protein (see Figure 59. Palmitate Modulates p38 in MIN6 Beta Cells on page 150). This increase (~1.5 fold) occurred in a time dependant manner from 6 to 72 hours. This was supported using densitometric analysis of the ratio of phosphorylated p38 protein to total p38 protein (see Figure 59 on page 150). Phosphorylated p38 indicates the activation of this stress-activated pathway (see Chapter3). This has shown that palmitate exposure negatively affects beta cells by activating such stress pathways.

Stimulation of the cells with Rosiglitazone however had a different effect. Western blot analysis (see Figure 60 on page 151, PANEL A) indicated a significant down-regulation in the phosphorylated p38 protein in ratio to the housekeeping gene GAPDH (see Figure 60 on page 151, PANEL B). A similar pattern was observed when investigating the total p38 protein levels. Both occurred in a time-dependant manner from as early as 6 hours until the last time point observed of 72 hours. This was supported using densitometric analysis of the ratio of phosphorylated p38 protein to GAPDH and the ratio of total p38 protein to GAPDH (see Figure 60 on page 151, PANEL C).
Figure 59. Palmitate Modulates p38 in MIN6 Beta Cells

MIN6 cells were cultured in 5 mM glucose DMEM alone (-) or in the presence of 0.4 mM palmitate as indicated. Cytoplasmic extracts were prepared and analyzed by western blotting using a specific phospho-p38 antibody or a specific total p38 antibody as indicated (PANEL A). Densitometric analysis of 3 separate experiments, with values expressed as a ratio of phospho-p38 to total p38, relative to untreated are represented (Panel B). Error bars represent standard deviation. *p<0.05 & **p<0.01 (Ali, Landy et al. 2008).
Figure 60. Rosiglitazone Modulates p38 in MIN6 Beta Cells

MIN6 cells were cultured in 5 mM glucose DMEM alone (-) or in the presence of 10 µM Rosiglitazone as indicated. Cytoplasmic extracts were prepared and analyzed by western blotting using a specific phospho-p38 antibody or a specific total p38 antibody, or GAPDH antibody as indicated (PANEL A). Densitometric analysis of at least 3 separate experiments, with values expressed as the ratio of phospho-p38 to GAPDH (PANEL B), or total p38 to GAPDH (PANEL C), relative to untreated are represented. Error bars represent standard deviation. *p<0.05,**p<0.01(Ali, Landy et al. 2008).
2.4 Modulation of Bcl2 mRNA Levels by Palmitate and Rosiglitazone in MIN6 Beta Cells

In terms of beta cell inflammation, death and apoptosis regulation we have seen pathways and proteins such as p38, AMPK, CK2, NFκB and UCP2 involved. Other apoptotic proteins such as the Bcl2 apoptotic protein family have long been implicated in cellular death and apoptosis and that is one of the causes of diabetes mellitus (O'Brien, Harmon et al. 1997) and that the fatty acid palmitate can induce apoptosis (De Pablo, Susin et al. 1999). Many studies have indicated that along with the decrease in Bcl2/Bax mRNA and protein levels, there was evidence of DNA fragmentation (Mizuno, Yoshitomi et al. 1998). Another question presents itself; can we mimic the effects of palmitate on Bcl2 mRNA levels in our model? More importantly will Rosiglitazone modulate these pathways in our model?

In order to determine any modulation of Bcl2 mRNA levels by palmitate and Rosiglitazone in MIN6 beta cells, real time PCR was carried out on MIN6 cells stimulated with 0.4 mM palmitate and 10µM Rosiglitazone either alone or in combination as indicated (Figure 61. Real-Time PCR Analysis of Bcl2 mRNA Expression Levels for MIN6 Cells Stimulated with Palmitate and Rosiglitazone, Either Alone or in Combination, for 24hours on page 153). In MIN6 cells treated for 24 hours with palmitate, Bcl2 mRNA levels were decreased significantly in comparison to untreated controls (p<0.01), an effect abolished by co-incubation with Rosiglitazone (p<0.05). Rosiglitazone addition alone showed Bcl2 expression levels similar to untreated controls indicating it alone has no effect. It has been previously suggested that free fatty acid induced beta cell defects are in part dependent on Bcl2 expression (Hockenbery, Nuñez et al. 1990). Our data therefore shows that in keeping with the literature we can see that palmitate does in fact decrease the levels of pro-apoptotic Bcl2 mRNA but more importantly that we have demonstrated that in our model the agent Rosiglitazone protects from this palmitate-induced decrease by modulating this pathway suggesting a therapeutic targeted effect.
Figure 61. Real-Time PCR Analysis of Bcl2 mRNA Expression Levels for MIN6 Cells Stimulated with Palmitate and Rosiglitazone, Either Alone or in Combination, for 24hours

MIN6 cells were cultured in 5mM glucose DMEM in the presence or absence of 0.5mM palmitate, 10μM Rosiglitazone either alone and in co-incubation. Figure is representative of total Bcl2 mRNA fold changes following Pfaffl method analysis of C<sub>T</sub> values following changes in raw C<sub>T</sub> values (delta C<sub>T</sub>. ΔC<sub>T</sub>). Values are expressed as a percentage of untreated controls and data represent at least 3 separate experiments with error bars representing standard deviation, **p<0.05.
We have investigated the modulation of Bcl2 protein by palmitate and Rosiglitazone and we have attempted to optimize anti-body concentrations to aid with visualizing the co-staining of Bcl2 and Bax proteins within our MIN6 beta cell model data not included). However, as we had been unable to optimize the visualization of distribution of these proteins using confocal microscopy, and as time was of the essence, we then decided to elucidate if we could observe any changes on the Bcl2/Bax ratio and detect any change in protein level via western blotting. This is because studies have suggested that it is the Bcl2/Bax ratio which carries much of the weight in the determination of cell fate (Mehmeti, Lenzen et al. 2011).

To decipher the effect of our stimuli on the Bcl2/Bax cellular ratios, MIN6 cells were cultured in 5 mM glucose DMEM and stimulated with palmitate and Rosiglitazone, either alone or in combination, for 24 hours as before (see Figure 62. Rosiglitazone Modulates the Ratio of Bcl2/Bax Protein Expression Levels in MIN6 Beta Cells on page 155). Cytoplasmic extracts were made and tested for Bcl2 levels which revealed a decrease in the palmitate sample (Lane 2), an effect blocked with Rosiglitazone co-incubation (Lane 4) which showed Bcl2 protein levels similar to untreated controls (Lane 1). The same membrane was stripped and re-probed for Bax. This showed uniform Bax protein expression in all treatment groups (Lanes 1-4); all relative to the housekeeping gene α-Tubulin. Stripping and re-probing the membrane to detect both Bcl2 and Bax on the same membrane (and thus on the same sample) allowed for the accurate comparison for changes on protein level. In the palmitate treated sample (Lane 2), the ration of Bcl2 to Bax seemed to possibly favour apoptosis with low anti-apoptotic Bcl2 protein levels and high pro-apoptotic Bax protein levels. This is consistent with our previous qPCR data highlighting that palmitate decreased Bcl2 mRNA and this effect was blocked by the addition of Rosiglitazone (see Figure 61 on page 153).
Figure 62. Rosiglitazone Modulates the Ratio of Bcl2/Bax Protein Expression Levels in MIN6 Beta Cells

MIN6 cells were incubated in 5 mM glucose DMEM in the presence of 0.4 mM palmitate or 10μM Rosiglitazone either alone or in combination as indicated. Cytoplasmic extracts were prepared and analyzed by western blotting using specific Bcl2, Bax and α-Tubulin specific antibodies as indicated. Protein sizes were marked against a Full Range Rainbow Marker; Bcl2 showing at ~28kDa, Bax at ~20 kDa and α-Tubulin at ~50kDa. The data represents at least 3 separate experiments.


3. Discussion

Thus far, we have discussed many proteins and their involvement in the regulation of beta cell processes varying from efficient cellular functionality to cellular death. Following from our previous investigations in the previous chapters, we have in this chapter investigated the effects of palmitate and Rosiglitazone on NFkB, p38MAPK (both the phosphorylated and total p38 protein levels), Bcl2 and Bax. We have dissected these pathways using both inhibitors and stimulators and utilized a multitude of techniques such as MTT assays, qPCRs, western blots and confocal microscopy in order to obtain a clearer picture as to how the stimuli actually affect the beta cell and what changes occur following any effect observed in our MIN6 model.

We have looked at the inflammatory protein NFκB and demonstrated that in MIN6 cells treated for 24 hours with palmitate, there was an observed nuclear localization of NFκB as it moved from the cytoplasm when compared to all the other treatments groups, particularly the palmitate and Rosiglitazone co-incubated groups (see Figure 55 on page 144). There was a marked decrease in the intensity of cytoplasmic staining in the palmitate treated sample whereas it was more uniform in all the other groups. It was very interesting to observe that in the sample co-incubated with both palmitate and Rosiglitazone, Rosiglitazone was able to prevent this palmitate-induced nuclear localization, which is indicative of the activation of NFκB, providing a more uniform distribution of NFκB in the cell as evident by the staining (see Figure 56 on page 145). The trend from our western blotting data supports this (see Figure 57. Rosiglitazone Modulates NFκB Protein Expression in MIN6 Beta Cellson page 146). The trend was consistent in all the blots carried out (data not included) but should time have allowed more repeats would have been done for a definitive statistical analysis following densitometry. The transcription factor NFκB regulates multiple pro-inflammatory genes that can contribute to islet destruction (Liuwantara, Elliot et al. 2006). NFκB is composed of a heterodimer of p65 and p50 subunits in most cell types and is sequestered in the cytoplasm by its inhibitor proteins, the IκBs (Hiroaki, Hiroaki et al. 2006).
1999). Upon the phosphorylation of I-κB by I-κB kinase, I-κB is degraded, allowing NFκB to move to the nucleus (Higa, Shimabukuro et al. 2005). A number of studies have shown that the blocking of NFκB activation or signalling protects beta cells against apoptosis (Maedler, Sergeev et al. 2002) and possibly prevent cytokine-induced destruction sequentially facilitating islet transplantation (Giannoukakis, Rudert et al. 2000). Thus our data suggests another mechanism by which Rosiglitazone can guard beta cell mass against free fatty acid induced damage and apoptosis. In the literature Rosiglitazone has been shown to reduce glucose-induced hypertrophy in other cell types, for instance in adipocytes (Han, Subramanian et al. 2007). Research on pancreatic beta cells from transgenic mice expressing a non-degradable form of IκBα (RIP-mlκBα mice) have also observed an effect associated with NFκB. These mice were more susceptible to killing by the pro-inflammatory cytokines tumour necrosis factor (TNF-α) plus interferon alpha (IFN-γ) but were more resistant to anti-inflammatory cytokine IL-1beta than normal beta cells (Kim, Millet et al. 2007). This also implicated the p38 mitogen-activated protein kinase highlighting the value of p38 since it is thought to be accountable for some of the pro-apoptotic function in pancreatic beta cells and that it may contribute to the pathogenesis of autoimmune type 1 diabetes as well as type 2 diabetes.

We have verified much exciting data. We have previously seen from our published work discussed in Chapter 3 that Rosiglitazone had rapidly down-regulated levels of the p38 total protein, ~50% decrease, occurring after 2 hours, potentially providing a level of beta cell protection (Ali, Landy et al. 2008) Our data in this chapter provide even more insight. Firstly that palmitate significantly increased the phosphorylation of p38 which is indicative of its activation. This was in a time dependant manner lasting up to 72 hours (see Figure 59 on page 150). We have then shown interestingly and contrastingly that Rosiglitazone caused the opposite effect and reduced significantly the phosphorylation of p38 also in a time dependant manner (see Figure 60 on page 151). The same marked and significant effect of Rosiglitazone was seen with the total
p38 protein where Rosiglitazone decreased the total protein expression level which was extremely exciting to see. In terms of cell viability, to investigate the involvement of the p38 pathway we treated cells and found that palmitate with the addition of the p38 inhibitor SB203580 had resulted in an increased cell viability when compared to palmitate alone but viability was not as high as the untreated controls (see Figure 58 on page 148). The addition of Rosiglitazone to the palmitate and SB203580 group caused a significant increase in cell viability; levels comparable with untreated controls. This leads us to deduce that the inhibition of the p38 pathway protects MIN6 beta cell viability from palmitate. As the p38 mitogen-activated protein kinase (p38MAPK) pathway which we touched on briefly in Chapter 3 is highly involved in cellular stress and apoptosis (Chai and Liu 2007) our data proved fascinating.

Looking more into the regulation of inflammation and death, we probed some of the cellular death pathways and regulatory proteins involved. Bcl2, Bax, and Bcl-X\textsubscript{L} are members of the expanding Bcl2 family that play key roles in the regulation of apoptosis as they are thought to regulate apoptosis via both homo- and hetero-dimerization (Hsu, Wolter \textit{et al.} 1997). Apoptosis is the process of programmed cellular self-destruction and death and genes such as Bcl2 and Bax respectively are known to both hinder and encourage apoptosis A wealth of literature has demonstrated that apoptosis induced in pancreatic beta cell lines involves the apoptotic pathways through the Bcl2 and Bax genes (Mizuno, Yoshitomi \textit{et al.} 1998). In this chapter we have seen that in MIN6 cells treated for 24 hours with palmitate Bcl2 mRNA levels were decreased significantly in comparison to untreated controls (p<0.01), an effect abolished by co-incubation with Rosiglitazone. Rosiglitazone addition alone showed Bcl2 expression levels similar to untreated controls indicating it alone has no effect (see Figure 61 on page 153). We then deciphered the western blot technique to see if any changes did occur following stimulations. This was because many studies aimed at scrutinizing apoptosis found that in most apoptotic insulin producing beta cells there were always detectable levels of Bacl2 and Bax and in the isolated
islets following culture with free fatty acids which resulted in both the insulin content and Bcl2 staining being decreased (Hanke 2001). Our data suggested that in the palmitate sample, there was a decrease in Bcl2 protein expression when compared to all other treatment groups. Bax was uniform throughout all the samples as was the housekeeping gene α-tubulin (see Figure 62 on page 155). With the Bcl2/Bax ratio in mind (Mehmeti, Lenzen et al. 2011) as mentioned previously, reduced Bcl2 expression and a higher Bax expression as seen in the palmitate treated sample will push the overall ratio in favour of the pro-apoptotic Bax protein and potentially pushing the cell towards apoptosis. This effect was blocked with Rosiglitazone Many blots were done (data not shown) with the same trend but densitometric analysis was not possible due to sometimes high background/noise on the photographic film skewing the reading. However, the data from both the qPCR and western blot techniques used support and complement each other. In addition to the consistency of the trends observed, the data provides confidence that Rosiglitazone was able to protect from much of the palmitate-induced damage.

As discussed in previous chapters protection of the vulnerable beta cell, which is subject to much stress stimuli in disease states such as diabetes, is needed. This necessitates the need to unveil more key mechanisms vital for beta cell function. In the previous chapters, we have identified key signalling pathways stimulated and the specific target proteins modified by our therapeutic agent Rosiglitazone which protected the beta cell from the palmitate-induced damage. Beta cell protection is crucial but is not the full story. Along with protection, there is a critical need to increase beta cell number and mass as a means of combating diabetes whether it is type1 (Emamaullee and Shapiro 2007) or type 2 diabetes (Asghar, Yau et al. 2006) as it is that loss of beta cells that ultimately leads to diabetes and encouraging a slow but steady rate of beta cell replication is the aspiration behind much of the research in therapeutics (Brennand and Melton 2009). With this in mind, we began to explore the effects of our stimuli on our MIN6 beta cell model and to determine what cell growth/proliferation pathways may be modified.
CHAPTER 6

Beta Cell Growth and Proliferation

1. Introduction

We have seen evidence from the literature and supported thus far by our studies that beta cell protection and rejuvenation are mandatory in combating diabetes. It therefore is reasonable to yearn for the discovery of agents that not only exert these positive effects but agents that may enhance beta cell longevity. We have established that agents such as Rosiglitazone have positive effects on beta cells; ranging from protection from detrimental effects of saturated free fatty acids such as palmitate, to stimulation of many key pathways essential for beta cell function. We therefore need to investigate these patterns further and draw more conclusions elucidating more of these desired effects. It is noteworthy to mention that this not only highlights the positive effects of such agents as the TZD Rosiglitazone but more importantly what pathways that are affected and how they affect the beta cell. This enhances our knowledge of what pathways to target and how to manipulate them, deciphering the mechanisms in the process, in order to ascertain new angles for therapy and combating diabetes.

When talking about diabetes both type 1 and type 2, beta cell growth and proliferation pathways are important. The inflammation and death of the beta cell is the ultimate problem which is present in both types of the disease state. Whether it is in a protection from elevated circulating lipid levels as in type 2 diabetes or from an autoimmune inflammation and death perspective, increasing beta cell numbers and mass or longevity is key in treatment. This is particularly interesting from a pre-transplant perspective, when an increase in beta cell number/mass is very much desired. It therefore was intriguing to look into whether agents such as Rosiglitazone with positive effects such as those observed and discussed in the last three chapters may affect beta cell
longevity in our model. We thus carried out preliminary investigations whereby cell viability was compared between MIN6 pancreatic beta cells treated and pre-treated with Rosiglitazone all in relation to the untreated controls over a total time course of 9 days.

2. Results

2.1 Effects of Rosiglitazone Treatment and Pre-treatment on Beta Cell Viability

For Rosiglitazone treatment versus pre-treatment studies, in order to determine the effect of Rosiglitazone treatment/co-incubation versus pre-treatment on beta cell viability, cell counts were carried out daily. MIN6 cells were cultured in 5mM glucose DMEM and there were two groups; one group was not pre-treated with Rosiglitazone for 72 hours prior to cellular counts (see Figure 63. Cell Viability Counts to Determine the Effects of Rosiglitazone Treatment on MIN6 Beta Cells on page 162) and the other group was pre-treated with Rosiglitazone for 72 hours prior to cellular counts (see Figure 64. Cell Viability Counts to Determine the Effects of Rosiglitazone Pre-treatment on MIN6 Beta Cells on page 163). Following the 72 hours, cells were either cultured in 5 mM glucose DMEM alone or with the addition of Rosiglitazone for 6 more days (144 hours) giving a total study time course of 9 days (216 hours).

Our initial preliminary study has revealed that the pre-treatment of beta cells with Rosiglitazone has increased beta cell number in comparison to the untreated controls. Rosiglitazone also increased beta cell number and longevity in the pre-treated group when compared to the co-incubated group. If time had allowed, more repeats and statistical analysis using ANalysis Of VAriance (ANOVA) would have been used to test for significance. The trend however is consistent within the experimental set up and the Rosiglitazone effects on beta cell viability are in line with what we have seen in our previous data. It is therefore feasible to comment on the trend observed bearing in mind the considerable indications of the data with regards to the positive effects of Rosiglitazone on beta cell viability and longevity.
Figure 63. Cell Viability Counts to Determine the Effects of Rosiglitazone Treatment on MIN6 Beta Cells

MIN6 cells were cultured in 5mM glucose DMEM alone for 72 hours then with the addition of 10μM Rosiglitazone for a further 6 days. Cell counts were then performed to determine cell viability. Values are mean ± SEM, expressed as a percentage of control. Results were obtained from 2 separate experiments.
Figure 64. Cell Viability Counts to Determine the Effects of Rosiglitazone Pre-treatment on MIN6 Beta Cells

MIN6 cells were cultured in 5mM glucose DMEM with the addition of 10µM Rosiglitazone for 72 hours then with or without the addition of 10µM Rosiglitazone for a further 6 days. Cell counts were then performed to determine cell viability. Values are mean ± SEM, expressed as a percentage of control. Results were obtained from 2 separate experiments.
In Figure 63, on page 162, showing the effect of Rosiglitazone treatment on MIN6 beta cells, following 72 hour culture of MIN6 cells in 5mM glucose DMEM only, Day 1 cell count was set to 100% and all other day cell counts were calculated to read as a percentage of this control. In Day 2, we observed an increase in cell number, slightly more evident in the Rosiglitazone treated group. Day 3 and Day 4 saw the same trend. By Day 6, the untreated cells continue to divide and grow but in the Rosiglitazone treated group, the cell numbers started to decline. This trend continues to Day 6 which was the end of the time course studied. In Figure 64, on page 163, showing the effect of Rosiglitazone pre-treatment on MIN6 beta cells, following 72 hour culture of MIN6 cells in 5mM glucose DMEM and Rosiglitazone, Day 1 cell count was set to 100% and all other day cell counts were calculated to read as a percentage of this control. In Day 2, we observed an increase in cell number, slightly more evident in the Rosiglitazone treated group. Day 3, Day4, Day 5 and Day6 have all shown a marked difference in the cell counts between the untreated and Rosiglitazone treated groups. The cell numbers did increase in the untreated group throughout the time course but the increase in cell numbers was not as high as the numbers for the Rosiglitazone treated group. Day 6 does saw a decrease in untreated cell count when compared to Day 5. In the Rosiglitazone treated group, there is a slight decrease in cell number but the cell numbers are still high; set between the cell numbers of Day 3 and Day4.

We can see that, as a whole, cells cultured in Rosiglitazone in the 5mM glucose DMEM 72 hours prior to the 6 day time course studies (see Figure 64 on page 163), have shown higher cell numbers when compared to cells that were not pre-treated with Rosiglitazone (see Figure 63 on page 162). It was also seen that the continual culture of MIN6 cells in Rosiglitazone after thief culture in Rosiglitazone for 72 hours had helped in increasing and maintaining cell numbers at high levels when compared to the untreated sample. In Figure 63, on page 162, where cells were not cultured with Rosiglitazone for 72 hours before the cell counts were carried, the untreated cells continued to grow and increase in number more that the Rosiglitazone treated group. In
Figure 64, on page 163, where cells were cultured with Rosiglitazone for 72 hours before the cell counts were carried, the untreated cells has failed to grow at the fast rate seen with the Rosiglitazone treated group. This further lends towards the argument that Rosiglitazone pre-treatment followed by continual Rosiglitazone treatment not only increases cell numbers more that in the untreated groups but that is aids in maintaining cell numbers at high levels at the end of the rather long time course studied of 9 days.

2.2 Effects of Casein Kinase 2 (CK2) on Beta Cell Viability

To determine the effects of CK2 on beta cell viability following palmitate and Rosiglitazone stimulations, MIN6 beta cells were incubated in 5mM glucose DMEM medium for 72 hours either alone (untreated controls) or with the addition of 0.4 mM palmitate, 10 µM Rosiglitazone and 20 µM TBB either alone or in combination as indicated (see Figure 65. MTT Viability Assays to Determine the Effects of Rosiglitazone and Palmitate on MIN6 Beta Cell Viability and the Possible Involvement of the CK2 Pathway on page 166). Cell viability was measured by MTT viability assay. As seen previously palmitate addition decreased cell viability significantly, an effect once again blocked by Rosiglitazone. TBB and Rosiglitazone co-incubation with palmitate indicated that Rosiglitazone was able to protect MIN6 cell viability from the detrimental effects of palmitate but although cell viability was significantly higher than in the presence of palmitate alone (p<0.05), when compared to the protective effects of Rosiglitazone alone, Rosiglitazone was not able to provide a complete protection of MIN6 viability in the presence of the CK2 inhibitor TBB (p<0.01). The data therefore lends towards the speculation that the protective effects of Rosiglitazone from the damaging effects of palmitate may be in part reliant on CK2 activation. Bearing in mind that CK2 activity assay was at a 2 hour time point and the viability effects are at 72 hours, keeping with previous viability assay time points, this leads to another suggestion. The data suggests that the means by which Rosiglitazone can overcome the observed inhibition of CK2 activity by TBB may be at least partly time dependent.
Figure 65. MTT Viability Assays to Determine the Effects of Rosiglitazone and Palmitate on MIN6 Beta Cell Viability and the Possible Involvement of the CK2 Pathway

MIN6 cells were incubated in 5 mM glucose DMEM with the addition of 0.4 mM palmitate, 10 µM Rosiglitazone and 20 µM TBB either alone or in combination as indicated. After 72 hours, cells were harvested and MTT analysis performed to determine cell viability. Results obtained from at least 3 separate experiments and values are expressed as a percentage of untreated control. Error bars represent standard deviation. *p<0.05, **p<0.01 (Ali, Landy et al. 2008).
2.3 Effects of Rosiglitazone on Serine/threonine p70S6 Kinase (S6K)/mTOR Activity

A pathway immensely involved in cellular proliferation is the mTOR pathway. The mammalian target of rapamycin complex 1 (mTORC1) pathway has integrating inputs from nutrients and growth factors required for cell growth. This kinase targets the substrate S6 ribosomal protein. Phosphorylation of S6 induces protein synthesis in a signalling pathway that includes mTOR which can be activated in distinct ways (Zamzami, Brenner et al. 1998). S6K is thought to control protein translation (Ferrari and Thomas 1994) and glucose-induced pro-insulin biosynthesis has been shown to be mediated through ATP modulation of the mTOR/p70S6K pathway in MIN6 cells and islets (Saxena, Houk et al. 2003). Rapamycin is an immunosuppressant used as an agent to treat islet transplant recipients but it has been known to have detrimental effects on islet growth and proliferation and in turn function (Aronovitz, Josefson et al. 2008). We therefore wanted to decipher whether this activation of mTORC1 and S6K occurs in our model following Rosiglitazone stimulation.

To determine the effects of Rosiglitazone on S6K/mTOR activity, MIN6 cells were incubated in 5 mM glucose DMEM alone (untreated controls) or treated with 10 μM Rosiglitazone for 1 hour. Cells treated with 25 mM glucose acted as a positive control and cells treated with 100 nM Rapamycin (an inhibitor of mTOR) acted as a negative control. Whole cell extracts were then prepared for western blot and densitometry analysis then followed for total S6K protein levels and activated phosphorylated S6K levels (see Figure 66. Effects of Rosiglitazone on the mTOR Pathway and the Phosphorylation of S6K in MIN6 Beta Cells on page 168). The addition of Rosiglitazone increased the pS6K/S6K ratio significantly (p<0.05) within 1 hour in comparison to both the untreated and the negative controls. These effects of Rosiglitazone on the phosphorylation of S6K phosphorylation were mimicked by the 25 mM glucose treated sample, i.e. the positive control. The addition of Rapamycin significantly reduced the ratio of phosphorylated S6K (pS6K) to total S6K protein when compared to untreated controls.
Figure 66. Effects of Rosiglitazone on the mTOR Pathway and the Phosphorylation of S6K in MIN6 Beta Cells

MIN6 cells were cultured in 5mM glucose DMEM (untreated); or stimulated for 1 hour with 25mM glucose (positive control), 100nM rapamycin (negative control) or 10μM rosiglitazone as indicated. Whole cell extracts were prepared and analysed by western blotting using specific antibodies for phospho S6K and total S6K. Densitometry was performed and values are expressed as a ratio of phospho S6K to total S6K, relative to untreated control. Data represent at least 3 separate experiments with error bars representing standard deviation, *p<0.05.
3. Discussion

We have demonstrated in this chapter the potentially positive effect of Rosiglitazone on beta cell number and how it not only is able to increase beta cell numbers but is able to maintain cellular growth and proliferation for a long period of time. In Figure 63, on page 162, the question of why at Day 5 and Day 6 the cell counts in the Rosiglitazone treated group were lower than the cell count for the untreated group could be explained by the fact that by Day 7 of the experimental set up (3days prior to cell count + Day1-4), there was no media change for the MIN6 beta cells. This will reduce the nutrients available for cellular growth.

In addition, physiologically, Rosiglitazone would be used to treat individuals with type 2 diabetes where beta cell function and number is compromised and not healthy untreated cells as within the experimental set-up. Therefore for the long duration of the study, incubation with the drug alone might have this effect. That said, the difference in question is not large as in Figure 63, on page 162, the maximum cell count for Day 6 is (~500 x 10^5 cells) for the untreated group and (~400 x 10^5 cells )for the Rosiglitazone treated group. It may still give rise to the question as to why this effect not observed in Figure 64 on page 163. As mentioned previously, the cell numbers on Figure 73 reflect cell counts of cells pre-treated with Rosiglitazone. As Rosiglitazone increases and protects beta cell number and viability as seen from our data in the previous chapters, the 72 hour incubation period where cells were pre-treated with Rosiglitazone could have potentially encouraged cell growth. That in addition to the change of media at the end of the 72 hour pre-treatment had a synergistic effect of increasing and maintaining beta cell number for the duration of the time course studied. By examining this pattern further we were able to determine some mechanisms implicated.

It is known that in a state of overweight and in the presence of the resultant increasing insulin demands as those associated with type 2 diabetes, compensatory beta cell growth occurs.
Proliferative pathways such as the CK2 and mTOR/S6K are therefore pivotal. The proliferative actions of insulin and insulin-like growth factors are mediated by more intricacies e.g. the complex IRS-2-PI3K-Akt pathway of pleiotropic insulin signalling (Huypens 2007). However, sustained activation leads to negative feedback via the mTOR-induced proteasomal degradation of insulin receptor substrate-2 (IRS-2). So in states of overweight or increased nutrition the mTOR pathway provides a control mechanism. Consequently the knowledge of how agents like Rosiglitazone modify pathways such as the CK2 and S6K/mTOR pathways still remains invaluable. Recent studies have shown that incretins like GLP and GLP-1 were found to increase IRS-2 levels in beta cells by acting through the cAMP-PKA pathway, whereas leptin inhibited PTEN activity via CK2-dependent pathways (Huypens 2007).

The literature has indicated that CK2 is part of this complex which has implications in cancer biology and general cellular metabolism, linking the latter to diabetes (Panova, Panov et al. 2006). We published that Rosiglitazone stimulation provoked a significant increase in CK2 activity, approximately 3 fold (p<0.01), an effect blocked by Compound C (Ali, Landy et al. 2008). This suggests that CK2 is a possible downstream target of AMPK in this pathway activated by Rosiglitazone. Contrastingly the addition of TBB completely blocked CK2 activity. Intriguingly, Rosiglitazone addition was able to overcome the inhibitory effects of TBB on CK2 activity which proposes that Rosiglitazone may be able to directly modulate the affinity of the CK2 enzyme to the TBB inhibitor and that Rosiglitazone stimulates CK2 enzyme activity in an AMPK dependent manner. In type 2 diabetes setting with increased circulating lipids, does this have an effect? Can this link between stimulating such pathways like CK2 and AMPK have any positive effects on the detrimental effects of palmitate on cell viability? With this in mind we carried out cell viability MTT assays to ascertain if there was any link and whether CK2 signalling plays a role in the longer term protective effects of Rosiglitazone against the detrimental effects of palmitate. Our data lends towards the speculation that the protective effects
of Rosiglitazone from the damaging effects of palmitate may be in part reliant on CK2 activation. Bearing in mind that CK2 activity assay was at a 2 hour time point and the viability effects are at 72 hours, keeping with previous viability assay time points, this leads to another suggestion. The data suggests that the means by which Rosiglitazone can overcome the observed inhibition of CK2 activity by TBB may be at least partly time dependent. We then explored what other pathways involved in cell growth and proliferation could be implicated? We therefore looked at the S6K/mTOR pathway.

In terms of type 1 diabetes and islet transplants, progressive decline in islet function is a major obstacle to transplant success, so again proliferative pathway stimulation is very much wished for. It therefore can be suggested that agents such as Rosiglitazone could potentially be used as tools to increase cellular growth and proliferation as well as increase beta cell longevity as seen from our pre-treatment versus co-incubation with Rosiglitazone investigations. The mTOR pathway was also modified by our agent Rosiglitazone. We have seen that the addition of Rosiglitazone increased the pS6K/S6K ratio significantly (p<0.05) within 1 hour in comparison to both the untreated controls and the negative controls treated with Rapamycin. These effects of Rosiglitazone on the phosphorylation of S6K were mimicked by the 25 mM glucose treated sample and contrasted by the addition of Rapamycin which significantly reduced the ratio of phosphorylated S6K (pS6K) to total S6K protein when compared to untreated controls. The data not only indicated that Rosiglitazone had activated S6K in MIN6 beta cells but it did so within a very short time span, 1 hour.

The ability of Rosiglitazone to cause all these significant changes and the speed by which the changes occur surely highlight not only how valuable these pathways are to cellular proliferation and longevity but also how effective agents such as Rosiglitazone are in activating such important pathways. We have now begun to have more of an understanding of some of the pathways
investigated, details that were previously yet to be explored, pathways that are so imperative for beta cell growth, proliferation, energy balance and viability. Knowing how agents like Rosiglitazone can affect these pathways is crucial in delineating target molecules and proteins in cellular pathways within the pancreatic beta cell. The enables us to possess the correct tools to best manipulate cellular pathways aiming for further beta cell rejuvenation and longevity and consequently improving our therapeutic agents in the process.

In all the chapters discussed thus far, we have unravelled many positive effects of Rosiglitazone on pancreatic beta cells and have unveiled a multitude of signalling pathways. We have explored numerous signalling molecules and pathways and elucidated whether they are PPARγ- dependant or PPARγ- independent. This is all very positive for type 2 diabetes therapies. What about type 1? We have mentioned previously that type 1 diabetes also suffers from the death and destruction of the beta cell and the ultimate resultant apoptosis (see, Type 1 Diabetes p 16). This in turn means that beta cell number and mass, as well as growth and proliferation, are compromised. We also know that in type 1 diabetes there is a persistent problem which is that of a consistent lack of donors (Baertschiger, Berney et al. 2008). This therefore means that the emphasis of the means to preserve pancreatic beta cells and enhance their longevity, and in turn function, is very strong. Even following transplantation there is one prevalent factor which determines greatly the success of the islet transplantation process; hypoxia.

We have discussed the effects of hypoxia in the introduction (see Hypoxia, p 66 pointing out the negative effects low oxygen levels have on beta cell viability). As we have deciphered many of the positive effects of Rosiglitazone on beta cells in the last few chapters, we were intrigued as to whether our agent is able to modify responses to hypoxia and if so what other molecular pathways may we be able to delineate. Prior to that, we had to establish some baseline responses.
CHAPTER 7

Hypoxia and Neogenesis

1. Introduction

We have seen evidence from the literature and supported thus far by our studies that beta cell protection and rejuvenation are mandatory in combating diabetes. We have continued with our fervent search of agents that not only exert positive effects on beta cells but agents that may enhance beta cell longevity and how they do so. From our data we have established that agents such as Rosiglitazone have positive effects on beta cells; ranging from protection from detrimental effects of saturated free fatty acids such as palmitate to stimulation of many key pathways essential for beta cell function. In the process we have also highlighted some proteins and pathways that are important for Type 1 Diabetes and islet transplant problems faced. Beta cell growth and proliferation along with re-vascularisation both remain a soaring priority. In this chapter we aim to investigate pathways that are of interest to this progress and follow on some ideas that seem to flow nicely from our work. These investigations hopefully shed more light on our search for pathways that benefit the beta cell and facilitate us to draw more conclusions elucidating more desired effects.

Hypoxia, or low oxygen levels, is a rate-limiting-step in pancreatic islet transplantation. Failure of grafted islets is often due to hypoxia and this is particularly crucial in the initial phase immediately following transplantation (Ko, Ryu et al. 2008; Kugelmeier, Nett et al. 2008; Maillard, Sanchez-Dominguez et al. 2008). Hypoxia along with many cellular inflammatory and apoptotic events all contribute to a poor islet yield post-transplantationally. Limiting this hypoxia might therefore limit the damage, especially by targeting the hypoxia-induced cellular pathways that cause apoptosis (Lau, Henriksnäs et al. 2009); supported immensely by re-vascularisation.
Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen that has an essential role in both vasculogenesis and angiogenesis (Ouchi, Shibata et al. 2005). VEGF has many isoforms and VEGF–A seems to be closely associated with beta cell revascularization, in particular the VEGF$_{121}$, VEGF$_{145}$ and VEGF$_{165}$ isoforms which are all secreted, the latter being the most abundant and biologically active (Cross, Richards et al. 2007). The islets of Langerhans naturally have a rich oxygen supply and hold an intense vascular structure where VEGF-A plays an indispensable role in the formation of this structure. VEGF has also been shown to promote the revascularization of transplanted islets, thereby reducing the initial number of beta cells required to prevent diabetes (Stagner, Mokshagundama et al. 2004).

Interestingly, it has been recently shown that Programmed Cell Death 4 (PDCD4) knockout non-obese diabetic mice are resistant to Type 1 Diabetes and have high levels of both pro-inflammatory and anti-inflammatory cytokines (Ruan, Kameswaran et al. 2009). PDCD4 is a gene encoding a protein localized to the nucleus in proliferating cells with an expression level modulated by cytokines in natural killer and T cells (GeneCards 2011). It functions as a tumour suppressor and inhibits tumour promoter-induced neoplastic transformation and it is a primary target and a limiting step for controlling protein translation as well as being involved in neogenesis and is potentially linked to VEGF (Ferris, Marriott et al. 2011).

Hypoxia-Inducible Factor 1 (HIF-1) has also been implicated in the link between VEGF and PDCD4 in terms of type 1 diabetes and islet revascularization as HIF-1 is activated by hypoxia (Cheng, Ho et al. 2010). Hypoxia activates this heterodymeric transcription factor consisting of an oxygen-dependent HIF-1α subunit and a constitutive partner HIF-1β (ARNT) causing its stabilization and its translocation from the cytoplasm to the nucleus and the HIF-1α sub-unit particularly is up-regulated to mediate this metabolic adaptation (Moritz, Meier et al. 2002).
It has been recently shown that PDCD4 knockout non-obese diabetic mice are resistant to type 1 diabetes and have high levels of both pro-inflammatory and anti-inflammatory cytokines (Ruan, Kameswaran et al. 2009). PDCD4 is also linked to neogenesis and VEGF (Ferris, Marriott et al. 2011). As VEGF is linked to HIF-1 and both to hypoxia, with the addition of the involvement of PDCD4 investigating the interactions of these proteins in beta cells is of great interest and importance when exploring therapy options in terms of islet grafts and transplants for individuals with type 1 diabetes. This is mainly because of the critical post-transplantation stage where the optimum and prolonged function and survival of the pancreatic beta cell is of utmost importance. This includes the need for a fast and sufficient revascularization (Kampf, Mattsson et al. 2006).

2. Results

2.1 Effects of Rosiglitazone Treatment on VEGF mRNA Expression

In order to determine the effects of Rosiglitazone treatment on VEGF mRNA expression semi-quantitative PCR was carried out on MIN6 cells stimulated with Rosiglitazone at the 24, 48 and 72 hour time point. We have shown that in our MIN6 model in vitro, 48 hour stimulations with Rosiglitazone have increased VEGF mRNA expression levels significantly in comparison to untreated controls, (p<0.05) (see Figure 67. Real-Time PCRs of VEGF mRNA Expression Levels for MIN6 Beta Cells Stimulated with Rosiglitazone on page 176). VEGF mRNA expression levels did not change significantly following 24 hour stimulation and by 72 hours had returned to control levels (Ferris, Marriott et al. 2011).

It has been previously suggested that post-transplantationally fast and sufficient revascularization is essential (Joseph, Koshkin et al. 2004). Our data does indeed suggest that the use of Rosiglitazone increases expression of VEGF potentiating vascularisation in our MIN6 pancreatic beta cell model which is extremely encouraging from a of Type 1 Diabetes perspective.
MIN6 cells were cultured in 5mM glucose DMEM and stimulated with 10µM Rosiglitazone as indicated for 24, 48 & 72 hours. Following RNA extraction and PCR analysis, fold change values were calculated. Values are expressed as a percentage of untreated controls and data represent at least 3 separate experiments with error bars representing standard deviation, *p<0.05.

Figure 67. Real-Time PCRs of VEGF mRNA Expression Levels for MIN6 Beta Cells Stimulated with Rosiglitazone
Moving on from this exciting piece of data we carried on and investigated whether there was an increase in mRNA expression only or whether there was a detectable change on a total protein level. We then used the visual route and investigated this further using confocal microscopy.

2.2 Effects of Rosiglitazone Treatment on VEGF Protein Expression: Normoxia vs Hypoxia

Hypoxia, or low oxygen levels, is a rate-limiting step in pancreatic islet transplants as they often fail due to this subjected hypoxia and this is particularly crucial in the initial phase immediately following transplantation (Ko, Ryu et al. 2008; Kugelmeier, Nett et al. 2008; Maillard, Sanchez-Dominguez et al. 2008). Hypoxia along with many cellular inflammatory and apoptotic events all contribute to a poor islet yield post-transplantationally (Lau, Henriksnäs et al. 2009). Limiting this hypoxia might therefore limit the damage, especially by targeting the hypoxia-induced cellular pathways that cause apoptosis. In MIN6 cells treated for 24 hours with 10 µM Rosiglitazone there was an observed difference in VEGF protein distribution under the normoxic conditions when compared to the hypoxic conditions (see Figure 62. Rosiglitazone Modulates the Ratio of Bcl2/Bax Protein Expression Levels in MIN6 Beta Cells on page 178). This was at the 1:50 dilution of the VEGF specific primary antibody. Under normoxic conditions there was a definite VEGF staining in the nucleus (PANEL A) and in the cytoplasm (PANEL B). The overall whole cell distribution was evident by the staining of the protein in the overlay image of the nuclear and cytoplasmic staining together in (PANEL C). It is clear that the protein is present throughout the whole cell. Under the hypoxic conditions however, using the same 1:50 dilution of VEGF antibody, a different pattern emerged. A nuclear staining was detected as previously (PANEL A) but the cytoplasmic staining not (PANEL B). The overlay of both (PANEL C) shows a distribution pattern similar to the nuclear staining in (PANEL A) which is clearly different by comparison to the pattern seen under normoxic conditions comparing both PANEL Cs.
Figure 68. Confocal Microscopy Imaging to Determine the Effect of Rosiglitazone on VEGF

Confocal microscopy imaging of MIN6 cells cultured for 24 hours in 5mM glucose DMEM with the addition of 10μM Rosiglitazone. Cells were stained using the VEGF primary antibody (at the 1:50 dilution) and TRITC-conjugated secondary specific antibodies in normoxic and hypoxic conditions. PANEL A shows blue nuclear DAPI staining, PANEL B shows cytoplasmic staining and PANEL C shows and overlay of both PANEL A & B. The data represents 3 separate experiments. Bar = 250μm.
We have mentioned that this endothelial cell mitogen that has an essential role in both vasculogenesis and angiogenesis (Ouchi, Shibata et al. 2005) and that VEGF has many isoforms (VEGF$_{121}$, VEGF$_{145}$, VEGF$_{165}$ and VEGF$_{189}$ which are all secreted (Cross, Richards et al. 2007). Is this then what we are seeing? Is hypoxia activating VEGF as a whole and causing it to be secreted? We thought we should try a different VEGF antibody dilution in case a different pattern arises.

Images where the 1:50 antibody dilution was used have shown a clear difference and more distinct patterns. Having optimized the antibody concentrations it seems that the data presented an interesting suggestion. Treatment of our MIN6 beta cell model with our agent Rosiglitazone seemed to have caused VEGF stimulation under hypoxic conditions. Mechanistically it would have been cleaved and sequestered from the cytoplasm resulting in the lack of its visualization as it was not stained. That said, we can begin to outline that the treatment with Rosiglitazone not only affects VEGF mRNA levels but affects the protein on a cellular level. The effects of hypoxia affect VEGF clearly and Rosiglitazone can modulate that. This is novel and exciting looking at Type 1 Diabetes and vascularisation. What about the other side, neogenesis and the involvement of PDCD4. Does Rosiglitazone have any effect on this?

2.3 Effects of Rosiglitazone Treatment on PDCD4 mRNA Expression

In order to determine the effects of Rosiglitazone treatment on PDCD4 mRNA expression semi-quantitative PCR was carried out on MIN6 cells stimulated with Rosiglitazone at the 24, 48 and 72 hour time point ($p<0.05$) (see Figure 69. PCR Analysis of PDCD4 mRNA Expression Levels for MIN6 Cells Stimulated by Rosiglitazone on page 180) (Ferris, Marriott et al. 2011). In our study, Rosiglitazone stimulation increased PDCD4 mRNA levels significantly at all the time points studied when compared to untreated controls; the effect however was more pronounced at 24 hours.
MIN6 cells were cultured in 5mM glucose DMEM in the presence or absence of 10μM for 24, 48 and 72 hours as indicated. Figure is representative of total PDCD4 mRNA fold changes following densitometrical analysis. Values are expressed as a percentage of untreated controls and data represent at least 3 separate experiments with error bars representing standard deviation, **p<0.01, (Ferris, Marriott et al. 2011).
From a PDCD4 point of view, perhaps collectively the data may suggest that one of the mechanisms of action of Rosiglitazone is via degrading PDCD4 in an mTORC1/S6K dependant manner allowing protein translation and improving the overall well-being of the beta cell. In cancer cells for instance, hypoxia inhibits mTORC1 activation (Guertin and Sabatini 2007) thus S6K cannot phosphorylate PDCD4, it cannot be degraded and thus will result in hindering protein translation. This is beneficial in cancer. If Rosiglitazone can reverse these hypoxic effects on S6K i.e. if it can still phosphorylate S6K causing PDCD4 phosphorylation and thus degradation allowing protein synthesis, then this will make Rosiglitazone an immensely beneficial agent for the management Type 1 Diabetes and the faced hypoxia which compromises greatly the already challenging islet transplantation process. Our studies with VEGF and PDCD4 and their activation by Rosiglitazone at the same time points confirms that the sustained increase in PDCD4 expression is not due to the general nonspecific upregulation of genes (Ferris, Marriott et al. 2011).

3. Discussion

In the literature it is indisputable that there is much evidence to signify that low revascularization and impaired oxygenation seems to prevail in intra-portal transplanted islets and this is clinically problematic (Emamaullee and Shapiro 2007). Hypoxia has a negative marked effect on the grafted islets’ non-functionality yet the mechanisms still remain unclear (Ko, Ryu et al. 2008). Many pathways are engaged in the regulation of cellular processes within the pancreatic beta cell. AMPK for instance being a regulator of energy homeostasis and activated by metabolic stresses such as hypoxia is thought to play a role in hypoxia regulation implicating oxidative stress roles in acute and severe hypoxic injury (Ryu, Lee et al. 2009). This is of considerable significance at the critical post-transplantation stage where the optimum and prolonged function and survival of the pancreatic beta cell is of utmost importance. This necessitates the need for a fast and sufficient revascularization (Kampf, Mattsson et al. 2006). This is also of particular consequence when
immuno-suppressants are to be taken into consideration for they are an essential part of the treatment process for individuals with type 1 diabetes undergoing islet transplants. In terms of type 1 diabetes, there is a problem of not enough VEGF released to overcome the hypoxic challenge (Stagner, Mokshagundama et al. 2004) thus there is still a rather large space and need for therapeutic agents that aid in the enhancement of this process. We have seen in our investigations that Rosiglitazone caused a significant increase in VEGF mRNA (see Figure 67 on page 176) and we have highlighted the importance of such a finding.

We then investigated further to elucidate whether the protein was translated utilizing western blotting techniques. Here we were faced with many difficulties. First, we wanted to ascertain the basic level of VEGF expression in our MIN6 beta cell model. We have preformed and analysed numerous blots (over 60 blots - data not included). We have tried untreated controls compared to samples treated with Rosiglitazone, palmitate, the PPARγ inhibitor GW9662 and the mTOR inhibitor Rapamycin. Rapamycin has been shown to potently inhibit early and mid stages of VEGF-A164–driven angiogenesis essential for mother vessel formation which increases vascular permeability (Xue, Nagy et al. 2009). We have also tried western blot analysis with cytoplasmic and whole cell extracts. We have tried a time course of Rosiglitazone stimulation (9 time points – maximum 72 hours) and we have also tried hypoxic samples and normoxic samples. We have even changed the primary antibody hoping for a better or clearer signal. To reduce background signals detected we blocked the membranes with high milk concentrations (up to 15%) and for long periods of time (up to 2 hours) as well as blocking with BSA. Different concentrations of acrylamide for the SDS-PAGE gel were tried but all with minimum and unconvincing outcomes. Should we have had the time and resource ELISA analysis would have been carried out. Eventually, we focused on the confocal imaging and the PCR amplifications.
Research has demonstrated that in pancreatic islet beta cell cultured and treated with exogenous VEGF there was a resultant observed dilation of the blood vessels (Olsson, Maxhuni et al. 2006). Animal studies concluded that the islet vascular system is fundamental for normal insulin secretion and also indicated that the roles of VEGF-A and islet vasculature in the regulation of beta cell mass depend on stimuli for the islets thus agents encouraging this, such as Rosiglitazone, are highly desirable (Watada 2010). Other animal studies have shown that mice deficient in VEGF-A have low insulin gene expression levels and consequently presented with impaired glucose tolerance (Jabs, Franklin et al. 2008). Slow healing wounds and wound infection are also a significant concern for individuals with diabetes as they can lead to limb amputations and that can also be attributed to the compromised blood vessel formation in response to ischemia (Thangarajah, Vial et al. 2010). Combined studies also indicated that smaller islets have a higher capacity to stimulate re-growth of blood vessels following transplantation and interestingly transplanted islets have increased concentrations of VEGF as a programmed defence and compensation mechanism (Johansson, Sandvik et al. 2006).

In terms of neogenesis, PDCD4 and VEGF are both very important. VEGF-A signalling is required to induce endocrine differentiation and in the developmental stages of the pancreas epithelial production of VEGF-A determines the spatial organization of endothelial cells which in turn limits acinar differentiation of the epithelium (Pierreux, Cordi et al. 2010). Endothelial cells are of course mandatory for initiating pancreas development from the endoderm as they control the endocrine islet function after birth as well. We have seen that in our investigations, that the thiazolidinedione Rosiglitazone specifically upregulated PDCD4 gene expression in beta cells in a time-dependent manner (see Figure 69 on page 180) (Ferris, Marriott et al. 2011). We have also published data indicating 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 by high glucose concentrations. However, intracellular localisation of the PDCD4 protein was
shown to be differentially regulated by these stimuli in beta and ductal cells. 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.

Studies have shown that the in vivo ablation of VEGF-A expression (of floxed VEGF-A alleles) caused a reduction in endothelial development and excessive acinar differentiation, an effect reversed by VEGF-A overexpression (Pierreux, Cordi et al. 2010). That said, overexpression of VEGF can conversely have undesirable effects where cancers are concerned. Vascularisation and thus growth of the tumour cellular clusters is unwanted. This is a link to the programmed cell death 4 (PDCD4). Tumour suppressor oncogenes such as PDCD4 were specifically involved in the promotion of malignant conversion of some cancers hereby these differentially expressed genes were found to be involved in apoptotic processes (along with MAPK, VEGF and beta cell receptor signalling pathways) and other functions associated with cell growth, signal transduction and immune system activation (Fang, Li et al. 2008).

As VEGF is linked to HIF-1 and both to hypoxia, tackling these angles is of great interest when exploring therapy options in terms of islet grafts and transplants for individuals with type 1 diabetes. In other cell types such as human breast cancer cells, human liver cells and kidney cells, it seems that HIF-1α has positive effects. This is because hypoxia induces target genes including VEGF, PGK-1 and GLUT-1 by stabilizing HIF-1α; the latter would otherwise be degraded by a ubiquity-dependent proteasome under normoxic conditions (Miao, Ostrowski et al. 2006). However in beta cells, increased HIF-1α in vitro has been associated with beta cell apoptosis (Moritz, Meier et al. 2002), and its suppression showed an opposite effect in islet transplants (Miao, Ostrowski et al. 2006).
In terms of beta cell differentiation, recent evidence suggests that hypoxia controls this process and these effects are mediated by HIF-1 (Heinis, Simon et al. 2010). Islet transplant hypoxia effects mediated by HIF-1 were also attributed to a link with the release of intracellular Ca\(^{2+}\) stores, which activates calpain-10-dependent apoptosis in beta cells subjected to prolonged block of ryanodine receptor (RyR)-gated channels. Blocking this calcium flux increased the expression of HIF-1 beta which provided a mechanism for the signalling pathways activated when intracellular Ca\(^{2+}\) homeostasis and metabolic activity are suppressed in diabetes and islet transplantation (Dror, Kalynyak et al. 2008). Elevated glucose levels detect the transactivation of HIF-1α which regulates VEGF expression resulting in ROS induction. HIF-1 also has an imperative role in beta cell function through regulation of ARNT expression, a gene important for beta cell function, indicating a potential therapeutic target for preventing beta cell dysfunction (Thangarajah, Vial et al. 2010). Transplanted islets that strongly express HIF-1 were associated with beta cell death and in turn a decrease in insulin production; however, that was the case until adequate revascularization was established. This was complementary to observations whereby the early suppression of HIF-1alpha resulted in less beta cell death, which had the desirable effect of minimizing early graft failure (Miao, Ostrowski et al. 2006). We began to investigate HIF-1 levels in our MIN6 beta cell model using nuclear extracts for western blots with and without Rosiglitazone to establish a baseline for a time course but in the optimization phase, time was a limiting factor. The aim was to establish a baseline then attempt to visualise levels using confocal microscopy as with VEGF. This however has led to a new exciting PhD project in our laboratory.

Contrary to the recent negative studies with Rosiglitazone, particularly in relation to cardiovascular complications, some studies have shown that Rosiglitazone facilitates Akt rephosphorylation and inhibits apoptosis in cardiomyocytes during hypoxia/reoxygenation and promotes cardiac myocyte survival (Kilter, Werner et al. 2009). This is excellent from a diabetic cardiovascular complication perspective. Studies have also indicated that PPARγ ligands
attenuated hypoxia-induced pulmonary vascular remodelling and hypertension by repressing the oxidative and proliferative signalling pathways (Nisbet, Bland et al. 2010). The use of Rosiglitazone prevented chronic hypoxia induced pulmonary hypertension by altering PPARγ endothelin-1 and VEGF expression levels (Kim, Lee et al. 2010). This is indicative of the beneficial use of Rosiglitazone, particularly in terms of VEGF and vascular benefits. Perhaps systemically over prolonged periods of time there might be some complications but this does not eradicate the benefits of such effective agents but in fact highlights the need for their further investigation and modifications to improve therapies further.

Previously in our studies, at the various time points examined (24, 48 and 72 hours), we have observed and demonstrated in the previous chapters that Rosiglitazone in our MIN6 model had increased cell viability and cellular organelle ultra-structure from palmitate-induced damage. We have demonstrated that Rosiglitazone treatment of pancreatic beta cells had restored the balance of the palmitate-induced stress as it modified inflammatory proteins such as NFκB and modified the balance of apoptotic proteins such as Bcl2 and Bax. We have also provided evidence that Rosiglitazone modulated energy, stress and transport systems through the modification of UCP-2 mRNA, p38 MAPK and GLUT2 mRNA levels respectively. Furthermore, we have confirmed how Rosiglitazone affected cellular growth and proliferation pathways via the CK2 and S6K/mTOR pathway. Here in this chapter we add to a growing body of evidence indicating the diversity of therapeutic agents such as Rosiglitazone. We have established that Rosiglitazone was able to increase mRNA levels of important vascularisation factors such as VEGF, potentially contributing to an overall much desired increase in vascularisation and that Rosiglitazone specifically upregulated PDCD4 gene expression in beta cells in a time-dependent manner, our study being the first to show PDCD4 expression in pancreatic beta cells (Ferris, Marriott et al. 2011). This has led to another new exciting PhD project in our laboratory.
CHAPTER 8

General Discussion

Main Outcome

Thus far, we have exemplified in this thesis that Rosiglitazone has many direct effects on our pancreatic MIN6 beta cell model, exhibited through various mechanisms by stimulating a multitude of signalling pathways; both PPARγ-dependant and PPARγ-independent.

Rationale and Findings

Obesity has become a common yet continually increasing problem worldwide (Haslam and James 2005; The National Institute for Health and Clinical Excellence (NICE) 2011). Diabetes mellitus, especially type 2 diabetes, is a multifactorial disease state. There is a wealth of literature supporting the involvement of the role of obesity and elevated saturated free fatty acids in the development and progression of the disease ultimately leading to beta cell death (El-Assad, Buteau et al. 2003; Welters 2006; Lupi and Del Prato 2008; Liang, Zhong et al. 2011). The regulation of lipogenesis and lipotoxicity affect pancreatic beta cell viability and insulin production (Morgan, Dhayal et al. 2008) and studies have demonstrated palmitic acid-induced apoptosis in other beta cell lines (Liang, Zhong et al. 2011). Lipotoxicity studies have also demonstrated that lipid-induced pancreatic beta cell dysfunction is likely to be one contributor to the complex collection of genetic and metabolic attack resulting in the relentless decline in pancreatic beta cell (Giacca, Xiao et al. 2011). Beta cell viability is affected greatly by free fatty acid toxicity as seen by our cell viability data thus far. The mechanisms underlying the toxicity of the saturated free fatty acids may reflect a decrease in protein processing triggering apoptotic responses by virtue of enhanced endoplasmic reticulum stress (Karaskov, Scott et al. 2006). In
terms of glucotoxicity, studies reveal that insulin resistance can impair glucose-stimulated insulin secretion, in the process, undermining beta cell compensation and leading to hyperglycaemia (Asghar, Yau et al. 2006). Both factors synergistically amount to the glucolipotoxicity theory whereby the development of progressive deterioration of beta cells accelerates via glucolipotoxicity causing loss of beta cell function and reduction in beta cell mass (Lencioni, Lupi et al. 2008). Understanding the causes for beta cell failure is therefore of capital importance to develop new and more effective therapeutic strategies.

Studies on beta cell lines demonstrated that unsaturated free fatty acids and their methyl-esters bring forth cytoprotection and relief of ER stress (Dhayal and Morgan 2011) which may be involved in mediating their effects on viability (Diakogiannaki and Morgan 2008). The studies propose that the cytoprotective effects are due to the presence of specific fatty acid binding sites having loose, but defined, structural criteria. This resultant ER stress is believed to contribute to insulin insufficiency connected with type 2 diabetes through a decrease in cell mass (Deguil, Pineau et al. 2011). The mechanism(s) which leads to these observed cytoprotective events following incubation with long-chain mono-unsaturates is thought to probably be receptor-mediated involving antagonistic actions on the effector arm of the ER stress pathway (Morgan 2009). Research utilizing INS-1E cells and human islets exposed to oleate and/or palmitate has shown that palmitate induced more apoptosis (markedly activated the IRE1, PERK and ATF6 pathways caused by sustained depletion of ER Ca^{2+} stores) whereas oleate caused a milder PERK and IRE1 activation and comparable ATF6 signalling (Cunha, Hekerman et al. 2008). Monounsaturates such as oleate are thought to potentially wield their anti-apoptotic actions via the regulation of the activity kinases involved in mediating the phosphorylation of eIF2 alpha (Diakogiannaki and Morgan 2008; Diakogiannaki, Welters et al. 2008) and that they may be able to promote cell viability and mitogenesis by a mechanism that does not require metabolism of triacylglycerol (TAG) (Diakogiannaki, Dhayal et al. 2007).
Both exogenous and endogenous fatty acids affect beta cell metabolism as well as gene and protein expression; long-chain acyl-CoA (formed from either endogenously synthesized or exogenous fatty acids) controls activation of specific isoenzymes of PKC (protein kinase C), modulation of ion channels, protein acylation, ceramide formation and/or NO-mediated apoptosis, and transcription factor activity (Newsholme, Keane et al. 2007). Studies propose that in vitro, beta cells respond differentially to long chain fatty acids, such that saturated species like palmitate, making them lipotoxic whereas long chain mono-unsaturated fatty acids, such as oleate, may provide cytoprotection (Morgan and Dhayal 2010). Research has associated chronic palmitate with caspase activation and induction of apoptosis (Welters, Diakogiannaki et al. 2006). Chronic administration of palmitoleic acid was found to decrease insulin resistance and hepatic lipid accumulation in KK-Ay Mice with genetic type 2 diabetes (Yang, Miyahara et al. 2011). Along with the lipotoxicity, glucotoxicity also has an effect as increased glucose levels induced ER stress markers in type 2 diabetes islet cells making the cells more susceptible to ER stress induced by metabolic perturbations (Marchetti, Bugliani et al. 2007). Pharmacological interventions that include Rosiglitazone such as Avandamet, a combination therapy of Rosiglitazone and Metformin, have been shown to be an effective and well tolerated therapy in patients with type 2 diabetes (Derosa, Tinelli et al. 2009; Derosa and Maffioli 2011). Rosiglitazone has also been shown to be positively synergistic with other drugs like glimepiride (Derosa, Gaddi et al. 2006). Along with the overall effects of Rosiglitazone on insulin sensitivity, alteration in adipocyte metabolism and hepatic gluconeogenesis our group had previously published Rosiglitazone has direct effects on specific beta cell gene expression (Richardson, Campbell et al. 2006).

We have seen significant in this study so far increases in the activation of vital fuel-sensing cellular systems, such as AMPK, supported by phosphorylation of downstream targets such as ACC. We have seen that Rosiglitazone was able to protect cellular ultra-structure from damage
by palmitate and mirrored by our cell viability data. Rosiglitazone also affected the glucose transport system by modulating GLUT2 mRNA levels. We have also reported that Rosiglitazone caused the decrease of many stress-related proteins and kinases such as p38MAPK (both the total and the phosphorylated protein) which were increased by palmitate. Rosiglitazone also provided protection from the palmitate-induced activation of inflammatory proteins such as NFκB. In addition, Rosiglitazone modulated mitochondrial enzyme levels such as UCP2 mRNA levels and apoptotic proteins such as Bcl2 and Bax. In this thesis we have provided visual evidence in our data of the extent of the palmitate induced damage on beta cell viability and ascertained that Rosiglitazone protected pancreatic beta cells from the time-dependant palmitate-induced damage which destroyed completely the internal cellular structure as evident from the TEM images. We have also confirmed that treatment with Rosiglitazone alone does not affect beta cell ultrastructure and the cellular morphology and structure is similar to untreated controls throughout all the time courses of the study. Functional organelles such as mitochondria, endoplasmic reticulum, ribosomes and golgi apparatus were destroyed following palmitate treatment in a time dependant manner. It is not therefore unreasonable to suggest that this might potentially have negative effects on insulin secretion, central for beta cell function. The data from our study make Rosiglitazone a desirable agent for the protection of pancreatic beta cells. In terms of cellular growth and proliferation Rosiglitazone caused a significant stimulation of the mTORC1 pathway and its downstream target S6K kinase. Interestingly, Rosiglitazone significantly increased VEGF mRNA levels; which is potentially beneficial from a type 1 diabetes vascularisation perspective, where the promotion and the maintenance of blood vessel formation is of utter importance. In terms of neogenesis, we have seen that in our MIN6 model, Rosiglitazone increased significantly the mRNA levels of PDCD4 in a time dependant manner. This published data by our group was the first study to confirm that the sustained increase in PDCD4 expression was not due to the general nonspecific upregulation of genes, as Rosiglitazone stimulates beta cell gene expression, but rather that the activation of VEGF and PDCD4 by Rosiglitazone occurred at specific time
points. Finally, as an overview of the total collective effects of Rosiglitazone on MIN6 beta cells, we have suggested that pre-treatment with Rosiglitazone can increase cell viability and longevity which are all very significant positive effects of Rosiglitazone. As Rosiglitazone is a pharmacological approach to the therapy of type 2 diabetes where high levels of circulating fatty acids is the main reason for beta cell death, we needed to establish a few baselines in our MIN6 model. We first had to establish to what extent was the cell viability affected by the fatty acid palmitate. We then ascertained the extent of the protective effects of Rosiglitazone. Mechanistically we wanted to confirm whether the palmitate-induced beta cell loss and viability was due to necrosis or apoptosis in our MIN6 model. Only when we established it was programmed cell death apoptosis that we began to elucidate more of the pathways involved.

With any study conducted, it is unreasonable to not have limitations. Cell viability and structure are essential for cell functionality and initial work by our group in 2006 established that following dose concentration-response analysis, 10 μM Rosiglitazone caused a maximal effect on the Ipf1 gene promoter over 24 hours and other markers of beta cell function (Richardson, Campbell et al. 2006). In this thesis it would have supported our work greatly if we had the time and resource to analyse beta cell function using ELISA and GSIS studies enabling us to attempt to close the link between the changes observed in cell viability and functionality. However, most of our work here was primarily structured to elucidate the extent of the palmitate-induced damage and what effects Rosiglitazone would have, and the pathways implicated. As most of the published work by our group looked previously at beta cell functionality, the core of the work in this thesis was at a cell signalling, cell pathway, transcription and translation scrutinizing level, thus proficiently the previous and current findings complement each other. The clinical significance of our study is noteworthy. Evidence from the literature as mentioned in previous chapters indicate that Rosiglitazone has many beneficial effects on the pancreatic beta cell, both short and long term, as we have demonstrated (Bell 2003) there has been much evidence to support positive
Rosiglitazone effects on beta cell growth and proliferation. However, we have mentioned previously, ‘Rosiglitazone and the Press’ section (p49), that the general overview in the literature and the resultant action to suspend Rosiglitazone from the drug market is thought provoking from a research point of view. This restricted use of Rosiglitazone was official in the later stage of our Rosiglitazone research. We therefore wanted to investigate other avenues by exploring its use as an agent for protecting beta cells and increasing growth and proliferation. This, as mentioned previously, is of great interest from a type 1 diabetes setting and might provide a different therapeutic use. Perhaps Rosiglitazone can be used as an agent to promote cellular growth, proliferation and vascularisation, decrease inflammatory cytokines, suppress stress-related proteins and increase beta cell protection especially in hypoxia. Previous literature does support the potential of Positive effects of Rosiglitazone (Fryer, Parbu-Patel et al. 2002; Bell 2003; Lupi, Del Guerra et al. 2003; Walter and Lubben 2005; Ye, Dzamko et al. 2006; Abaraviciene, Lundquist et al. 2008; Han, Kang et al. 2008; Ji-Wom, Ji-Hun et al. 2009; Schindler, Rieger et al. 2009) but not collectively or not in our MIN6 model with all the optimizations achieved in this study.

Successfully our group had published the study ‘Effects of rosiglitazone and metformin on pancreatic beta cells gene expression’ and other supporting work establishing the direct effects on beta cell specific gene expression by Rosiglitazone (Macfarlane, Campbell et al. 2000; Campbell and Macfarlane 2002; Richardson, Campbell et al. 2006). This had lead to the intriguing questions in this study as to whether Rosiglitazone can provide protection of the beta cell via various mechanisms and pathways. We have been successful in generating interesting data of significance and have presented and published much of our work (Ali, Landy et al. 2008; Ferris, Marriott et al. 2011). So, looking at the title of the research ‘Protection of pancreatic beta cells by Rosiglitazone - mechanisms and pathways’, we can say we have reached our goal. In addition we expanded our study onto other perspectives of the area first examined and managed to highlight...
other potential therapeutic uses outside the type 2 diabetes area for which this agent was originally intended, for instance with our published work regarding proliferation and neogenesis (Ferris, Marriott et al. 2011).

We have tried to keep all variables as physiological as possible such as the concentration of free fatty acids and glucose concentrations. It is known however that the MIN6 pancreatic beta cell model originates from a tumour cell line model. It would have been intriguing to use other cell lines such as human/primary cell lines. It is also unquestionable that beta cells exist as islets in the Islets of Langerhans and thus islets would have been an even better option. It was hoped that the most optimum stimuli concentrations, time scales and conditions would be used at the end of the study cumulatively on the pseudo islets available in the group as pseudo islets provide a more 3-dimensional structure when compared to the MIN6 cell monolayer. Pseudo islets are still considered the primary islet replacement model used in research (Persaud, Arden et al. 2010). There are many cell to cell interactions that govern cellular process (Luther, Davies et al. 2005) as well as important islet cell surface molecules (Flatt, Bone et al. 1985) absent in monolayer studies and these are important. There would be the need to also scrutinize what concentrations are affecting the outer layer of the islet when compared to the inside cell clusters in the middle of the islet. This is very true in the case of hypoxia and was a big part of the thought process when investigating the effects of low oxygen levels on beta cells. Another perspective is that there are also intrinsic humoral and hormonal responses to stimuli which can only exist in vivo. It would have been extremely interesting to use animal models of diabetes. Islets in a dish or bio-reactor still are a step further from islets in an organism because many of the natural intrinsic pathways that are only fully functioning when present in a whole organism basis. However the last publication on our work with PDCD4 was intriguing and took some time. Time and resource aside, looking at diabetes as a disease state, much of the effects of high fat on diabetes are well established and the general anti-diabetic effects of Rosiglitazone are becoming well established.
What we set out to elucidate was the investigations of the specific mechanisms by which stimuli affect the beta cell and explicate more of the beta cell specific responses. It therefore was essential to start at more basic levels, establish baselines for our targets, proteins and pathways, and only then can we justify the use of a more complex system such as islets (pseudo, animal or primary) or animal models as a whole organism. Studies often set out to discover and explore and not necessarily to prove, yet the work presented here is a good testament of a mixture of both.

**General Discussion Elaboration**

Diet and exercise are still the first line non-pharmacological approach to combating diabetes (MacDonald, Philp *et al.* 2006; Wylie-Rosett and Davis 2009; Abete, Goyenechea *et al.* 2011) and pharmacological intervention alone is not the only option. Nevertheless, if it was that simple, diabetes would not still be one of the vastly expanding uncontrollable epidemics worldwide (Diabetes UK 2010; World Health Organization 2011). Obesity has become a common yet continually increasing problem worldwide (Haslam and James 2005; The National Institute for Health and Clinical Excellence (NICE) 2011). However, not everyone can diet and exercise to a level that can prevent type 2 diabetes onset, especially if individuals are predisposed to the disease state, let alone maintain that level of diet and exercise over prolonged periods of time. Mainly this is because such individuals generally suffer from a combination of genetic and environmental factors (Dean and McEntyre 2004; Pereira, Guedes *et al.* 2009) as well as lifestyle factors (Blackburn, Wollner *et al.* 2009; Harrington, Perry *et al.* 2010). As mentioned previously in the Introduction (see Obesity, Morbid Obesity, Exercise on p10) modern times and the sedentary nature of lifestyle it brings with it are both not helping in pointing people in the right direction to manage their diet and exercise (Jones, Roche *et al.* 2009; Zanuso, Balducci *et al.* 2009). Nevertheless, awareness of this issue is increasing and efforts are being made to encourage a reversal of this situation but in the meantime this can only mean one thing; along with
progression of current research, the use of medication and pharmacological intervention is a necessity. Therefore, this dictates the need for highly-specific targeted agents. Therefore, this therapeutic search remains the aim for our research. We have emphasized the positive protective effects of Rosiglitazone on pancreatic beta cells. Despite the fact that the licence for use of Rosiglitazone limits its use, it does not take away from the magnitude of the positive effects observed on beta cells that are much desired for combating diabetes consequently adding great value to the work presented in this thesis.

In view of the fact that diabetes is a multi-factorial disease state, the study presented has delineated a multitude of signalling pathways and mechanisms involved and affected by our stimuli. As we have discussed the pharmacological interventions, we need to understand the pharmacology and electrophysiology of the beta cell. There have been many studies aiming to delineate this and have looked at stripping the functioning of the beta cell to the basic and complex level of ions and ion channel electrophysiology. Research has looked at investigating beta cell ion channels on isolated cells and the role of calcium and potassium channels (Rorsman, Eliasson et al. 2011) and the role of Ca\(^{2+}\)/CaMK(IV)/CREB cascade playing a critical role in the regulation of the insulin receptor 2 (IRS2) expression in beta cells (Persaud, Liu et al. 2011). In the literature, reports suggested that arachidonic acid metabolites and a Ca\(^{2+}\)-independent phospholipase A(2) (iPLA(2)) activity play a role in beta cell apoptosis (Lei, Barbour et al. 2010). The PLA(2) family of enzymes catalyse the hydrolysis of the sn-2 substituent (i.e. arachidonic acid) of membrane phospholipids. As beta cells are enriched in arachidonate-containing phospholipids and express the group VIA iPLA(2)\(\beta\), it has been proposed that iPLA(2)\(\beta\) participates in ER stress-induced beta cell apoptosis (Lei, Zhang et al. 2007) and thus a novel mechanism, involving ceramide generation and triggering of mitochondrial abnormalities, by which iPLA(2)\(\beta\) participates in the beta cell apoptosis process was elucidated (Ramanadham, Hsu et al. 2004; Lei, Barbour et al. 2010). This also affected beta cell mass as it is governed by the
balance between beta cell growth and beta cell death, due to apoptosis (Lei, Zhang et al. 2007). Recent work also highlighted that the activation of phospholipases A(2) (PLA(2)s) leads to the generation of biologically active lipid mediators that can affect numerous cellular events and that iPLA(2)beta-derived products trigger pathways that may lead to beta cell apoptosis during the development of diabetes (Lei, Zhang et al. 2010). Our group has previously published work highlighting the importance of such ions for beta cells by proposing that, like the insulin promoter, glucose regulation of the IAPP promoter is dependent on the activity of PDX1, but unlike the insulin promoter, it additionally requires the activity of another factor, the activity of which is calcium-dependent (Macfarlane, Campbell et al. 2000). The physiology of glucose homeostasis requires the close co-operation of a number of organ systems, humoral secretions and neural signalling complexes which is in addition to the predisposing risk factors (obesity, poor diet, lack of exercise as well as genetic factors) of which many require elucidation as they all elevate the risk of developing type 2 diabetes (Campbell 2003). Preservation of beta cell function is now gaining recognition as a critical target in the management of type 2 diabetes as it has the potential to reduce or stabilise the progression of the disease and to decrease the need for additional oral glucose-lowering agents and/or insulin therapy (Standl 2007).

We have then investigated the palmitate-induced stress-activated p38 MAPK pathway and the potential effects it may have on cell viability. The p38 mitogen-activated protein kinases are kinases belonging to the mitogen-activated protein kinase family and they respond to stress stimuli as well as being involved in cell differentiation and apoptosis (Chai and Liu 2007). We have observed that the p38 inhibitor (SB203580) had no effect on the ability of Rosiglitazone to protect MIN6 cells from the detrimental effects of palmitate indicating this effect of Rosiglitazone on beta cell viability is independent of the p38 pathway (see Figure 15 on page 86). This was very intriguing as literature supports the notion of p38 involvement in palmitate-induced beta cell death (Chai and Liu 2007). Our data provides more evidence of the multitude of pathways
affected by Rosiglitazone; firstly what these pathways are and secondly how exactly they are being affected to ultimately enable us to further understand how to protect the beta cell.

We have mentioned the pivotal role of the fuel sensing AMPK system within the cell (See Introduction p54). Energy level regulation is essential for the functioning of the beta cell and it is inevitable that some of this energy will come from glucose. Some gene regulation occurs in response to glucose (Greenman, Gomez et al. 2007). The protein kinase RNA-like endoplasmic reticulum kinase (PERK) can induce cell death (Herbert 2007). PERK serves as a critical effector of unfolded protein response (UPR)-induced G1 growth arrest due to the loss of cyclin D1 and is activated at low glucose concentrations and that this plays an important role in glucose-regulated protein synthesis in pancreatic beta cells (Gomez, Powell et al. 2008). However glucolipotoxicity exhibits synergistic detrimental effects on ER stress along with the impairment of insulin receptor substrate signalling (Tanabe, Liu et al. 2011) in addition to the shunting of excess amounts of glucose toward reactive oxygen species production worsening beta cell death (Maris, Waelkens et al. 2011).

The saturated free fatty acid palmitate has been known to cause de-differentiation of beta cells and evidence shows that highly abundant beta cell genes like GLUT2 and mGAPDH are down-regulated after lipid treatment (Busch, Cordery et al. 2002). In beta cells, PPARγ and TZDs activate the genes encoding GLUT2 (Kim, Kim et al. 2000). Studies with diabetic or glucose-intolerant mice treated with the PPAR-gamma agonist Pioglitazone and/or a control indicated that isolated islets of Pioglitazone treated mice exhibited significantly improved glycaemic control corresponding to increased serum insulin and enhanced glucose-stimulated insulin release and calcium $\text{Ca}^{2+}$ responses from isolated islets thus showed improved islet function and this was at least partially accredited to the significant up-regulation of the islet genes including GLUT2 (Evans-Molina, Robbins et al. 2009). MIN6 cells stimulated with Rosiglitazone alone for 24, 48
and 72 hours indicated a significant reduction in GLUT2 mRNA levels which occurred in a time
dependent manner (see Figure 34 on page 110). In beta cells, PPARγ and thiazolidinediones
activate GLUT2 encoding genes (Kim, Kim et al. 2000). Some literature suggests that
Rosiglitazone could stimulate the release and synthesis of insulin through the upregulation of
GLUT2 (Hyo-Sup, Jung-Hyun et al. 2008). High doses of Rosiglitazone (100 μM) were shown to
increase GLUT2 mRNA, paralleled by an increase of GLUT2 protein after 24 hours of treatment
(Blumentrath, Neye et al. 2001). In such studies however, non-physiological concentrations were
used to highlight the effects Rosiglitazone might have on GLUT2 levels, whereas we have
attempted to mimic what might be happening under physiological conditions in our beta cell
model.

On being prescribed Rosiglitazone an individual would typically be a person with type 2 diabetes,
with excess circulating glucose levels, thus treatment with agents such as Rosiglitazone will
usually endeavour to reduce glucose levels in a system where there is an existing malfunction in
glucose regulation. As glucose uptake by tissues in a hyperglycaemic state is not much needed,
we could speculate that less GLUT2 expression/activity would be needed. Thus Rosiglitazone
reducing GLUT2 gene expression may not necessarily be seen as a disadvantage. Also, GLUT2
provides facilitated glucose transport, needing energy in the form of ATP involving the AMPK
fuel sensing pathway. In a diabetes setting, the beta cell is already under much stress to meet the
demands for insulin availability thus needing energy for important processes such as insulin
release and exocytosis, which require ATP. Rosiglitazone modulates beta cell energy as we have
seen from our data, for instance with the electron transfer chain and the regulation of UCP2. This
further lends towards the argument that when Rosiglitazone caused the down-regulation of
GLUT2 mRNA levels, it not only potentially reduced glucose uptake which is harmful in a
diabetes setting, but in the process Rosiglitazone was able to ‘save’ beta cell energy levels and
perhaps prioritized where the energy and ATP would needed most. Thus reducing the energy used by GLUT2 facilitated transport for other pathways may not necessarily be a negative effect either.

Other proteins that are involved in energy regulation and oxidative balance are important, especially those present in the energy house of the cell; the mitochondrion. These proteins include the uncoupling proteins. These inner mitochondrial membrane transporters are involved in the control of energy conversion and there is evidence, both experimental and genetic, suggesting that their dysfunctions are linked with metabolic syndrome and obesity (Villarroya, Iglesias et al. 2007). Studied revealed that the simultaneous elevation of glucose and fatty acids is usually sensed by AMPK but in a state of fuel oversupply causes the secondary release of endogenous fatty acids from triglyceride stores, the stimulation of UCP2 expression, mitochondrial uncoupling and the resultant inhibition of GSIS (Poitout 2004). The UCP2 gene interestingly is under the control of PPARs even in tissues such as liver and is the predominantly expressed isoform in the pancreas (Villarroya, Iglesias et al. 2007). Mutations in UCP2 were linked to the incidence of type 2 diabetes (Niño Fong, Fatehi-Hassanabad et al. 2011; Xu, Zhang et al. 2011) and UCP-2 knockout mice had higher levels of reactive oxidative species (Lee, Robson-Doucette et al. 2009). UCP2 seems to be labelled as a negative insulin secretion modulator (Chan, Saleh et al. 2004) and is a major link between obesity, beta cell dysfunction, and type 2 diabetes (Zhang, Baffy et al. 2001). We have shown that in MIN6 cells treated for 72 hours with palmitate UCP2 mRNA levels were elevated significantly in comparison to untreated controls (~4 fold) (see Figure 50 on page 131), an effect abolished by co-incubation with Rosiglitazone. It has been previously suggested that free fatty acid induced beta cell defects are in part dependent on UCP2 expression (Joseph, Koshkin et al. 2004). This in turn leads to impaired insulin secretion (Chan, Saleh et al. 2004). In keeping with current literature trends, our data showing that Rosiglitazone protects from this palmitate-induced increase in UCP2, makes agents such as Rosiglitazone more desirable for the treatment of type 2 diabetes. From a cell signalling perspective, we have also demonstrated that
the addition of the p38 inhibitor (SB203580) alone decreased UCP2 mRNA levels below levels of untreated controls (~0.8 fold), an effect overcome by Rosiglitazone. In our study we have investigated the link between palmitate and p38MAPK induced stress with changes in UCP2 mRNA expression levels and interestingly found that the p38 pathway is not involved in the protective effects of Rosiglitazone from palmitate-induced increases in UCP2 mRNA level. However, using western blots, followed by densitometric analysis, our laboratory had shown that Rosiglitazone rapidly down-regulated total protein levels of the stress activated protein kinase p38 (see Figure 20 on page 93), with a ~50% decrease occurring after 2 hours, potentially providing a level of beta cell protection (p<0.005) (Ali, Landy et al. 2008). Moreover, our data suggested that the protective effects of Rosiglitazone from the palmitate-induced increase in UCP2 mRNA were significantly blocked by the PPARγ antagonist GW9662 while no effect was observed with the AMPK inhibitor Compound C. This suggested that the protective effects of Rosiglitazone on decreasing the palmitate-induced UCP2 expression were PPARγ-dependent. So not only have we examined mitochondrial proteins that are affected by our stimuli but in the process we have highlighted which pathways are affected and which are not. This shows how agents such as Rosiglitazone can modulate multiple pathways including the more traditional PPARγ pathway highlighting the potential diverse use of such agents.

It is evident thus far from our presented data and the literature that mitochondrial energy homeostasis and oxidative stress do impact on beta cell function. Recent research supported the use of anti-oxidants to improve diabetic metabolic alterations by counteracting beta cell dysfunction and loss caused by oxidative stress (Novelli, D'Aleo et al. 2007). Studies reported marked reduction in lipid peroxidation and an improvement in endothelial function as well as endothelial-dependent vasodilatation (Cuerda, Luengo et al. 2011). In our group, we have published work to support the potential positive effects of supplements and minerals, particularly the trace mineral Selenium. We have shown that Selenium had specifically upregulated Ipfl
(insulin promoter factor 1) gene expression, increased both Ipf1 and insulin mRNA levels in MIN6 cells as well as stimulated increases in insulin content and insulin secretion in isolated primary rat islets of Langerhans. This is the first study to implicate Selenium in the regulation of specific target genes suggesting the potential promotion of the overall improvement in islet function (Campbell, Aldibbiat et al. 2008). Along with energy balance, mitochondria and ER function in the regulation of oxidative stress as mentioned above there is also a great need to examine in detail the effects on inflammation and inflammatory markers in diabetes. We have in our study investigated inflammatory markers such as NFκB utilizing confocal microscopy and western blotting techniques. We have observed under confocal microscopy that in MIN6 cells treated for 24 hours with palmitate, there was an observed nuclear localization of NFκB from the cytoplasm when compared to all the other treatments groups (see Figure 55 on page 144 and Figure 56 on page 145), an effect blocked by Rosiglitazone. The transcription factor NFκB regulates multiple pro-inflammatory genes that can contribute to islet destruction (Liuwantara, Elliot et al. 2006). A number of studies have shown that the blocking of NFκB activation or signalling protects beta cells against apoptosis (Maedler, Sergeev et al. 2002). Recently, palmitate has been reported to induce NFκB activation in INS-1 cells (Rakatzi, Mueller et al. 2004) and blocking the NFκB pathway is thought to possibly prevent cytokine-induced beta cell destruction sequentially facilitating islet transplantation (Giannoukakis, Rudert et al. 2000). Using our MIN6 beta cell model, our data therefore suggests another mechanism by which Rosiglitazone can guard beta cell mass against free fatty acid induced damage and apoptosis. Furthermore, the literature has suggested that palmitate increased NFκB activation, ceramide and diacylglycerol mass, and apoptosis and AICAR activation of AMPK or overexpression of AMPK had prevented this effect whereas interestingly, the palmitate induced oxidative stress was not affected by AMPK stimulation (Cacicedo, Benjachareonwong et al. 2011). This adds to the importance of our data highlighting the very fast ability of Rosiglitazone to stimulate AMPK and phosphorylate ACC
and how the use of Compound C has prevented the protective effects of Rosiglitazone on cell viability in our MIN6 model (see Figure 14 on page 84).

Along with NFκB other inflammatory molecules and proteins have been involved in beta cell damage and apoptosis such as interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα) as they have been shown to induce nitric oxide formation (Dunger, Cunningham et al. 1996). It is noteworthy to mention that some cytokines such as interleukin-4 (IL-4) have been reported to provide a protective effect against beta cells death (Kaminski, Welters et al. 2009). Studies also have implicated that the blockade of pathways stimulated by INF and TNFα by the nitric oxide synthase inhibitor N(G)-monomethyl-L-arginine (NMMA) prevented much nitric oxide-induced DNA damage (Rosales, Cunningham et al. 2004). However, as inflammation has a significant impact on beta cell dysfunction in type 2 diabetes data show that some of these defects might be directly counteracted, at least, in part via pharmacological intervention (Marchetti, Lupi et al. 2010). This highlights again how crucial our investigations are in terms of deciphering how agents such as Rosiglitazone are working and elucidating these important inflammatory pathways aiming to unveil new mechanism of action for these therapeutic agents.

As we continued to unveil the mechanisms behind the effects we observed of our stimuli on our beta cell model, we have established many pathways feeding into the regulatory cycle within the beta cell. We have observed effects on cell viability, growth, inflammation and we have also looked at beta cell stress by investigating markers such as the p38MAPK. MAPKs are a family of serine/threonine kinases of three main subgroups; ERK (Extracellular signal–Regulated Kinase), p38 MAPK and JNK (c-Jun N-terminal Kinases) which participate in signalling cascades controlling cellular responses to cytokines and stress involved in cell differentiation and apoptosis (Chai and Liu 2007). In type 2 diabetes many of the detrimental effects of glucolipotoxicity on beta cells are mediated by reactive oxygen species (ROS) and nitrogen free radicals (RNS) and
these molecules have been associated with the regulation of cellular function along with the p38 pathway (Lopes, Oliveira et al. 2008). In this thesis, investigations using western blots indicated a significant up-regulation in phosphorylated p38 protein in ratio to total p38 protein following palmitate treatment which occurred in a time dependant manner, supported by densitometric analysis of the ratio of phosphorylated p38 protein to total p38 protein (see Figure 59 on page 150). Phosphorylated p38 indicates the activation of this stress-activated pathway (see Chapter 3 on page 93). Stimulation with Rosiglitazone indicated a significant down-regulation in the phosphorylated p38 protein, especially in ratio to the housekeeping gene GAPDH (see Figure 60 on page 151). We have also documented that Rosiglitazone rapidly down-regulated p38 levels, ~50% decrease occurring after 2 hours, proposing a level of protection from palmitate-induced p38 detrimental events (Ali, Landy et al. 2008).

We have investigated the more negative effects of the stress and inflammatory pathway, but what about the more positive pathways involving cellular growth and proliferation? In our studies we mentioned the observed Rosiglitazone provoked stimulation of CK2 causing a marked increase in CK2 activity, an effect blocked by Compound C, at a time point and concentration also shown to block ACC phosphorylation, potentially suggesting that AMPK activity lies upstream of CK2 in the signalling events being regulated by Rosiglitazone. CK2 has been implicated in exocytosis of insulin secretory granules, regulating NFκB signalling and crosstalk with the p38 signalling cascade during inflammatory responses to viral infection in vitro and in vivo and that CK2 is a ubiquitous and highly pleiotropic protein kinase (Bortolato, Cozza et al. 2008). This protein kinase targets an immense assortment of substrates positioned in a number of cellular compartments (Filhol and Cochet 2009). It is a stress-activated serine/threonine protein kinase that is oncogenic and frequently over expressed in human tumours of multiple histological origins (Scaglioni, Yung et al. 2008). However, our study is the first to link Rosiglitazone with the
modulation of CK2 signalling (Ali, Landy et al. 2008). This again emphasizes the diversity of the agent Rosiglitazone in regulating beta cell processes.

It has been documented that the onset and progression of type 2 diabetes is caused mainly by beta cell dysfunction participating in the reduction in beta cell mass and number (Ritzel 2009). Apoptotic death of pancreatic beta cells is one of the causes of diabetes mellitus (O'Brien, Harmon et al. 1997). The fatty acid palmitate can induce apoptosis (De Pablo, Susin et al. 1999). It has been suggested that the overall balance is more reliant on controlling apoptosis as the increased apoptosis is eventually not compensated by sufficient beta cell regeneration (Marchetti, Lupi et al. 2010). Beta cell fate is also determined by other proteins that regulate cell death to include the Bcl2 family of proteins which encode a family of mammalian genes that govern apoptosis by controlling mitochondrial outer membrane permeabilization (MOMP) (Lupi, Dotta et al. 2002). They can be either pro-apoptotic (Bax, BAD, Bak and Bok) or anti-apoptotic (Bcl2 proper, Bcl-xL, and Bcl-w) and prolonged exposure to free fatty acids has pro-apoptotic effects on human pancreatic beta cells (Lupi, Dotta et al. 2002). Immunolocalization studies revealed that Bcl2 is an integral inner mitochondrial membrane protein and its over-expression obstructs apoptotic death (Hockenbery, Nuñez et al. 1990). We have found from our HPI studies that beta cell death in our model was through apoptosis and not necrosis (see Figure 13 on page 83). We have shown that in MIN6 cells treated for 24 hours with palmitate, there was a significant decrease in Bcl2 mRNA expression levels in comparison to untreated controls, an effect abolished by Rosiglitazone (see Figure 61 on page 153). The data was mirrored by the trend seen in our western blot analysis of protein expression under the same stimuli pattern whereby Rosiglitazone treatment protected from the palmitate-induced decrease in the anti-apoptotic protein Bcl2. It has been previously suggested that free fatty acid induced beta cell defects are in part dependent on Bcl2 expression (Hockenbery, Nuñez et al. 1990). Our data therefore shows that in keeping with the literature (Lupi and Del Prato 2008; Gurzov and Eizirik 2011) we can see that palmitate does
in fact decrease the pro-apoptotic levels of Bcl2 mRNA but more importantly that we have demonstrated that in our model the agent Rosiglitazone can protect from this palmitate-induced decrease by modulating this pathway suggesting a therapeutic targeted effect.

Electron microscopy on human beta cells specified positive healthy morphological appearances that correlate with the presence of good basal levels of the pro-apoptotic protein Bcl2 in beta cells (Landau, Forti et al. 2006). Studies have demonstrated that apoptosis induced in pancreatic beta cell lines involves the apoptotic pathways through the Bcl2 and Bax genes (Mizuno, Yoshitomi et al. 1998). Studies have highlighted that the Bax/Bcl2 ratio is the responsible factor for the liberation of pro-apoptotic mitochondrial factors which in turn activate the caspase-9 and ultimately caspase-3 causing cell death (Mehmeti, Lenzen et al. 2011). The literature also supports the fact that along with the decrease in Bcl2/Bax mRNA and protein ratio, DNA fragmentation was gradually observed (Mizuno, Yoshitomi et al. 1998). We then took an interest in the Bcl2/Bax ratio. Using western blotting techniques, we observed a noted decrease in the Bcl2 protein in the palmitate sample, an effect blocked with Rosiglitazone. The same membrane stripped and re-probed for Bax showed uniform Bax protein expression in all treatment groups and our data suggested a trend whereby the ratio of Bcl2 to Bax seemed to possibly favour apoptosis; low anti-apoptotic Bcl2 protein levels and high pro-apoptotic Bax protein levels (see Figure 62 on page 155). This is consistent with our previous qPCR data highlighting that palmitate decreased Bcl2 and this effect was blocked by the addition of Rosiglitazone. Our work is relevant and in support of current literature (Mehmeti, Lenzen et al. 2011) highlighting that the determination of cell fate is regulated by apoptosis.

Early work elucidating the damage caused by toxicity to beta cells suggested that the mechanisms and pathways by which agents may provide protection are the same pathways implicated in the pathogenesis of diabetes (Pipeleers and Van de Winkel 1986). It is therefore the aim of much
research, including our own, to encourage the stimulation of the pathways with positive effects on beta cells, ultimately, aiming to increase beta cell number and mass and to protect this increase from potential antagonistic actions of any agents. Growth and proliferation pathways include the S6K/mTOR pathway. Glucose-induced pro-insulin biosynthesis has been shown to be mediated via ATP modulation of the mTOR/p70S6K pathway in MIN6 cells and islets (Saxena, Houk et al. 2003). The phosphorylation levels of ribosomal protein S6 and eukaryotic initiation factor 4E binding protein 1, by the downstream effectors for mTORC1, were up-regulated in transgenic beta cells from transgenic mice that over-express Rheb in beta cells (Hamada, Hara et al. 2009). Studies have shown that ribosomal protein S6 is phosphorylated in vivo by isoforms of p70 S6 protein kinase and p90 ribosomal S6 kinase, and there is good evidence it plays a encouraging role in having influence over pancreatic beta cell size and function (Moore, Xie et al. 2009). Research also indicated that the immunosuppressant Rapamycin has inhibited beta cellulin and insulin-like growth factor-I (IGF-I) which are important for islet cell survival and/or proliferation in INS-1 cells; the effects, in part, were mediated through the mTOR signalling pathway via possible downstream targets such as S6K (Aronovitz, Josefson et al. 2008).

We have shown that in MIN6 cells treated for 1 hour with 100nM Rapamycin, an mTOR inhibitor, the ratio of phosphorylated S6K (pS6K) to total S6K protein was decreased significantly compared to untreated controls (see Figure 66 on page 168). The addition of Rosiglitazone increased the pS6K/S6K ratio significantly within 1 hour in comparison to both the untreated and the negative controls. The data supports current literature showing that high glucose and nutrients stimulate the mTOR pathway (Guim, Marshall et al. 2006). This could be useful for individuals with type 2 diabetes where there is a reduced beta cell mass (Pipeleers, Chintinne et al. 2008) or those with type 1 diabetes where approximately 90% destruction of pancreatic beta cells had occurred (Pipeleers, Veld et al. 2008) to potentially sustain transplants (Purdy 2006). Interestingly our data suggests that Rosiglitazone activates the mTOR pathway within an hour
suggesting the importance of this pathway and suggesting a potential strong clinical therapeutic implication in terms of increasing beta cell mass in islet grafts and transplants.

In pancreatic beta cells sustained AMPK activation exerts some effect on apoptosis mainly under conditions of glucose limitation. In terms of mechanism, it still remains unclear whether Akt activation can counteract AMPK-mediated apoptosis, or whether mTOR activation, which is downstream of Akt, can mediate survival signals. Akt is also known as protein kinase B (PKB) and is made up of three isoforms Akt1, Akt2 and Akt3 (or PKBα/β/γ respectively) which are activated by growth factors and by oncogenic mutations in important upstream regulatory proteins such as Ras, PI3-kinase and tumour suppressor PTEN (Hers, Vincent et al. 2011). Studies indicate that Akt substrates contribute to cell proliferation, survival and metabolism. Therefore, Akt malfunction can contribute to diseases such as cancer and diabetes. Recent research indicated that expression of a constitutively active form of Akt increases mTOR activity and prevents apoptosis upon AMPK activation, an effect abolished by Rapamycin. Expression of a constitutively active form of the mTOR target S6K or of translation factor eIF4E reduced apoptosis by glucose limitation, and co-expression of S6K and eIF4E protected beta cells to the same extent as active Akt. The protective effects were associated with increased cellular protein synthesis activity. Thus Akt stimulation of mTOR and subsequent activation of the targets by which mTOR affects protein translation are required and sufficient mechanisms for Akt-mediated survival of beta cells undergoing sustained AMPK activation (Cai, Wang et al. 2008).

cAMP and mTOR signalling pathways control a number of critical cellular processes including metabolism, protein synthesis, proliferation and cell survival. Pharmacological elevation of intracellular concentrations of cAMP in mouse embryonic fibroblasts (MEFs) and human embryonic kidney 293 (HEK293) cells have indicated an inhibition of mTORC1 activation via a PKA-dependent mechanism via the PKB, MAPK and AMPK signalling cascades that inhibit
TSC1/2, an upstream negative regulator of mTORC1. However, cAMP may act independently of known regulatory inputs into mTOR. This provides valuable insight into how cAMP signals to mTOR and down-regulates its activity, potentially leading to the identification of novel drug targets to inhibit mTOR thus used for treatment and prevention of human diseases such as cancer (Xie, Ponuwei et al. 2011). The mTOR downstream target S6K is also involved in cAMP stimulations. The ribosomal protein S6 (rpS6) is phosphorylated in vivo by isoforms of p70 S6 protein kinase and p90 ribosomal S6 kinase, and there is good evidence that it plays a positive role in controlling pancreatic beta cell size and function. Studies on MIN6 cells and pancreatic islets show that agents which stimulate increases in cAMP lead to the phosphorylation of rpS6 independently of the activation of the currently known in vivo rpS6 kinases via a pathway sensitive to inhibitors of cAMP-dependent protein kinase (protein kinase A - PKA). Therefore it follows that the PKA pathways provides a potentially central link between cAMP signalling and the protein synthesis regulation (Moore, Xie et al. 2009).

Beta cell size and protein synthesis all are imperative for beta cell function. Pancreatic cell proliferation and replication is pivotal in both type 1 and type 2 diabetes. This is mainly due to the fact that following all interventions, pharmacological and non-pharmacological, when islet transplants are required there is a shortage of donors. In addition, the quality of islets also depends on the donor. Recent research indicates that increased beta cell replication was most often noted in relatively young donors and was associated with decreased inflammatory infiltration of the pancreas (Veld, De Munck et al. 2010; Richardson, Willcox et al. 2011). Thus the quality of transplanted islets should be assessed prior transplantation for beta cell function and survival (Morgan, Diakogiannaki et al. 2009; D'Aleo, Del Guerra et al. 2010). Recent evidence in the literature supports that cells from transformed cell lines such as MIN6 cells have higher proliferation rates in comparison to primary islets but this is often accompanied by lower levels of insulin content and release although the secretory functions are not often influenced by
experimental reduction of proliferation (Reers, Hauge-Evans et al. 2011). Although pseudo islets are the primary islets used currently for research (Persaud, Arden et al. 2010) xenografts from other species, tissue stem cells and embryonic stem cells are also all utilized however, of all these groups used, the xenografts are the closest to clinical use but embryonic stem cells are better in providing much higher numbers of cells required for transplantation therapy (Jones, Courtney et al. 2008). Thus, there is a huge positive therapeutic potential for the use of stem cells in diabetes therapy (Burns, Persaud et al. 2006). This becomes more critical for therapies for type 1 diabetes due to the almost total destruction of beta cells.

We have discussed previously the significant negative effects of hypoxia in islet transplants. Hypoxia, or low oxygen, is a rate-limiting-step in pancreatic islet transplants however the mechanisms still remain unknown (Ko, Ryu et al. 2008; Kugelmeier, Nett et al. 2008; Maillard, Sanchez-Dominguez et al. 2008). Pancreatic islets face hypoxia during transplantation as they are separated from their vascular system and here HIF-1α is up-regulated to mediate this metabolic adaptation (Moritz, Meier et al. 2002). VEGF has been shown to promote the revascularization of transplanted islets, thereby reducing the initial number required to prevent diabetes (Stagner, Mokshagundama et al. 2004). Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen that has an essential role in both vasculogenesis and angiogenesis (Ouchi, Shibata et al. 2005). VEGF has many isoforms and VEGF–A seems to be closely associated with beta cell revascularization, in particular VEGF_{121}, VEGF_{145} and VEGF_{165} which are all secreted, the latter being the most abundant and biologically active. VEGF_{189} was also detected in human islets however this isoform is mainly cell attached and membrane anchored unless sequestered or cleaved (Cross, Richards et al. 2007). We have shown that in our MIN6 model in vitro, 48hour stimulations with Rosiglitazone have increased VEGF mRNA expression levels significantly (see Figure 67 on page 176). This is a great indication that Rosiglitazone can be used as an agent to
increase VEGF dependant re-vascularisation which is highly desirable for islet transplants in the treatment of Type 1 Diabetes.

Beta cell mass is governed by numerous processes including cell differentiation, neogenesis, cell proliferation and/or apoptosis as well as increased or decreased cell size i.e. cell hypertrophy or atrophy (Höglund, Mattsson et al. 2009; Masini, Bugliani et al. 2009) in addition to insulin signalling activation (Yesil and Lammert 2008). The specific mechanisms by which beta cells recognise and transform in mass in response to physical demand is a rather complex matter highlighting how exceptionally specialised the beta cell is (Khalaileh, Gonen-Gross et al. 2008). Animal studies show that mice recover spontaneously from diabetes following beta cell regeneration alone (Khalaileh, Gonen-Gross et al. 2008) suggesting that regenerative therapy for diabetes may be a realistic goal. Mechanistically, some procedures involve forced expression of beta cell-specific transcription factors and mature beta cells, although somewhat compromised, are able to proliferate, increasing cell mass (Jun 2008). We have demonstrated that Rosiglitazone modulates multiple signalling pathways; some are PPARγ-dependent and others are PPARγ-independent. Yet, PPARγ still remains a valuable therapeutic target and novel advances in designing selective agonists that fit both alpha and delta but not the gamma subunit of the PPARs were utilized recently aiming to evade the possible side effects when targeting obesity and stroke (Huang, Lee et al. 2010). This explores new avenues in therapy approaches. Due to the mentioned roles of the PPARs as regulators of lipid and glucose metabolism and as the aim in diabetes therapy is to accomplish a range of metabolic effects reducing morbidity and mortality via improvements in levels of insulin resistance, hyperglycaemia, and atherogenic dyslipidaemia. Another perspective is dual action PPARγ agonists. The fibrate class of hypolipidaemic agents for instance acts through PPARα (Lever, George et al. 2010) while the thiazolidinedione class of anti-diabetic agents act through PPARγ thus exploring a dual PPARα/γ agonists may present a promising combination strategy for type 2 diabetes (Charbonnel 2009).
In this thesis, we have aimed to investigate and demonstrate that the thiazolidinedione Rosiglitazone can protect MIN6 beta cells from the detrimental effects of palmitate-induced apoptosis and the resultant loss of pancreatic beta cell viability through the effects on a multitude of signalling pathways and essential beta cell transcription factors. Molecular dissection of the pathways regulating these events has newly identified effects of Rosiglitazone and we have begun to determine the exact intracellular signalling events that are regulating these effects. We have shown that Rosiglitazone modified the expression of several key beta cell genes imperative for beta cell viability and function. We have also provided evidence that some effects of Rosiglitazone were exhibited through the traditional drug target PPARγ but more interestingly we have successfully unveiled other pathways fundamental for beta cell function. We have verified in this present study, and added to a growing body of evidence, data indicating that Rosiglitazone can have direct protective effects on beta cell viability and function that occur through both PPARγ-dependent and PPARγ-independent signalling pathways. This highlights the importance of protecting pancreatic beta cells and enhancing beta cell mass. Our studies with VEGF and PDCD4 and their activation by Rosiglitazone at the same time points are the first to confirm that the sustained increase in PDCD4 expression is not due to the general nonspecific up-regulation of genes. The effect of VEGF on angiogenesis also plays a significant role in overcoming the challenge of hypoxia in transplanted islets, a process limited by the progressive loss of beta cell mass post transplantation due to the combination of immunosuppressant drugs, hypoxic conditions and lack of available donors. We also report that Rosiglitazone specifically up-regulated PDCD4 gene expression in beta cells in a time-dependent manner. Therefore, collectively, our data not only suggests that Rosiglitazone can protect beta cells in patients with type 2 diabetes, but signifies how Rosiglitazone may also serve as an agent to enhance survival, growth, proliferation and vascularization of beta cells and thus transplanted islets in patients with type 1 diabetes. We propose the following model as per page 212.


Diabetes UK (2010). "Diabetes in the UK 2010 (Mar 2010);Key statistics on diabetes."


The Medical Biochemistry Page (2007). "AMPK Figure." from http://themedicalbiochemistrypage.org/ampk.html.


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